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MTA Doktori Pályázat

Tézisei

**Neuropeptidek és mellékvese szteroid hormonok
kölcsonhatásai az adrenokortikotropin elválasztás
hipotalamikus szabályozásában.**

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A Tézisekben használt rövidítések:

AC7	adenilil cikláz 7
AC9	adenilil cikláz 9
ACTH	adrenokortikotróp hormon
AVP	arginin-vazopresszin
BK-csatorna	nagy vezetőképességű Ca^{2+} aktiválta K^{+} csatorna.
cAMP	adenozin-3':5'-monofoszfát, ciklikus AMP
cGMP	guanozin-3':5-monofoszfát, ciklikus GMP
CRF	bioassay-el mért corticotropin releasing-factor
CRF41	A 41 aminosavból álló corticotropin releasing-factor
hERG	Humán Ether-à-go-go-related gene K^{+} csatorna
HPA	Hipotalamusz-hipofízis-mellékvese rendszer
mRNS	Hírvivő (messenger) RNS
NO	nitrogén monoxid
STREX	Stress responsive exon
TREK-1	TWIK-related K^{+} channel 1

1. BEVEZETÉS

Kandidátusi értekezésemben, amelyet 1984-ben védtem meg, többek között a CRF41-et termelő hipofízeotróp neuronok elhelyezkedésével és axonjaik lefutásával foglalkoztam (Antoni *et al.*, 1983). Röviddel ezután három munkacsoport szinte egyszerre közölte, hogy a hipofízeotróp CRF41-t termelő sejtek egy másik neuropeptidet, nevezetesen a már az 1960-as évek legelején CRF-nek tartott AVP-t is termelik, amelyet a CRF41-el együtt szekretálnak (Kiss *et al.*, 1984; Sawchenko *et al.*, 1984; Tramu *et al.*, 1983).

A doktori értekezésemben összefoglalt munkák a fenti alapvető megfigyelésből kibontakozó kérdéseket vizsgálták. Többségükben *in vitro* rendszerekben arra kerestünk választ, hogy levezethető-e sejtszintű jelenségekből miért szabályozza két, külön génről átiródó serkentő neuropeptid a hipofízis ACTH elválasztását? Ahol megfelelő lehetőség kínálkozott, ott az *in vitro* megfigyeléseket célzott *in vivo* vizsgálatokkal egészítettük ki.

2. A KISÉRLETEK IRODALMI HÁTTERE ÉS FŐBB CÉLKITÚZÉSEI

2.1 Az arginin-vazopresszin (AVP) szerepe az adrenokortikotróp hormon (ACTH) hipotalamikus szabályozásában:

A neuroendokrin szabályozás alapeleme a hipotalamo-hipofízeális rendszer. Ennek egyik fontos működési módja, hogy hipotalamuszban elhelyezkedő hipofízeotróp idegsejtek axonjai az eminentia mediana külső rétegében végződnek. Az axonvégződésekből felszabaduló mediátorok a hipofízeális portális vérkeringés útján kerülnek az elülső hipofízis lebenyben elhelyezkedő célsejtjeikhez. A célsejtekből felszabaduló hormonok pedig a szisztémás vérkeringéssel jutnak el célszerveikhez. A HPA tengely esetében a hipofízisből felszabaduló ACTH serkenti a mellékvesekéregben a szteroid hormonok termelését aminek következtében nő ezen hormonok vérszintje. A homeosztatisz egyensúly fenntartása érdekében a mellékvese szteroidok általában gátolják a HPA tengely működését, mind a hipofízeotróp CRF41 neuronok, mind az ACTH-t termelő adenohipofízeális kortikotróp sejtek szintjén.

A hipotalamo-hipofízeális rendszer további működési módját a hipofízis hátsó lebenyében, másik nevén neurohipofízisben találjuk. Itt, az ún. magnocelluláris neuroszekretoros sejtek rostjai végződnek amelyek az eminentia mediana belső rétegében futnak le rendeltetési helyükre. Ezek a rostok AVP-t vagy oxytocint tartalmaznak, többszörös varikozitásaikból a szisztémás keringésbe jutnak a neuropeptidek és hormonnként működnek. A szisztémás vérben keringő AVP koncentrációja mind rágcsálokban mind emberben 1-50 pmol/L között mozog, ami nem elegendő az ACTH elválasztás serkentéséhez. Ezenfelül a neurohipofízeális AVP szerepe mint antidiuretikus hormon az élettani szabályozásban jól ismert. Végül, többen kimutatták nem AVP jellegű CRF aktivitással rendelkező anyag jelenlétét a hipotalamuszban, ami látszólag megerősítette azt a felfogást, miszerint minden hipofízeális sejtípushoz egyetlen hipotalamikus serkentő hormon rendelhető. Tehát az 1970-es évek végén az AVP CRFszerű hatását kétkedés övezte. A CRF41 felfedezése (Vale *et al.*, 1981) nyomán lehetőség nyílt az AVP CRF szerepének újraértékelésére. Világossá vált, hogy *in vitro* a CRF41 és az AVP szinergisztikusan serkentik az ACTH leadást és a

CRF41 sejtek AVP-t is termelnek (Antoni, 1986). Ugyanakkor AVP receptor antagonistákkal végzett *in vivo* vizsgálatok nem támasztották alá az AVP ACTH elválasztásban játszott szerepét. Saját munkánkban ezért arra kerestünk választ az AVP milyen receptorokon keresztül fejti ki hatását az adenohipofízisre? Valóban csak a parvicelluláris CRF41 sejtekből származó neuropeptidek szabályozzák az ACTH leadást? Befolyásolja-e az AVP a glukokortikoid visszacsatolós gátlást a hipofízis szintjén?

A kísérletek eredményei a következők (a zárójelben lévő számok a 5.1 bekezdésnél található cikkeket jelzik):

2.1.1 Az AVP hatása az elülső hipofízis szintjén újszerű, a kísérletek elvégzésekor még nem ismert membrán receptoron keresztül megy végbe.

(1-2)

2.1.2 Az adenohipofízis oxytocin receptorokat tartalmaz, azonban valószínűtlen, hogy ezek a kortikotróp sejtekben találhatók.

(3-5)

2.1.3 Patkányban a hipotalamusz magnocelluláris neuronjaiból származó AVP depolarizáció nyomán Ca^{2+} -függő módon szekretálódik az izolált emimentia mediana-ban valamint kimutatható a hipofízeális portális vérben.

(6-8)

2.1.4 A vazopresszin egyik fontos szerepe a glukokortikoid visszacsatolás hatékonyságának szabályozása a kortikotróp sejtek szintjén. Intenzív stresszhelyzetre jellemző vazopresszin koncentrációk mellett a glukokortikoid gátlás korai késleltetett fázisa gyakorlatilag nem érvényesül. A glukokortikoid rezisztencia alapja a jelentősen megnövekedett intracelluláris cAMP szint.

(9)

Összefoglalva, mind a parvicelluláris, mind a magnocelluláris eredetű AVP befolyásolhatja az ACTH elválasztást. Magas AVP szintek mellett, a HPA rendszerben glukokortikoid rezisztencia mutatható ki. A mellékvese-szteroid visszacsatolós gátlás AVP által előidézett hipofízis szintű módosulása szervezeti szinten kortikoszteroid túltermelést eredményezhet. A fenti eredmények részletes tárgyalása az 5.3-as bekezdés alatt felsorolt 34, 35, 36, 38, 43 számú összefoglaló közleményekben található.

2.3 A glukokortikoid visszacsatolós gátlás mechanizmusa az adenohipofízis szintjén.

Az ACTH elválasztás szabályozásának tanulmányozása világossá tette, hogy az AVP egyik fontos hatása kortikotróp sejteken érvényesülő visszacsatolós glukokortikoid gátlás hatékonyságának módosítása. A visszacsatolós gátlás időbeli lefolyásának elemzése során általában három fázist különböztetnek meg. Leírtak gyors gátlást amelynek hatása az alkalmazott mérési módszerek feloldásától függően akár másodpercek alatt kimutatható. *In vivo* a glukokortikoid hatás a szteroid adagolás sebességétől függőnek mutatkozik, ezért gyors, sebesség-függő gátlásnak nevezik (Dallman *et al.*, 1992) (fast, rate-sensitive feedback inhibition). Mechanizmusa nem tisztázott, de feltehetően a

plazmamembrán közvetlen közelében elhelyezkedő glukokortikoid-receptor fehérjéken keresztül jön létre. Patkány kortikotróp sejteken végzett kísérleteinkben ilyen jellegű gátlást nem találtunk – az irodalom szerint is elsősorban a hipotalamusz szintjén érvényesül. A glukokortikoid gátlás második fázisa a hipofízis szintjén in vitro a szteroid adagolás kezdetétől számított 20 percen belül kimutatható. Korai, késleltetett gátlásnak nevezik (early delayed feedback inhibition) és alapvető fiziológiás szerepet játszik a HPA rendszer hormon szintjeinek beállításában. Korai, késleltetett glukokortikoid gátlás a hippokampusz, a hipotalamusz és az adenohipofízis szintjén egyaránt kimutatható. A glukokortikoid hatás harmadik szakasza a késői gátlás (delayed feedback inhibition) amely a hormon elválasztásban az adagolástól számított két órán túl érvényesül, bár a génátírás szintjén 5 percen belül észlelhető. A késői gátlás elsősorban a krónikus stressz állapotokhoz valamint a mesterséges szteroid adagolással előidézett tartós szteroid szint növekedéshez kapcsolható. A késői gátlás egyik fontos megnyilvánulása a célsejtek differenciált funkcióinak glukokortikoid hormonok által történő fojtása a génkifejezés szintjén. Ezzel szemben a korai késleltetett gátlás gén és fehérje indukción alapul és a differenciált funkciókat a célsejtek jelátviteli folyamatainak szintjén módosítja. Kísérleteinkben arra kerestünk választ hogyan működik a korai késleltetett gátlás és mi az AVP gátlást módosító hatásának lényege?

A főbb eredmények a következők:

2.3.1 A glukokortikoid gátlás korai késleltetett fázisa új mRNS és fehérje szintézisét igényli.

(10, 11)

2.3.2 A korai késleltetett gátlás megfelelően időzített, intracelluláris adenosin 3':5' monofoszfát termelést indukáló ingerrel felfüggeszthető.

(12)

2.3.3 A korai késleltetett glukokortikoid hatás membrándepolarizációval felfüggeszthető.

(13, 14)

2.3.4 A korai késleltetett gátlás egér kortikotróp tumor sejt modellben a nagy vezetőképességű Ca^{2+} -aktiválta K^{+} -csatornák (BK-csatorna) foszforillációs állapotát változtatja meg, és a jelenség humán embrionális vesesejtekben szintén megfigyelhető.

(15, 16, 17, 18)

2.3.5 Meglepő módon normális patkány kortikotróp sejtekben a BK-csatorna nem játszik jelentős szerepet a glukokortikoid gátlásban: a szteroid hatás valószínűleg hERG vagy TREK-1 típusú K^{+} csatornákon keresztül jön létre.

(9, 14)

2.3.6 A glukokortikoid visszacsatolási gátlás fontos permisszív tényezője az intracelluláris Ca^{2+} ciklikus AMP bioszintézist gátló hatása, amelynek célpontja az adenilil cikláz 9.

(19,20)

2.3.7 Az AVP által indukált glukokortikoid rezisztencia alapja a cAMP anyagcsere utak AVP hatására bekövetkező módosulása. Az adenilil cikláz 9 helyett egy protein kináz C által serkentett, intracelluláris Ca^{2+} által nem befolyásolt adenilil cikláz – valószínűleg adenilil cikláz 7 – kerül előtérbe. Ennek nyomán nagyságrenddel magasabb intracelluláris cAMP szintek jönnek létre.

(9, 19, 20)

A fenti eredmények részletes tárgyalása az 5.3-as bekezdés alatt felsorolt 42, 48, 58, 60 számú összefoglaló közleményekben található.

2. 3 A guanozin 3':5' monofoszfáton (cGMP) keresztül működő gátlás szerepe az ACTH elválasztás szabályozásában.

Az 1970-as évek közepe táján fedezték föl, hogy a NO serkenti a szolubilis guanilil cikláz enzimet és ezáltal növeli a cGMP intracelluláris koncentrációját. Az is ismert volt, hogy a glukokortikoid hormonok a NO szintáz enzim egyik altípusának kifejezését serkentik. A mellékvesekéreg hormonok hatásmechanizmusának vizsgálata során az egyik munkahipotézisünk az volt, hogy a szteroidok a NO szintáz indukciója révén fejtik ki hatásukat. Sikerült kimutatnunk, hogy a sejtmembránon áthatoló cGMP analógok jelentősen gátolják a CRF41 és AVP fiziológias kombinációjával serkentett ACTH leadást. Azonban nem találtunk összefüggést a hipofízéális cGMP termelés és a glukokortikoid gátlás között. Ezért megvizsgáltuk milyen cGMP szintézist serkentő anyagok jöhetnek szóba mint fiziológias ACTH leadást gátló tényezők?

A kísérletek főbb eredményei a következők:

2.3.1 A cGMP analógok valamint a sejten belüli cGMP bioszintézist aktiváló anyagok gátolják a neuropeptidek által serkentett ACTH leadást in vitro.

(21, 22)

2.3.2 Atriopeptin infúziója gátolja a CRF41 és AVP kombinációjával serkentett ACTH elválasztást éber patkányokban.

(23)

2.3.3 Éber patkányokban az endogén atriopeptin immun-neutralizációja növeli az immobilizációs stressz indukálta plazma ACTH és kortikoszteron választ , tehát ezen körülmények között az atriopeptin fiziológias ACTH leadást gátló neurohormon.

(24)

A fenti eredmények összefoglalása az 5.3-as bekezdés alatt felsorolt 43-as számú összefoglaló közleményekben található. Mivel ez az összefoglaló munka meglehetősen régi keletű, az alábbiakban a két előző témakörhöz képest részletesebben tárgyalom az atriopeptinnel kapcsolatos eredményeket.

Irodalmi szinten a kísérletek kivitelezésekor az atriopeptin hipofízeotróp hatásairól ellentmondó eredmények voltak forgalomban. Saját munkánk kiindulási pontja a cGMP

hatására létrejövő gátlás amely akutan izolált és perifundált patkány adenohipofízis sejteken jól reprodukálható volt. Mások több napos túlélő kultúrákban próbálkoztak sztatisztikus inkubálásban és valószínűsíthetően ezért nem észlelték a cGMP illetve a cGMP szintet fokozó anyagok serkentett ACTH elválasztást gátló hatását. A későbbiek során akut egér hipofízis szeletkéken (Guild *et al.*, 1999) és foetalis patkány hipofízis kultúrákban (Chatelain *et al.*, 2003) mások is megerősítették az atrialis peptidek ACTH elválasztást gátló hatásáról tett megfigyeléseinket. Ezzel szemben birka (Bowman *et al.*, 1997) illetve ló (Mulligan *et al.*, 1997) hipofízis sejtekben az atriopentin ACTH elválasztást gátló hatását nem sikerült kimutatni. A német klinikai irodalomban két, egymástól független munkacsoport (Bierwolf *et al.*, 1998; Strohle *et al.*, 1998; Wiedemann *et al.*, 2000) is kimutatta az atriopentin plazma ACTH szinteket és következésképpen plazma kortizol szinteket is csökkentő hatását. Tudomásom szerint a NO donorokkal végzett munkánk úttörő jellegű volt az adenohipofízis működésének vizsgálatában. Később, az adenohipofízeális cGMP NO-szolibilis guanilil cikláz keletkezési útjával számos munkacsoport foglalkozott de senki nem vizsgálta az ACTH elválasztást. Mivel minden valószínűség szerint a NO is elsősorban cGMP-n keresztül befolyásolja az ACTH leadást, az atriopentinnel kapcsolatban említett technikai problémák is közrejátszhattak abban, hogy ez a kérdéskör nem kapott nagyobb figyelmet. Végül, de nem utolsósorban hangsúlyozni szeretném, hogy akut *in vitro* modellben a serkentett ACTH leadás cGMP-n keresztül történő gátlása a glukokortikoid visszacsatolástól függetlennek látszik (Antoni *et al.*, 1990b). Összefoglalva, a rendelkezésre álló frissebb irodalom megerősítette az atriopentin hipofízis szintjén kifejtett, ACTH leadást csökkentő hatásával kapcsolatos megfigyeléseinket, és kiterjesztette azokat humán vizsgálatokra is.

A hipotalamikus atriopentin hipofízeotróp szerepét számos neuranatómiai megfigyelés is valószínűsíti. Patkány agyban az eminentia mediana zona externában atriopentin immunreaktív rostokat figyeltek meg, a periventrikuláris és paraventrikuláris hipotalamikus magokban pedig jelentős számú atriopentint tartalmazó idegsejt található (Kawata *et al.*, 1985; Skofitsch *et al.*, 1986; Standaert *et al.*, 1986). Ezen idegsejtek sebészi léziója nyomán az eminentia mediana atriopentin tartalma drasztikusan lecsökken (Palkovits *et al.*, 1987). Altatott patkányok hipofízeális portális vérében 10x magasabb a radioimmuoassay-el mért atriopentin koncentráció mint a perifériás vérben (Lim *et al.*, 1990), ezenfelül a hipofízeális portális vérben az atriopentin 5-28 a domináló forma míg a perifériás keringésben az 1-28 változat található (Lim *et al.*, 1994). Saját vizsgálatainkban az atriopentin 5-28 serkentette leghatékonyabban izolált patkány adenohipofízis sejtek cGMP termelését [ref(22)]. Az endogén atriopentin ACTH elválasztást gátló szerepét Fink és mtsai is megfigyelték – az általunk alkalmazotthoz hasonló, de más anti-testtel végrehajtott immun-neutralizációs módszerrel (Fink *et al.*, 1991). Végezetül, ismert, hogy a mellékvesekéreg szteroidok serkentik az atriopentin gén kifejezését (Gardner *et al.*, 1986) ami beleillik a két hormon egymást ellensúlyozó szerepébe. Sajnos szelektív, kismolekulasúlyú atriopentinerg anyagok hiányában nem terjedhettek el az atriopentin és rokon peptidjeinek (brain natriuretic factor) neuroendokrin és pszichotróp hatásait vizsgáló kísérletek.

3. A TUDOMÁNYOS EREDMÉNYEK JELENTŐSÉGE

3.1 A hipofízeális AVP receptor

Az általunk eredetileg az adenohipofízisből kimutatott újszerű vazopresszin receptor kézikönyvi szinten, V_{1b} vazopresszin receptorként szerepel az irodalomban. A receptor gén klónozása nyomán kiterjedt agyi lokalizációjáról számoltak be, valamint magas mRNS szintekről az immunrendszerben (Lolait *et al.*, 1995). A szervezetben játszott funkcionális szerepének vizsgálata mára kiterjedt irodalommal rendelkezik (Roper *et al.*, 2011). Két nagy gyógyszercég, a Sanofi/Aventis (Griebel *et al.*, 2002) és az Organon (Craighead *et al.*, 2008) is kidolgozott kis molekulású, szelektív V_{1b} receptor antagonistát. A Sanofi vegyület fejlesztését fázis II klinikai vizsgálatok nyomán 2008-ban leállították. Az Organon vegyület alighanem áldozatául esett az Organon-ScheringPlough-Merck láncfúciónak, illetve felvásárlásnak. Legutóbb Koob munkacsoportja (Edwards *et al.*, 2012) számolt be arról, hogy patkányban V_{1b} antagonistával megakadályozható az alkohol-dependencia kifejlődése. Összefoglalva, a V_{1b} receptor mint anxiolitikus és esetleges anti-depresszáns terápiás célpont továbbra is érdeklődésre tarthat számot.

3.2 AVP és glukokortikoid hormonok kölcsönhatása adenohipofízeális kortikotróp sejtekben

Három lényeges megfigyelést tettünk: Először, adataink alapján megalapozottnak látszik a következtetés, hogy patkányban a hipotalamikus magnocelluláris AVP neuronok hipofízeotróp funkciót töltenek be, azaz a magnocelluláris sejtekből leadott AVP befolyásolja az elülső hipofízissejtek működését. Másodszor, adenohipofízeális kortikotróp sejtekben az AVP jelentősen csökkenti a glukokortikoid visszacsatolós gátlás hatékonyságát. Harmadszor, a glukokortikoid rezisztencia sejtszintű magyarázata a kortikotróp sejtek cAMP jelátvitelének AVP hatására bekövetkező módosulása.

3.2.1 Kísérleteinkben a hipotalamikus CRF41 és AVP leadás *in vitro* és *in vivo* követése alapján bizonyítottuk a magnocelluláris AVP sejtek hipofízeotróp szerepének lehetőségét. Munkánk nyomán a magnocelluláris AVP fizikai stresszre adott HPA válaszban játszott szerepét több, döntően rágcsáló modellekben végrehajtott vizsgálat igazolta, (összefoglalásukat lásd (Engelmann *et al.*, 2004; Ueta *et al.*, 2011)). Mint arra egy korábbi összefoglaló munkámban rámutattam (Antoni, 1993), a kísérletes gyakorlatban elterjedt nagyobb testű fajokban, pl. birka és macska, a periférián mért AVP szintek nanomoláris nagyságrendűek, ami egyértelműen amellett szól, hogy a hipofízis működését is befolyásolhatják. Emberben a perifériás vérben mérhető AVP szintje a rágcsálókhoz hasonlóan 20 pmol/L fölé ritkán emelkedik, viszont a plazma koncentráció változásai jelzik a magnocelluláris neuronok aktivitását. A klinikai gyakorlatban a HPA rendszer glukokortikoid gátlásra nem érzékeny (non-suppresszor) állapotát dexamethasone adagolással diagnosztizálják. Mai ismereteink szerint (Meijer *et al.*, 1998) a dexamethasone elsősorban a hipofízis szintjén hat a HPA rendszerre, tehát az AVP glukokortikoid gátlást csökkentő hatására a teszt várhatóan kiemelten érzékeny. A plazma AVP szintek követése és a dexamethasone suppressziós teszt kombinációja alapján Chrousos és Gold munkacsoportja arra a következtetésre jutott, hogy extrém

fizikai megterhelés során az egyedek mintegy 50%-ban neuroendokrin glukokortikoid rezisztencia alakul ki és ennek oka a magnocelluláris neuronok magasabb AVP leadása. (Deuster *et al.*, 2000; Deuster *et al.*, 1998). Egy másik klinikai vizsgálatban (Inder *et al.*, 1997) depresszióban szenvedő betegek két csoportját különböztették meg: magas perifériás AVP szintek esetén hiperkortizolémia is fellépett míg normális perifériás AVP mellett a plazma kortizol szintek sem mutattak eltérést. Ezek a megfigyelések is a magnocelluláris AVP elválasztás hipofízeotróp szerepe mellett szólnak.

Nyitott kérdés, hogy a magnocelluláris AVP a hipotalamusz szintjén befolyásolja-e a hipofízeotróp parvicelluláris CRF41/AVP neuronok működését? Számos megfigyelés valószínűsíti az igenlő választ. A magnocelluláris neuronok dendritjeiből AVP szabadul föl (Ludwig *et al.*, 2002) a CRF41 sejttestek és magnocelluláris dendritek között sajátos szinaptikus kapcsolatok vannak (Léránth *et al.*, 1983), a hipofízeális portális vérben mért CRF41 koncentráció AVP agykamrába történő adagolása nyomán csökken (Plotsky *et al.*, 1984). Ez a kérdés ma már CRF41-GFP egerekből nyert agyszeletekben valamint csatornarodopszint szelektíven kifejező magnocelluláris neuronok ingerlésével közvetlenül vizsgálható.

3.2.2 Az AVP glukokortikoid rezisztenciát okozó hatásában mind a parvi- mind magnocelluláris rendszerből származó AVP szerepe fölmerül. Számos közleményben számoltak be olyan kísérleti beavatkozásról amely jelentősen, akár tízszeresen is, növelte a parvicelluláris CRF41 sejtekben az AVP bioszintézist. A közelmúlt epigenetikai kutatásai alapján úgy tűnik, hogy a HPA stressz-válasz egyik fő meghatározója lehet a születés utáni életesemények tartós hatása a hipotalamikus AVP gén kifejezésre (Bunck *et al.*, 2009; Murgatroyd *et al.*, 2009). Humán poszt-mortem anyagban Swaab és munkatársai, de mások is (Mouri *et al.*, 1993) vizsgálták a hipotalamikus AVP kifejezést (Purba *et al.*, 1996; Raadsheer *et al.*, 1994). Eredményeik alapján fölvetődik, hogy melankólikus depresszióban a magnocelluláris és parvicelluláris rendszerben egyaránt szignifikánsan magasabb hipotalamikus AVP kifejezés okozza az ehhez a kórképekhez gyakran társuló kortizol túltengést (Holsboer, 2001). Idős korban szintén megnő a hipotalamuszban a CRF41-AVP ko-lokalizáció ami oka lehet az idősokban megfigyelhető, stressz helyzet után lassabban lecsengő plazma kortizol válasznak. Az idősokban megnövekedett stressz-indukálta plazma kortizol válasznak patogenetikus szerepe lehet, elsősorban a gyakran fellépő szorongásos rendellenességekben, illetve súlyosabb esetekben progresszív hippocampus atrófiában (Holsboer, 2001; Sapolsky *et al.*, 2000). A károsnak tűnő pszichotróp hatásokkal ellentétben autoimmun modellekben a tartós kortizol hiperszekréció életmentő hatású (Mason, 1991). Beltenyésztett, az immunreakció fő génjeire (HLA rendszer homológok) szingenetikus patkány és egértörzsek vizsgálatával kimutatható, hogy mellékvesekéreg hormon túltengés öröklődhet és javítja az autoimmun gyulladás túlélésének esélyeit (Mason *et al.*, 1990). Mai tudásunk alapján nem zárható ki azonban, hogy ezeknél a HPA „hiperreaktív” állattörzseknél is epigenetikus tényezők dominálnak azaz további vizsgálatokra van szükség az állatok HPA reaktivitásának korai életkorban történő „programozását” illetően (Holmes *et al.*, 2006). További fontos közlés, hogy Freund adjuvánssal indukált artritiszben szenvedő patkányok hipofízeális portális vérében ötször magasabb AVP szinteket találtak mint a kontrollokban (Chowdrey *et al.*, 1995). Ezzel párhuzamosan a hipofízeotróp CRF41 sejtekben kimutatható AVP mRNS mennyisége is jelentősen emelkedett míg a CRF41

mRNS szintje változatlan maradt (Chowdrey *et al.*, 1995). Tehát nagy valószínűséggel ebben az állapotban a parvicelluláris AVP idézi elő a tartós hiperkortizolémiát. Összefoglalva, adataink rámutattak az AVP glukokortikoid visszacsatolást módosító hatására, amelynek jelentős szerepe lehet különböző emberi kórképek patogenezisében és lefolyásában.

3.2.3 A HPA rendszerbeli glukokortikoid korai késleltetett gátlás mechanizmusát és hatékonyságának meghatározóit elemeztük a hipofízeális kortikotróp sejtek szintjén. Megállapítottuk, hogy a gátlás 20 percen belül kifejlődik, új mRNS és fehérje szintézisét igényli, és nagyrészt a plazma membrán szintjén zajlik. Egér hipofízis tumor sejtekben kimutattuk, hogy a BK-csatorna STREX variánsa játszik szerepet a glukokortikoid hatásban, a hatás lényege a csatorna cAMP-dependens foszforillációjának megakadályozása. A glukokortikoidok által indukált gátló mechanizmus nem sejtspecifikus, mivel humán embrionális vesesejtekben is reprodukálható volt amennyiben azokban kifejeztük a BK-csatorna STREX változatát. Azaz a BK-STREX variáns alkalmas lehet a glukokortikoid hatás különféle sejttrendszerekben, így például limfocitákban történő kísérleti elemzésére.

A hipotézist, miszerint glukokortikoid kezelés hatására a BK-csatornához kapcsolódó fehérje foszfátáz (PPA2) mennyisége változik meg nem sikerült igazolni. Ko-immunprecipitációs adataink szerint a csatornafehérjéhez konstitutívan kapcsolódik a PPA2 és ebben glukokortikoid kezelés nyomán változást nem találtunk (Yazdi *et al.*, 2005). Elképzelhető, hogy a PPA2 valamely szabályozó alegysége indukálódik, amely a csatornán horgonyzó katalitikus alegységet aktiválja. Azonban az is felerősít, hogy korábbi kísérleteinkben, amelyekben okadátal és calyculin A-val gátolhatóknak bizonyult a BK-csatornákra kifejtett glukokortikoid hatás, valójában a kettős specifitású fehérje foszfátázok (DUSP) valamelyikét bénítottuk, amelyek a kísérleteink végzése idejében nem voltak kellően jellemezve. A BK-csatornához konstitutívan kapcsolódó PPA2 pedig vagy más szabályozó szerepet tölt be, vagy a fehérje túlkifejezésből eredő műtermék. A frissebb irodalomban a DUSP-k szerepe a glukokortikoid hormonok hatásában széles körben elfogadott (Beck *et al.*, 2009; Clark *et al.*, 2008; Tchen *et al.*, 2010), de neuroendokrin kontextusban nem közöltek ilyen irányú vizsgálatokat.

További meglepetésként szolgált, hogy patkány adenohipofízis primér kultúrákban a BK-csatornák bénítása nem módosította sem a kortikoszteron sem a dexamethasone ACTH elválasztást gátló hatását. Ugyanakkor két, K^+ -csatorna gátlóként is működő, klinikai farmakoterápiában is megfordult anyaggal, nevezetesen astemizollal illetve clofiliummal a kortikoszteron gátlás megszűnt illetve jelentősen gyengült. Mások reprodukálták az astemizol hatását (Yamashita *et al.*, 2009) ugyanakkor velünk szemben szelektív hERG csatorna bénítókkal is fel tudták függeszteni a kortikoszteron serkentett ACTH leadást gátló hatását. Mivel az adenohipofízeális kortikotróp sejtek számos, az ACTH szekréciós választ befolyásoló K^+ -csatornát tartalmaznak (Antoni *et al.*, 1990a; Kuryshev *et al.*, 1997; Liang *et al.*, 2011), ezeket a látszólagos ellentmondásokat akután készült adenohipofízis szelektékből azonosított kortikotróp sejteken végzett elektrofiziológiai vizsgálatokkal kellene feloldani. További érdekes kérdés, hogy a hippocampalis CA1 régió piramis sejtjeiben is kimutatható, és az adenohipofízeális szintű kortikoszteron

hatással szoros párhuzamot mutató glukokortikoid hatás (Joëls, 1997) milyen típusú K^+ -csatornán jön létre? Mint az AtT20-as sejtek példáján láttuk, szoros analógiákat mutató „fenotípus” dacára a glukokortikoid hatás molekuláris célpontja sejtenként változhat annak ellenére, hogy nagy valószínűséggel a szteroid adagolás nyomán ugyanaz a fehérje készlet indukálódik.

Az AVP hipofízeális glukokortikoid gátlást felfüggesztő hatását illetően újszerű jelenséget figyeltünk meg – „agonist-induced plasticity” (Antoni, 2012) – amely kítűnő példa a jeltáviteli rendszerekben tapasztalható molekuláris szintű változatosság élettani szerepének szemléltetésére. Lényege, hogy agonista hatású anyagok az intracelluláris jeltáviteli rendszerek módosítása nyomán még akkor is alapvetően másfajta tulajdonságú sejtválaszt hozhatnak létre, amikor a lényeges intracelluláris hírvivő molekulák, esetünkben a cAMP és a Ca^{2+} változatlanok. Éppen ezért ez más jelenség mint a „biased agonism”, amely esetben egyazon receptor különböző állapotai eltérő típusú jeltáviteli utakat aktiválnak. A konkrét példa a kortikotróp sejt, ahol a fiziológiás koncentrációjú CRF41-el serkentett cAMP termelést az intracelluláris Ca^{2+} hatékonyan gátolja, ezáltal visszacsatolási kört alkotva a receptor aktiválódás plazma-membránbeli hatásai felé. A fiziológiás szinteket jelentősen meghaladó CRF41 koncentráció alkalmazása esetén a Ca^{2+} visszacsatolás cAMP bioszintézisre gyakorolt hatása eltűnt – a cAMP szintek a CRF41 koncentrációval egyenesen arányosan növekedtek. A stresszorokra jellemző 2-3 nmol/L AVP-vel fiziológiás CRF41 koncentrációk mellett is előhívható volt a Ca^{2+} gátlás alól felszabadult cAMP bioszintézis, ezenfelül a jelenséget protein kináz C-t aktiváló forbol-dibutirát-észterrel is utánozni tudtuk. Az intracelluláris Ca^{2+} cAMP szintézist gátló hatásának célpontja az AC9. Magas, afiziológiás CRF41 koncentrációk esetében, illetve AVP hatására bekövetkező protein kináz C-vel történt foszforilláció nyomán valószínűsíthetően az AC7 kerül előtérbe mint cAMP bioszintetikus enzim. Az AC7 paralóg sajátossága, hogy Ca^{2+} nem befolyásolja, protein kináz C hatására aktív $G_{s\alpha}$ -val jelentősen erősebben serkentődik mint defoszforillált állapotban. Érdekes módon az AC7-t a frissebb irodalom depressziós kórképek és alkohol dependencia kapcsán vizsgálja (Desrivieres *et al.*, 2011; Pronko *et al.*, 2010). További farmakológiai vizsgálataink azt mutatták, hogy AVP jelenlétében a cAMP lebontásban Ca^{2+} /calmodulin aktivált, kis affinitású, nagy kapacitású PDE1 dominál, szemben az alacsony intenzitású CRF41 ingerrel ahol a PDE4 nagy affinitású, azonban korlátozott kapacitású altípusának közreműködése valószínű.

Összegezve, adataink azt mutatják, hogy az AVP kortikotróp sejtekre gyakorolt hatásának fő eleme a cAMP anyagcsere utak módosulása: AC9 mellett az AC7 végzi a cAMP bioszintézis nagyobb hányadát, a Ca^{2+} visszacsatolós gátlás célpontja ezért áttevődik az AC9-ről a PDE1A-ra. Mivel a PDE1A cAMP K_m -je 20 μ mol/L szemben a PDE4 család 1 μ mol/L K_m -jével, a sejtek AVP jelenlétében nagyságrenddel magasabb cAMP mellett működnek. Ilyen magas cAMP koncentrációk mellett a cAMP által szabályozott fehérjék további fajtái pl. a ciklikus nukleotidok által aktiválható ioncsatornák lépnek működésbe, amelyeknek kinyílása jelentős depolarizációt eredményez és kézenfekvően magyarázhatja az AVP glukokortikoid gátlást felfüggesztő hatását. Ennek a mechanizmusnak kísérleti bizonyítéka egyelőre nincs, bár ismert, hogy ciklikus nukleotidokkal aktiválható ioncsatornák kifejeződnek az adenohipofízisben (Kaupp *et al.*, 2002).

3.3 Atriopeptin mint ACTH elválasztást gátló hormon.

A gerinces élőlényeknél az „ős-stressz“ a só- és vízvesztés amelynek következménye az ion- és folyadékháztartás összeomlása lehet. Ennek érdekében az angiotenzin, az AVP, CRF41 és a mellékvesekéreg szteroid hormonok számos szervben összehangoltan a víz és sóretenciót segítik elő – még az AVP izo-oszmotizis fenntartó hatásait is folyadéktérfogat csökkentéssel semlegesíteni lehet. Ezzel szemben, az atriopeptin ACTH elválasztást csökkentő hatása beleillik abba az általános képbe, hogy az atriopeptinek számos szervben összehangoltan működve, a stressz által kiváltott reakciókat ellensúlyozzák. Tehát az atriopeptin „nyugi“ hormon amely ellensúlyozza a fentebb felsorolt „izgik“ hatását, ez által helyreállítja a homeosztázist. Mint már fentebb jeleztem, számos munkacsoport, így Telegdy és mtsai is (Biró *et al.*, 1996; Biró *et al.*, 1995), kimutatta az atriopeptin pszichotróp, ezen belül anxiolitikus hatását. Mások ebből kiindulva pánik betegségben, általános szorongási betegségben és alkohol megvonási tünetek enyhítésében javasolták terápiás szerként (Kiefer *et al.*, 2002; Kiefer *et al.*, 2011) (Kellner *et al.*, 2003). Az atriopeptin és a glukokortikoid gátlás viszonylagos súlyát nem vizsgálták. Logikusnak tűnik a feltételezés, hogy az atriopeptin vagy egyéb, hatását cGMP-n keresztül kifejtő gátló tényező a glukokortikoid non-szuppresszor állapotban kerül előtérbe az ACTH elválasztás szabályozásában.

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Novel Ligand Specificity of Pituitary Vasopressin Receptors in the Rat

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Key Words. V_1 antagonists · Oxytocin · dDAVP

Abstract. The ligand specificities of sites binding tritium-labeled arginine vasopressin (^3H -AVP) were investigated in membrane preparations of rat anterior pituitary gland and liver. Labeled AVP interacted with high-affinity, low-capacity binding sites in liver as well as in pituitary membrane fractions. Binding displacement studies showed that AVP was a potent ligand for liver ($K_1 = 0.23 \text{ nM}$) and pituitary ($K_1 = 0.4 \text{ nM}$) receptors. Oxytocin and the antidiuretic (V_2) agonist dDAVP had low affinities ($K_1 > 10 \text{ nM}$) both for liver and pituitary binding sites. The pressor (V_1) antagonist $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ was equipotent with AVP in liver membranes, but was 1,000-fold less potent on pituitary receptors. Another V_1 antagonist, $\text{dPenTyr}(\text{Me})\text{AVP}$, displaced ^3H -AVP with K_1 values of 3 and 8 nM from pituitary and liver receptors, respectively. These data are in reasonable agreement with recent studies of the *in vitro* biological activities of the analogs tested, and suggest that the ligand specificity of rat pituitary AVP receptors is distinct from those of previously characterized V_1 , V_2 and oxytocin binding sites in the rat.

The secretory activity of anterior pituitary corticotrope cells is weakly stimulated by arginine vasopressin (AVP) alone; however, there is powerful synergism between AVP and 41-residue corticotropin-releasing factor (CRF-41) to increase the secretion of ACTH/ β -endorphin [10]. The possible physiological significance of the latter phenomenon is indicated by the fact that AVP and CRF-41 are produced in the same hypothalamic nerve cells [12, 19].

The nature of the receptors that mediate the actions of AVP on pituitary corticotrope cells has not been clearly established. Earlier studies, examining the CRF activity of vasopressin analogs *in vivo*, gave discrepant results, some suggested that pressor (V_1) receptors are involved [1, 8], while other failed to confirm this [2, 4, 9]. More recently, several investigators reported that V_1 receptor antagonists inhibit ACTH release induced by AVP *in vivo*, thus supporting the V_1 receptor hypothesis [1, 13, 16, 17]. Contrast experiments, in which the release of ACTH/ β -endorphin by rat pituitary tissue incubated *in vitro* was examined [3, 5, 14], failed to confirm this hypothesis. The present study was undertaken to investigate the problem further by compar-

ing the ligand specificity of receptors binding radiolabeled AVP in rat anterior pituitary tissue with that of V_1 receptors [6, 15] in rat liver.

Materials and Methods

Randomly cycling female Osborne-Mendel rats (225–270 g body weight) were decapitated, the liver and the anterior pituitary gland were removed and placed in ice-cold 0.15 M NaCl. Crude membrane fractions of the tissues were prepared as described earlier [11]. The pelleted membrane fraction was resuspended in 0.05 M Tris-HCl pH 7.4, containing 1 mM EGTA (Sigma), 3 mM MgSO_4 , 1 mg/ml bovine serum albumin, and 0.1 mg/ml bacitracin (Sigma). Aliquots of membrane suspension (80–100 μg protein of pituitary membranes, 180–220 μg liver) were incubated with increasing amounts of tritium-labeled AVP (^3H -AVP; New England Nuclear) or with 0.5 nM ^3H -AVP and varying concentrations of unlabeled peptides in a final volume of 250 μl . The reaction was allowed to proceed for 45–60 min at 22 °C, bound and free peptide were subsequently separated by vacuum-filtration over Whatman GF/C filters. The filters were dried and counted for radioactivity in 5 ml of Hydrofluor (National Diagnostics) in a Beckman LS 2800 liquid scintillation counter with 45% efficiency. Nonspecific binding was determined in the presence of 1 μM AVP and was found to be between 10 and 18% of total binding when 0.5 nM ^3H -AVP was used.

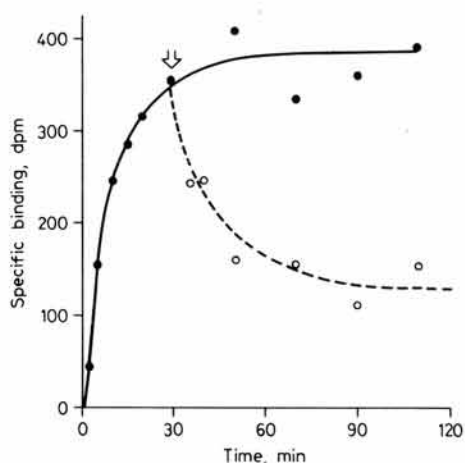


Fig. 1. time course of the association and dissociation of ^3H -AVP (0.5 nM) to pituitary membrane suspensions. AVP (10^{-6} M) was added at 30 min to some of the tubes (arrow). Data are means of triplicates from a representative experiment.

The peptides used in displacement studies were AVP, oxytocin, $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$, $\text{dPenTyr}(\text{Me})\text{AVP}$ (all from Bachem), and dDAVP (Spofa, Prague, Czechoslovakia). (Abbreviations are defined in table I.) Binding displacement curves were linearized by LOGIT-log transformation, the inhibitory constants (K_i) of the displacing ligands were calculated using the concentration of peptide giving 50% displacement [7].

Results

In pituitary membrane suspensions the binding reaction reached equilibrium by 30 min, and the amount of radioactivity bound to the membrane fraction remained stable up to 2 h (fig. 1). The formation of the ligand-receptor complex was reversible, addition of AVP (10^{-6} M) decreased the amount of bound ^3H -AVP in a time-dependent manner. The dissociation constant estimated on basis of the on- and off-rates of the reaction was 1 nM . Incubation of pituitary membrane suspensions with increasing amount of labeled peptide showed that specific binding was saturable while nonspecific was not (fig. 2). Similar results were obtained with membrane suspensions of liver tissue (data not shown). Scatchard plots of the data indicate that ^3H -AVP interacts with high-affinity, low-capacity binding sites in pituitary as well as in liver membrane suspensions (fig. 2; pituitary: $K_d = 0.32 \pm 0.02 \text{ nM}$, $B_{\text{max}} = 64 \pm 2 \text{ fmol/mg protein}$, $n = 3$; liver: $K_d = 0.27 \pm 0.04 \text{ nM}$, $B_{\text{max}} = 158 \pm 21 \text{ fmol/mg protein}$, $n = 4$; mean $\pm$ SEM values). A summary of the results of displacement studies with various structural analogs of AVP is presented in table I. In liver membranes, AVP and $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ were equally potent in displacing ^3H -AVP, while $\text{dPenTyr}(\text{Me})\text{AVP}$ was

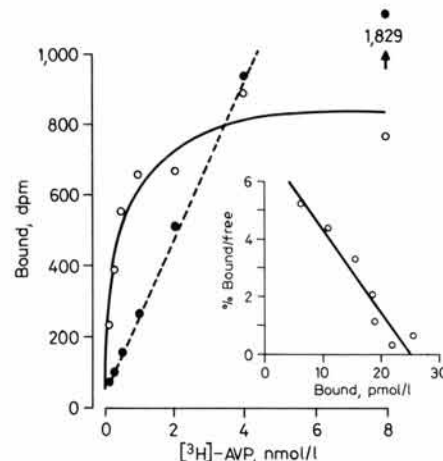


Fig. 2. Isotherm of the binding of ^3H -AVP to pituitary membrane suspensions. $\circ$ = Specific binding. $\bullet$ = Nonspecific binding. The insert shows the Scatchard plot of the data; $K_d = 0.36 \text{ nM}$, $B_{\text{max}} = 67 \text{ fmol/mg protein}$. Points are the means of duplicate determinations.

Table I. Comparison of the ability of various AVP analogs to displace ^3H -AVP from pituitary and liver binding sites¹

Compound	Liver	Pituitary
8-Arginine vasopressin (AVP)	0.23 ± 0.03 (5)	0.40 ± 0.10 (5)
Oxytocin	363 ± 99^b (3)	$100 \pm 26^{a, b}$ (3)
1-Deamino, 8-D-arginine vasopressin (dDAVP)	193 ± 43^b (3)	$34 \pm 5^{a, b}$ (3)
[1-Mercapto- β , β -cyclopentamethylene propionic acid(<i>o</i> -methyl-tyrosine)8-arginine]vasopressin ($\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$)	0.19 ± 0.02 (3)	$313 \pm 116^{a, b}$ (3)
1-Deamino, penicillamin(<i>o</i> -methyl-tyrosine)8-arginine vasopressin ($\text{dPenTyr}(\text{Me})\text{AVP}$)	8.1 ± 2.1 (3)	3.3 ± 1.1^b (3)

Means $\pm$ SEM of the K_i (nM) values are shown; the numbers in parentheses indicate the number of experiments.

^aSignificantly different ($p < 0.05$) from the respective value in liver membranes, or ^bfrom the K_i of AVP in the respective tissue.

considerably less effective. Oxytocin and dDAVP had low affinities for liver receptors. The endogenous ligand AVP also proved to be the most potent peptide tested in pituitary membranes, while $\text{dPenTyr}(\text{Me})\text{AVP}$ and $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ were 10- and 1,000-fold less potent, respectively. Oxytocin as well as dDAVP had K_i values greater than 10 nM in pituitary membranes; however, these proved to be significantly lower than the corresponding values in liver membranes.

Discussion

The pituitary, as well as the liver sites binding ^3H -AVP had low affinities for dDAVP and oxytocin, potent antidiuretic (V_2) and oxytocic agonists, respectively [18]. This is in agreement with the previous classification of pituitary and liver AVP receptors in the rat as V_1 receptors [1, 6, 8, 15, 18].

The novel feature of pituitary AVP binding sites is their low affinity for $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$, which is the most potent antagonist of the V_1 -type effects of AVP on vascular smooth muscle and on liver cells [18]. In liver membranes, $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ behaved as expected on the basis of previous observations, displacing ^3H -AVP as potently as AVP. In sharp contrast, $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ was about 1,000-fold less effective than AVP in pituitary membranes. Another well-characterized V_1 antagonist, $\text{dPenTyr}(\text{Me})\text{AVP}$, had reasonably high affinities for liver as well as for pituitary receptors.

These findings parallel recent studies of AVP-induced ACTH/ β -endorphin release by rat pituitary tissue in vitro, which indicated that (1) $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ is a poor inhibitor of the action of AVP [3, 5, 14]; (2) $\text{dPenTyr}(\text{Me})\text{AVP}$ is a relatively effective antagonist [3]; (3) there is about a 100-fold difference between the potencies of $\text{dPenTyr}(\text{Me})\text{AVP}$ and $\text{d}(\text{CH}_2)_5\text{Tyr}(\text{Me})\text{AVP}$ to interact with pituitary AVP receptors.

Thus, it appears that the ligand specificity of rat pituitary AVP receptors does not conform with the specificities of previously characterized V_1 , V_2 and oxytocin receptors.

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Evidence That the Effects of Arginine-8-Vasopressin (AVP) on Pituitary Corticotropin (ACTH) Release are Mediated by a Novel Type of Receptor

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ANTONI, F. A., M. C. HOLMES, G. B. MAKARA, M. KÁRTESZI AND F. A. LÁSZLÓ. Evidence that the effects of arginine-8-vasopressin (AVP) on pituitary corticotropin (ACTH) release are mediated by a novel type of receptor. *PEPTIDES* 5(3) 519-522, 1984.—Ovine corticotropin releasing factor (oCRF-41) and AVP act synergistically to stimulate pituitary ACTH secretion. In the present study we have investigated whether the effect of AVP, either in the presence or in the absence of oCRF-41 (0.5 nmol/l), could be blocked by V_1 (pressor)-antagonists. Furthermore, oxytocin, and [1-deamino,8-D-arginine] vasopressin (dDAVP) were tested for their ability to release ACTH. All experiments were carried out *in vitro*, using segments of rat anterior pituitary glands. The V_1 -antagonist [1-deamino,penicillamine(o-methyl-tyrosine)]AVP inhibited ACTH release induced by AVP or AVP + oCRF-41. However, it also had some agonistic activity which was more pronounced in the presence of oCRF-41. An equally potent V_1 -antagonist, [1- β -mercapto- β , β -cyclopentamethylenepropionic acid (o-methyl-tyrosine)]AVP, failed to inhibit AVP-stimulated ACTH secretion, and also had weak agonist potency. The relatively selective V_2 (antidiuretic)-agonist dDAVP was 20-30 fold less potent than AVP. Oxytocin, a weak V_1 - and V_2 -agonist was only 4-8 fold less potent than AVP. These data are compatible with the suggestion that AVP receptors on pituitary corticotrope cells are neither classical V_1 - nor V_2 -receptors.

Vasopressin-antagonists Oxytocin 1-deamino,8-D-arginine-vasopressin Corticotropin-releasing factor

THE release of ACTH by the anterior pituitary gland is stimulated by AVP (cf., [12]). Some earlier studies of this effect suggested that it is mediated by V_1 (pressor)-receptors [1, 8, 9, 17, 22, 25]. Vasopressin also potentiates the ACTH-releasing effect of ovine corticotropin-releasing factor (oCRF-41) [13]; the receptors involved in this action of AVP have not been characterized. More recently, potent, relatively selective V_1 -antagonists have been synthesized and characterized (cf., [28]). Two such antagonists, [1-deamino-penicillamine(o-methyl-tyrosine)]AVP (dPenTyr[Me]AVP) and [1- β -mercapto- β , β -cyclopentamethylenepropionic acid (o-methyl-tyrosine)]AVP (d[CH₂]₅Tyr[Me]AVP) blocked the increase of plasma ACTH levels caused by systemic injections of AVP [1, 17, 22, 25], and it has been generally assumed that the site of inhibition is at the level of the pituitary gland. Based on this assumption V_1 -antagonists have been used in several studies to establish the physiological role of AVP in the hypothalamic regulation of ACTH secretion [6,

10, 17, 22, 25]. The results of these studies have been variable, in part perhaps because V_1 -antagonists may not directly inhibit the action of AVP on pituitary ACTH secretion. Therefore, we have investigated the effects of d[CH₂]₅Tyr[Me]AVP and dPenTyr[Me]AVP on ACTH release *in vitro*.

METHOD

Male Wistar rats (220-280 g body weight) from G. Richter Ltd., Budapest, Hungary were decapitated, and the anterior lobe of the pituitary gland was removed. Segments of the anterior pituitary gland were preincubated for 4 hr in Krebs-Ringer bicarbonate medium (KRBG), containing L-ascorbate (10⁻⁴ mol/l) and bovine serum albumin (1 g/l), as described previously [3]. Subsequently, various peptides were added for another hour in fresh medium. The peptides used included oCRF-41, dPenTyr[Me]AVP (courtesy of Dr. J. Rivier, La Jolla, CA), d[CH₂]₅Tyr[Me]AVP (courtesy of

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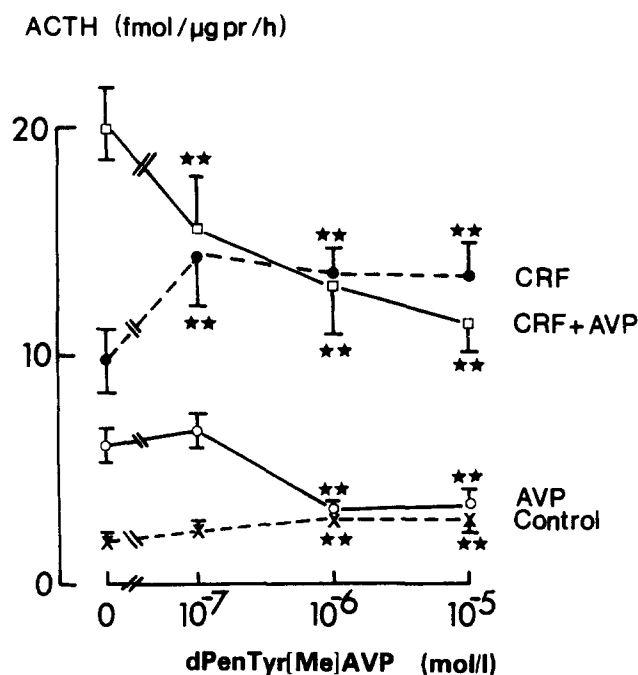


FIG. 1. Effect of dPenTyr[Me]AVP on the release of ACTH by incubated pituitary segments: (X) basal, and in the presence of (○) AVP 10^{-8} mol/l; (●) oCRF-41 5×10^{-10} mol/l; (□) AVP 10^{-8} mol/l and oCRF-41 5×10^{-10} mol/l. Values are geometrical means $\pm$ R.S.E.M. (indicated by bars), $n=8$ per group. Three-way ANOVA gave significant interaction, $F(3,112)=11.9$, $p<0.01$. ** $p<0.01$ when compared to the respective value in the absence of antagonist. Dunn's test for multiple comparisons.

Dr. M. Manning, Toledo, OH) and AVP (Peninsula, San Carlos, CA). The above were dissolved in acetic acid (0.1 mol/l), then diluted further in KRBG. Ovine CRF-41 was used at a concentration of 5×10^{-10} mol/l as this is a close estimate of the amount of endogenous 41-residue CRF in rat pituitary portal plasma [11]. Oxytocin (G. Richter Ltd.) and [1-deamino,8-D-arginine]vasopressin (dDAVP; Ferring A. B., Malmö, Sweden or SPOFA, Prague, Czechoslovakia), were obtained in solution. The molar concentration of oxytocin was calculated assuming that the bioactivity of the pure peptide is 486 IU/mg [21,28]. The ACTH content of the medium was determined by radioimmunoassay [20], the protein content of the pituitary segments was measured according to Peterson [24]. Hormone release is expressed as fmol immunoreactive ACTH released per μ g pituitary protein. Data were evaluated after log-transformation to achieve homogeneity of variances [3], by 2- or 3-way analysis of variance (ANOVA). Relative potencies for agonistic activity were obtained after linearization of the dose-response curves by LOGIT-transformation, and measuring the distance between the regression-lines along the X-axis. Geometrical means $\pm$ the retransformed standard error of the mean (R.S.E.M.) are presented.

RESULTS

The effect of AVP in the absence and in the presence of oCRF-41 was markedly reduced by dPenTyr[Me]AVP in a dose dependent manner (Fig. 1). However, the antagonist itself enhanced basal as well as oCRF-41-stimulated secre-

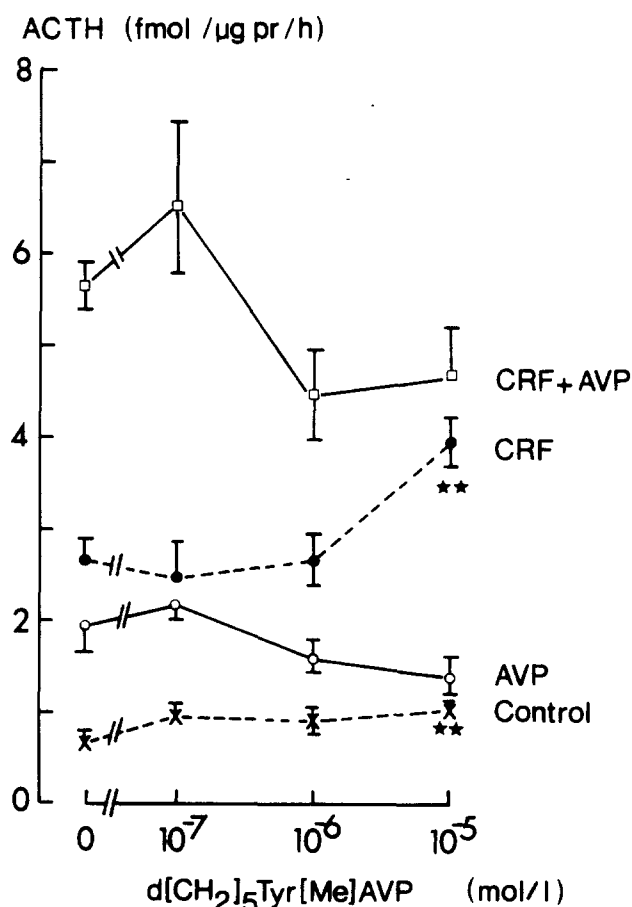


FIG. 2. Same as Fig. 1 except that d[CH₂]₅Tyr[Me]AVP was used as the antagonist. Three-way ANOVA gave significant interaction between AVP and the antagonist, $F(3,112)=7.0$, $p<0.01$. ** $p<0.01$, when compared to the respective value in the absence of antagonist. Dunn's test for multiple comparisons.

tion of ACTH (Fig. 1). When increasing concentrations of AVP (10^{-10} to 2×10^{-6} mol/l) were combined with oCRF-41, or with oCRF-41 and dPenTyr[Me]AVP (10^{-6} mol/l), it was found that the antagonist had agonistic activity approximating that of AVP at 2.5×10^{-10} mol/l, and that the effects of higher concentrations of AVP (up to 3.2×10^{-8} mol/l) were attenuated (data not shown). However, the maximal stimulation of ACTH release achieved was the same in the presence of the antagonist as in its absence (oCRF-41 plus AVP 8×10^{-9} mol/l: 17.4 ± 1.09 ; oCRF-41 plus AVP 1.28×10^{-7} mol/l plus dPenTyr[Me]AVP: 19.1 ± 1.13 , ACTH fmol/ μ g protein, geometrical means $\pm$ R.S.E.M., $n=8$ per group).

The other antagonist, d[CH₂]₅Tyr[Me]AVP had no significant effect on AVP induced ACTH release. In contrast, weak but significant agonist activity could be demonstrated (Fig. 2). Preincubation of the tissue (30 min) with the antagonist did not increase its inhibitory potency (data not shown). Arginine-vasopressin oxytocin, dDAVP and dPenTyr[Me]AVP all enhanced oCRF-41 stimulated release of ACTH (Fig. 3). The relative potencies were 0.14 and 0.03 for oxytocin and dDAVP respectively, while dPenTyr[Me]AVP did not have full agonist activity. Similar relative potencies (oxytocin 0.24, dDAVP 0.05) were obtained for these compounds when they were applied without oCRF-41 (Fig. 4).

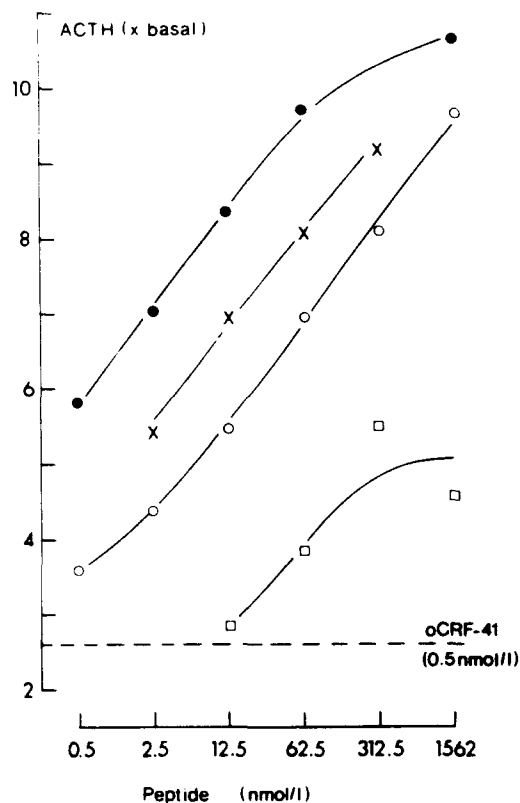


FIG. 3. Effect of AVP related peptides on ACTH release in the presence of oCRF-41 (5×10^{-10} mol/l). Data were standardized by taking basal release as unity. (●) AVP the average R.S.E.M. of the curve is: 1.17; (X) oxytocin: 1.20; (○) dDAVP: 1.26; (□) dPenTyr(Me)AVP: 1.2; $n=8$ for all data points.

DISCUSSION

The data presented above suggest that receptors for AVP on pituitary corticotropes are different from V_1 - or V_2 -receptors. We have shown that dPenTyr[Me]AVP inhibits the effect of AVP on pituitary ACTH release in the presence or absence of 5×10^{-10} mol/l oCRF-41. However, the antagonist was found to have significant, albeit partial agonist activity. Increasing the concentration of AVP in the presence of an inhibitory concentration of dPenTyr[Me]AVP resulted in a maximal ACTH release which was identical to that obtained without antagonist. Moreover, oCRF-41 enhanced the effects of dPenTyr[Me]AVP just as those of other analogs of AVP tested. These observations suggest that dPenTyr[Me]AVP is in fact a partial-agonist, competitive antagonist on pituitary AVP receptors.

In the present study d[CH₂]₅Tyr[Me]AVP had no statistically significant effect on AVP-stimulated ACTH release. Nevertheless it is possible that a minor inhibitory action demonstrated in cultures of fetal pituitary tissue [4] might have been masked by the relatively high variation of the present data. Recently, Knepel and coworkers have reported [18] that d[CH₂]₅Tyr[Me]AVP is a weak antagonist of AVP-induced β -endorphin release by corticotrope cells of the anterior pituitary gland, however, they could achieve a substantial inhibition by high a concentration of antagonist, which proved to have agonist activity in our hands. Nevertheless, it seems clear that dPenTyr[Me]AVP is a more po-

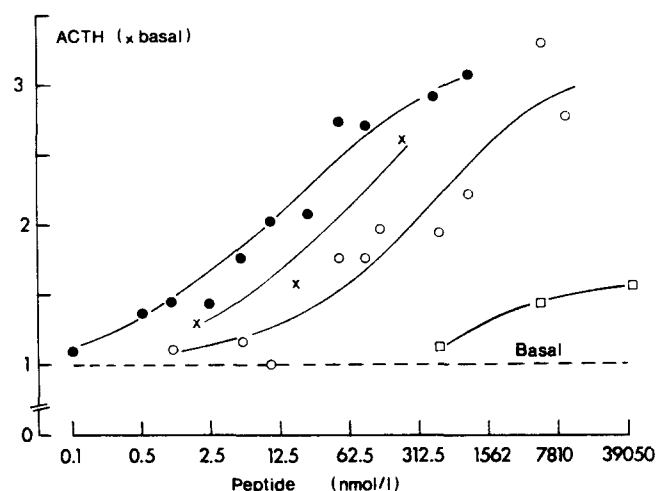


FIG. 4. Effect of AVP related peptides on ACTH release, oCRF-41 was not applied. Pooled data from several experiments standardized by taking basal release in respective experiments as unity. (●) AVP the average R.S.E.M. of the curve is: 1.19; (X) oxytocin: 1.15; (○) dDAVP: 1.16; (□) dPenTyr(Me)AVP 1.22.

tent antagonist of AVP in the pituitary than d[CH₂]₅Tyr[Me]AVP. The converse is usually found when responses mediated by V_1 -receptors are studied [28].

Both dPenTyr[Me]AVP and d[CH₂]₅Tyr[Me]AVP had low, but significant agonist activity and dPenTyr[Me]AVP was more potent in this regard. The agonist effect of these compounds on pituitary ACTH release could be demonstrated in other systems, such as isolated anterior pituitary cells and cultures of fetal rat pituitary [4]. In contrast, neither of the antagonists used in this study has been shown to have V_1 -agonist activity in any system tested [16,28]. Thus it seems unlikely that the effect of AVP on pituitary corticotrope cells is mediated by classical V_1 -receptors.

Both dPenTyr[Me]AVP and d[CH₂]₅Tyr[Me]AVP possess some V_2 (antidiuretic) activity [28], hence the ACTH-releasing effect could be mediated by V_2 -receptors. Accordingly, we examined the effects of dDAVP, a peptide which is as potent as AVP for V_2 -type effects, but has very low V_1 -type activity [7, 16, 27, 30]. Both preparations of dDAVP used (Ferring and SPOFA) were approximately 25-fold less effective than AVP in stimulating the release of ACTH. This suggests that the response is not mediated by V_2 -receptors either. Recent data on the secretion of β -endorphin by rat anterior pituitary tissue *in vitro* are in agreement with this suggestion [18]. Therefore, it could be argued that both V_1 - and V_2 -type activities are required for full ACTH releasing potency. However, oxytocin a peptide with about 1/100 or less of the V_1 - or the V_2 -type activities of AVP [2, 7, 16, 21, 28] was only approximately 5–8-fold less potent than AVP, corroborating earlier findings [3,5]. Moreover, it appears that similar relative potencies for the ACTH releasing activity of AVP analogs are found in the presence or in the absence of oCRF-41 ([3, 5, 18], present study). Hence, it seems reasonable to suggest that the potentiation of oCRF-41 and the stimulation of ACTH release by AVP alone are mediated by the same type of receptor. This receptor seems to be distinct from classical V_1 - or V_2 -receptors, and is clearly not

identical to the adenylate-cyclase coupled binding sites that mediate the action of o-CRF-41 [15,29], Holmes, Antoni and Szentendrei, submitted).

Our data obtained *in vitro* are at variance with a number of recent *in vivo* observations showing that dPen-Tyr[Me]AVP as well as d[CH₂]₅Tyr[Me]AVP potentially antagonize the stimulation of ACTH secretion by a systemic injection of AVP [1, 17, 22, 25]. Since these observations supported earlier findings [8, 9, 23], it was usually concluded that the pituitary actions of AVP are mediated by V₁-receptors. However, the main site of action of AVP in such

cases is not necessarily at the pituitary gland. For instance, functional as well as morphological evidence suggests that AVP may influence the release of 41-residue CRF from the hypothalamus [14, 19, 26], either by a direct central nervous action, or through a secondary mechanism, e.g., the "stress" of a pressor response.

In summary, we have presented evidence that AVP receptors on pituitary corticotrope cells may represent a novel type of receptor. Studies with more specifically designed AVP analogs are warranted to investigate further the nature of the effect of AVP on pituitary ACTH secretion.

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OXYTOCIN RECEPTORS IN RAT ADENOHYPOPHYSIS: EVIDENCE FROM RADIOLIGAND BINDING STUDIES.

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ABSTRACT: The nature of purported receptors for oxytocin (OT) in the rat adenohypophysis was investigated by means of radioligand binding. Tritium-labeled oxytocin (^3H -OT) was used as the radioligand. Particulate fractions prepared from the adenohypophysis of estrogen-treated female rats bound ^3H -OT reversibly and with high affinity ($K_d = 1.5 \text{ nM}$). Scatchard plots indicated the presence of a single, low-capacity binding-site ($B_{\text{max}} = 33 \text{ fmol/mg protein}$). However, ligand displacement experiments suggested that ^3H -OT labels OT- as well as vasopressin-preferring sites in adenohypophysial membrane-suspensions. When the number of pituitary vasopressin receptors was reduced by adrenalectomy, it was revealed that the ligand-specificity of the high-affinity OT binding site is similar to that of classical OT receptors. The results show the presence of separate receptors for OT and vasopressin in the rat adenohypophysis, and hence reinforce the notion that OT may function as a hypophyseotrophic hormone.

Oxytocin (OT) is a hypothalamic neurohormone which, apart from its well-defined actions in peripheral tissues, stimulates the secretion of ACTH and PRL by a direct action on pituitary cells (1-5). The mechanism of action of OT in the pituitary gland is poorly characterized, for instance, it is not known whether OT activates pituitary-type vasopressin receptors (6-9), or acts via genuine uterine-type oxytocin receptors. The aim of the present work was to examine the nature of purported oxytocin receptors in the anterior pituitary lobe.

MATERIALS AND METHODS

Female Wistar rats (200-250g BW), bred in the Animal House of the Department of Human Anatomy, Oxford, were used throughout. It was recognized in the preliminary stage of the experiments that the treatment of the animals with estrogen (diethylstilbestrol, SIGMA Co, St Louis, MO) increased the amount of radiolabeled OT bound by adenohypophysial membrane-suspensions. Therefore, all animals were injected with 20 μg of estrogen dissolved in dimethylsulfoxide 24 and 48 h before decapitation. In some cases the rats were ovariectomized and/or adrenalectomized 5 days before the day of the experiment. There were no marked differences in the characteristics of the binding of radiolabeled OT to pituitary membranes prepared from estrogen-treated or ovariectomized and estrogen-treated rats, hence these results are reported together.

Anterior pituitary lobes and uteri were collected in ice-cold saline to be homogenized in 20 mM NaHCO_3 and 5 mM Na_2EDTA (10,11). The homogenates were spun at $600 \times g$ for 5 min at 4°C; the supernatant was centrifuged $2 \times 20 \text{ min}$ at $30,000 \times g$. The resulting pellets were resuspended in 50 mM TRIS-HCl, pH 7.4, containing 5 mM MgSO_4 , 100 mg/liter bacitracin (SIGMA) and 0.5 g/liter BSA (SIGMA No.7906) (7,11). The concentration of protein was 80-140 $\mu\text{g/tube}$.

The membrane suspensions were incubated in a volume of 250 μl at 22°C either in the presence of increasing concentrations of tritium-labelled OT (^3H -OT) or 8-arginine-vasopressin (^3H -AVP) both from Dupont U.K., SA 40 and 67 Ci/mmol, respectively, or with a constant concentration of radioligand, and increasing amounts of non-radioactive peptide analogues. These analogues were: OT, AVP (Bachem, Torrance, CA), 1-deamino-8-D-Arginine AVP (dDAVP, courtesy of Ferring AB, Mölndal, Sweden), hydroxy-4-threonine-7 glycine OT (OH-Thr⁴-Gly⁷-OT, courtesy of M. Manning,

Toledo, OH), 1-deamino-4-lysine AVP (courtesy of G. Tóth, Szeged, Hungary).

Bound and free hormone were separated by vacuum filtration over Whatman GF/C filters. The amount of radioactivity retained by the filters was measured by liquid scintillation counting at 45% efficiency. Non-specific binding, the amount of radioactivity attached to the filters when the incubation buffer contained 1 μM OT, was 30-40% of total bound radioactivity when 2.5 nM of ^3H -OT was used. Binding displacement curves were linearized by LOGIT-log transformation, and inhibitory constants (K_i) were calculated according to Cheng and Prusoff (12).

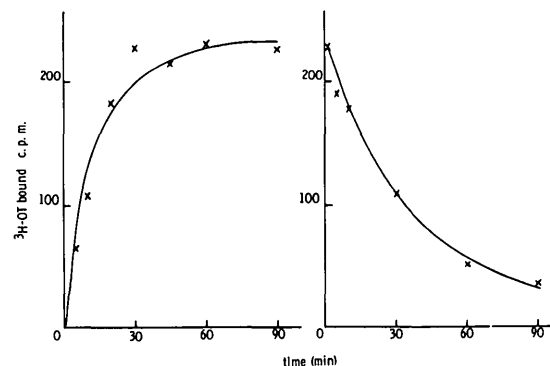


Fig 1. The time-course of association (left panel) and dissociation (right panel) of ^3H -OT (2.5 nM) in adenohypophysial membrane-suspensions. Means of duplicate measurements.

RESULTS

The association of ^3H -OT to pituitary membrane suspensions reached a steady state by 60 min (Fig 1). Virtually complete dissociation of the radioligand was observed upon addition of 5 ml of assay buffer containing 1 μM OT (Fig. 1). Estimation of the "on"- and "off"-rates of the reaction (13) yielded a dissociation constant (K_d) of 1.4 nM. In the steady state (60 min) the specific component of the binding of ^3H -OT was saturable (Fig. 2) and Scatchard plots suggested the presence of a single site with $K_d = 1.5 \pm 0.1 \text{ nM}$ and $B_{\text{max}} = 33 \pm 4 \text{ fmol/mg protein}$ ($n = 3$, mean $\pm$ SEM).

When the specificity of the binding reaction was examined, OT and AVP appeared equipotent (Fig 3A), with a K_i of 1.4 and 1.5 nM, respectively, while the selective antidiuretic agonist dDAVP was 30-times less

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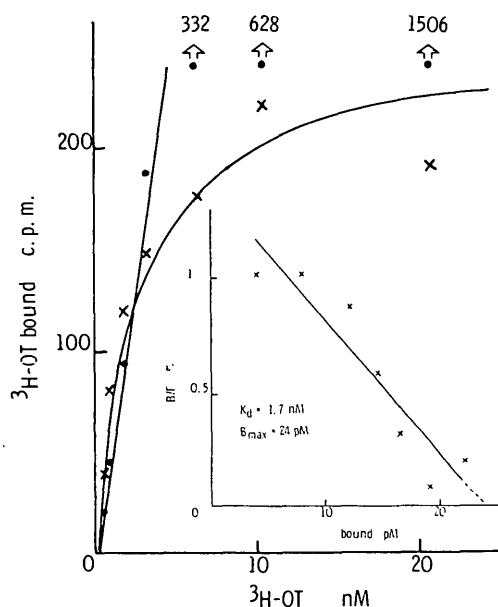


Fig 2. The specific (x) and non-specific (o) components of the binding of ^3H -OT (2.5 nM) to adeno-hypophyseal membrane fraction. Means of duplicates from a representative experiment. Inset: Scatchard-plot of the data, K_d 1.7 nM, $B_{\max}$ 39 fmol/mg protein.

potent than the former compounds (data not shown). The selective oxytocin agonist, OH-Thr⁴Gly⁷OT, appeared slightly more potent than OT, but did not displace ^3H -OT completely (Fig. 3A): in 3 experiments the maximal displacement was 37±3% (mean±SEM). In the same concentration-range, OH-Thr⁴Gly⁷OT failed to influence the binding of ^3H -AVP to pituitary membranes (Fig. 3B), but was, as expected, the most potent displacer of ^3H -OT in rat uterus membranes (Fig. 3C). The peptide dLys⁴AVP appeared to provide the mirror-image of OH-Thr⁴Gly⁷OT: it displaced only about 30% of the ^3H -OT bound by pituitary membrane suspensions (Fig. 3A). It was almost as potent as AVP on the pituitary AVP receptor (Fig. 3B) and proved to be a low-affinity ligand for uterine OT receptors

(K_i = 0.5 μM , Fig. 3C). Put together, the data suggested that ^3H -OT interacts with two types of receptor in rat adeno-hypophysis: a uterine-type OT receptor and the pituitary AVP receptor.

This hypothesis was tested by examining the ligand profile of ^3H -OT binding in pituitary membranes obtained from adrenalectomized, ovariectomized and estrogen-treated rats. Adrenalectomy reduced the binding of ^3H -AVP by 85% (data not shown, see ref. 10). After adrenalectomy (Fig. 4), the relative potencies of the analogues tested were the same in the pituitary membrane-suspension as on the uterine OT receptor, and, furthermore, OH-Thr⁴Gly⁷OT completely displaced ^3H -OT.

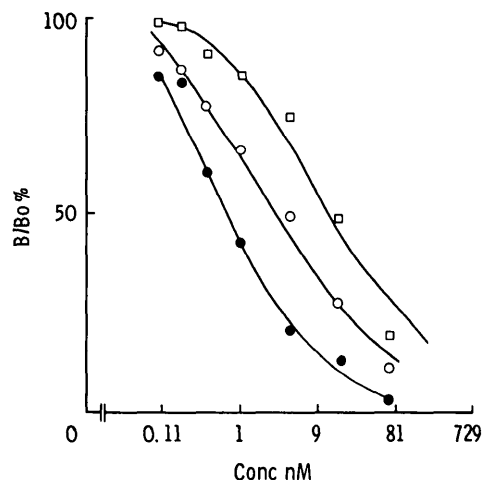


Fig 4. Displacement of ^3H -OT (2.5 nM) by peptide analogues in pituitary membrane-suspensions obtained from ovariectomized, adrenalectomized and estrogen-treated rats. Symbols as in Fig 3. Means of duplicates.

DISCUSSION

The concentration of OT in hypophyseal portal blood is in the nanomolar range (14,15), which is sufficient to stimulate the secretion of ACTH and PRL by pituitary tissue *in vitro* (1-5). The results pre-

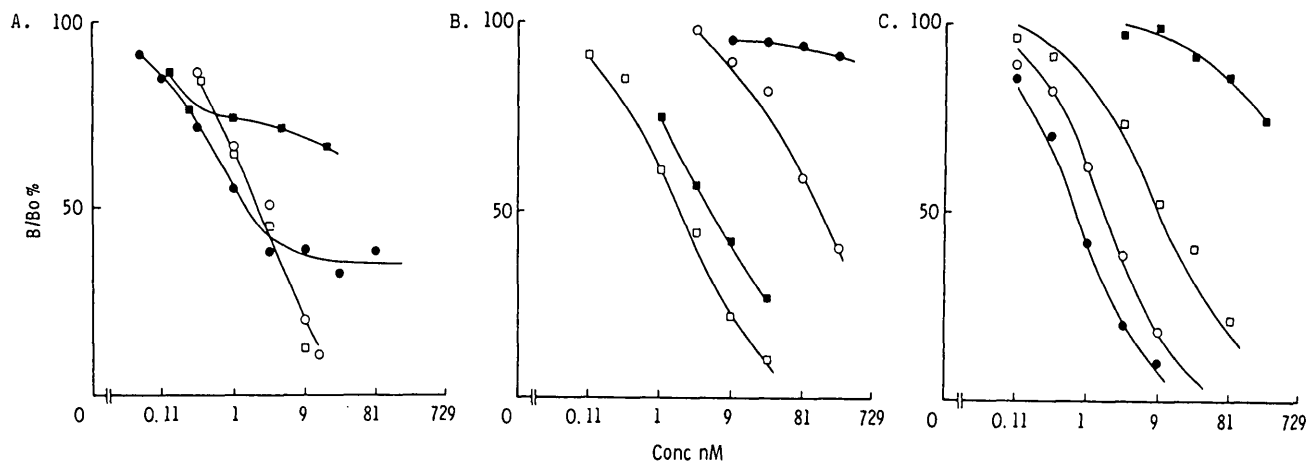


Fig 3. Displacement of ^3H -OT (2.5 nM) by OH-Thr⁴Gly⁷OT (●), OT (○), AVP (□) or dLys⁴AVP (■) in adeno-hypophyseal (A) and uterine (C) membrane fractions obtained from estrogen-treated rats. Panel B shows the displacement of ^3H -AVP (1.5 nM) by the same analogues in adeno-hypophyseal membrane fractions from estrogen-treated rats. Means of duplicates from representatives of at least 3 similar experiments.

sented here suggest that the hypophysiotrophic actions of OT may be mediated by typical OT receptors, which have been previously characterized in the uterus and the mammary gland (11).

In pituitary membrane-suspensions the major technical difficulty was to characterize the binding of ^3H -OT to the high-affinity site, in the face of distorting interference from pituitary-type AVP receptors. These bind OT with a K_d of cca. 100 nM (7) and are about 5 times more abundant in rat adeno-hypophysis than OT receptors (Antoni, unpublished). Hence the experimental approach: i) pretreatment of rats with estrogen, which increased OT receptor activity, (it could not, however, be established with certainty whether receptor affinity as well as capacity were increased, unpublished data); ii) using relatively low radioligand concentrations; and iii) adrenalectomy, which selectively down-regulated AVP receptors, was chosen to minimize the interference from AVP receptors. Despite these maneuvers, it is likely that the K_d of the OT receptor is overestimated. Because of the low ratio of specific to non-specific binding, and limitations on the amount of tissue that could be used, the "single-site" indicated by the Scatchard-analysis is in all probability comprised of two components - a high affinity OT receptor and the pituitary AVP receptor.

Nevertheless, the data clearly show that the adeno-hypophysis of the estrogen-treated female rat contains high-affinity sites which bind ^3H -OT. The ligand-specificity of these sites closely resembles that of classical OT receptors.

Lactotroph and corticotroph cells are the strongest candidates for cells which may express the OT receptor. The modulation by estrogen favors the involvement of lactotrophs which respond to OT, but not to AVP, by increased PRL release (5). Furthermore, a preliminary study has reported that immunoneutralisation of OT attenuated the hypersecretion of PRL induced by treatment with estrogen or suckling (16). It should be noted, however, that gonadal steroids may also modulate the corticotroph response to the cocktail of hypothalamic ACTH-releasing peptides (CRF-41, OT, AVP etc.) found in median eminence extracts (17). Immunoneutralization of OT *in vivo* reduces the ACTH response to some, but not all, types of stress (18), suggesting the involvement of OT in the regulation of ACTH release. It remains to be established whether or not these effects are mediated through the OT receptor described in the present study. Finally, OT levels in pituitary portal blood are highest on the afternoon of proestrus (19), a period which is preceded by a prolonged elevation of the concentration of estrogens in blood. It is therefore attractive to speculate that pituitary OT receptor activity may be increased just in time to mediate the effects of the surge of hypothalamic OT release.

In summary, the presence of specific, high-affinity receptors for OT in the adeno-hypophysis further strengthens the notion that OT may function as a hypophysiotrophic hormone.

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Characterization of oxytocin receptors in rat adenohypophysis using a radioiodinated receptor antagonist peptide

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ABSTRACT

Oxytocin may function as a hypothalamic releasing hormone for prolactin and ACTH secretion in the rat. In the present study we have investigated the properties of putative oxytocin receptors in the rat adenohypophysis by radioligand-binding assay.

A novel oxytocin receptor antagonist [1-(β -mercapto- β , β -cyclopentamethylene propionic acid),2-(ortho-methyl)-Tyr²-Thr⁴-Orn⁸-Tyr⁹-NH₂]-vasotocin (OTA) was radioiodinated by the iodogen method to a specific activity of 0.6 nCi/fmol. The radioiodinated derivative ¹²⁵I-labelled OTA (¹²⁵I-OTA) was reacted with membrane suspensions prepared from the uterus or adenohypophysis of female rats which were (a) ovariectomized for 7 days, (b) ovariectomized and treated with 5 μ g oestradiol-17 β 48 h before death or (c) implanted with a piece of silicone elastomer tubing containing 50 mg diethylstilboestrol (DES) 5 days before death. In uterine as well as the pituitary membrane suspensions, the radioligand was bound reversibly and with high affinity (dissociation constants 0.2 ± 0.1 and 0.1 ± 0.01 nmol/l respectively; means $\pm$ S.E.M., $n=3$) to a single class of sites with limited binding capacity, which varied with the type of

pretreatment. Oestradiol-17 β increased the binding capacity fivefold in the uterus in ovariectomized rats, but only very low specific radioligand binding was found in pituitary preparations from the same animals. Treatment with DES markedly increased the number of receptors in both the uterus and the adenohypophysis. Studies with several agonist and antagonist analogues revealed no difference in the ligand specificity of the uterine and adenohypophysial sites binding ¹²⁵I-OTA, indicating that they are the same species of receptor. Furthermore, ligand-binding studies, carried out with tritiated vasopressin as tracer in pituitary membrane preparations, showed that OTA is not bound by pituitary vasopressin receptors at concentrations below 1 μ mol/l.

In summary, ¹²⁵I-OTA is a highly specific radio-ligand suitable for the analysis of pituitary oxytocin receptors. The low number of oxytocin receptors in the adenohypophysis of rats treated with oestradiol-17 β suggests that the prolactin-stimulating action of oxytocin is mediated by highly efficient transmembrane signalling.

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INTRODUCTION

Substantial evidence indicates that in the rat, oxytocin may function as a hypothalamic releasing hormone for prolactin and corticotrophin (ACTH) (Gibbs, 1986; Samson, Lumpkin & McCann, 1986; Antoni, Kovács, Dohanics *et al.* 1988; Johnston & Negro-Vilar, 1988). Stimulation of prolactin release by oxytocin has also been demonstrated in man (Coiro, Gnudi, Volpi *et al.* 1987), suggesting that such a functional role of oxytocin may not be restricted to rats.

Similar to other peptide hormones, oxytocin exerts its actions by binding to cell-surface receptors on tar-

get cells. Specific, high-affinity receptors for oxytocin have been identified in several tissues including the anterior pituitary gland (Soloff, 1979; de Kloet, Voorhuis & Elands, 1985; van Leeuwen, van Heerikhuizen, van der Meulen & Wolters, 1985; Antoni, 1986a). The study of pituitary oxytocin receptors is tedious because of the limitations imposed by the low number of receptors present and the relatively low specific activity of the radioligand, tritium-labelled oxytocin. Furthermore, tritiated oxytocin binds to vasopressin as well as oxytocin receptors in the adenohypophysis, making it necessary to deplete vasopressin receptors before any analysis of genuine oxytocin receptors can be performed (Antoni, 1986a).

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These problems may be overcome by the use of radioligands having higher specific activity as well as greater pharmacological selectivity.

In the present study, we report the pharmacological characterization of adeno-hypophysial oxytocin receptors using a novel, highly selective radioiodinated oxytocin receptor antagonist peptide (Elands, Barberis, Jard *et al.* 1988).

MATERIALS AND METHODS

Animals

Female Wistar rats (200–300 g body weight; Harlan Olac, Bicester, Oxon, U.K.) were used. Under pentobarbitone anaesthesia (40 mg/kg, i.p.) the animals received s.c. implants of diethylstilboestrol (50 mg; Sigma Chemical Co., Poole, Dorset, U.K.) in Silastic tubing (Dow Corning, Midland, MI, U.S.A.; outer diameter 5 mm, inner diameter 4 mm) 5–6 days before decapitation. This treatment markedly increased the amount of radiolabelled oxytocin bound by adeno-hypophysial membrane suspensions (S. E. Chadio & F. A. Antoni, unpublished data). Other groups of rats were bilaterally ovariectomized under anaesthesia, injected s.c. with 5 µg oestradiol-17β (Sigma) 7 days afterwards and killed 48 h later.

Preparation of membrane suspensions for receptor-binding assay

Anterior pituitary lobes and uteri were removed and placed in ice-cold saline. Uteri were cut into small pieces and homogenized in NaHCO₃ (20 mmol/l) containing EDTA (5 mmol/l) for 30 s with an Ultra-Turrax homogenizer (IKA-Sartorius Ltd, Belmont, Surrey, U.K.). The homogenate was spun at 600 g for 5 min at 4 °C and the supernatant filtered through a Whatman no. 1 filter paper (Maidstone, Kent, U.K.) whilst the pellet was rehomogenized and centrifuged again at 600 g. This procedure was repeated three times and the supernatants were pooled. Anterior pituitary lobes were homogenized in a Teflon-glass homogenizer and the homogenate was spun at 600 g for 5 min at 4 °C. The supernatant fractions of uterus and adeno-hypophysis were finally centrifuged twice for 30 min at 30 000 g at 4 °C. The resulting pellets were resuspended in Tris-HCl buffer (50 mmol/l, pH 7.4) containing MgSO₄ (3 mmol/l), and protein content was determined according to the method of Bradford (1976).

Analogues of oxytocin

The oxytocin agonist peptides used were: oxytocin, arginine vasopressin (AVP) (Ferring A.B., Malmö,

Sweden), [Thr⁴-Gly⁷]-oxytocin (TG-oxytocin) (provided by Professor M. Manning, Medical College of Ohio, Toledo, OH, U.S.A.) and [Ala⁷]-oxytocin (A-oxytocin) (provided by Professor V. Hruby, University of Arizona, Tucson, AZ, U.S.A.). The antagonists used were: [1-(β-mercapto-β,β-cyclopentamethylene propionic acid),2-(ortho-methyl)-Tyr²-Thr⁴-Orn⁸-Tyr-NH₂]-vasotocin (OTA) (provided by Professor M. Manning) and [1-penicillamine,2-(para-methyl)-Phe²-Thr⁴-Orn⁸]-oxytocin (PPT-oxytocin) (provided by Professor V. Hruby).

Radioiodination of oxytocin antagonist

Equimolar (0.25 nmol) amounts of OTA and Na¹²⁵I (IMS-30; Amersham International, Amersham, Bucks, U.K.) were reacted for 15 min at room temperature in 40 µl sodium phosphate buffer (0.5 mol/l, pH 6.0) in a polypropylene vial previously coated with 10 µg Iodogen (Pierce Eurochemie, Oud-Beijerland, The Netherlands) at its bottom (Salacinski, McLean, Sykes *et al.* 1981). The reaction was stopped by the addition of 1 ml 0.1% (v/v) trifluoroacetic acid (TFA), and the mixture was injected through a Sep-Pak octadecasil silica cartridge (Waters, Harrow, Middx, U.K.). The cartridge was washed with 8 ml 0.1% TFA and developed with 2 ml each of a 10% stepwise gradient of acetonitrile (HPLC grade; BDH, Atherstone, Warwicks, U.K.) in 0.1% TFA (10–80%, v/v). Typically 50–60% of the radioactivity appeared in the void volume (i.e. in the TFA washes), and a peak containing 30–40% of the activity eluted at 40 and 50% acetonitrile. Both fractions could be used in the binding assay. The specific activity of the tracer was estimated by measuring the radioactivity eluting at 40 and 50% acetonitrile (peptide recovery was assumed to be 100%) as well as by autodisplacement (Morris, 1976) using the uterine membrane suspension. Values ranged between 0.6 and 0.7 nCi/fmol; all calculations were performed using an initial specific activity of 0.6 nCi/fmol. In some experiments, tritium-labelled AVP (New England Nuclear, Boston, MA, U.S.A.; sp. act. 67 Ci/mmol) was also used.

Receptor-binding assay

Aliquots of membrane suspension (15 µg protein for uterus, 30 µg protein for pituitary membranes) were incubated in 250 µl Tris-HCl containing MgSO₄ (3 mmol/l), 0.1% (w/v) bovine serum albumin (Sigma) and 0.01% (w/v) bacitracin (Sigma) at 22 °C. For analysis by the method of Scatchard (1949) ¹²⁵I-labelled OTA (¹²⁵I-OTA; 0.05 nmol/l) was used, and increasing concentrations of OTA were added to achieve saturation of the binding sites. In ligand-displacement studies, varying concentrations of unlabelled peptides were added to 0.08 nmol ¹²⁵I-

OTA/l. When [^3H]AVP was used as radioligand in displacement studies, 0.8 nmol tracer/l was used.

After incubation for 60 min, bound and free ligand were separated by filtration over GF/C filters (Whatman) under reduced pressure (Antoni, 1984). The amount of radioactivity attached to the filters was measured in a gamma counter at 70% efficiency. Non-specific binding was determined in the presence of 1 μmol oxytocin antagonist/l, and was typically 10 (uterus) and 30% (pituitary) of the total amount of radioactivity bound. Binding-displacement curves were linearized by log-Logit transformation, and inhibitory constants were calculated using the concentration of peptide giving 50% displacement (Cheng & Prusoff, 1973).

RESULTS

Characterization of the binding reaction in pituitary and uterine membrane suspensions

The binding of ^{125}I -OTA to the pituitary membrane suspension was time dependent, reaching apparent steady-state at 60 min (Fig. 1). Dilution of the tracer at 60 min by adding 3 ml assay buffer containing 1 μmol OTA antagonist/l decreased the amount of radioactivity specifically bound. All subsequent incubations were terminated at 60 min.

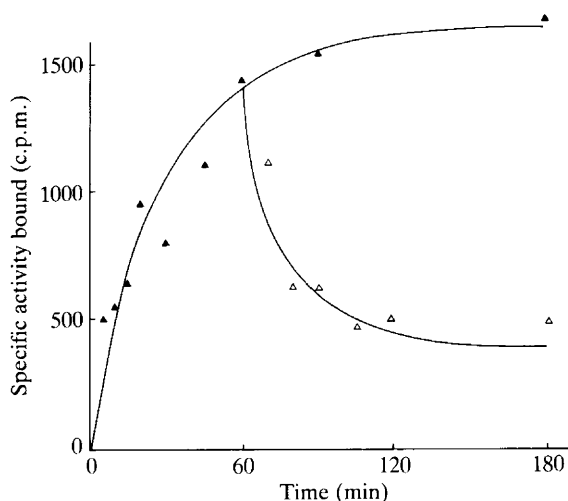


FIGURE 1. Time-course of the association (▲) and dissociation (△) of ^{125}I -labelled [1-(β -mercapto- β , β -cyclopentamethylene propionic acid),2-(ortho-methyl)-Tyr ^{2} -Thr ^{4} -Orn ^{8} -Tyr ^{9} -NH $_2$]-vasotocin (OTA) in adenohipophysial membrane suspensions prepared from oestrogen-pretreated rats. Dissociation was induced by adding 3 ml assay buffer containing 1 μmol OTA/l. The points are means of triplicate measurements.

Divalent cations of Mg, Mn, Co or Ca did not modify the binding of ^{125}I -OTA to the particulate fraction; however, Mg^{2+} was included in the assay buffer in all experiments since it was required for the high-affinity binding of agonists.

The specific binding of ^{125}I -OTA to pituitary membranes was saturable (Fig. 2). Scatchard plots of the binding isotherms were linear, indicating the presence of a single class of sites with high affinity (dissociation constant (K_D) 0.1 ± 0.01 nmol/l; mean $\pm$ S.E.M., $n=3$) and low capacity (maximum binding capacity (B_{max}) 48 ± 2 fmol/mg protein; $n=3$) (Fig. 2, insert). The findings with uterine membrane suspensions were analogous in all respects, except that the K_D was 0.2 ± 0.1 nmol/l ($n=3$) and the capacity was much higher (B_{max} 1453 ± 22 fmol/mg protein).

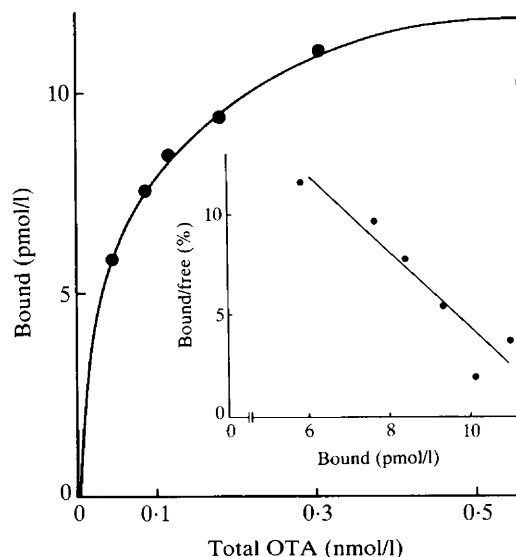


FIGURE 2. The specific component of the binding of ^{125}I -labelled [1-(β , β -cyclopentamethylene propionic acid),2-(ortho-methyl)-Tyr ^{2} -Thr ^{4} -Orn ^{8} -Tyr-NH $_2$]-vasotocin (OTA) to the adenohipophysial membrane suspension prepared from oestrogen-pretreated rats. Increasing amounts of OTA were added to tubes containing a constant amount of radiolabel (0.08 nmol/l). Non-specific binding was 30% of total bound when only tracer was present. Points are means of duplicate measurements from a representative experiment. The insert shows the Scatchard plot of the dose-dependent binding curves.

Comparison of the ligand profiles of uterine and pituitary sites labelled with ^{125}I -OTA

A summary of the results of displacement studies is presented in Table 1. In pituitary membranes, oxytocin and the selective oxytocin receptor agonist

TG-oxytocin were equipotent, while AVP and A-oxytocin were substantially less effective. The antagonist OTA proved to be the most potent ligand of all the compounds tested, while PPT-oxytocin was equipotent with oxytocin.

TABLE 1. Potency of oxytocin analogues to compete with ^{125}I -labelled [1-(β -mercapto- β , β -cyclopentamethylene propionic acid),2-(ortho-methyl)-Tyr²-Thr⁴-Orn⁸-Tyr⁹-NH₂]-vasotocin (OTA) binding to receptor sites in membrane suspensions prepared from adenohipophyses and uteri of female rats pretreated with oestradiol-17 β . The data are geometrical means with the retransformed S.E.M. in brackets; $n=3-5$ rats per group

Analogue	K_i (nmol/l)	
	Adenohipophysis	Uterus
Antagonists		
OTA	0.1 (1.09)	0.1 (1.1)
PPT-oxytocin	1.5 (1.1)	1.4 (1.2)
Agonists		
Oxytocin	0.8 (1.1)	1.2 (1.2)
TG-oxytocin	0.6 (1.3)	2.3 (1.2)
A-oxytocin	3.1 (1.6)	10.3 (1.3)
AVP	7.4 (1.6)	15.5 (1.2)

K_i , inhibitory constant; PPT-oxytocin, [1-penicillamine,2-(para-methyl)-Phe⁴-Thr⁸-Orn]-oxytocin; TG-oxytocin, [Thr⁷-Gly⁴]-oxytocin; A-oxytocin, [Ala⁷]-oxytocin; AVP, arginine vasopressin.

In uterine membranes, the rank order of relative potencies was the same as in pituitary membrane suspension: OTA > oxytocin $\geq$ TG-oxytocin $\geq$ PPT-oxytocin > A-oxytocin $\geq$ AVP. Moreover, the absolute values of the respective inhibitory constants were similar.

Finally, OTA had a very low affinity for pituitary receptors for vasopressin (Table 2).

Effect of low-dose oestrogen on binding sites for ^{125}I -OTA in the adenohipophysis and uterus

There was no appreciable specific binding of the radioligand by pituitary membranes prepared from randomly cyclic female rats or ovariectomized rats. In contrast, binding sites could be detected still in the uterus of ovariectomized rats: in two experiments K_D was 0.1 and 0.2 nmol/l and $B_{\max}$ 140 and 86 fmol/mg protein. Injection of 5 μg oestradiol-17 β in ovariectomized rats increased the concentration of binding sites in the uterus at 48 h ($B_{\max}$ 434 ± 20 fmol/mg protein, $n=3$), while the K_D was not altered. This type of

TABLE 2. Displacement of [^3H]arginine vasopressin (AVP) (0.8 nmol/l) by AVP and [1-(β -mercapto- β , β -cyclopentamethylene propionic acid),2-(ortho-methyl)-Tyr²-Thr⁴-Orn⁸-Tyr⁹-NH₂]-vasotocin (OTA) adenohipophysal membrane preparations. Data are means of duplicate measurements

Analogue	Concentration (nmol/l)	% Specific binding	IC ₅₀ (nmol/l)
AVP	0.33	97	7.4
	1	88	
	3	62	
	9	43	
	27	20	
	81	11	
OTA	11	93	> 1000
	33	98	
	100	95	
	300	99	
	1000	97	

IC₅₀, concentration producing 50% inhibition.

oestrogen treatment had no consistent effect on the specific binding of ^{125}I -OTA in the adenohipophysis, which was not sufficiently high to be characterized.

DISCUSSION

The results show that ^{125}I -OTA labels uterine-type oxytocin receptors in the adenohipophysis of rats exposed to a pharmacological dose of oestrogen.

The properties of the interaction of ^{125}I -OTA with uterine and pituitary membrane suspensions fulfil the classical receptor criteria of high affinity, reversibility and pharmacological specificity. With the latter, we found no marked difference between the ligand profile of the pituitary and uterine receptors labelled by ^{125}I -OTA, suggesting that the radioligand labels genuine oxytocin receptors in both types of membrane preparation. The high-affinity binding of the selective oxytocin agonist TG-oxytocin (Manning & Sawyer, 1985) in both systems is the most important finding in this regard. Moreover, the rank order of binding potencies of agonists is largely in agreement with their reported biological activity in the myometrium (Manning & Sawyer, 1985; Hruby, 1986).

An unusual property of the binding of ^{125}I -OTA is the lack of a requirement for divalent cations (e.g. Mg^{2+}), which is a prominent feature of the binding of tritiated oxytocin to its receptors (Pearlmutter & Soloff, 1979; Antoni, 1986a). In experiments reported elsewhere (Antoni & Chadio, 1989), we have studied this phenomenon in detail and found that similar to

some other receptor systems (Bird & Maguire, 1978; Williams, Mullikin & Lefkowitz, 1978), Mg^{2+} modulates the affinity of the oxytocin receptor for agonists, but not antagonists.

We also observed that OTA had a very low affinity for pituitary vasopressin receptors which is in keeping with previous observations (Antoni, 1987), showing that ligands with the bulky hydrocarbon ring attached to their N-terminal Cys residue have very low affinity for this type (V_3) of vasopressin receptor. Thus ^{125}I -OTA is a useful tool for the assay of oxytocin receptors in the anterior pituitary gland.

An interesting characteristic of various biological responses elicited by oxytocin, including the stimulation of prolactin secretion, is the modulation of the response by oestrogens, which is at least partly due to the regulation of the number of oxytocin receptors in target tissues (Fuchs, Periyasamy, Alexandrova & Soloff, 1983; Fahrbach, Morrell & Pfaff, 1985; de Kloet *et al.* 1985; Sheldrick & Flint, 1985).

The concentration of oxytocin receptors in the uterus as well as the adenohypophysis was markedly increased upon prolonged exposure to a pharmacological dose of synthetic oestrogen, confirming previous observations (Soloff, 1979; Antoni, 1986a). A treatment regimen with oestradiol-17 β , which is a closer approximation of the physiological situation, effectively increased oxytocin receptor concentration in the uterus, but was without detectable effect in the pituitary gland. It is, however, well documented that oxytocin stimulates prolactin secretion in rats given the same treatment with oestradiol (Samson *et al.* 1986). Thus it is more likely that we failed to detect oxytocin receptors in the anterior pituitary gland under these conditions because of the limitations of the binding assay. In particular, ^{125}I -OTA is a hydrophobic peptide, and increasing the concentration of membrane protein in the assay reduces the ratio of specific to non-specific binding quite dramatically, thus limiting the amount of membranes that can be used.

Furthermore, it is possible that upon exposure to oestradiol, oxytocin secretion from the hypothalamus increases and oxytocin receptors are down-regulated, whilst transmembrane signalling becomes amplified, and thus a substantial hormonal response is found despite the scarcity of cell-surface receptors for oxytocin. A similar situation exists in adrenalectomized rats where pituitary receptors for vasopressin and corticotrophin-releasing factor are virtually undetectable (Wynn, Hauger, Holmes *et al.* 1984; Antoni, 1987), the post-receptor inositol lipid and adenylate cyclase responses are reduced (Wynn *et al.* 1984; Todd & Lightman, 1987), yet the amount of ACTH secreted upon stimulation with vasopressin is actually enhanced (Antoni, 1986b). Further studies

are needed to clarify these issues for the case of oxytocin-stimulated prolactin release.

In summary, we show that ^{125}I -OTA is a highly specific radioligand for the detection of oxytocin receptors in the anterior pituitary gland.

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Specific oxytocin agonist stimulates prolactin release but has no effect on inositol phosphate accumulation in isolated rat anterior pituitary cells

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ABSTRACT

We have previously characterized specific oxytocin receptors in the rat anterior pituitary gland, using a highly selective oxytocin receptor antagonist as radioligand. The aim of the present study was to examine whether occupation of these receptors by oxytocin produces a stimulation of prolactin release and a rise in the accumulation of total inositol phosphates in the rat adenohypophysis. Anterior pituitary cells harvested from randomly cycling and diethylstilboestrol (100 µg s.c.)-treated rats were perfused with Dulbecco's minimal essential medium at a rate of 0.3 ml/min. Oxytocin and the specific oxytocin agonist [Thr⁴-Gly⁷]-oxytocin (TG-OT) both stimulated a significant prolactin release at concentrations of 10⁻⁶ and 10⁻⁷ M. Oestrogen treatment did not affect the response to oxytocin, indicating that there is no straightforward correlation between receptor number and prolactin secretory response in the rat pituitary gland.

The involvement of phosphoinositide hydrolysis

was investigated in dispersed anterior pituitary cells and uterine tissue from randomly cycling rats. Oxytocin and arginine-vasopressin stimulated a significant ($P < 0.05$) and dose-related increase in total inositol phosphates, vasopressin being more potent. The specific oxytocin agonist TG-OT had no effect on total inositol phosphate production in pituitary cells, but when tested in uterine tissue it significantly ($P < 0.05$) stimulated the accumulation of total inositol phosphate at all concentrations tested (10⁻⁵ to 10⁻⁹ M). In conclusion, the data show that oxytocin has prolactin-releasing activity, acting on specific receptors in the anterior pituitary gland. Furthermore, although oxytocin receptors in the rat uterus are coupled to the inositol phospholipid cycle, it would appear that this is not a prerequisite for the stimulation of prolactin secretion when specific oxytocin receptors in the rat adenohypophysis are activated.

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INTRODUCTION

Oxytocin is one of several neuropeptides which stimulate the secretion of prolactin by a direct action on anterior pituitary cells (Lumpkin *et al.* 1983; Abe *et al.* 1985; Horn *et al.* 1985; Samson *et al.* 1986; Schramme & Morrison, 1986). Oxytocin has been detected in rat hypophysial portal blood at levels which exceed those in peripheral blood by a factor of 50–100 (Gibbs, 1984), suggesting that it may contribute to the regulation of the release of anterior pituitary hormones. Oxytocin receptors in the rat adenohypophysis resemble uterine receptors with

respect to ligand pharmacology (Chadio & Antoni, 1989). However, the conditions for the oxytocinergic stimulation of adenohypophysial hormone secretion have not been fully defined. The formation of inositol phosphates (IPs) has been implicated in various target tissues as a mediator of the action of oxytocin (Marc *et al.* 1986; Schrey *et al.* 1986), but the mechanism of action of oxytocin in the anterior pituitary gland has not been investigated.

The objectives of this study were to investigate the prolactin-releasing activity of oxytocin *in vitro* and to examine whether oxytocin receptors in the rat anterior pituitary gland increase the production of IPs when activated by the hormone.

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MATERIALS AND METHODS

Animals

Female Wistar rats (200–300 g body weight) were purchased from Harlan Olac, Bicester, Oxon, U.K. For perfusion studies, two groups of rats were used: a) randomly cycling and b) injected s.c. with 100 µg diethylstilboestrol (Sigma Chemical Co., Poole, Dorset, U.K.) and killed 48 h later. For the measurements of formation of IPs randomly cycling rats were used.

Chemicals

Oxytocin and arginine-vasopressin were provided by Ferring A. B. (Malmö, Sweden). [Thr⁴-Gly⁷]-Oxytocin (TG-OT) and [1-(β-mercapto-β,β-cyclopentamethylene propionic acid),2-(ortho-methyl)-Tyr²-Thr⁴-Orn⁸-Tyr⁹-NH₂]-vasotocin (OTA) were

kindly provided by Prof. M. Manning (Medical College of Ohio, Toledo, OH, U.S.A.). Hepes, DNase I, lima bean trypsin inhibitor and other reagents were purchased from Sigma Chemical Co. and trypsin TRK from Lorne Laboratories (Reading, Berks, U.K.). Myo-[2-³H]inositol was from Amersham International plc (Amersham, Bucks, U.K.) and AG 1 X8 was obtained from Bio-Rad (Watford, Herts, U.K.).

Perfusion of isolated rat anterior pituitary cells

Isolated cells were prepared from adenohypophyses by modifying previously published procedures (Gillies & Lowry, 1978; Dobson & Brown, 1985). The anterior pituitary lobe was separated from the posterior lobe and minced with a razor blade to segments of approximately 0.5 mm diameter or

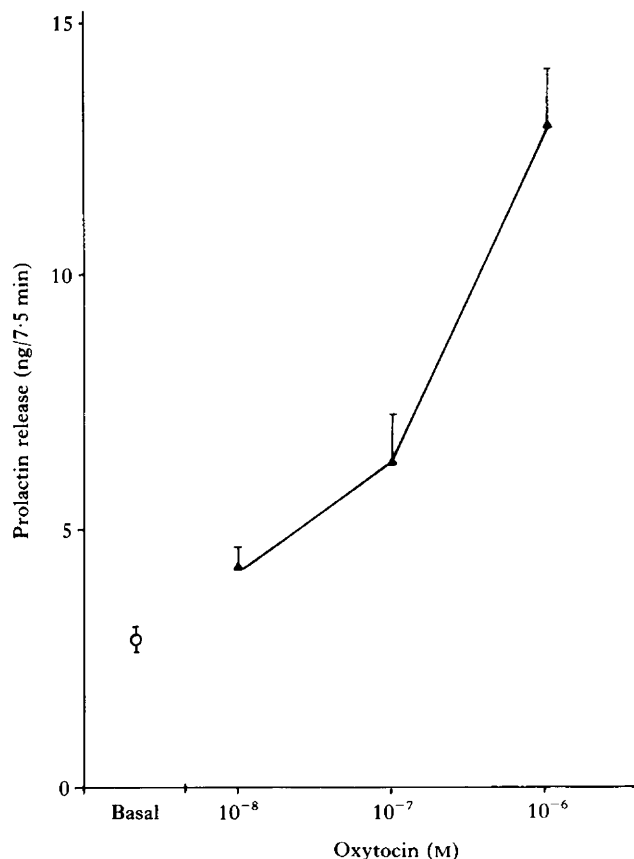


FIGURE 1. The release of prolactin by perfused anterior pituitary cells from randomly cycling rats in response to oxytocin. Four columns of cells were exposed to various concentrations of oxytocin for 2.5 min in a randomized complete block design. Data are means $\pm$ S.E.M. ($n=4$ /group).

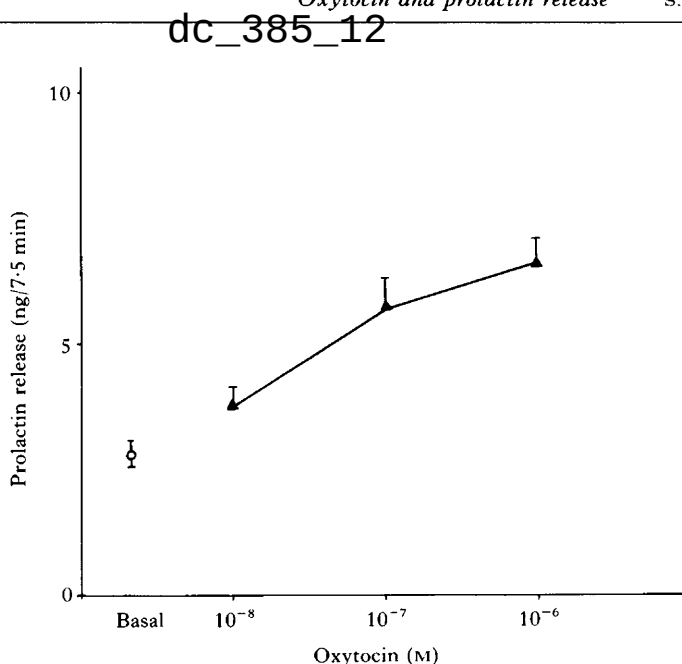


FIGURE 2. Effects of oxytocin on prolactin release by perfused anterior pituitary cells from animals treated with diethylstilboestrol. Values are means $\pm$ S.E.M. ($n = 4/\text{group}$).

smaller. The tissue fragments were placed in Dulbecco's minimal essential medium (DMEM; Gibco, Paisley, Strathclyde, U.K.), buffered with 22 mM Hepes at pH 7.4 containing 2.5 g trypsin/l, 2.5 g bovine serum albumin (BSA)/l (ICN Bio-medicals Ltd, High Wycombe, Bucks, U.K.), 1 mg DNase I/l (0.5 ml solution per gland) and incubated at 37 °C for 20 min. To facilitate dispersion, the tissue segments were aspirated into a 5 ml Pipetman tip (Gilson, Worthington, OH, U.S.A.) every 5 min, and after 20 min they were triturated with a plastic pipette (Sterilin, Hounslow, Middlesex, U.K.) equipped with a 1 ml Pipetman tip. The suspension was filtered over 100 μm nylon mesh and centrifuged at 100 g for 10 min at room temperature. The pellet was resuspended in 1 ml DMEM containing 2.5 g BSA/l, 1 mg DNase/l, 10^5 kallikrein units of aprotinin/l, and centrifuged again. The final pellet was resuspended in 1 ml DMEM containing 2.5 g BSA/l, mixed with preswollen Sephadex G-10 (Pharmacia, Milton Keynes, Bucks, U.K.) and loaded onto columns. Two pituitaries per column were used in experiments with randomly cycling rats and one pituitary per column for experiments with diethylstilboestrol-treated rats. The columns were perfused overnight at 37 °C, because a variable prolactin release in response to oxytocin in acutely dispersed cells had been observed. Similar findings have been reported by others (Luque *et al.* 1986).

The volume of the columns was adjusted to 0.5 ml and perfusion was started by a multichannel peristaltic pump at a rate of 0.3 ml/min. Test materials, dissolved in the perfusion medium, were applied to the system as 2.5-min pulses.

Fractions of the column effluent were collected at 2.5-min intervals and 50 μl aliquots of the effluent were assayed for prolactin, using the kit provided by the National Hormone and Pituitary Program of NIADDK (Bethesda, MD, U.S.A.). Results were expressed relative to rat PRL RP-3 reference preparation. Iodination of the rat prolactin was performed using the oxidant 1,3,4,6-tetrachloro-3 α ,6 α -diphenylglycouril (Iodogen; Pierce Eurochemie, Oud-Beijerland, The Netherlands) according to the method described by Salacinski *et al.* (1981). Equimolar (0.25 nmol) amounts of rat prolactin and Na¹²⁵I (IMS-30; Amersham International plc) were reacted for 15 min at room temperature in 40 μl sodium phosphate buffer (0.5 M, pH 7.5) in a polypropylene vial previously coated at its bottom with 10 μg Iodogen. The reaction was stopped by the addition of 0.5 ml phosphate buffer (0.5 M) and the reaction mixture was separated on a Sephadex G-25 M column (Pharmacia, Uppsala, Sweden) in 0.05 M sodium phosphate. Fractions (1 ml) were collected and counted on a gamma counter. The sensitivity of the assay was 0.05 ng/tube. The inter- and intra-assay coefficients of variation were 12.5 and 6.5% respectively.

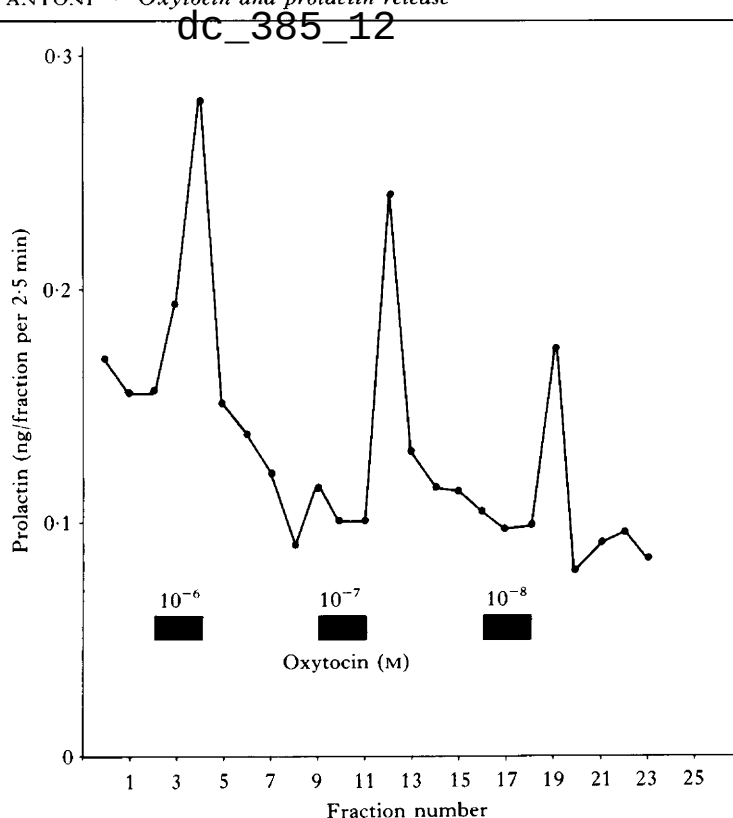


FIGURE 3. A representative experiment illustrating the stimulation of prolactin release by perfused anterior pituitary cells from diethylstilboestrol-treated rats in response to oxytocin. The solid boxes show the application of various concentrations of oxytocin.

Measurement of the accumulation of IPs

To study the effect of oxytocin on the hydrolysis of phosphoinositides, 5×10^5 dispersed anterior pituitary cells or myometrial strips, free of endometrium, (200 mg wet weight/ml) were incubated overnight in Krebs-Ringer medium supplemented with 2.5 mM Ca^{2+} , 0.2% (w/v) BSA, 11 mM glucose and 20 mM Hepes, pH 7.4 at 37°C , with 25 μCi myo- $[\text{}^3\text{H}]$ inositol. After being washed in radioisotope-free medium, samples of the cell or tissue slice suspension were incubated for 10 min in the presence of 10 mM LiCl, which has been reported to promote IP accumulation (Berridge *et al.* 1982). Peptides to be tested were subsequently added for up to 30 min at 37°C . The reaction was terminated by adding 200 μl ice-cold 15% (w/v) trichloroacetic acid. After rapid centrifugation of the acid extracts, the total IP content of the supernatant was determined as described by Berridge *et al.* (1983), using anion-exchange chromatography on Dowex AG 1 X8 columns. Results are expressed as percentages of stimulation compared with control.

Data analysis

The data are expressed as the means $\pm$ S.E.M. and analysed statistically by analysis of variance followed by Duncan's multiple range test, using the SPSS computer package (Version M, 1979). Differences where $P < 0.05$ were considered to be significant. Net release was defined as $(X + Y) - 2B$, where X is the 2.5-min fraction collected during the presence of stimulus (a 70-s transit-time sample was discarded), Y is the next 2.5-min fraction and B the basal value immediately before application of the stimulus.

RESULTS

Effects of oxytocin on prolactin release

The effects of increasing concentrations of oxytocin on prolactin release by perfused anterior pituitary cells obtained from randomly cycling rats are shown in Fig. 1. Oxytocin caused a concentration-dependent stimulation of prolactin secretion. In two independent experiments, oxytocin at 10^{-6} and

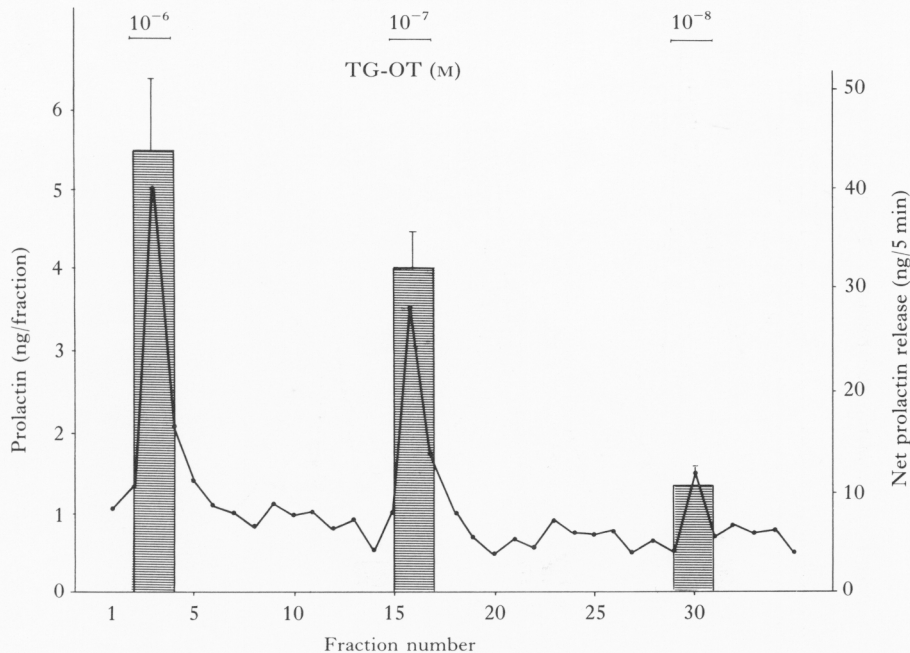


FIGURE 4. Effects of various concentrations of [Thr⁴-Gly⁷]-oxytocin (TG-OT) on prolactin release by perfused anterior pituitary cells. The solid line illustrates a representative experiment. The hatched bars show the mean net prolactin release in four separate columns. Values are means $\pm$ S.E.M. ($n=4$).

10^{-7} M significantly ($P<0.05$) stimulated prolactin release. Significant ($P<0.05$) stimulation of prolactin release by oxytocin was also observed in experiments with cells from animals treated with diethylstilboestrol (Fig. 2). When the data from the two experiments were analysed, a greater ($P<0.05$) response was observed at 10^{-6} M oxytocin in cells from untreated rats, while the mean response to oxytocin did not differ ($P<0.17$) between experiments with cells from untreated and diethylstilboestrol-treated rats, despite the well documented increase in pituitary oxytocin receptor number in the latter group (Chadio & Antoni, 1989). Figure 3 illustrates a representative example of the effects of oxytocin on prolactin release by perfused anterior pituitary cells from diethylstilboestrol-treated rats.

Effects of oxytocin analogues on prolactin release

In order to obtain further information about the specificity of the response and the nature of the receptor which mediates the action of oxytocin, the highly specific oxytocin agonist TG-OT (Lowbridge *et al.* 1977) and the selective oxytocin antagonist OTA (Elands *et al.* 1987) were used in experiments with cells from diethylstilboestrol-treated rats. The secretion of prolactin was stimulated by TG-OT in a

concentration-dependent manner. Significant effects were observed at 10^{-6} and 10^{-7} M TG-OT (Fig. 4). A 5-min pulse of oxytocin at 10^{-6} M released 3.14 ± 0.35 ng prolactin (mean $\pm$ S.E.M., $n=8$) and this was reduced to 0.26 ± 0.07 ng prolactin (mean $\pm$ S.E.M., $n=8$) in the presence of 10^{-6} M OTA.

Accumulation of IPs

Figure 5 illustrates the effects of oxytocin, vasopressin and TG-OT on the total accumulation of IP in dispersed anterior pituitary cells from randomly cycling female rats. Stimulation of anterior pituitary cells by increasing concentrations of vasopressin and oxytocin produced a dose-dependent increase in total IP production, vasopressin appearing to be considerably more potent. In contrast, TG-OT had no effect on IP accumulation in pituitary cells, but when tested in uterine slice suspensions it significantly ($P<0.05$) stimulated the production of total IP at all concentrations tested (Fig. 6).

DISCUSSION

This study demonstrates that oxytocin stimulates the release of prolactin from anterior pituitary cells by acting on oxytocin receptors. Furthermore, the activation of the inositol phospholipid cycle did not

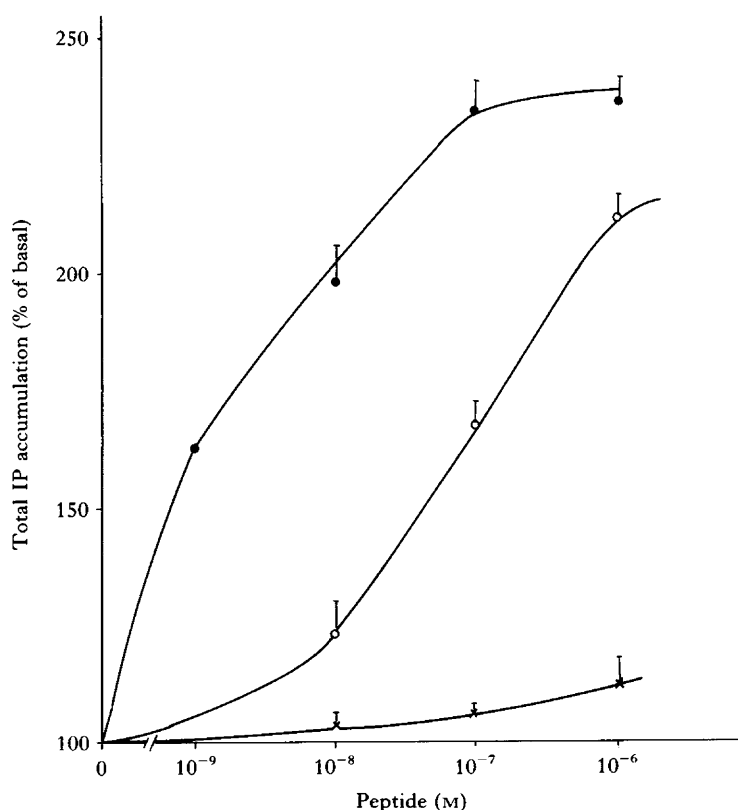


FIGURE 5. Dose-response curves for the accumulation of total inositol phosphate (IP) in anterior pituitary cells from randomly cycling rats after treatment with oxytocin (○), arginine-vasopressin (●) and [Thr⁴-Gly⁷]-oxytocin (×). Results are expressed as percentages of corresponding controls. Values are means $\pm$ S.E.M. ($n = 4-6$).

appear to be essential for the stimulation of prolactin release, since the specific agonist TG-OT increased prolactin secretion but had no effect on the accumulation of IPs.

It is well established that oestrogens increase the number of oxytocin receptors in the rat adenohypophysis (Chadio & Antoni, 1989). Prolactin secretion in response to oxytocin was not affected in cells harvested from diethylstilboestrol-treated animals in the present study, suggesting that processes downstream from receptor activation are also influenced by treatment with oestrogen. A dissociation between receptor regulation and cell responsiveness has been reported previously in lactotrophs (Platia *et al.* 1986). Treatment with oestrogen *in vitro* decreases the number of angiotensin II receptors without affecting the response of prolactin to angiotensin II. Moreover, in pituitary corticotrophs corticotrophin-releasing factor (CRF)-stimulated adrenocorticotrophin (ACTH) release is increased after adrenalectomy,

despite a marked decrease in pituitary CRF receptors (Wynn *et al.* 1985).

The mode of action of oxytocin in the pituitary gland has not been previously investigated. In the main target tissues, the uterine smooth cells and the mammalian myoepithelial cells, it has been shown that oxytocin enhances the breakdown of membrane polyphosphoinositides, leading to the production of IPs, which cause Ca²⁺ mobilization from intracellular stores (Marc *et al.* 1986; Schrey *et al.* 1986; Soloff *et al.* 1988). In the present study, oxytocin and vasopressin both enhanced the accumulation of IPs in dispersed anterior pituitary cells, vasopressin being more potent than oxytocin, in agreement with previous studies on pituitary vasopressin receptors (Antoni, 1984; Raymond *et al.* 1985; Todd & Lightman, 1987).

The anterior pituitary gland contains receptors for vasopressin which are coupled to the inositol phospholipid cycle and bind oxytocin. Vasopressin

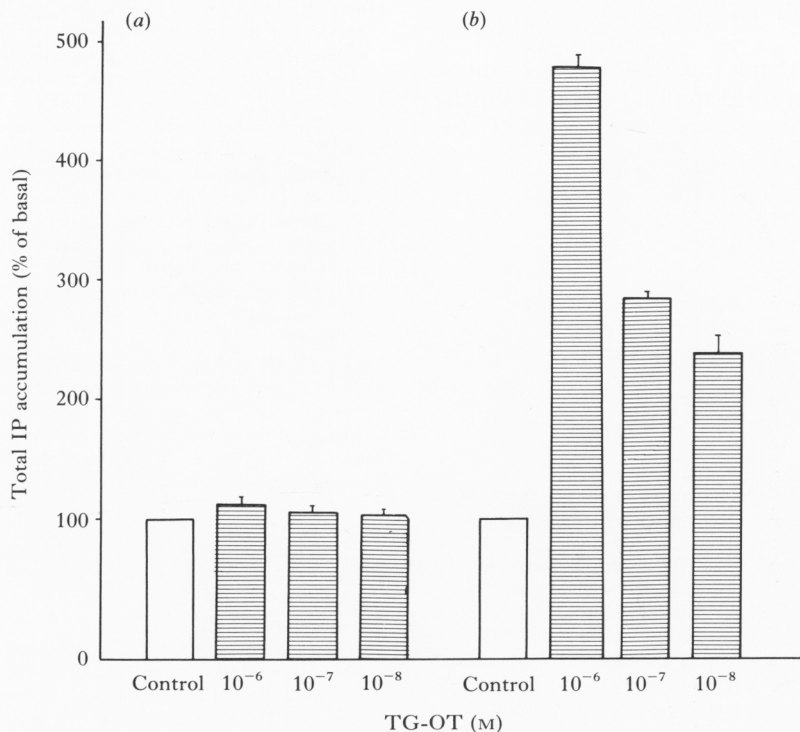


FIGURE 6. Dose-response plots for the accumulation of total inositol phosphate (IP) in rat (a) anterior pituitary cells and (b) uterine tissue preparations after treatment with the specific oxytocin agonist [Thr⁴-Gly⁷]-oxytocin (TG-OT). Values are means $\pm$ S.E.M. ($n=4-6$). In the rat uterus all concentrations tested (10^{-6} to 10^{-8} M) resulted in peak IP values significantly ($P<0.05$) different from the control, while in anterior pituitary cells no significant accumulation of the IPs was detected.

receptors are confined to corticotrophs (about 4–8% of the total number of hormone-producing pituitary cells) (Baker *et al.* 1970; Childs *et al.* 1982) and possibly thyrotrophs (2–6% of hormone-producing cells) (Childs, 1983; Dada *et al.* 1984). The higher potency of vasopressin with respect to the stimulation of IP accumulation suggests that oxytocin in fact acts through the pituitary vasopressin receptor to activate inositol lipid breakdown. This suggestion is further supported by the finding that the highly specific oxytocin agonist TG-OT did not affect the production of IPs at any of the concentrations used (10^{-9} to 10^{-5} M). As lactotrophs constitute about 50% of hormone-producing cells (Dada *et al.* 1984), and in view of the marked effect of vasopressin on the accumulation of IPs derived from a maximum of 14% of the cells, it seems unlikely that a change in pituitary accumulation of IPs evoked by TG-OT eluded detection because of the low proportion of cells responding. The same agonist peptide, when tested in uterine tissue preparations, produced a dose-

dependent increase in total IPs and was also active in stimulating pituitary prolactin release.

Taken together, the present data confirm previous findings that oxytocin has prolactin-releasing activity, although at relatively high concentrations. Whether or not oxytocin is physiologically important in regulating prolactin secretion at the pituitary level remains to be established. It is of note, however, that prolactin is regulated by multiple factors of hypothalamic origin which may interact synergistically to stimulate hormone release (Delaescalera & Weiner, 1992). A well-known example is the synergistic interaction of vasopressin and CRF (Antoni, 1986), in which case vasopressin alone produces little or no ACTH release at physiological concentrations, but is strongly stimulatory in the presence of CRF. Finally, it appears that while oxytocin receptors are coupled to the inositol phospholipid cycle in the rat uterus, this is not a prerequisite for the stimulation of prolactin secretion upon the activation of specific oxytocin receptors in the adenohypophysis.

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dent, electrogenic ion pump resolved in the path-clamp experiments¹⁶ is a H⁺ pump. The results support a chemi-osmotic mechanism as the basis of ion uptake by guard cells¹.

Proton pumping in plant cells is usually attributed to the activity of a plasmalemma ATPase¹⁷, with its activity reported to be inhibited by vanadate¹⁸. However, vanadate failed to affect the blue light-induced acidification, even at 2.5 mM (Table 1). Because this finding could imply that blue light-activated H⁺ extrusion is mechanistically different from other H⁺-pump systems previously described¹⁷, we tested the vanadate sensitivity of the fusicoccin-induced H⁺ extrusion, which is known to be mediated by a plasmalemma ATPase¹⁹. The high rate of H⁺ extrusion elicited by fusicoccin in guard-cell protoplasts was also not affected by vanadate (Table 1). Inability of guard-cell protoplasts to take up vanadate could explain the results¹⁸. On the other hand, blue light-induced acidification was abolished by diethylstilboestrol, a putative inhibitor of plasmalemma ATPase²⁰ (Table 1). Carbonyl cyanide-*m*-chlorophenylhydrazone (CCCP) also completely suppressed both the blue light- and fusicoccin-dependent acidification (Table 1). CCCP is a protonophore that dissipates H⁺ gradients; it can also suppress ATPase activity by reducing ATP levels in guard-cell protoplasts²¹. Although the results from the inhibitor experiments described above are in accord with the possible mediation of blue light-induced H⁺ extrusion by a plasmalemma ATPase, conclusive demonstration awaits further investigation.

The acidification response to blue-light pulses has further implications for the role of blue light in the activation of the H⁺ pump. The observed high rates of H⁺ release for minutes after pulse application argues against a direct energetic contribution by blue light for H⁺ extrusion. Furthermore, conservatively assuming a H⁺/ATP ratio of 2 (ref. 17), a calculation of the maximal efficiency of light quanta for H⁺ extrusion, estimated from the initial slope of the data shown in Fig. 1C, indicates that this blue light response is about 15-fold more efficient, based on a unit chlorophyll weight, than the ATP formation from non-cyclic photophosphorylation, measured in *Vicia* guard-cell chloroplasts¹⁰. Thus, the activated, blue light receptor probably functions as a modulator of H⁺-pumping activity, and not as a source of energy for the pump. Further characterization of sensory transduction in guard cells should increase our understanding of the photobiological and bioenergetic properties of the blue light response⁷.

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Magnocellular axons in passage through the median eminence release vasopressin

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Vasopressin (arginine vasopressin, AVP) is present in two types of nerve fibres in the median eminence (ME)¹⁻³. First, it is found in nerve terminals that originate in the parvicellular neurones of the hypothalamic paraventricular nucleus (PVN) and abut on the pericapillary space surrounding the fenestrated capillaries of the primary pituitary portal plexus in the external zone (EZ) of the ME. These neurones also synthesize corticotropin-releasing factor (CRF)⁴⁻⁶, which acts synergetically with vasopressin to stimulate release of adrenocorticotropin (ACTH) from the pituitary gland (see ref. 7). Second, vasopressinergic axons of the magnocellular neurosecretory system pass through the internal zone (IZ) of the ME to terminate in the neurohaemal contact zone of the neurohypophysis. The involvement of vasopressinergic magnocellular neurones in the control of ACTH secretion is much debated⁷. Of particular interest in this context is the origin of the vasopressin found in pituitary portal blood^{1,8,9}. Although it has been demonstrated that vasopressin and CRF are present in the same neurosecretory granules of EZ fibres³, parallel determinations of vasopressin and CRF in pituitary portal blood have shown alterations of the concentration of vasopressin without a concomitant change in that of CRF^{8,9}. Such a dissociation suggests that either differential release of vasopressin and CRF can occur from a single population of nerve endings, or there are fibres in the pituitary-stalk ME which release vasopressin but not CRF. Here we present evidence for the latter. Our results indicate that stimuli causing depolarization of the axonal membrane *in vitro* elicit release of vasopressin from nerve fibres in the external and internal zones of the ME.

We have reported that K⁺ ions and veratridine stimulate secretion of CRF and vasopressin from the ME *in vitro* in a dose-dependent manner, and that this process depends on extracellular Ca²⁺ (ref. 10). Immunocytochemical and radio-immunoassay studies have shown that the amount of CRF in the magnocellular fibre system of the ME is at least one order of magnitude lower than that in the EZ parvicellular fibres (see ref. 7). Hence, the release of CRF appears to be an appropriate marker for the secretory activity of the CRF/AVP nerve endings of the EZ; we have used this to analyse the possible compartments of vasopressin release by the ME *in vitro*, after impairing the function of the fibres in the EZ by various experimental manipulations.

First, rats were pretreated with reserpine, which activates the hypothalamo-pituitary-adrenocortical system by inducing hypersecretion of CRF¹¹. As a result, the peptide content of the CRF/AVP terminals in the EZ is dramatically decreased but there is no apparent change in the vasopressin content of the IZ magnocellular fibres^{12,13}. In our hands, pretreatment with reserpine decreased the CRF content of the ME by 64% but had no effect on the amounts of vasopressin in either the ME or the neurointermediate lobe of the pituitary (Fig. 1). The latter result suggests that the quantity of vasopressin in the EZ system is close to that of CRF, that is, small compared with the total ME vasopressin content, and hence minor changes of EZ

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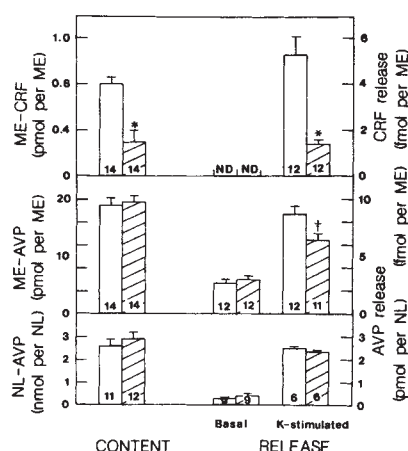


Fig. 1 Effects of pretreatment with reserpine on the content (left) and *in vitro* release (right) of vasopressin and CRF in ME or neurointermediate lobes of the pituitary gland (NL). Tissues were obtained from male Sprague-Dawley albino rats (200–300 g body weight) injected s.c. with either 10 mg per kg reserpine dissolved in dimethyl sulfoxide (DMSO) (hatched columns) or 2 ml per kg of the vehicle alone (open columns) 24 h before killing by decapitation. The columns represent the mean $\pm$ s.e.m., and the numbers of observations are shown at the bottom of each column. The piece of tissue referred to as median eminence also contained variable portions of the pituitary stalk but never any part of the neural lobe of the pituitary gland. The relative amounts of vasopressin released from the two pools of vasopressin in the ME may be estimated as follows: assuming that a proportionate amount of vasopressin is co-released with CRF from a population of parvocellular nerve endings³, the secretion of vasopressin from these fibres should decrease to the same extent as the release of CRF, that is, by 75%. Hence, 75% of AVP released from the CRF/AVP compartment is equivalent to the 44% decrease in the total K^+ -stimulated release of AVP (release in the presence of K^+ minus basal release) provided that reserpine does not affect the release of vasopressin from a second compartment that has no CRF. From this, the amount of vasopressin released by the CRF/AVP compartment in untreated rats can be estimated as 44/75, which is ~60% of the total release of vasopressin. The remaining 40% of the vasopressin secreted would originate from the reserpine-insensitive compartment.

Methods. The incubations were performed in 350 μ l of Krebs-Ringer-bicarbonate (KRBG) medium, containing 11.1 mM D-glucose 0.1 mM L-ascorbate and bovine serum albumin (1 g l⁻¹), at 37 °C in an atmosphere of O₂/CO₂ (95:5). After dissection from the brain, the median eminences (6–7 per tube) were preincubated for 30–45 min, after which the medium was carefully removed and changed every 10 min during the period of the experiment for any one of the following: fresh KRBG, KRBG with 48 mM K^+ , from which an appropriate amount of NaCl was omitted to maintain iso-osmotic conditions; KRBG with 10 μ M veratridine (supplied by Dr G. Brown, University of Alabama, Birmingham); KRBG containing 1 mM EGTA and no added calcium; KRBG containing 1 μ M tetrodotoxin. The concentrations of CRF and vasopressin in the incubation medium were determined by specific radioimmunoassays as described elsewhere¹⁰. After the incubation, the tissues were homogenized in 100 μ l of 0.1 M HCl containing 1 mM ascorbate, and after appropriate dilution in 0.05 M sodium phosphate buffer containing 0.1% Triton X-100 (v/v), the peptide content was determined by radioimmunoassay. * $P < 0.01$; † $P < 0.05$ by Student's *t*-test in comparison with the group treated with vehicle alone.

vasopressin content are unlikely to be detected. In parallel with the decrease of CRF content, the K^+ -stimulated release of CRF was reduced by 75% in rats treated with reserpine (Fig. 1). Pretreatment with reserpine also diminished the K^+ -evoked release of vasopressin from the ME, but to a lesser extent (44%) than that of CRF. In contrast, no change of the K^+ -induced release of vasopressin from neurointermediate lobes was found (Fig. 1). These findings are consistent with the suggestion that

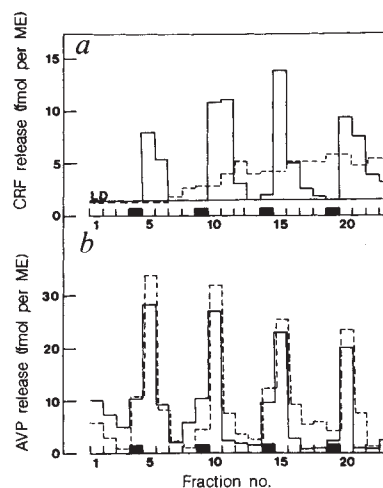


Fig. 2 Potassium-evoked release of CRF (a) and vasopressin (b) by median eminences obtained from sham-operated (solid line) or 7-day PVN-lesioned (broken line) rats. The solid bars indicate the application of KRBG containing 48 mM KCl. The abscissa shows the number of 10-min fractions collected. In a, the continuous line at the bottom of the figure, labelled LD, indicates the limit of detection in the radioimmunoassay. Data are representative of six pairs of similar experiments.

Methods. Lesioning of the PVN was performed in rats anaesthetized with chloral hydrate (4 g per kg body weight) using a specially designed microknife¹⁵. After killing by decapitation on post-operative day 7, the site of the lesion in each brain was examined in serial coronal sections (300–400 μ m thick)¹⁵ prepared during the period of preincubation of the median eminences in individual wells of a 24-well polystyrene tissue-culture plate. Subsequently, the tissues from sham-operated rats or those with complete lesions of the PVN were pooled (6–7 per tube) and incubated as described in Fig. 1 legend. The CRF content of each ME was measured after the incubation as an additional indicator of the completeness of the lesion.

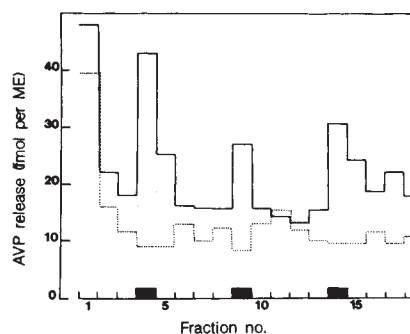


Fig. 3 Potassium-evoked release of vasopressin by median eminences obtained from sham-operated rats (solid line) or from animals subjected to anterolateral transection of the hypothalamus 7 days before the experiment (dotted line). Solid bars indicate the application of KRBG containing 48 mM KCl. The abscissa shows the number of 10-min fractions collected. The data are representative of three pairs of similar experiments. Three ME were incubated per tube as described in Fig 1 legend. Anterolateral cuts were made with a bayonet-shaped knife, in rats anaesthetized with chloral hydrate as described previously¹⁸. Each brain was examined to determine the site of the cut as described in Fig 2 legend.

secretion of vasopressin occurs from two compartments in the ME. One of these compartments also releases CRF and is depleted of peptides by reserpine, whereas the other is not affected by the drug and neither contains nor releases CRF. Neuroanatomical evidence^{1–7,12,13} indicates that the CRF/AVP fibre compartment is present in the EZ, whereas the compartment containing only vasopressin and which is not affected by reserpine is localized in the IZ of the ME.

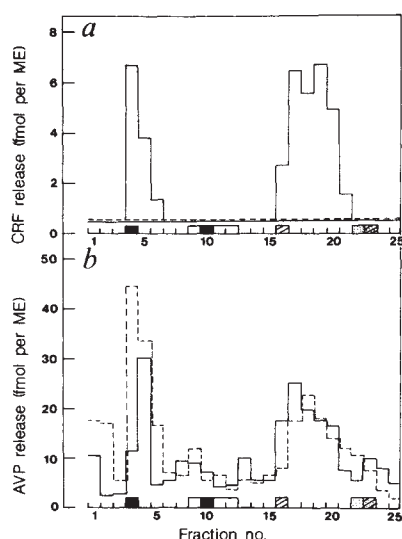


Fig. 4 Release of CRF (a) and vasopressin (b) *in vitro* by median eminences of PVN-lesioned (broken line) and sham-operated (solid line) rats. The abscissa indicates the number of 10-min fractions collected. Solid bars indicate the application of 48 mM KCl; open bars, Ca²⁺-free medium; hatched bars, 10 μM veratridine; stippled bars, 1 μM tetrodotoxin. Data are representative of four pairs of similar incubations.

To obtain further evidence for the release of vasopressin from magnocellular axons in the IZ, we examined the secretion of vasopressin and CRF by ME obtained from rats which had been subjected to surgical ablation of the PVN. It has been shown previously that such lesions decrease drastically the CRF content of the ME, as well as the numbers of the nerve fibres that are immunoreactive for CRF and vasopressin in the EZ (see, for example, refs 1, 7). In contrast, the magnocellular neurones of the supraoptic nucleus and the supraoptico-hypophyseal tract remain intact after PVN lesions. In agreement with these findings, we observed a marked decrease in the CRF content of the ME 7 days after hypothalamic surgery: from 848 ± 88 fmol in sham-operated controls to 75 ± 16 fmol in lesioned animals (mean $\pm$ s.e.m., $n = 11$ in both groups). The vasopressin content of the ME was also decreased, from 22.9 ± 2.9 pmol in controls to 12.0 ± 0.8 pmol in lesioned rats (mean $\pm$ s.e.m., $n = 10$ and 11, respectively). These results are consistent with the neuro-anatomical observation that ~40% of the vasopressinergic magnocellular neurones lie in the region destroyed by the PVN lesion¹⁴. There was no release of CRF in response to K⁺ in ME obtained from PVN-lesioned rats (Fig. 2). In contrast, K⁺ released substantial amounts of vasopressin from the same segments of tissue that showed no CRF response. In three series of experiments, the average amounts of vasopressin released by a 10-min exposure to high-K⁺ medium were similar in sham-operated and PVN-lesioned rats (15.0 ± 2.2 and 16.0 ± 3.0 fmol per ME; mean $\pm$ s.e.m., $n = 15$ and 13, respectively). This finding contrasts with the 40% decrease of vasopressin content mentioned above, and suggests that a compensatory increase of releasable vasopressin occurs in the ME after PVN lesions. The nature of this apparently compensatory response is not clear, however it was not inhibited by dexamethasone (250 μg per kg given to PVN-lesioned rats subcutaneously (s.c.) for 7 days; data not shown), making it unlikely that the build-up of vasopressin occurs in surviving cells of the parvocellular CRF/AVP system, which are strongly inhibited by glucocorticoid hormones^{1,15}. Moreover, to compensate for the loss of 90% of the CRF/AVP neurones, the cells spared by the lesion would have to increase their ability to secrete vasopressin by 900% in 7 days; this is also improbable, as in the absence of glucocorticoid feedback following bilateral adrenalectomy, it takes 3–4 weeks for ME vasopressin release to increase by

500%¹⁰. Moreover, PVN-lesioned rats have high concentrations of circulating corticosterone (~40% of controls)^{16,17}, and circadian rhythms of corticosterone are also apparent¹⁶.

To demonstrate that degenerating magnocellular fibres cannot be the source of the vasopressin release in response to depolarization, ME obtained from rats with anterolateral hypothalamic transections¹⁸ were tested for their ability to release the peptide (these lesions transect all vasopressinergic axons, including those in the supraoptico-hypophyseal tract, that project to the ME). Seven days after the lesion, the vasopressin content of the ME was decreased by ~90% (2 ± 0.6 pmol per ME, $n = 9$ compared with 18 ± 3 , $n = 9$ in controls; mean $\pm$ s.e.m.) and there was no detectable release of vasopressin in response to a high K⁺ concentration (Fig. 3).

The release of AVP from the ME of sham-operated or PVN-lesioned rats had similar properties (Fig. 4): the release evoked by K⁺ was calcium-dependent, and that elicited by veratridine was blocked by tetrodotoxin. The activity of the cytoplasmic enzyme lactate dehydrogenase¹⁹ (EC 1.1.1.27) was not above background (0.001 extinction units per 120 s) in high-K⁺ medium bathing ME from PVN-lesioned rats, but on addition of 0.1% Triton X-100, enzyme activity increased 14-fold, indicating leakage of the enzyme from the cytoplasm. Taken together, these data suggest that the release of vasopressin from the ME of PVN-lesioned rats occurs from magnocellular fibres of the supraoptico-hypophyseal tract in the IZ of the ME, via a physiologically relevant exocytotic process.

The magnocellular axons in passage through the IZ are varicose unmyelinated fibres with distinct swellings, called Herring bodies, in which mitochondria and neurosecretory vesicles containing immunoreactive vasopressin are abundant^{20,21}. It appears that the cellular machinery necessary for neurotransmitter release is present along the entire length of the axon, albeit at lower density than at nerve terminals (compare with refs 22, 23), and it is well documented that most of the vasopressin found in the ME is in the biologically active form (see refs 24, 25). Hence, a small but significant fraction of the peptide present in IZ fibres may be released in response to depolarizing stimuli, perhaps preferentially at Herring bodies. The released peptide could gain access to the pituitary portal circulation via the extensive network of fenestrated primary portal capillaries which penetrates the IZ^{20,21} and is not separated by any morphologically evident diffusion barrier from the fibres in passage^{21,22}. Because the amount of vasopressin in the IZ fibre system is about 10–20-fold higher than in the EZ, release from the IZ neurites may account for a substantial proportion (~40%; see Fig. 1 legend) of the vasopressin in pituitary portal blood.

Although we have no direct evidence that the release process described above operates *in vivo*, a considerable amount of functional data favours the existence of two independent sources of ME vasopressin secretion. It has been noted that the concentrations of vasopressin and CRF may vary independently in pituitary portal blood (see refs 8, 9). Furthermore, PVN lesions prevent the increase of plasma ACTH after adrenalectomy, a manoeuvre that stimulates parvocellular CRF/AVP neurones, but not, or only slightly, the magnocellular system^{1,25,26}. In contrast, PVN lesions merely attenuate the pituitary-adrenocortical response to stressful stimuli such as ether-venesection and ether-laparotomy^{16,17}, which are known to activate the parvocellular CRF/AVP system⁷ as well as the magnocellular vasopressinergic neurones^{8,27}. Anterolateral hypothalamic transections that destroy all vasopressinergic fibres (parvocellular as well as magnocellular) reaching the ME completely block the ACTH response to adrenalectomy or the stressful stimuli mentioned above^{7,17}.

Intriguingly, these functional findings are paralleled by studies on the release of vasopressin from the ME *in vitro*, in which lesions of the PVN abolished the enhancement by bilateral adrenalectomy of the depolarization-induced release of

vasopressin²⁶. Here we have reported that lesioning of the PVN in otherwise intact rats did not diminish substantially the release of vasopressin from the ME, whereas anterolateral cuts abolished the vasopressin response to depolarization.

Thus, our data suggest that the original, and now largely rejected, proposal that magnocellular neurones contribute to the regulation of anterior pituitary hormone secretion by releasing neurohormones in the ME²⁸, should be reconsidered with respect to the control of ACTH secretion.

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Excitatory amino acids inhibit stimulation of phosphatidylinositol metabolism by aminergic agonists in hippocampus

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Since the initial observations in the 1950s^{1,2} a large number of neurotransmitters and hormones have been shown to influence phosphatidylinositol (PI) metabolism in brain and peripheral ganglia (see ref. 3 for review). This has led to the suggestion that PI is part of an intracellular second messenger system for some types of diffusible chemical factors^{4,5}. Consistent with this are recent reports that one of the products of PI turnover (diacylglycerol) stimulates the Ca-dependent phospholipid-dependent protein kinase (kinase C) while a second (inositol trisphosphate) causes the release of calcium from intracellular stores^{6,7}. Thus it is possible that at least some brain neurotransmitters utilize the PI system to produce functional effects that are in addition to and which outlast the very brief physiological responses they elicit. Although it had been anticipated that another class of receptors might inhibit receptor-mediated stimulation of PI breakdown, no clear examples of such effects have been described. We now report that acidic amino acids, which are thought to be excitatory neurotransmitters at the majority of brain synapses⁸, strongly inhibit the stimulation of PI metabolism elicited by carbachol, histamine, or by potassium-induced depolarization, without changing the response to noradrenaline. As well as indicating a novel function for the excitatory amino acids, these results suggest that the central nervous system possesses cell-cell interactions of a previously unsuspected type.

The experiments were performed on transverse rat hippocampal slices prepared as described previously⁹. Following preincubation for 1 h at 33 °C in Krebs-bicarbonate medium under a constant stream of O₂/CO₂ (95:5), the slices were further preincubated for 1 h at 33 °C in the presence of ³H-*myo*-inositol (NEN, 15-20 Ci mmol⁻¹). The accumulation of ³H-inositol phosphate derivatives (³H-InsP) induced by various agonists was then determined in the presence of 5 mM lithium chloride as described by Brown *et al.*¹⁰ (see Fig. 1). The elution profile of the ³H-inositol phosphates obtained by stepwise addition of formate solutions of increasing strength indicated that more than 90% of these derivatives corresponded to *myo*-inositol-1-phosphate, a result in good agreement with that of Brown *et*

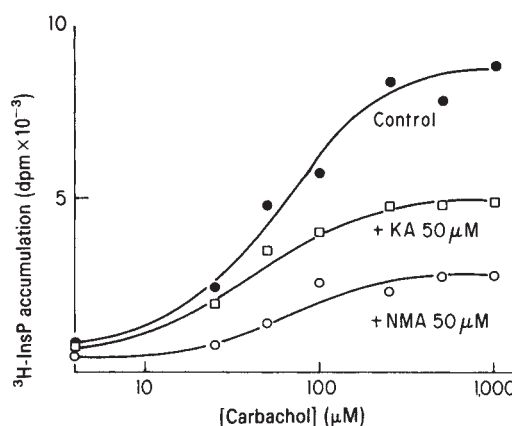


Fig. 1 Effects of *N*-methylaspartic acid (NMA) and kainic acid (KA) on basal and carbachol-stimulated accumulation of ³H-InsP in rat hippocampal slices. Transverse hippocampal slices (0.4 mm thick) were cut with a McIlwain tissue chopper and preincubated for 60 min at 33 °C in Krebs-Ringer bicarbonate medium containing (final concentration in mM): NaCl 124, KCl 3.33, Mg₂SO₄ 2.42, CaCl₂ 3.1, KH₂PO₄ 1.25, NaHCO₃ 25, D-glucose 10, urea 2 and ascorbic acid 2, under a constant stream of O₂/CO₂ (95:5). After three washes with freshly oxygenated medium, slices were further incubated for 60 min at 33 °C in the same medium containing ³H-*myo*-inositol (NEN, 15.8 Ci mmol⁻¹, 5 μCi ml⁻¹). Slices were washed again three times with freshly oxygenated medium and were distributed in incubation vials (two or three slices per vial) and incubated for 10 min at 33 °C in the presence of 5 mM LiCl and various concentrations of carbachol and excitatory amino acids (final volume 0.25 ml). At the end of the incubation 940 μl of a mixture of chloroform/methanol (1:2) were added, slices were sonicated briefly and 310 μl of chloroform and 310 μl of H₂O were added to separate the phases. Aliquots of the upper aqueous phase (0.75 ml) were transferred to a column containing ~175 mg of an anion-exchange resin (Bio-Rad AG 1X-8, 100-200 mesh, formate form). The columns were washed with 10 ml of H₂O to remove ³H-*myo*-inositol. Labelled ³H-inositol phosphate derivatives (³H-InsP) were eluted with 5 ml of 200 mM ammonium formate/100 mM formic acid and counted in a liquid scintillation counter, and are expressed as d.p.m. of ³H-InsP accumulated and represent means of 4-6 experiments with s.e.m. less than 10% of the means.

*al.*¹⁰. Figure 1 indicates that two different excitatory amino acids, *N*-methylaspartic acid (NMA) and kainic acid (KA) had a small although not statistically significant inhibitory effect on basal accumulation of ³H-InsP. As previously reported by various groups^{10,11}, carbachol caused a large and concentration-dependent stimulation of ³H-InsP accumulation. Both NMA (50 μM)

Predominant Release of Vasopressin vs. Corticotropin-Releasing Factor from the Isolated Median Eminence after Adrenalectomy

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Key Words. Corticotropin-releasing factor · Vasopressin · Median eminence · Adrenalectomy

Abstract. In an *in vitro* system, we have demonstrated concomitant release of corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) from the median eminence (ME) of normal and adrenalectomized rats. The ME were incubated in a Krebs-Ringer bicarbonate medium, CRF and AVP released into the medium were measured by radioimmunoassay. The release of both neuropeptides was stimulated by increasing concentrations of potassium (28–56 mM) in the incubation medium, or by addition of veratridine (5–20 μ M). In both cases the release process was dependent on the presence of calcium in the incubation medium. Interestingly, potassium-induced release was found to be relatively insensitive to calcium channel antagonists that are potent inhibitors in smooth and cardiac muscle. The sodium channel antagonist, tetrodotoxin (1 μ M), completely blocked the effect of veratridine, while no change was seen in the response to potassium. Adrenalectomy increased the ratio of AVP:CRF release from 2:1 in ME removed from sham-operated rats to 8:1 in ME from the adrenalectomized group. We suggest that this ratio of AVP:CRF release may also pertain *in vivo*, and that AVP could be the predominant corticotropic stimulus in adrenalectomized rats.

Corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) appear to be the major mediators of the hypothalamic control of anterior pituitary ACTH secretion [18, 26, 28]. In the hypothalamic paraventricular nucleus (PVN), these peptides are co-localized in parvocellular neurons that project to the external zone of the median eminence (ME) [13, 24, 27, 31]. Hence, AVP and CRF can be released from nerve terminals of the external zone and reach the anterior pituitary via the pituitary portal blood, in which both neurohormones are present at relatively high concentrations [6, 20].

The increase in plasma levels of ACTH following adrenalectomy (ADX) is known to depend upon hypothalamic stimuli originating from the PVN [17], but the extent to which CRF and AVP participate in the ACTH response to ADX is not clear. Immunoneutralization of CRF *in vivo* reduces the plasma ACTH levels in ADX rats [4, 21], but it has not been established that CRF release from the hypothalamus is indeed enhanced by ADX. The concentration of AVP in hypophyseal portal blood has been reported to be unaltered [10] or increased [15] in ADX rats. The release of

AVP from the ME incubated *in vitro* is markedly augmented by ADX [14, 29], consistent with immunohistochemical evidence for activation of the hypophyseotrophic CRF/AVP neurons by ADX [13, 24, 27, 31]. In the present study, we performed simultaneous measurement of CRF and AVP release from the ME *in vitro*, and observed that ADX results in a remarkable increase (about 4-fold) of the molar ratio of AVP:CRF released by depolarizing stimuli.

Materials and Methods

Animals

Male Sprague-Dawley rats, weighing 250–275 g, were obtained from Charles River and housed in a light-controlled room (lights on 07.00–19.00 h) with rat chow and tap water available *ad libitum*. Some of the rats were bilaterally or sham-adrenalectomized under ether anesthesia, allowed to recover and then housed for 4 days or 4 weeks prior to each experiment. They were given tap water and 0.9% NaCl to drink *ad libitum*.

Incubation of ME

The rats were decapitated and the ME with a small portion of the pituitary stalk attached was removed under a dissecting microscope. The wet weight of each ME was 0.5 mg or less. Five to eight

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ME were collected in a tube containing Krebs-Ringer bicarbonate glucose (KRBG) medium, and were incubated in 350 μ l KRBG medium at 37 °C in an atmosphere of 95% O₂/5% CO₂. After 40 min preincubation, the medium was carefully removed and then changed every 10 min during the experimental period for fresh KRBG with and without high potassium, veratridine or test drugs as described in the individual figure legends. In high potassium medium the concentration of NaCl was lowered to maintain normal osmolality. The incubation medium was collected on ice, and CRF and AVP were measured by direct radioimmunoassays (RIA).

To study the effect of calcium channel antagonists on peptide release, the ME were first stimulated with 48 mM K⁺ (S₁) for 10 min followed by three 10-min periods where KRBG medium was added, and a fourth period when either a calcium antagonist in KRBG or KRBG alone were present. This was followed by a second challenge with high K⁺ medium with or without the calcium channel antagonist at various concentrations (S₂). The amount of peptide released upon stimulation by K⁺ was calculated as follows: In the case of AVP, basal release (the amount released in the period prior to the K⁺ stimulus) was subtracted from the amount of AVP released in the periods when K⁺ was present and the next 10-min period. The K⁺-evoked release was divided by the number of ME times 2 to obtain the net amount of AVP released in 10 min by a single ME. For CRF no correction was made to obtain the net K⁺-evoked release, because basal release was usually undetectable. The ratio of peptide released in response to S₂ and S₁ (S₂/S₁) was calculated for AVP and CRF, respectively, for the whole series of experiments to obtain the control values, and these were compared to the ratios obtained in the presence of the Ca channel antagonists by subjecting the data to one-way analysis of variance (Anova) followed by Duncan's test for multiple comparisons.

The effects of ADX were determined by incubating a tube containing 5–8 ME from sham-adrenalectomized rats in parallel with a similar tube containing ME removed from adrenalectomized rats. Each pool of ME was stimulated with 48 mM K⁺ on three occasions, for 10 min each, and the stimulation periods were separated by four periods of incubation in normal KRBG (total 40 min). The amounts of CRF and AVP released by K⁺ were calculated as above, and the mean value of the amount of peptide released upon three challenges with K⁺ was treated as a single observation. The data were analyzed using Student's *t* test.

Tissue Peptide Content

At the end of the incubation the ME were individually placed in 100 μ l of 0.1 M HCl containing 1 mM ascorbic acid. The tissue was then rapidly frozen on dry-ice and stored at –20 °C. Prior to the assay of peptide content, the tissue was dispersed for 30 s with an ultrasound homogenizer and the volume was made up to 1.5 ml with 50 mM sodium phosphate buffer. CRF and AVP were measured directly by RIA as described below. The peptide contents obtained were averaged for each pool of ME to give the content per 1 ME, and was treated as a single observation. Fractional release (the ratio release:content) of CRF was obtained by dividing the mean of potassium-induced peptide release by the mean CRF content per ME for each pool of ME that was tested.

Reagents

Rat CRF was purchased from Peninsula Labs (San Carlos, Calif.) and vasopressin from Bachem (Torrance, Calif.). Verapamil

was provided by Dr. E.B. Kirsten of the Knoll Pharmaceutical Co. (Whippany, N.J.), nitrendipine by Dr. A. Scriabine of Miles Pharmaceutical Co., and diltiazem by Marion Labs (Kansas City, Mo.). Veratridine was kindly supplied by Dr. G. Brown (University of Alabama, Birmingham) and tetrodotoxin (TTX) was obtained from Sigma (St. Louis, Mo.).

Peptide Radioimmunoassays

The antiserum against AVP was kindly supplied by Dr. G. Rougon (LCB, NIMH, Bethesda, Md.) and has been characterized previously [3]. AVP was iodinated with carrier-free Na¹²⁵I (Amersham) by the transfer Iodogen method [23]. The RIA consisted of a 2-day incubation at 4 °C followed by precipitation of the antibodybound peptide with 0.7 ml of 20% polyethylene glycol (PEG 7,000 MW) and 0.2 ml 50% normal rabbit serum in phosphate-buffered saline.

The anti-rat CRF antiserum (GA-13) was raised in rabbits by subcutaneous injections of 100 μ g rCRF. Iodination of rCRF was performed by the transfer Iodogen method [16, 23]. The CRF RIA was carried out by incubating the samples with antiserum (1:80,000 dilution) for 24 h at 4 °C followed by a further 24-hour incubation at 4 °C with ¹²⁵I-CRF (8,000 cpm). Bound CRF was separated by PEG as described for the RIA of AVP. Under these conditions, the assay had a working range between 1.0 and 64 fmol rCRF/tube and showed no cross-reaction with AVP, GnRH, TRH and oxytocin up to 1 pmol/tube.

Results

Effect of Potassium and Veratridine on CRF and AVP Release

The basal release of CRF from 7 ME incubated in vitro was near or below the limit of detection of the RIA. Increasing concentrations of K⁺ from 28 to 56 mM in the incubation medium caused a dose-dependent increase in CRF release. A lower concentration of 14 mM K⁺ failed to elicit a detectable CRF response (fig. 1a). Veratridine (5–20 μ M) also stimulated CRF release in a dose-dependent manner (fig. 1b), and had similar effects upon AVP release. The effects of 10 μ M veratridine on both CRF and AVP release were abolished by 1 μ M TTX, a sodium channel antagonist; however, this dose of TTX failed to exert any effect on K⁺-stimulated CRF or AVP release (table I).

Involvement of Extracellular Calcium in K⁺-Stimulated CRF and AVP Release

Substitution of the normal incubation buffer by calcium-free KRBG containing 2 mM EGTA caused a marked reduction of both the K⁺-stimulated (48 mM) and veratridine-induced (10 μ M) release of CRF and AVP (table I). We next investigated the effects of three structurally different voltage-sensitive calcium channel antagonists on the K⁺-stimulated release of AVP and CRF. At very high concentrations, verapamil (10^{–5} M) and nitrendipene (10^{–4} M) re-

Table I. The effects of Ca-free medium and tetrodotoxin (1 μ M) on potassium (48 mM) and veratridine (10 μ M) stimulated CRF and AVP release

	CRF release fmol/ME	AVP release fmol/ME
Control		
K ⁺	5.67 $\pm$ 0.85	5.5 $\pm$ 0.5
Veratridine	4.03 $\pm$ 0.53	17.9 $\pm$ 11.4
Ca-free		
K ⁺	n.d.	1.3 $\pm$ 0.7*
Veratridine	0.29 $\pm$ 0.15*	3.2 $\pm$ 1.3*
TTX		
K ⁺	6.6 $\pm$ 0.84	6.0 $\pm$ 1.5
Veratridine	0.34 $\pm$ 0.12*	2.2 $\pm$ 0.8*

Values are mean $\pm$ SEM, n = 6 in each group. *p < 0.01 compared to respective control values.

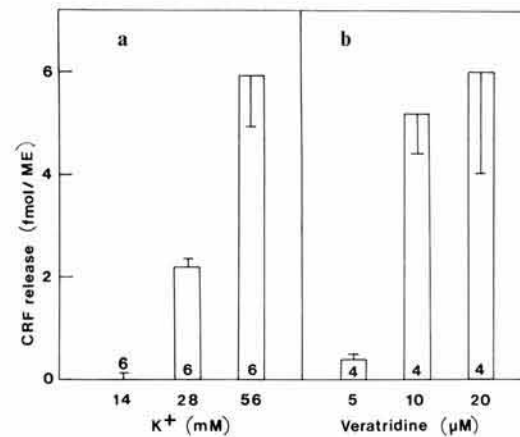


Fig. 1. The effect of increasing concentrations of potassium (a) and veratridine (b) on CRF release from ME in vitro. Columns represent means $\pm$ SEM with the number of observations given at the base of each column.

duced the K⁺-stimulated CRF response whereas diltiazem was without effect (fig. 2). None of the calcium channel antagonists had a significant effect on AVP release (data not shown).

Effects of Adrenalectomy on CRF and AVP Release

Short-Term Adrenalectomy

The effects of 48 mM K⁺ upon the release of CRF and AVP were compared in the ME of sham- and 4-day adrenalectomized rats. The data of CRF and AVP release in a representative experiment are shown in figure 3. In all experiments, the CRF response to K⁺ was blunted after ADX, whereas the release of AVP was increased. Over a series of experiments (n = 19) the mean ratio of AVP:CRF released from control ME in response to 48 mM K⁺ was 1.8, the 99.9% limits of confidence of the mean were: 1.4–2.3. In the 4-day ADX experiments, the ratio of AVP:CRF release was increased from a value of 2.4:1 seen in the sham group to 9.2:1 in the adrenalectomized group (table II). Analysis of the peptide content of the ME after the incubation showed a 33% decrease in CRF content and no change in AVP content (table II). The fractional release of CRF (the ratio of peptide release to content) was not altered 4 days after ADX. In two experiments, adrenalectomized rats were given daily injections of 50 μ g dexamethasone s.c. for 4 days. The CRF released in response to 48 mM K⁺ was re-

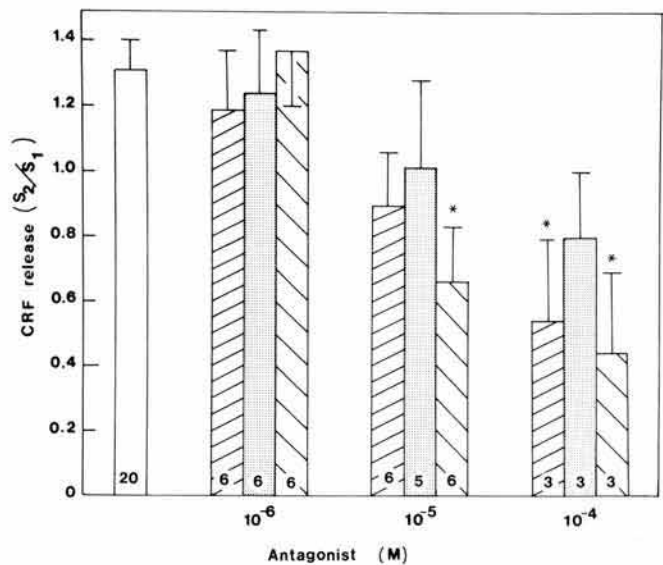


Fig. 2. The effects of various calcium channel antagonist compounds on potassium (48 mM)-stimulated CRF release in vitro. The open column shows the ratio S₂/S₁ for two subsequent challenges with high potassium without Ca channel antagonists. The other columns show the effects of nitrendipine (□), diltiazem (▨) and verapamil (▩) on S₂/S₁. Mean $\pm$ SEM with the number of observations in each group depicted at the base of the column. *p < 0.05 compared to control as determined by one-way Anova followed by Duncan's test for multiple comparisons.

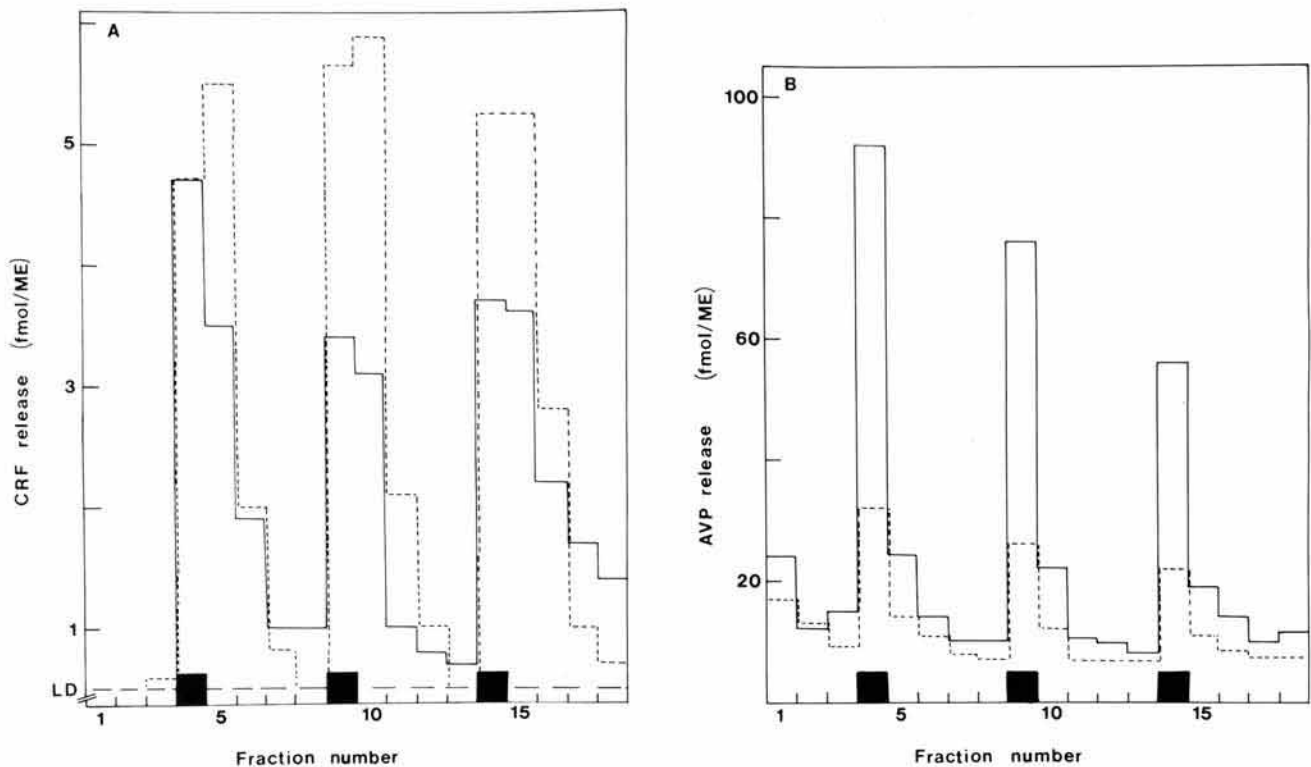


Fig. 3. The effect of adrenalectomy on the potassium-evoked release of CRF (A) and AVP (B) from ME in vitro. The data shown are from a representative experiment; each tube contained 6 ME, which were either from rats that were sham-operated (dotted line) or were adrenalectomized (solid line) 4 days prior to the experiment. The abscissa shows the number of 10-min fractions collected (fraction number); ■ = application of 48 mM K^+ . LD = Limit of detection of the CRF radioimmunoassay.

Table II. The effect of adrenalectomy on the potassium-induced release and the content of CRF and AVP in median eminence

	Release, fmol/ME		Ratio AVP:CRF	Content, pmol/ME		n
	AVP	CRF		AVP	CRF	
4-day adrenalectomy						
Sham	14.8 ± 2.0	6.2 ± 0.1	2.4 ± 0.3	18.9 ± 1.9	0.62 ± 0.06	4
ADX	37.5 ± 2.8**	4.1 ± 0.2**	9.2 ± 0.5**	20.5 ± 2.2	0.42 ± 0.02*	5
4-week adrenalectomy						
Sham	11.0 ± 1.4	4.7 ± 0.2	2.4 ± 0.4	19.4 ± 2.5	0.85 ± 0.05	3
ADX	65.3 ± 3.1**	7.9 ± 0.2**	8.5 ± 0.7**	28.0 ± 3.5	1.03 ± 0.06	3

Values are mean ± SEM. n = Number of experiments. * $p < 0.05$, ** $p < 0.01$, by Student's *t* test.

duced from 5.2 and 4.1 fmol/ME in the sham groups to 2.6 and 1.2 fmol/ME in the adrenalectomized groups. In ADX rats treated with dexamethasone the release of CRF was 5.7 and 4.7 fmol/ME. Similarly, dexamethasone inhibited the changes in the ME content of CRF caused by ADX: instead of a 57 and 58% decrease in the two experiments, respectively, there was either no change or only a 27% decrease in dexamethasone-treated rats.

Long-Term Adrenalectomy

Four weeks after ADX, both CRF and AVP release from ME in vitro were increased. However, the increase in AVP (6-fold) release was markedly greater than that of CRF (1.7-fold) giving an increased ratio of AVP:CRF (8.5:1) similar to that observed 4 days after ADX. The content of both CRF and AVP in the ME tended to increase 4 weeks following ADX but the changes were not statistically signif-

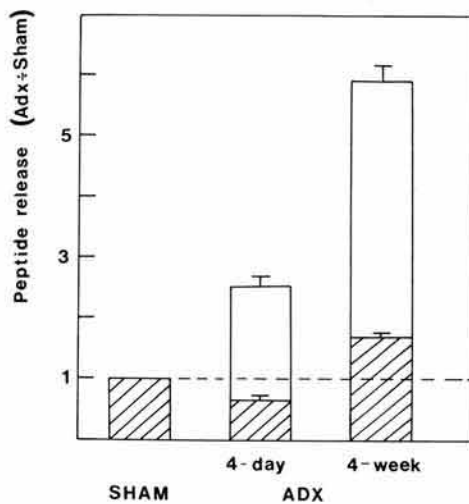


Fig. 4. Summary of the effects of adrenalectomy on AVP (□) and CRF (▨) release from the ME in vitro. The release induced by K^+ is expressed as multiples of the release found in sham-operated rats. Mean $\pm$ SEM, the data are from the experiments in table II.

icant ($p < 0.2$). The fractional release of CRF was 5.6 ± 0.5 in the sham-operated group and 7.7 ± 0.2 in the ADX group (fmol released/fmol content $\times 1,000$, mean $\pm$ SEM, $n = 3$ in both groups, $p < 0.05$).

Discussion

We have shown that the relative amount of AVP vs. CRF released from the ME in vitro by depolarizing stimuli is markedly increased by ADX. Basal levels of CRF were usually below the limit of detection of the RIA but the basal release of AVP from the same tissues was much higher, possibly due to the leakage of AVP from the cut fibers of the hypothalamo-neurohypophyseal pathway passing through the internal zone of the ME. However, the stimulated release of both peptides has the characteristics of a physiologically relevant membrane-mediated event, as shown by the calcium dependence of the responses to potassium and veratridine.

The effect of veratridine was blocked by TTX, a known inhibitor of Na^+ channels, consistent with the generally accepted view that veratridine activates calcium channels by opening TTX-sensitive Na^+ channels [19]. In contrast, TTX had no effect on K^+ -induced neurohormone release, in agreement with the direct depolarizing action of potassium on the plasma membrane with consequent activation of calcium influx.

The characteristics of the in vitro responses of the ME in these experiments suggest the participation of voltage-dependent calcium channels in eliciting the secretory response to depolarizing stimuli. However, when the nature

of the putative calcium channels was investigated by the use of calcium channel antagonists, K^+ -evoked CRF release was inhibited only by $10 \mu M$ verapamil and $100 \mu M$ nifedipine, and there was no significant inhibition of the release of AVP. At such high concentrations, the inhibitory effect may not be due to the specific calcium channel antagonist properties of the drugs. These results indicate that the calcium channels involved in AVP and CRF release from the ME are largely insensitive to calcium channel blockers that are potent inhibitors of calcium influx and biological responses in other tissues, e.g. smooth muscle and heart muscle [11]. The findings, obtained by examining the depolarization-induced release of two endogenous neuropeptides, support and extend observations on the release of radioligands from neural tissues prelabeled with exogenous radioactive neurotransmitters [7, 8, 12, 25]. Such unresponsiveness to calcium channel antagonists may be common to neural membranes and could reflect heterogeneity of the voltage-dependent calcium channels in different tissues.

The most remarkable effect of ADX was the increase in the ratio of AVP:CRF released from the ME, a change that was already attained at 4 days after ADX. While this increase was observed after short- as well as long-term ADX, the underlying mechanisms appear to be somewhat different (fig. 4).

Four days after ADX the release of CRF was reduced by about 30%, whereas that of AVP was more than doubled. In contrast, 4 weeks following ADX both CRF and AVP release were enhanced, but this was much more pronounced for AVP (5.7-fold over sham ADX) than for CRF (1.6-fold).

Such changes in the ratio of AVP:CRF may be of functional significance because increasing the ratio of AVP:CRF (with no change in the concentration of CRF) markedly stimulates ACTH release when both peptides are added to rat anterior pituitary tissue in vitro [1, 2]. As noted earlier, AVP and CRF are synthesized by hypophyseotrophic neurons of the PVN that project to the external zone of the ME. In fact, these peptides are co-localized in the same neurosecretory granules within the nerve terminals of AVP/CRF neurons [30]. Moreover, in rats with surgical lesioning of the PVN, ADX fails to enhance the release of AVP by the ME in vitro [14] and there is no detectable release of CRF [9]. It seems, therefore, reasonable to suggest that the AVP and the CRF released by the ME in vitro after ADX originate from the same nerve terminals and, furthermore, the increase of the ratio of AVP:CRF released in vitro reflects the relative proportion of these neurohormones in pituitary portal blood. Thus, it appears that AVP is the predominant ACTH-releasing stimulus in ADX rats, while the presence of CRF could be essential to allow the action of AVP. Functional evidence supporting this conclusion has been recently presented by Dallman et al. [5]: The administration of CRF to ADX rats with hypothalamic lesions that deplete CRF as well as AVP from the ME will increase the

pituitary levels of the messenger RNA that encodes pro-opiomelanocortin to the same extent as ADX does in intact rats. However, CRF only caused a 2.5-fold elevation of plasma ACTH in lesioned ADX rats, whereas ADX in intact animals brought about a 12-fold increase.

It should be noted that our studies, which were performed on tissue containing nerve terminals isolated from their cell bodies, do not exclude the possibility that ADX substantially increases the hypothalamic secretion of CRF in vivo, as opposed to the biphasic and relatively small changes of release in vitro. Further experiments are warranted to examine this issue.

The mechanism of the enhancement by ADX of the release of AVP from the ME in vitro is not known. While it appears established that ADX increases the delivery as well as the storage of AVP in the external zone of the ME [22, 32], the actual size of these changes has not been determined, due to the presence of two distinct pools (internal zone and external zone fibers) of AVP in the ME. In contrast, about 95% of the CRF is found in the external zone fibers, and the changes of its release can be related to the alterations in ME content. From an analysis of these data, it appears that 4 days after ADX the decrease in CRF release follows the decrease in content. In contrast, at 4 weeks the increase in release is apparently not accompanied by a proportional change of CRF content, indicating an alteration in the function of the nerve terminals in the external zone following ADX. However, until it is determined which molecular forms of CRF are recognized by our antiserum, and what proportion of this is actually available for release, these data should be interpreted with a degree of caution.

In summary, our data suggest that AVP could be the major stimulus of ACTH release in adrenalectomized rats, while CRF may act as a trophic or permissive factor in the control of corticotropin secretion by AVP.

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Corticotrophin-releasing peptides in rat hypophysial portal blood after paraventricular lesions: a marked reduction in the concentration of corticotrophin-releasing factor-41, but no change in vasopressin

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ABSTRACT

Previous data show that corticotrophin-releasing factor-41 (CRF-41), arginine vasopressin (AVP) and oxytocin are released into hypophysial portal blood. It has been presumed that the CRF-41 originates mainly from parvicellular neurones of the paraventricular nuclei (PVN); however, AVP and oxytocin could also be derived as a consequence of preterminal release from magnocellular projections to the neurohypophysis. The latter has been suggested to be the case for AVP as assessed by studies of the median eminence *in vitro*. Here we have investigated the source of CRF-41, AVP and oxytocin in hypophysial portal blood of adult male Wistar rats 8–10 days after surgical lesioning of the PVN. In PVN-lesioned animals the output of CRF-41 into hypophysial portal blood was reduced by about 90%, and that of oxytocin by about 40%; however, the output of AVP into portal blood

was reduced only by about 10%. The release of AVP into portal blood increased after adrenalectomy; this increased release could be returned to normal by treatment with dexamethasone. No change of AVP release occurred after adrenalectomy in animals in which the PVN had been lesioned. These results show (i) that most of the CRF-41 released into hypophysial portal blood is derived from the PVN, (ii) that in PVN-lesioned animals AVP and oxytocin release remains at near normal or 60% of normal respectively, suggesting that a substantial amount of both neuropeptides in portal blood is derived as a consequence of preterminal release from supraoptic nuclei projections in the median eminence, and (iii) that glucocorticoid feedback inhibition of AVP release is exerted at the level of the PVN.

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INTRODUCTION

The secretion of hormones by the anterior pituitary gland is controlled by neurohormones released into hypophysial portal blood from nerve terminals in the median eminence. Corticotrophin-releasing factor-41 (CRF-41) and arginine vasopressin (AVP) appear to be the principal hypothalamic neurohormones that regulate the secretion of adrenocorticotrophin (ACTH) (for reviews see Antoni, 1986; Rivier & Plotsky, 1986; Jones & Gillham, 1988). Current evidence indicates that these neuropeptides are transported to the median eminence in four different hypothalamic neural pathways (Antoni, 1986; Whitnall, 1988).

First, some nerve cells in the parvicellular subdivisions of the hypothalamic paraventricular nuclei

(PVN) (Armstrong, Warach, Hatton & McNeill, 1980) synthesize both CRF-41 and AVP, and give rise to an axonal projection that terminates in the external layer of the median eminence (Tramu, Croix & Pillez, 1983; Kiss, Mezey & Skirboll, 1984; Sawchenko, Swanson & Vale, 1984a). Secondly, it has been suggested that there is an anatomically similar CRF-41-containing pathway that does not normally contain AVP (Whitnall, 1988). Thirdly, it is well known that preterminal axons of vasopressinergic magnocellular neurones traverse the median eminence in its internal layer and terminate in the neural lobe of the pituitary gland. The cell bodies of these axons are found in the magnocellular part of the PVN, the supraoptic nucleus and some accessory cell groups scattered in the anterior lateral hypothalamus.

Fourthly, oxytocinergic magnocellular neurons also contain CRF-41 (Bulet, Tonon, Tankosic *et al.* 1983; Sawchenko, Swanson & Vale, 1984b); their axonal projection to the median eminence is similar to that of magnocellular vasopressinergic cells.

Functional studies have suggested the involvement of all four of these pathways in the regulation of the hormonal response to stressful stimuli (for reviews see Antoni, 1986; Gibbs, 1986; Antoni, Kovács, Dohanits *et al.* 1988). However, with the exception of the parvocellular CRF-41/AVP system the evidence is indirect and inconclusive (Antoni, 1986).

The origin of AVP in hypophysial portal blood remains controversial because it has not been established with certainty whether or not magnocellular axons release AVP into hypophysial blood. The majority of previous studies suggest that the concentration of AVP in hypophysial portal blood is not altered after removal of the neurohypophysis (Recht, Hoffman, Haldar *et al.* 1981; Gibbs, 1985; Horn, Robinson & Fink, 1985; Plotsky, 1985). Hence, it was inferred that AVP released by magnocellular neurones, which have axon terminals in the neurohypophysis, does not reach the adenohypophysis in significant amounts. However, *in-vitro* studies (Holmes, Antoni, Aguilera & Catt, 1986a) indicate that preterminal magnocellular axons in passage through the median eminence may secrete AVP. In addition, exocytosis of neurosecretory granules along vasopressinergic axons in the internal layer of the median eminence has been reported also using the tannic acid fixation method (Buma & Nieuwenhuys, 1988).

In the present study we have examined whether AVP released by preterminal magnocellular axons is found in hypophysial portal blood. The hypothesis was tested by examining neuropeptide release into pituitary stalk blood in animals in which the PVN (the parvocellular source of AVP and the major source of CRF-41) were lesioned.

MATERIALS AND METHODS

Animals

Male albino Wistar rats (200–280 g body weight; Harlan-Olac, Bicester, Oxon, U.K.) were used throughout. The animals were kept under controlled conditions of lighting (on between 08.00 and 20.00 h in Oxford, and between 05.00 and 19.00 h in Edinburgh) and temperature (22 °C), and had free access to pelleted food and tap water.

Paraventricular lesions and collection of hypophysial portal blood

Under sodium pentobarbitone anaesthesia (Sagittal; May & Baker, Dagenham, Essex, U.K.; 40 mg/kg i.p.),

the PVN were bilaterally destroyed with a specially designed microknife as described by Makara, Stark, Kapócs & Antoni (1986). Sham lesions were performed by lowering the tip of the microknife in the midline to 3 mm below the surface of the skull. Some animals were bilaterally adrenalectomized or sham-adrenalectomized 24 h after the hypothalamic surgery under ether anaesthesia.

Between 8 and 10 days after the hypothalamic surgery, the animals were anaesthetized with sodium pentobarbitone and surgically prepared for the collection of hypophysial portal blood as well as peripheral venous blood from the jugular vein according to previously published procedures (Horn *et al.* 1985). Briefly, collection of hypophysial portal blood was started shortly (3–5 min) after the exposure and transection of the stalk, 30–45 min after the induction of anaesthesia. Portal blood was collected during two consecutive 30-min periods. Peripheral blood was sampled immediately before the pituitary stalk was cut for portal blood collection and again at the end of the second 30-min period of portal blood collection. Blood samples were collected in chilled plastic tubes containing aprotinin (Fink, Robinson & Tannahill, 1988).

Adrenalectomy and dexamethasone treatment

In a separate experiment, rats were bilaterally adrenalectomized under ether anaesthesia. Dexamethasone (250 µg/kg; Sigma, Poole, Dorset, U.K.) or its vehicle, dimethylsulphoxide, were injected s.c. once daily for 7 days at 17.30 h. Animals were caged singly 24 h before being killed by decapitation between 09.00 and 10.00 h. Trunk blood was collected in chilled polypropylene tubes containing Na₂ EDTA.

Hormone assays

Plasma was separated from blood cells by centrifugation, and analysed for CRF-41, AVP, oxytocin, ACTH and corticosterone content by specific radioimmunoassays (Fink *et al.* 1988).

Corticotrophin-releasing factor-41

The antiserum used in the radioimmunoassay was an anti-rat(r)CRF-41 purchased from IgG Corporation (Nashville, TN, U.S.A.) used at a dilution of 1/11 000, and ¹²⁵I-labelled[Tyr⁰]-rCRF-41 was used as tracer. Bound was separated from free antigen by precipitation with anti-rabbit γ-globulin (Scottish Antibody Production Unit, Carlisle, Lanarkshire, U.K.). Specificity of the assay was tested by determining cross-reaction with several hypothalamic and related peptides; dynorphin, β-endorphin, luteinizing hormone-releasing hormone (LHRH), somatostatin-14, thyrotrophin-releasing

hormone, sauvagine, ovine CRF-41 and -41. Cross-reaction was less than 0.1% for all these peptides at concentrations of up to 1 µg/ml. The sensitivity of the assay was 0.5–1.0 fmol/tube at 90% tracer bound, the interassay coefficients of variation (C.V.) were 13.5 and 9.4% for the low- (16 fmol/tube) and high- (64 fmol/tube) quality control samples respectively, and the intra-assay coefficients of variation were 10.9 and 3.4% for the low- and high-quality control samples respectively.

Arginine vasopressin

Antiserum RAS-8103 from Peninsula Laboratories (St Helens, Merseyside, U.K.) was used. Cross-reactions were: [Lys⁸]-vasopressin, 3%; pressinoic acid, <0.1%; α-human atriopeptin, <0.1%; oxytocin, <0.1%; vasopressin metabolite neuropeptide, <0.1% and CRF-41, <0.1%. Intra- and inter-assay coefficients of variation were 6.5 and 9.8% respectively. Bound was separated from free hormone as for CRF-41. Sensitivity was 1 fmol/tube of standard obtained from Cambridge Research Biologicals, Harston, Cambridge, U.K.

Oxytocin

Antiserum AF106 from Cambridge Research Biologicals gave the following cross-reactions: mesotocin, 0.36%; isotocin, <0.01%; lysine vasopressin, <0.01%; AVP, <0.01%; Pro-Leu-Gly NH₂, <0.01%, LHRH, <0.01%; ACTH, <0.01%; CRF-41, <0.01%. Bound was separated from free hormone as for CRF-41. Sensitivity was 1.2 fmol/tube of standard obtained from Cambridge Research Biologicals. Intra- and inter-assay coefficients of variation were 9.4 and 10.2% respectively.

Adrenocorticotrophin

The rabbit anti-ACTH serum was purchased from IgG Corporation and used at a final dilution of 1/30 000. This antiserum cross-reacted at less than 0.5% with α-melanocyte-stimulating hormone (α-MSH), β-MSH, corticotrophin-like intermediate lobe peptide, β-lipotrophic hormone (β-LPH), γ-LPH and the endorphins. The antigen-primary antibody complex was precipitated with anti-rabbit γ-globulin (Scottish Antibody Production Unit). The lower limit of sensitivity of the assay was 0.13–0.25 fmol/tube and the inter- and intra-assay coefficients of variation were 13.4 and 5.4% respectively.

Corticosterone

The primary corticosterone antiserum was raised in rabbits and generously provided by Dr D. Kime (Sheffield). The cross-reaction of the corticosterone

antiserum was: corticosterone, 100%; cortisol, 10%; aldosterone, 0.4%; deoxycorticosterone, 18.2%; progesterone, 4.8%; compound S, 6.1% and dexamethasone, <0.01%. The sensitivity was 3 pmol/tube at 90% tracer bound and the inter- and intra-assay coefficients of variation were 10.8 and 6.7% respectively.

Histology

The brain of each PVN-lesioned animal was fixed in 10% (v/v) formalin, embedded in Paraplast, and serial coronal sections of the hypothalamus (15 µm) were cut on a microtome. Every fifth section was mounted and stained with cresyl-violet. The configuration of the lesion was inspected at 40× magnification. Only animals with a complete lesion of the PVN are included in the results. Typically, the anterior commissural nucleus was partly or completely destroyed; caudally the rostral portions of the dorsomedial nucleus were affected in some cases. Laterally, the lesion does not extend beyond the fornix (Makara, Stark, Kárteszi *et al.* 1981; Makara *et al.* 1986).

RESULTS

Methodological considerations

The contents of CRF-41, AVP and oxytocin in portal plasma and the concentration of ACTH and corticosterone in peripheral plasma as well as the volumes of blood collected in animals subjected to sham PVN lesion, or sham lesion plus sham adrenalectomy were similar to each other and to the values in intact animals. Therefore the values were analysed together as the same 'control' group. The haematocrit values for hypophyseal portal blood increased in rats given dexamethasone. There was considerable variation in the volume of blood collected from the transected stalk within the groups of animals (typical range 200–1000 µl). In the case of PVN-lesioned and adrenalectomized rats the average blood volume collected was significantly different from the control value (Table 1). Examination of the concentrations of AVP and CRF-41 in the hypophyseal stalk plasma of all control animals revealed significant ($P < 0.01$) inverse linear correlation with the volume of portal blood collected (for AVP, $r = -0.66$; for CRF-41, $r = -0.64$; $n = 33$). Furthermore, a similar relationship was apparent between portal plasma AVP levels and blood volume in PVN-lesioned rats ($r = -0.63$; $n = 20$; $P < 0.01$). In contrast, the total amount of peptide released during the sampling period did not show such correlations, presumably because the secretory rate of peptides into hypophyseal portal blood is not dependent on blood flow in the portal system. Hence, in the following sections the values of neuropeptide contents in hypophyseal portal blood are considered throughout.

TABLE 1. Content and concentration of corticotrophin-releasing factor-41 (CRF-41), arginine vasopressin (AVP) and oxytocin in rat hypophyseal stalk blood after various treatments: PVN-L, paraventricular hypothalamic lesions; DEX, dexamethasone treatment; ADX, adrenalectomy. Data shown are geometrical means with the retransformed S.E.M. in parentheses, except for haematocrit values which are means $\pm$ S.E.M.

Treatment	Blood vol. (μ l/30 min)	Haematocrit (%)	Peptide content (fmol/30 min)			Peptide concn (nmol/l)			n
			CRF-41	AVP	Oxytocin	CRF-41	AVP	Oxytocin	
Control									
Period 1	510(1.14)	33.7 $\pm$ 1.8	43(1.11)	614(1.10)	235(1.14)	0.12(1.15)	1.7(1.19)	0.7 (1.28)	13
Period 2	447(1.14)		35(1.10)	354(1.16)	112(1.18)	0.10(1.10)	1.1(1.18)	0.4 (1.16)	
PVN-L									
Period 1	536(1.23)	36.9 $\pm$ 3.1	4** (1.11)	493(1.22)	103*(1.10)	0.01(1.10)	1.5(1.34)	0.31(1.23)	8
Period 2	428(1.24)		4(1.11)	277(1.15)	73(1.15)	0.01(1.11)	0.8(1.25)	0.3 (1.21)	
PVN-L + DEX									
Period 1	696(1.11)	40.5* $\pm$ 1.4	6** (1.12)	735(1.22)	97*(1.09)	0.01(1.12)	1.8(1.23)	0.2 (1.12)	6
Period 2	515(1.12)		5(1.17)	373(1.17)	65(1.14)	0.01(1.15)	1.1(1.14)	0.2 (1.17)	
ADX									
Period 1	400(1.17)		16** (1.20)	960(1.10)		0.06(1.20)	3.6(1.16)		12
Period 2	302(1.20)	32 $\pm$ 1.7	9(1.23)	508(1.13)		0.05(1.20)	2.5(1.20)		
ADX + DEX									
Period 1	619(1.17)		29*(1.13)	686(1.14)		0.08(1.17)	1.9(1.17)		8
Period 2	529(1.23)	44** $\pm$ 2.2	20(1.12)	438(1.19)		0.08(1.15)	1.4(1.33)		
ADX + PVN-L									
Period 1	301(1.17)	30.2 $\pm$ 2.7	3** (1.11)	708(1.12)		0.01(1.12)	3.4(1.19)		6
Period 2	277(1.27)		2(1.24)	385(1.19)		0.01(1.18)	2.0(1.26)		

Hypophyseal stalk blood was collected from surgically prepared rats under anaesthesia with pentobarbitone for two consecutive 30-min periods designated 1 and 2.

* $P < 0.05$, ** $P < 0.01$ compared with respective control values (Kruskal–Wallis test, followed by Mann–Whitney U test), (only data for content in period 1 were analysed).

The trends of the changes in the content of peptides in stalk plasma brought about by the various treatments were the same during both periods of portal blood sampling, except for the case of oxytocin in the control group.

Effects of PVN lesions on CRF-41, AVP and oxytocin in hypophyseal portal blood

The amount of CRF-41 in hypophyseal portal blood was markedly reduced by the PVN lesions (Table 1), while AVP levels were not affected. Treatment of PVN-lesioned rats with 250 μ g dexamethasone/kg per day had no significant effect on the content of CRF-41 or AVP in hypophyseal portal blood. The data on CRF-41 and AVP for the first collection period are also presented in Fig. 1a as percentiles of the respective controls.

In control rats the amount of oxytocin in portal blood during the first 30 min of sampling was significantly higher than in PVN-lesioned rats (Table 1), but this difference was not apparent during the second 30 min of collection. Treatment with dexamethasone had no significant effect on the oxytocin content of hypophyseal portal blood in PVN-lesioned rats.

CRF-41 and AVP in hypophyseal portal blood from adrenalectomized rats

In order to validate the effectiveness of the PVN lesion and dexamethasone treatment in suppressing parvocellular AVP and CRF-41 secretion, experiments were also performed in adrenalectomized rats.

Between 7 and 9 days after bilateral adrenalectomy, the content of AVP in portal blood rose significantly, whilst the levels of CRF-41 were reduced to less than half the control value (Table 1 and Fig. 1a). Lesioning of the PVN blocked the increase of portal blood AVP concentration that was observed in adrenalectomized animals (Table 1 and Fig. 1a). Treatment with dexamethasone (250 μ g/kg) also suppressed the effect of adrenalectomy on portal blood AVP content, but failed to return the content of CRF-41 in hypophyseal portal blood to the control value.

Plasma ACTH and corticosterone concentrations in rats prepared for collection of hypophyseal portal blood

The plasma ACTH levels in PVN-lesioned animals with an intact pituitary stalk were 40% lower than in sham-operated 'control' rats (Table 2 and Fig. 1b). After the sectioning of the stalk, the levels of ACTH fell to a similar level in both groups (compare values 1

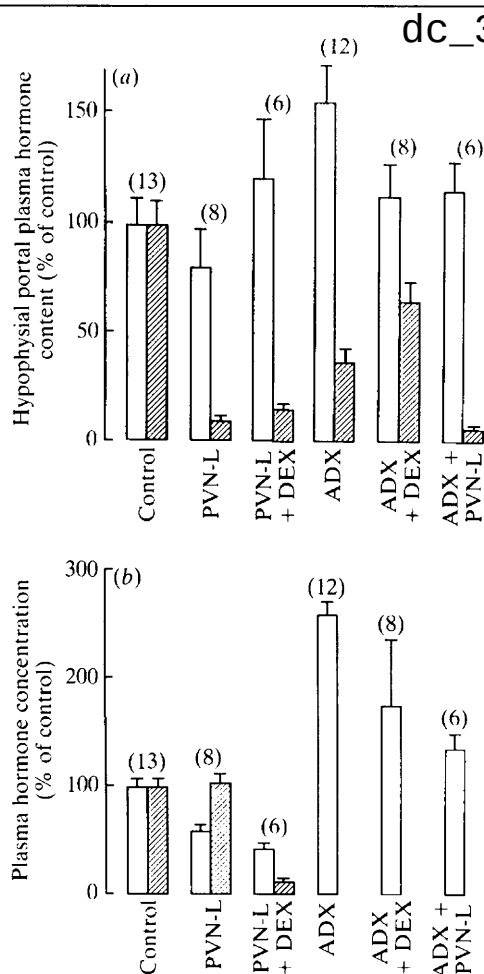


FIGURE 1. Effect of paraventricular hypothalamic lesions (PVN-L), adrenalectomy (ADX) and dexamethasone treatment (DEX) on (a) the content of 41-residue corticotrophin-releasing factor (hatched bars) and arginine vasopressin (open bars) in hypophyseal portal plasma, and (b) the concentration of ACTH (open bars) and corticosterone (hatched bars) in peripheral plasma of rats under anaesthesia with pentobarbitone. Data (period 1 values) are expressed as percentage of the respective control groups from Tables 1 and 2. The bars represent the retransformed S.E.M., the numbers in parentheses show the numbers of observations.

and 2 in Table 2). The plasma concentration of corticosterone was the same in both groups of animals and also decreased to a similar level upon transection of the pituitary stalk (compare values 1 and 2 in Table 2). Treatment with dexamethasone reduced plasma ACTH as well as corticosterone concentrations in PVN-lesioned rats (Table 2).

Adrenalectomy increased plasma ACTH, and this was largely prevented by lesioning of the PVN, whilst treatment with dexamethasone was less effective (Table 2).

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TABLE 2. Concentration of ACTH and corticosterone in peripheral plasma in rats prepared for collection of hypophyseal stalk blood, after various treatments: PVN-L, paraventricular hypothalamic lesions; DEX, dexamethasone treatment; ADX, adrenalectomy. The first sample (1) was taken immediately before the transection of the stalk, the second (2) was collected after 65 min at the end of the stalk blood collection. Data are geometrical means with the retransformed S.E.M. in parentheses.

	ACTH (pmol/l)	Corticosterone (nmol/l)
Treatment		
Control		
Period 1	93(1.06)	1055(1.07)
Period 2	28(1.08)	341(1.11)
PVN-L		
Period 1	52(1.11)††	1083(1.06)
Period 2	18(1.16)	286(1.17)
PVN-L + DEX		
Period 1	17(1.49)*††	88(1.23)*††
Period 2	<4.5	20(1.17)
ADX		
Period 1	244(1.10)††	<40
Period 2	106(1.13)	<40
ADX + DEX		
Period 1	167(1.37)†	<40
Period 2	22(1.37)	<40
ADX + PVN-L		
Period 1	127(1.12)†	<40
Period 2	33(1.19)	<40

* $P < 0.01$ compared with PVN-L values; † $P < 0.05$, †† $P < 0.01$ compared with control (Kruskal-Wallis test followed by Mann-Whitney U-test).

Basal levels of plasma ACTH in adrenalectomized and dexamethasone-treated rats

In the sham-adrenalectomized control group plasma ACTH was 7.1 ± 2.2 pmol/l, (mean $\pm$ S.E.M. $n = 6$) and increased to 114 ± 33 pmol/l ($n = 6$) 7 days after adrenalectomy. Dexamethasone (250 μ g/kg per day) decreased plasma ACTH to below control levels (1.8 ± 0.7 pmol/l).

DISCUSSION

The most striking findings of this study are that the integrity of the PVN is not necessary to maintain normal levels of AVP in hypophyseal portal blood, but that lesioning of the PVN resulted in a marked reduction in the amount of CRF-41 released into hypophyseal portal blood. Thus, after PVN lesion, the bulk of AVP found in the hypophyseal portal blood from pentobarbitone-anaesthetized rats probably originates from the preterminal axons of supraoptic magnocellular neurones.

Validity of the experimental model

Various techniques have been developed for the assessment of the activity of hypothalamic CRF-41 or

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AVP neurones (see Antoni, 1990 for review). All the methods are invasive, in that the normal functioning of the hypothalamo-hypophyseal pathways has to be perturbed. The degree of the disruption of normal function is the major factor determining the utility of a particular technique as applied to a particular problem.

Comparison of the anaesthetized rat models (Gibbs, 1985; Plotsky, 1985; Eckland, Todd, Jessop *et al.* 1988; Fink *et al.* 1988) in which animals are exposed to acute surgical trauma, with the recently published observations in conscious sheep prepared with chronic cannulas (Caraty, Grino, Locatelli & Oliver, 1988; Engler, Pham, Fullerton *et al.* 1989) confirm that the initial levels of CRF-41 and AVP in the anaesthetized rat model are relatively high. However, mild stress in the sheep increases the concentrations of these peptides to the levels found in anaesthetized rats. Thus the concentrations of CRF-41 and AVP measured in anaesthetized rats are not unrealistically high.

In the present study, rats were used since hypothalamic neuroanatomy is known in considerable detail in this species. Pentobarbitone anaesthesia was chosen because it causes a less severe activation of the hypothalamo-pituitary-adrenocortical axis than urethane (Fink *et al.* 1988). Interestingly, the concentrations of CRF-41, AVP and oxytocin in the pituitary portal blood of pentobarbitone-anaesthetized rats are not markedly different from the levels found in urethane-anaesthetized rats. Some of the responses, e.g. the increase of AVP after adrenalectomy, do appear blunted; the possible reasons for this are discussed in a subsequent section of the paper.

Origin of AVP in hypophyseal portal blood of PVN-lesioned rats

Disparate changes of the concentrations of CRF-41 and AVP in the hypophyseal portal blood of rats and sheep have been reported previously (Gibbs, 1985; Caraty, Grino, Locatelli & Oliver, 1988; Plotsky, 1985; Eckland, Todd, Jessop *et al.* 1988; Engler, Pham, Fullerton *et al.* 1989; Fink *et al.* 1988), but analysis of the functional anatomy underlying such phenomena has not been attempted *in vivo*. Given the current level of understanding of the hypothalamic topography outlined in the Introduction, the dissociation of AVP and CRF release may have two alternative explanations: either parvicellular neurones spared by the lesion increase the production of AVP and effectively compensate the loss of over 90% of the cell population, or the AVP found in hypophyseal portal blood after PVN lesions is released by preterminal magnocellular axons of supraoptic origin.

With respect to the first alternative, it is well established that the region of the PVN contains practically all

the neurones that synthesize CRF-41 as well as AVP and send axons to the external lamina of the median eminence (Antoni, 1986; Swanson, Sawchenko, Lind & Rho, 1987). Consonant with this, the lesion performed here depleted at least 90% of the CRF-41 content of the median eminence (Antoni, Palkovits, Makara *et al.* 1984; Bruhn, Plotsky & Vale, 1984; Holmes *et al.* 1986a; Makara *et al.* 1986) and abolished depolarization-induced release of CRF-41 from the median eminence *in vitro* (Holmes *et al.* 1986a).

Despite the precipitous fall in portal blood CRF-41, it is clear that some parvicellular CRF-41 neurones, particularly those in a perifornical location, are spared by the PVN lesion (Makara, Antoni, Stark & Kártesz, 1984). These cells can respond to glucocorticoid deficiency by enhancing AVP synthesis several-fold, while the production and release of CRF-41 is not markedly altered (Holmes, Antoni, Catt & Aguilera, 1986b; Plotsky & Sawchenko, 1987). Since PVN lesions do reduce the amplitude of circadian peaks of corticosterone in the circulation (Ixart, Alonso, Szafarczyk *et al.* 1982), i.e. the overall glucocorticoid feedback signal is diminished, it is possible that the surviving parvicellular neurones may switch to high AVP production, and thus produce the pattern of hypophyseal portal CRF-41 and AVP levels observed in the present study. However, treatment of PVN-lesioned rats with the synthetic glucocorticoid dexamethasone failed to influence the amount of AVP in portal blood, while the same treatment prevented the increase of portal blood AVP content brought about by bilateral adrenalectomy. It is therefore highly unlikely that the AVP in the portal blood of PVN-lesioned rats is released by parvicellular neurones that are inhibited by glucocorticoid hormones.

More plausibly, AVP may be derived from the preterminal axons of supraoptic magnocellular neurones in the median eminence, as suggested on the basis of studies *in vitro* (Holmes *et al.* 1986a; Antoni, 1986). Further support for this is provided by recent studies showing, directly at the ultrastructural level, that both AVP and oxytocin are released by exocytosis from preterminal magnocellular axons in the internal layer of the median eminence (Buma & Nieuwenhuys, 1988). Finally, the data on oxytocin levels in portal blood indicate that a proportion of oxytocin may be also released from preterminal axons in the median eminence in PVN-lesioned rats.

Portal blood AVP and CRF-41 in adrenalectomized rats

These experiments were performed to verify the effectiveness of PVN lesions combined with dexamethasone treatment in suppressing parvicellular CRF-41/AVP neurones.

A course of dexamethasone injections that effectively prevented the increase of portal blood AVP in adrenalectomized rats had only a variable, statistically insignificant effect on ACTH. These findings are complementary to those of Plotsky, Otto & Sapolsky (1986) who reported that the acute removal of glucocorticoid feedback had no effect on CRF-41 or AVP in rat pituitary portal blood, whilst ACTH in peripheral plasma was increased. Thus, under the conditions of portal blood collection, glucocorticoid action at the pituitary level appears to be a major determinant of plasma ACTH levels. Alternatively, changes in the rate of release of other CRFs (see Antoni, 1986, for review) or the putative corticotrophin release-inhibiting factor (see Engler *et al.* 1988) may occur.

A somewhat surprising finding is the decrease of CRF-41 in portal blood 7 days after adrenalectomy, in contrast to the increase in AVP. Such a pattern of changes is not without precedent; previous studies on *in-vivo* (Plotsky & Sawchenko, 1987) or *in-vitro* experimental models (Holmes *et al.* 1986b) gave similar results, although the time-intervals after adrenalectomy, and the method of achieving reduced adrenocortical function were somewhat different. All studies concur, however, that adrenalectomy produces a preponderance of AVP over CRF-41 in the hypophysiotrophic CRF-41/AVP system.

It seems possible that anaesthesia with pentobarbitone suppresses stimulatory inputs to CRF-41/AVP neurones. This would explain why the increase in the AVP content of hypophyseal portal blood after adrenalectomy was relatively small, about 1.6-fold, compared with the five-fold increase reported by Koenig, Meltzer, Devane & Gudelsky (1986) who used a similar 5-day time-interval after adrenalectomy but anaesthesia with urethane, and also why CRF-41 levels were actually reduced after adrenalectomy. This possibility is all the more plausible because enhancement of the synthesis as well as the release of CRF-41 appears to occur after adrenalectomy (Alonso, Szafarczyk & Assenmacher, 1986; Young, Mezey & Siegel, 1986; Berkenbosch & Tilders, 1988), and there is evidence that this requires neural inputs which may be suppressed by pentobarbitone (Sawchenko, 1988; Murakami, Akana, Dallman & Ganong, 1989).

The PVN lesions blocked the rise of AVP content in portal blood after adrenalectomy, and this correlated well with the markedly reduced rise of plasma ACTH in these animals. However, AVP content in hypophyseal portal blood was not reduced by the lesion when compared with controls. These findings confirm previous reports measuring AVP release *in vitro* (Knepel, Nutto, Meyer & Vlaskovska, 1984; Antoni, 1986) or AVP mRNA and immunoreactivity *in situ* (see Antoni, 1986, for review) in suggesting that the AVP secretory response to adrenalectomy takes place

in the parvicellular CRF-41/AVP neurones of the PVN.

Treatment with dexamethasone prevented the rise of AVP in portal blood caused by adrenalectomy, but failed fully to return the concentration of CRF-41 to the control value. This conforms with an earlier report (Fink *et al.* 1988), and indicates that the long-term control of CRF-41 and AVP secretory capacity by parvicellular neurones may be differently sensitive to glucocorticoids.

Taken together, the evidence obtained here shows that the combination of PVN lesions and dexamethasone treatment should be sufficient to suppress any compensatory responses in parvicellular CRF-41/AVP neurones that are spared by the PVN lesion.

Plasma ACTH and corticosterone levels during the collection of hypophyseal portal blood

Plasma ACTH and corticosterone levels in peripheral blood were measured to assess the possible impact of the PVN lesion on pituitary-adrenocortical function, and to establish whether there are any differences in the glucocorticoid feedback signal during the periods of portal blood collection. Plasma ACTH was reduced by 40% after PVN lesions, a change that is not proportional to the 90% reduction of the CRF-41 content of hypophyseal portal blood. It is likely that under the conditions of the experiment, pituitary corticotroph cells are driven to near the maximum of their secretory capacity (plateau of dose-response to CRF-41 and AVP). Thus the large drop in CRF-41 levels may not translate into a similar reduction in ACTH levels. A reduced clearance of ACTH from the circulation seems unlikely as an alternative explanation, because ACTH levels declined at a similar rate in PVN-lesioned and sham-lesioned animals after the transection of the pituitary stalk.

Lesioning of the PVN had no appreciable effect on plasma corticosterone levels during portal blood collection. Thus changes of glucocorticoid feedback during the sampling are not responsible for the marked dissociation of AVP and CRF-41 release into hypophyseal portal blood. Substantial reductions (50–60%) of stimulated ACTH levels may occur without any change in plasma corticosterone (e.g. see Szafarczyk, Malaval, Laurent *et al.* 1987), which readily explains why, despite the decrease of plasma ACTH concentration, the PVN lesion had no effect on plasma corticosterone levels.

In summary, the data on ACTH and corticosterone suggest that under the conditions of portal blood sampling, hypothalamic ACTH-releasing factors as well as pituitary ACTH are secreted in excess over what is required for maximal stimulation of their respective target cells.

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Implications of the present observations for hypothalamic function in intact animals

An important issue for consideration is whether the release of magnocellular AVP into hypophyseal portal blood is unique to the PVN-lesioned rat, or does it also occur in intact animals, and if so, is it of functional significance? The results of a number of previous studies where hypophyseal stalk blood was collected from otherwise intact rats can only be satisfactorily explained by assuming that the AVP measured is derived from magnocellular AVP-ergic neurones (Antoni, 1990). The possibility that AVP of magnocellular origin actually enters hypophyseal portal capillaries has been addressed in the past under the assumption that the AVP is of neurohypophyseal origin (Oliver, Mical & Porter, 1977; Recht *et al.* 1981). These studies gave diametrically opposite results. As far as preterminal magnocellular hormone secretion is concerned, it is possible that the AVP released enters the lumen of fenestrated subependymal capillary loops (Page & Dovey-Hartman, 1984) and may contribute to the regulation of adenohypophyseal corticotrophs. As noted earlier, direct morphological demonstration of AVP release by preterminal magnocellular axons in the median eminence has been presented (Buma & Nieuwenhuys, 1988). If magnocellular neurones do indeed participate in the regulation of ACTH secretion, then it should be expected that osmotic stimulation increases plasma ACTH concentration. Unfortunately, this issue is highly controversial (for review see Irving, Alexander & Donald, 1989), primarily because of the variety of experimental approaches used. Importantly, however, in a carefully controlled study, Irving *et al.* (1989) show that in conscious horses acute infusion of hypertonic saline increases plasma ACTH levels, and this increase is highly correlated with the rise of AVP in pituitary venous blood. These data therefore favour the hypothesis of the magnocellular regulation of pituitary ACTH release.

In summary, the destruction of the parvocellular CRF-41/AVP neurosecretory system in the PVN fails to reduce the concentration of AVP in rat hypophyseal stalk blood, while 90% of CRF-41 is eliminated. We conclude that the bulk of AVP in rat hypophyseal stalk blood originates from preterminal axons of supraoptic magnocellular neurones.

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Posttranslational Modulation of Glucocorticoid Feedback Inhibition at the Pituitary Level

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Diagnostic tests of hypothalamic-pituitary-adrenocortical function in psychiatric illness largely report the interaction of hypothalamic secretagogues with glucocorticoids at the pituitary level. This study investigated whether the efficiency of glucocorticoid inhibition is subject to modulation by intracellular processes that enhance cAMP accumulation and/or facilitate membrane depolarization. The secretion of ACTH induced by corticotropin-releasing factor (CRF; 0.1 nM) in primary cultures of rat anterior pituitary cells was markedly inhibited upon a 2-h exposure to 100 nM corticosterone. Arginine vasopressin (2 nM) enhanced the cAMP as well as the ACTH responses to CRF and reduced the efficiency of glucocorticoid inhibition of ACTH release. The action of arginine

vasopressin was mimicked by rolipram, an inhibitor of cyclic nucleotide phosphodiesterase type 4. Application of the broad specificity K⁺ channel blockers clofilium and astemizole produced minor or no significant enhancement of CRF-induced ACTH release, respectively, but opposed the inhibitory effect of corticosterone. Specific blockers of HERG, KCNQ, and Isk channels had no effect on ACTH release under any condition examined. In summary, these data reveal multiple sites of posttranslational modulation of adrenal corticosteroid action at the level of the pituitary gland, which appear important for the outcome of diagnostic tests of hypothalamic-pituitary-adrenocortical function. (*Endocrinology* 143: 3796–3801, 2002)

CORTICOSTEROID feedback inhibition of the hypothalamic-pituitary-adrenocortical (HPA) axis is a key process in the maintenance of body homeostasis (1, 2). It is well established that the anterior pituitary corticotrope cell is a pivotal target of corticosteroid feedback (3, 4). Diagnostic tests of HPA axis function in major psychiatric disorders essentially report secretagogue-glucocorticoid interaction at the anterior pituitary corticotrope cell (5, 6). The cellular mechanisms underlying corticosteroid feedback action remain to be clarified (3, 4, 7). Previous studies of rat anterior pituitary tissue and the mouse corticotrope tumor cell line AtT20 *in vitro* have helped to delineate a model of glucocorticoid inhibition of corticotropin releasing-factor (CRF)-induced ACTH release (3, 4). In AtT20 cells, CRF inhibits large conductance Ca²⁺-activated K⁺ channels (BK channels) through protein kinase A, resulting in sustained depolarization and enhanced exocytosis (8). Protein(s) rapidly (30 min) induced by glucocorticoids enhances protein phosphatase activity in the vicinity of BK channels and thus blocks the action of protein kinase A (9). As a result, intracellular free Ca²⁺ transients are clamped at levels that activate the stress hormone regulated BK channel variant expressed in AtT20 cells (10), but are insufficient for substantially enhanced secretory activity (11). The nature of the rapidly induced protein(s) remains unclear; current candidates include annexin I (7) and dex-ras 1 (12).

Several physiological studies indicate that the efficiency of glucocorticoid feedback inhibition undergoes plastic

changes depending on the stressor stimulus. For example, glucocorticoid feedback inhibition of plasma ACTH levels was not apparent in rats subjected to hemorrhage (13). In rats with chronic autoimmune inflammation, exaggerated and prolonged increases in plasma corticosteroids are vital for survival and involve a resetting of the negative feedback effect on the HPA axis (14, 15). In humans, strenuous exercise gives rise to a dexamethasone nonsuppressor phenotype (16, 17). A common feature of all of the aforementioned paradigms is activation of the hypothalamic vasopressinergic neurosecretory system (18). Indeed, several groups have reported that rat pituitary cells exposed to high, nonphysiological levels of arginine vasopressin (AVP) showed signs of markedly reduced glucocorticoid feedback (19–21). Escape from dexamethasone inhibition upon injection of lysine vasopressin has also been reported in humans (22). The present study investigated the hypothesis that physiological levels of AVP are sufficient to bring about glucocorticoid resistance at the pituitary level *in vitro*. Furthermore, pharmacological tests were carried out to delineate the possible mechanism(s) of action of AVP and corticosterone. The data demonstrate that physiological levels of AVP in combination with CRF induce glucocorticoid resistance of stimulated ACTH release. The reduction of glucocorticoid inhibition correlated with the magnitude of the cAMP response induced by the secretagogues. Finally, clofilium and astemizole, which are blockers of several recently characterized K⁺ channels, also reversed the inhibitory action of corticosterone, but had no apparent effect on cAMP accumulation. Taken together, the data reveal multiple sites of posttranslational modulation of corticosteroid action at the level of the pituitary gland, which appear important for the outcome of diagnostic tests of HPA function.

Abbreviations: AVP, Arginine vasopressin; CRF, corticotropin-releasing factor; HPA, hypothalamic-pituitary-adrenocortical; SOC, store-operated Ca²⁺ channel.

Materials and Methods

Reagents

Reagents for tissue culture and RIAs were previously described (23). Synthetic human 41-amino acid residue CRF and AVP were obtained from Bachem (St. Helens, UK). Astemizole was purchased from Sigma-Aldrich (Gillingham, UK). Clofilium tosylate was obtained from RBI (Sigma, Poole, UK). The following compounds were generously provided by the manufacturers: Rolipram—Schering AG (Berlin, Germany); E4031—Eisai Chemical Co. Ltd. (Kashima-gun, Ibaraki-ken, Japan); dofetilide (UK-68,798)—Pfizer Central Research (Sandwich, UK); WAY-123,398—Wyeth-Ayerst Research (Princeton, NJ); and chromanol 293B—Hoechst-Marion (Frankfurt/Main, Germany).

Cell cultures

Dispersed rat anterior pituitary cells were prepared from the pituitary glands of male Wistar rats (30–40 d postnatally; Charles River Ltd., Margate, UK) as previously described (23). Approximately 5×10^4 cells/well were plated in 24-well tissue culture plates (Falcon, B&D, Le Point de Claix, France) and cultured in DMEM containing 2.5% fetal bovine serum (Harlan-Sera-lab, Belton, Loughborough, UK) and 7.5% horse serum (Sigma, H-1138). Monitoring of the ACTH response to various stimuli was as detailed previously (23). Briefly, corticosterone or vehicle was applied in fresh, serum-free medium 2 h before stimulation with CRF. Blockers of K^+ channels were applied 30 min before and during the 60-min incubation with CRF. In some experiments cellular cAMP content was also measured. The cells were pretreated as described for ACTH release, and the incubation was terminated by aspiration of the medium, immediately followed by the addition of 0.1 M HCl. Cellular cAMP was extracted into 0.1 M HCl by repeated freeze-thawing, acetylated, and quantified by RIA (24).

Data analysis

Data were evaluated by one- or two-way ANOVA, followed by multiple comparisons as appropriate. Results presented as a percentage of control ACTH release were derived from the raw data according to the formula: $(X - B)/(C - B) \times 100\%$, where X is the value analyzed, B is the basal ACTH secretion, and C is the amount of ACTH observed in the presence of a control stimulus.

Results

Vasopressin (2 nM) enhanced the response to CRF (0.1 nM), but failed to stimulate ACTH when given alone (Fig. 1A). Pretreatment with corticosterone produced a dose-dependent inhibition of CRF-induced ACTH release (Fig. 1B). The efficiency of this inhibition was markedly reduced by AVP. The cAMP phosphodiesterase inhibitor rolipram mimicked the effects of AVP on the ACTH response as well as its inhibition by corticosterone (Fig. 2). AVP enhanced the cAMP response to CRF at both 10 and 60 min (Fig. 3A). A series of similar studies with various CRF and AVP concentrations revealed that the ability of 100 nM corticosterone to suppress ACTH secretion markedly diminished as the agonist-evoked cAMP response increased (Fig. 3B).

Plasma membrane K^+ channels are prominent targets of cAMP-dependent phosphorylation (25) and have been previously implicated in the action of glucocorticoids in corticotrope cells (8) as well as in hippocampal pyramidal neurons (26, 27). As previous results with the blocker tetraethylammonium (23) excluded the involvement of a number of well characterized K^+ channels in glucocorticoid action in our experimental system, the class III antiarrhythmic agent clofilium and the antihistamine drug astemizole were tested. These drugs are blockers of HERG, KCNQ, and Isk type channels, all of which are expressed in pituitary cells

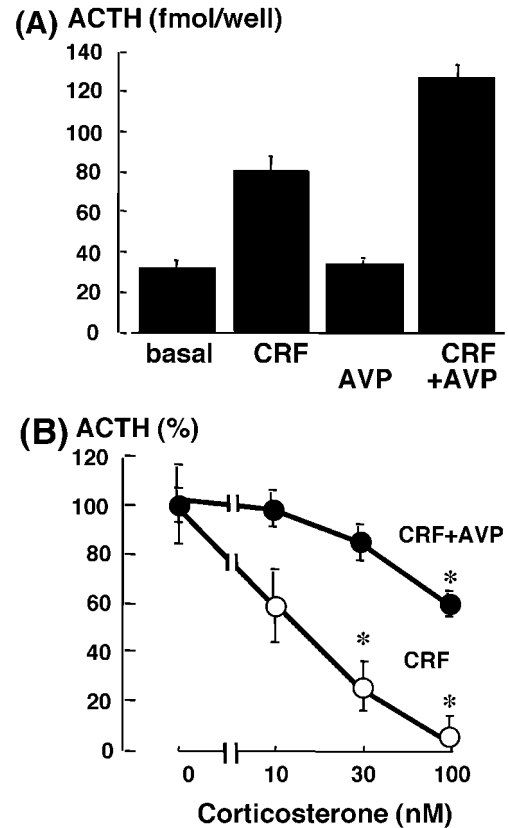


FIG. 1. AVP reduces the efficiency of glucocorticoid feedback inhibition. A, ACTH response elicited by 0.1 nM CRF and 2 nM AVP. Columns represent means; bars indicate the SEM ($n = 4/\text{group}$). The effect of CRF was significant ($P < 0.01$), and there was a significant ($P < 0.05$) interaction between CRF and AVP (by two-way ANOVA). B, Concentration-dependent inhibition of 0.1 nM CRF-evoked ACTH release by corticosterone in the absence and presence of 2 nM AVP. Data are expressed as a percentage of the net ACTH release evoked by the respective control stimulus. Values are the mean $\pm$ SEM ($n = 4/\text{group}$). *, $P < 0.05$ compared with corresponding vehicle-treated group by one-way ANOVA, followed by Dunnett's test. Results shown are representative of eight experiments.

(28). Both agents significantly reduced the efficiency of glucocorticoid inhibition, (Fig. 4), but when applied above $10 \mu\text{M}$ inhibited CRF-induced ACTH release (not shown) and thus were not tested further at concentrations higher than $10 \mu\text{M}$. Most notably, $10 \mu\text{M}$ astemizole was without effect on basal and CRF-induced ACTH release (Fig. 5A), but markedly reduced the inhibitory effect of up to $1 \mu\text{M}$ corticosterone (Fig. 5B). Clofilium modestly enhanced the ACTH response to CRF and also reduced the ability of corticosterone to inhibit CRF-stimulated ACTH release (Fig. 5, C and D). Importantly, neither drug altered the cAMP response to 0.1 nM CRF at 10 or 60 min (not shown), suggesting that the site of action is downstream of cAMP. The involvement of potential targets of clofilium and astemizole, namely, HERG-, KCNQ-, and Isk-type channels, was addressed by applying the selective blockers E4031, dofetilide, WAY-123,398 (all at $1\text{--}10 \mu\text{M}$), and chromanol 293B ($5\text{--}50 \mu\text{M}$), respectively. None of these drugs had a statistically significant effect on basal or CRF-induced ACTH release, and there was also no alteration of the inhibition of 0.1 nM CRF-induced ACTH release by 100 nM corticosterone (data not shown).

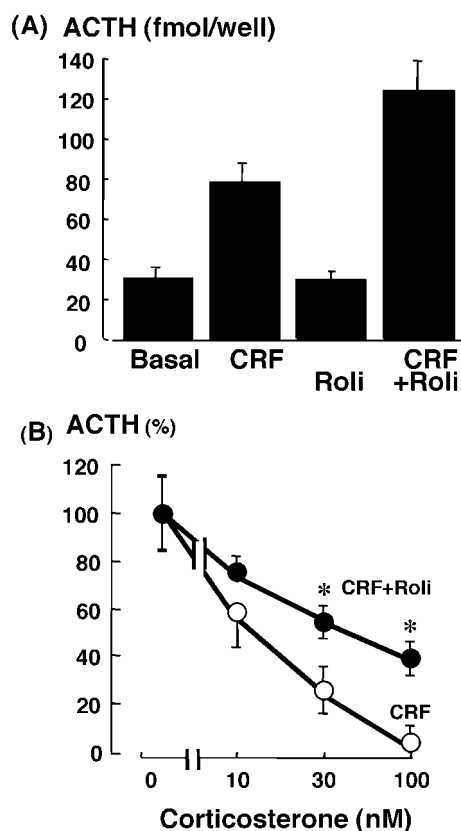


FIG. 2. The cyclic nucleotide phosphodiesterase inhibitor rolipram mimics the effects of AVP. A, ACTH response to 0.1 nM CRF and 0.1 mM rolipram (Roli). Solid columns indicate means; bars indicate the SEM ($n = 4/\text{group}$). The effect of CRF was significant ($P < 0.01$), and there was a significant ($P < 0.05$) interaction between CRF and rolipram by two-way ANOVA. B, Concentration-dependent inhibition of 0.1 nM CRF-evoked ACTH release by corticosterone in the absence and presence of 0.1 mM rolipram. Data are expressed as a percentage of the net ACTH release evoked by the respective control stimulus. Values are the mean $\pm$ SEM ($n = 4/\text{group}$). *, $P < 0.05$ compared with corresponding CRF group by one-way ANOVA, followed by orthogonal contrasts. Results shown are representative of four experiments.

Discussion

These data demonstrate that the agonist-induced cAMP response is a major determinant of the outcome of early gene induction by glucocorticoids. Furthermore, they raise the possibility that novel plasma membrane K^+ channels mediate corticosteroid negative feedback in anterior pituitary corticotrope cells. Collectively, the results highlight the potential for posttranslational modulation of the negative feedback action of adrenal corticosteroids at the pituitary level.

The idea that AVP reduces glucocorticoid feedback was previously proposed (see Ref. 18 for review). However, the issue has not been addressed directly using physiological levels of secretagogue and by examining the effect of a range of corticosteroid concentrations. In a similar *in vitro* model Vale and co-workers (29) showed that the efficiency of inhibition of ACTH release by a 16-h exposure to dexamethasone was dependent on the size of the stimulus: ACTH release induced by 0.01–0.1 nM CRF was significantly more suppressed than that evoked by 1–100 nM CRF. Further *in vitro* studies reported reduced inhibition of ACTH release

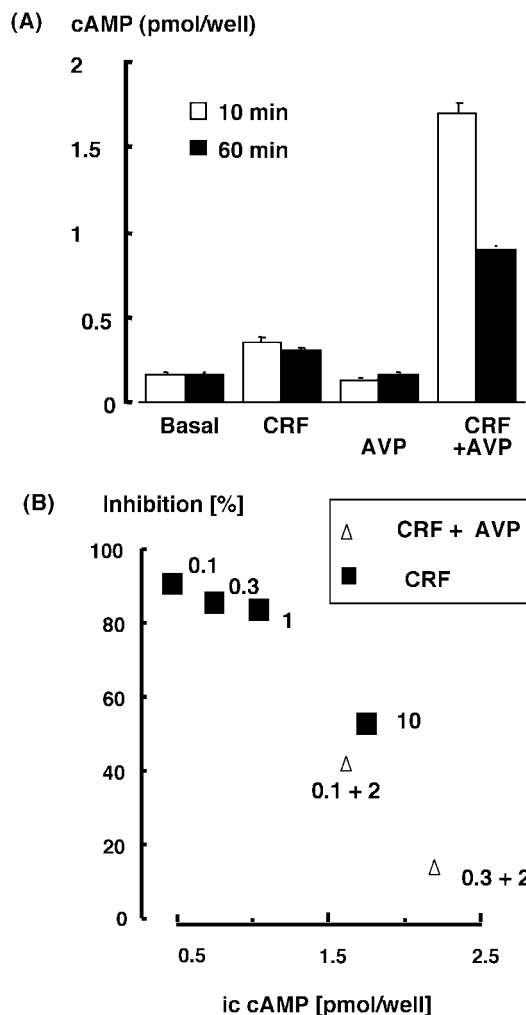


FIG. 3. Intracellular cAMP levels are a principal determinant of the efficiency of glucocorticoid feedback inhibition. A, Potentiation of the cAMP response to CRF by AVP. Columns are the means; bars indicate the SEM ($n = 4/\text{group}$). The basal cAMP level measured at time zero was 153.11 ± 7.44 fmol/well. The effect of CRF was significant ($P < 0.01$) at both time points, and there was a significant ($P < 0.05$) interaction with AVP at both time points by two-way ANOVA. B, The degree of inhibition of stimulated ACTH secretion by 100 nM corticosterone plotted against the intracellular cAMP levels measured at 60 min. CRF or CRF and AVP in combination were used. The numbers indicate the nanomolar concentrations of the respective secretagogues. Data points are the average of four determinations.

upon the introduction of AVP with CRF compared with CRF alone, but the concentrations of the neuropeptides were grossly pharmacological (20, 21). The present study shows for the first time, using physiologically relevant concentrations of CRF as well as AVP, that AVP induces resistance to corticosterone feedback inhibition.

The level of CRF in pituitary portal plasma is maximally 0.3 nM; at this concentration corticotropes are far from maximally stimulated. *In vivo*, further enhancement of the hypothalamic drive is achieved by AVP, which is copackaged and cosecreted with CRF in parvocellular hypophysiotropic neurons of the hypothalamic paraventricular nuclei (18, 30–32). In addition, there is evidence that AVP is released into hypophysial portal blood by the anatomically distinct vaso-

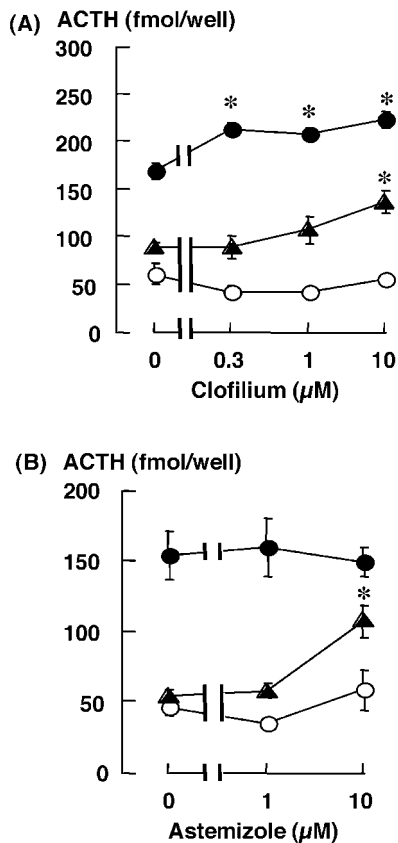


FIG. 4. Clofilium and astemizole attenuate the inhibition of CRF-stimulated ACTH release by 100 nM corticosterone. The effects of clofilium (A) and astemizole (B) on basal (○) or 0.1 nM CRF-evoked ACTH release in the absence (●) and presence (▲) of 100 nM corticosterone (CORT) pretreatment are shown. Data are expressed as a percentage of the net ACTH release evoked by the respective control stimulus. Values are the mean $\pm$ SEM ($n = 3$ –7/group). *, $P < 0.05$ compared with corresponding stimuli in the absence of clofilium or astemizole. Where error bars are not visible, the SEM is less than the space required for the symbol. Results shown are representative of three and two experiments for A and B, respectively.

pressinergic magnocellular neurons of the supraoptic and hypothalamic paraventricular nuclei, which do not synthesize CRF (see Ref. 18 for review). The synthesis of AVP by parvicellular cells has been shown to change dramatically in animal models; for example, chronic stress produces marked increases in AVP synthesis with little or no change in CRF (18, 33). The level of AVP in the hypophysial portal blood of various species ranges between 0.3 and 2 nM (18). Somewhat lower, up to 0.4 nM levels have been reported for human inferior petrosal sinus blood (34). Thus, the concentration of AVP used in the present study represents the upper extreme of the physiological range, characteristic of magnocellular activation (e.g. hemorrhage) or a state of enhanced parvicellular AVP synthesis.

Postmortem analysis of the hypothalamus of patients with depression indicated an increased degree of coexpression of CRF and AVP in the PVN (35). Similar findings were reported with aging (36), which is also characterized by a reduced sensitivity toward glucocorticoid feedback inhibition (37, 38). Acute paradigms, which have indicated an

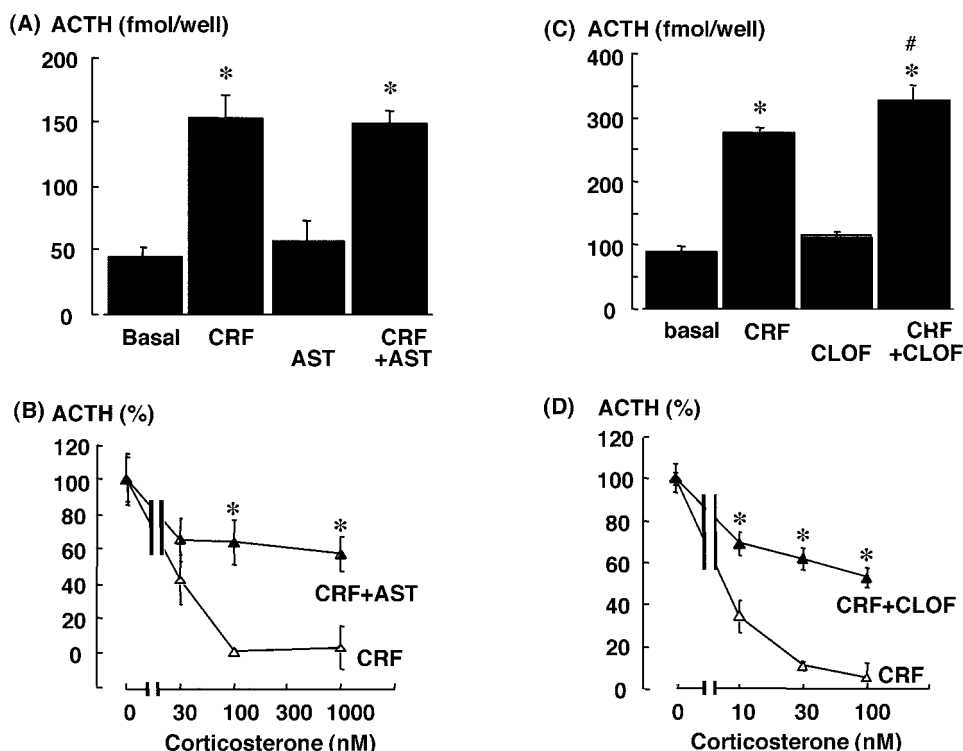
association of dexamethasone nonsuppression in humans with elevated plasma AVP, were listed in the introduction. Taken together, these studies have provided evidence that AVP counteracts glucocorticoid feedback in humans.

The principal mode of action of AVP in corticotroph cells is amplification of the cAMP response to CRF through protein kinase C (18, 39). The enhancement of the cAMP response to low physiological concentrations of CRF by AVP is dramatic, up to 10- to 15-fold in the physiological range, and is characterized by acute plastic changes in the cAMP signaling cascade (40, 41). The augmentation of CRF-induced ACTH release by AVP is modest by comparison with the enhancement of the cAMP response. Thus, it is reasonable to suggest that amplification of the CRF-evoked cAMP has downstream targets other than CRF-evoked exocytosis. Indeed, the present study shows directly that the efficiency of glucocorticoid feedback on stimulated ACTH release is inversely related to the secretagogue-induced cAMP response.

The finding that exaggerated cAMP accumulation and consequently augmented activation of protein kinase A can override glucocorticoid inhibition is in agreement with the hypothesis that glucocorticoid feedback action is mediated by a protein phosphatase that opposes the action of protein kinase A (42). Plasma membrane K^+ channels are common targets of protein kinase A and glucocorticoid inhibition (8, 23). In AtT20 and transfected HEK293 cells, glucocorticoids modulate the activity of a protein phosphatase in the vicinity of the STREX variant BK channel to counteract protein kinase A stimulated by CRF-induced cAMP (42, 43). In rat anterior pituitary cells, depolarization of the membrane potential abrogated glucocorticoid feedback inhibition of stimulated ACTH release (3, 23, 44), which is consonant with the hypothesis that feedback is mediated by plasma membrane K^+ channels. However, no evidence for the involvement of BK or other tetraethylammonium-sensitive K^+ channels could be demonstrated in cultured rat anterior pituitary cells (23). The findings with clofilium and astemizole presented here suggest that atypical, as yet uncharacterized plasma membrane K^+ channels may mediate glucocorticoid feedback inhibition in cultured rat anterior pituitary corticotropes. Specific blockers of HERG-, KCNQ-, and Isk-type K^+ channels, all of which are reportedly expressed in the adenohypophysis (28), failed to alter the effect of corticosterone. It is of note that the concentration of astemizole required to oppose glucocorticoid inhibition was similar to that required for the blockage of store-operated Ca^{2+} channels (SOCs) in GH₃ pituitary tumor cells (45). Intriguingly, dofetilide was inactive in this respect, similar to the present findings (45). It is not immediately obvious how SOCs would be important for early inhibition by glucocorticoids. However, Ca^{2+} entry via SOC could be intricately linked to subplasmalemmal changes in intracellular Ca^{2+} and could be important for the activity of Ca^{2+} -activated K^+ channels that mediate glucocorticoid feedback inhibition (26, 27, 46). Clofilium as well as astemizole inhibit more than one type of K^+ channel (47–50). Thus, it cannot be excluded that their action to oppose glucocorticoid feedback is due to generalized depolarization, which is known to reduce the efficiency of glucocorticoid inhibition (3, 23).

In summary, these data show that the efficiency of corti-

FIG. 5. Efficiency of corticosterone feedback inhibition is reduced by astemizole and clofilium. A, ACTH response to 0.1 nM CRF and 10 μ M astemizole (AST). Solid columns are means; bars indicate the SEM (n = 3–5/group). *, $P < 0.05$ compared with basal by one-way ANOVA, followed by Newman-Keuls test. B, Inhibition of 0.1 nM CRF-evoked ACTH release by corticosterone in the absence and presence of 10 μ M astemizole (n = 4–5/group). *, $P < 0.05$ compared with corresponding CRF group by one-way ANOVA, followed by orthogonal contrasts. The results shown are representative of four experiments. C, ACTH response to 0.1 nM CRF and 10 μ M clofilium (CLOF). Solid columns are means; bars indicate the SEM (n = 4–5/group). *, $P < 0.05$ compared with basal; #, $P < 0.05$ compared with CRF (by one-way ANOVA, followed by Newman-Keuls test). D, Corticosterone inhibition of 0.1 nM CRF-evoked ACTH release in the absence and presence of 10 μ M clofilium (n = 4–5/group). *, $P < 0.05$ compared with the corresponding CRF group by one-way ANOVA, followed by orthogonal contrast of means. Results shown are representative of three experiments.



corticosteroid feedback inhibition may be markedly modified by agents acting on the cellular targets of glucocorticoid-induced proteins. A fundamental determinant of the efficiency of feedback inhibition is the magnitude of the cAMP response evoked by hypothalamic secretagogues. Finally, whether the observed effects of clofilium and astemizole on glucocorticoid feedback are clinically relevant requires further investigation.

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Rapid as well as Delayed Inhibitory Effects of Glucocorticoid Hormones on Pituitary Adrenocorticotrophic Hormone Release Are Mediated by Type II Glucocorticoid Receptors and Require Newly Synthesized Messenger Ribonucleic Acid as well as Protein*

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ABSTRACT. Glucocorticoid hormones suppress the release of ACTH by the anterior pituitary gland: rapid feedback inhibits hormone secretion within 30 min of steroid application, delayed feedback is most effective at 1–2 h, and slow feedback becomes manifest in several hours. The aim of the present study was to determine the type of glucocorticoid receptor that mediates the rapid and delayed feedback actions of glucocorticoids and whether genomic activation occurs during the rapid and delayed time domains.

Rat anterior pituitary cell columns were perfused with Dulbecco's minimum essential medium, 41-residue CRF (10^{-9} M) was used as the secretagogue, which stimulated ACTH secretion to a peak of about 8- to 10-fold of basal release. The amount of ACTH released upon repeated 5 or 10 min stimulation with CRF was constant. Treatment with 10^{-7} M corticosterone for 20 min immediately before and for 10 min during stimulation with CRF reduced ACTH release by about 50% (rapid feedback), while at 1 h and 2 h after the initial exposure to corticosterone the secretory response was 33% and 15% of control, respectively. The effect of corticosterone was prevented by the type II glucocorticoid/progesterone antagonist RU 38486 (10^{-6} M). The selective type II receptor agonist RU 28362 (10^{-7} M) was even more potent than corticosterone in inhibiting ACTH release; the time course of action was similar. When actinomycin D (10^{-4} M) was applied in conjunction with RU28362 or corticosterone, no inhibitory effects appeared up to 2 h after the exposure to steroid. Puromycin (10^{-4} M), given during and for 1 h after the administration of the steroid prevented the rapid as well as the delayed (1 h) inhibitory action of RU28362. When puromycin was removed from the system, a 75% inhibition of stimulated ACTH release developed at 2 h after the application of the steroid, indicating that translatable messenger RNA (mRNA) was still present in the cells. Cycloheximide (10^{-4} M) was only partially effective at inhibiting rapid or delayed feedback, and increasing its concentration impaired the ACTH response to CRF-41.

In summary, at the pituitary level the rapid as well as the delayed feedback inhibition of ACTH secretion by adrenal corticoids is exerted via type II glucocorticoid receptors. Furthermore, both rapid and delayed feedback require the synthesis of new mRNA and protein. (*Endocrinology* 125: 308–313, 1989)

THE NEUROENDOCRINE control loop that regulates the activity of the hypothalamo-pituitary-adrenocortical axis is closed by the negative feedback actions of adrenocortical glucocorticoid hormones (1–3). The inhibitory action of glucocorticoids is exerted at multiple target sites, of which only two have been unequivocally identified to date: neurons in the hypothalamic paraventricular nucleus that produce CRFs and the corticotrope cells in the adenohypophysis (*cf.* Ref. 2).

The suppression of ACTH secretion by glucocorticoid

hormones has been divided into three time domains: rapid, evident within 30 min, delayed, manifest later than 30 min but within 2 h, and slow, apparent after several hours of exposure to glucocorticoids (*cf.* Refs. 1–3). The rationale of discerning these time domains is the assumption that the effects of steroids during these intervals are exerted through different intracellular processes. For instance, the generally held view is that rapid feedback is mediated by a nongenomic action of glucocorticoids, whereas delayed effects are brought about via the well defined cytoplasmic receptors that bind to chromatin and regulate messenger RNA (mRNA) transcription (*cf.* Refs. 1–4).

A further basis for the discrimination of the actions of steroids in target cells is the identification of various types of receptors for these hormones. For instance, the

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actions of glucocorticoids are exerted through at least two types of structurally different receptor molecules designated type I (mineralocorticoid) and type II (glucocorticoid) (5–7). Recently, it has been suggested that rapid and delayed feedback are mediated by different types of receptors (8).

At the pituitary level, slow feedback is known to suppress the expression of the gene that encodes the POMC precursor molecule (4), and to affect aspects of transmembrane signaling by some but not all ACTH secretagogues (9). The exact nature of the cellular actions of rapid and delayed feedback is not known.

In the present study we have examined the type of glucocorticoid receptor involved in rapid and delayed feedback and studied the effects of the inhibition of mRNA and protein synthesis on the action of the steroids.

Materials and Methods

Chemicals

Rat CRF-41 was supplied by Peninsula (St. Helen, U.K.); actinomycin D, aprotinin, corticosterone, cycloheximide, DNase I, HEPES, lima bean trypsin inhibitor, puromycin were from Sigma (St. Louis, MO); trypsin TRK came from Worthington Biochemical Corp (Freehold, NJ); and BSA (Miles Tissue culture grade) from Miles Laboratories (Elkhart, IN). The synthetic steroids RU38486, a type II glucocorticoid/progestin antagonist, and RU28362, a selective type II agonist, were generously supplied by Roussel Uclaf, Romainville, France.

Perfusion of anterior pituitary cell columns

For each experiment 8–10 randomly cycling female Wistar rats 250–350 g body wt (Harlan-Olac, Bicester, U.K.) were killed by decapitation.

Anterior pituitary cells were isolated by modifying previously published procedures (10–12). The anterior pituitary lobe was separated from the posterior lobe and minced with a razor blade to approximately 0.5 mm diameter or smaller segments. The tissue fragments were placed in Dulbecco's minimum essential medium (MEM) (GIBCO, Grand Island, NY) buffered with 22 mM HEPES at pH 7.4 containing 2.5 g/liter trypsin, 2.5 g/liter BSA, 1 mg/liter DNase I (0.5 ml solution per gland) and incubated at 37°C for 20 min. To facilitate dispersion the tissue segments were aspirated into a 5 ml Pipetman (Gilson, Worthington, OH) tip every 5 min, and after 20 min they were triturated with a 5 ml plastic pipette (Sterilin, Hounslow, U.K.) equipped with a 1 ml Pipetman tip. The suspension was filtered over 100 μ m nylon mesh and centrifuged at $100 \times g$ for 10 min at room temperature. The pellet was resuspended in 5 ml Dulbecco's MEM-HEPES containing 2.5 g/liter BSA, 1 mg/liter DNase I, 0.5 g/liter lima bean trypsin inhibitor, 10^6 kallikrein units/liter aprotinin, and centrifuged again. The final pellet was resuspended in 1 ml Dulbecco's MEM-HEPES and 2.5 g/liter BSA and mixed with 1 ml swollen Sephadex G-10

cell columns (11) which were kept in a thermostated incubator at 37°C. The volume of the column was adjusted to 0.6 ml, and perfusion was started with a multichannel peristaltic pump at a rate of 1 ml/5 min. Fractions of the effluent were collected every 5 min on ice. The transit time from the medium reservoir to the fraction collector was 1.5 min. After an equilibration period of 2 h, various treatments were applied.

Fifty microliter aliquots of the effluent were assayed for immunoreactive ACTH content using antiserum no. 6 (courtesy of G. B. Makara, Budapest, Hungary) by RIA (13) with double antibody precipitation. The assay standard human ACTH₁₋₃₉ was kindly provided by CIBA-Geigy A.G. (Basel, Switzerland).

Data analysis

After a 5 min exposure to secretagogues the concentration of ACTH in the column effluent increased in three consecutive 5 min fractions. Net ACTH release is therefore defined as $(X + Y + Z)/3(B)$, where X is the fraction collected during the presence of stimulus (1.5 min transit time effluent discarded), Y and Z are the next consecutive 5 min fractions, and B is the basal value immediately before the application of a stimulus. In the case of 10 min stimulation with CRF, net ACTH release was calculated similarly from four consecutive 5 min fractions. There was substantial variation in the net release of ACTH induced by CRF-41 between columns in different experiments which made it necessary to standardize the data.

In cases where data analysis was between columns, net ACTH release is expressed as percentage of the net ACTH release elicited by 1 nM CRF-41, which was applied to all columns. Statistical evaluation was by one-way analysis of variance and Dunnett's test for multiple comparisons.

Comparisons within columns were made in experiments depicted in Figs. 2 and 3. Here, data are expressed as percentage of the total amount of ACTH released by the respective column during the entire period of sampling. Statistical evaluation was by one-way analysis of variance with correction for repeated measures followed by Dunnett's test.

Results

Response to CRF-41

Figure 1 shows the dose-response relationship of net ACTH release and 5 min pulses of CRF-41 (10^{-10} to 10^{-8} M). In all subsequent experiments CRF-41 was used at the intermediate concentration of 10^{-9} M. Repeated applications of 10^{-9} CRF-41 released similar amounts of ACTH up to 6 h (Fig. 2).

Characterization of the receptor mediating glucocorticoid action

Application of corticosterone for 25 min suppressed stimulated but not basal ACTH release in a time-dependent manner; the effect at 25 min was not statistically significant but was marked at 1 and 2 h after the withdrawal of the steroid (Fig. 3). If CRF-41 was applied for 10 min instead of 5, the rapid inhibitory action was more

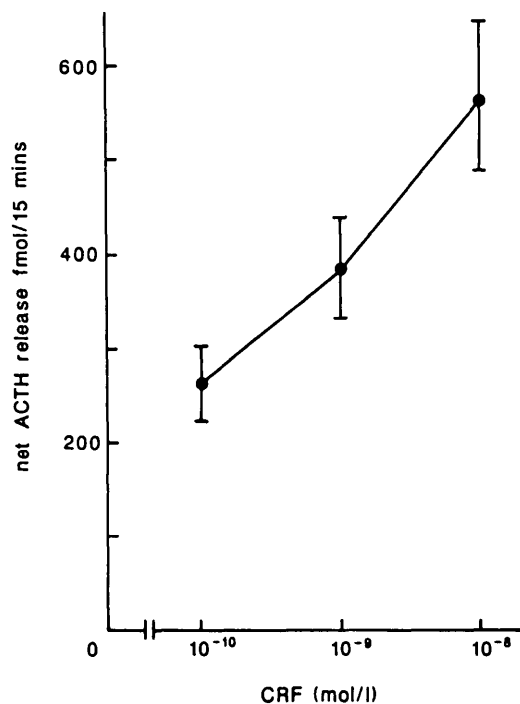


FIG. 1. The release of ACTH by perfused anterior pituitary cells in response to CRF-41. Four columns of cells were exposed to various concentrations of CRF-41 for 5 min in a randomized complete block design. Data are means of the net release of hormone; bars indicate the SE. The basal rate of secretion in this experiment was 130 ± 10 fmol/15 min ($n = 12$).

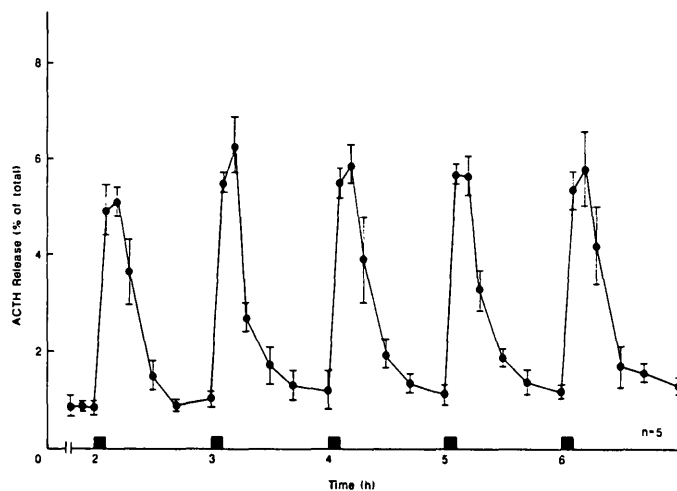


FIG. 2. The release of ACTH by perfused anterior pituitary cells in response to repeated stimulation with 10^{-9} M CRF-41. Data are expressed as the percentage of the total amount of ACTH detected in the fractions assayed from the respective columns. This procedure was necessary to compensate for the interexperimental variability of ACTH release. Data are means $\pm$ SEM; ■, application of CRF-41.

prominent, reducing net ACTH release by 50% (Fig. 4). The type II glucocorticoid/progesterone antagonist RU38486 blocked the action of corticosterone (Fig. 4). The highly selective type II glucocorticoid agonist RU28362 potently suppressed CRF-41-induced ACTH release by 60% within 25 min of application, the degree of inhibition increasing to 90% by 2 h (Fig. 5).

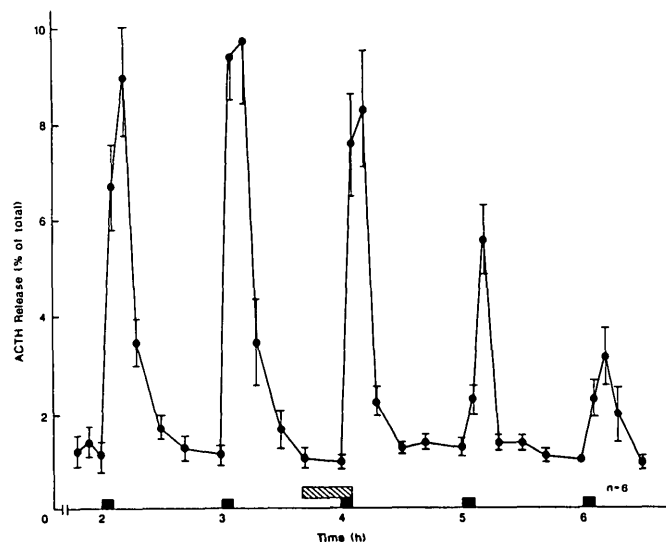


FIG. 3. Effect of 10^{-7} M corticosterone on CRF-41-stimulated ACTH release by perfused anterior pituitary cells. Corticosterone was applied for 25 min as indicated by ■; ■ show application of 10^{-9} M CRF-41 for 5 min. Data are expressed as percentage of the total amount of ACTH detected in the fractions assayed from the respective columns. The points represent means; the bars show the SE. The effect of corticosterone was significant ($P < 0.01$) at 5 and 6 h.

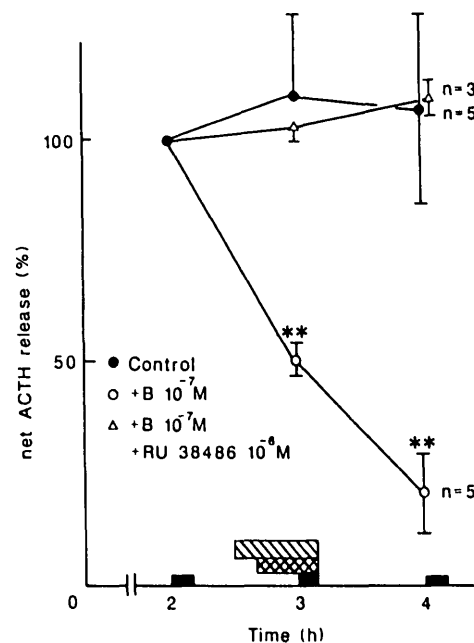


FIG. 4. Antagonism of the feedback action of corticosterone by RU38486 in perfused anterior pituitary cells. Data are expressed as percentage of the net amount of ACTH released during the first 10 min exposure to 10^{-9} M CRF-41. The points are means; the bars show the SE. ■, RU38486 (10^{-6} M), ■, corticosterone (10^{-7} M); ■, CRF-41; **, $P < 0.01$ when compared with the control group.

Effects of inhibitors of mRNA and protein synthesis on rapid and delayed feedback.

As shown in Fig. 6, the lag time between the application of RU28362 and the appearance of the inhibition of ACTH release is greater than 15 min even if the dose of steroid is increased.

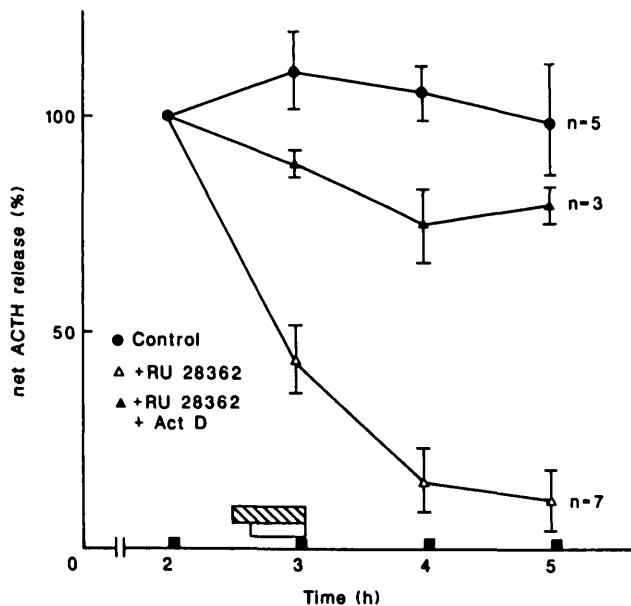


FIG. 5. Effect of RU28362 on ACTH release induced by CRF-41 in perfused anterior pituitary cells. Data are expressed as percentage of the net amount of ACTH released after the first 5 min exposure to 10^{-9} M CRF-41. ▨, Actinomylin D 10^{-4} M, 35 min; □, RU28362 (10^{-7} M) 25 min; ■, CRF-41 (10^{-9} M) 5 min. The glucocorticoid effect was significant ($P < 0.01$) at all time points. At 4 h the RU28362 + actinomycin D values are significantly different ($P < 0.05$) from control.

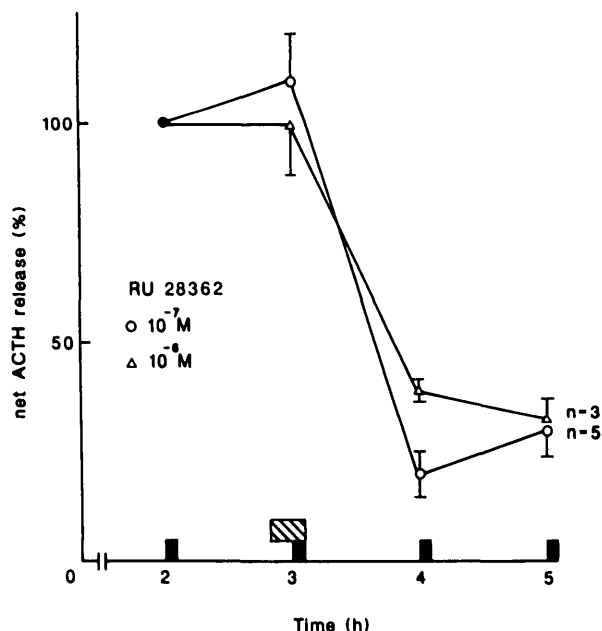


FIG. 6. Evidence for a lag time in the action of RU28362 on stimulated ACTH release in perfused anterior pituitary cells. The points are means; the bars show the SE. ▨, RU28362 for 15 min; ■, CRF-41 10^{-9} M for 5 min.

The rapid as well as the delayed inhibitory action of RU28362 could be almost completely abolished by treatment with an inhibitor of mRNA synthesis, actinomycin D (Fig. 5). Similar data were obtained using corticosterone (not shown).

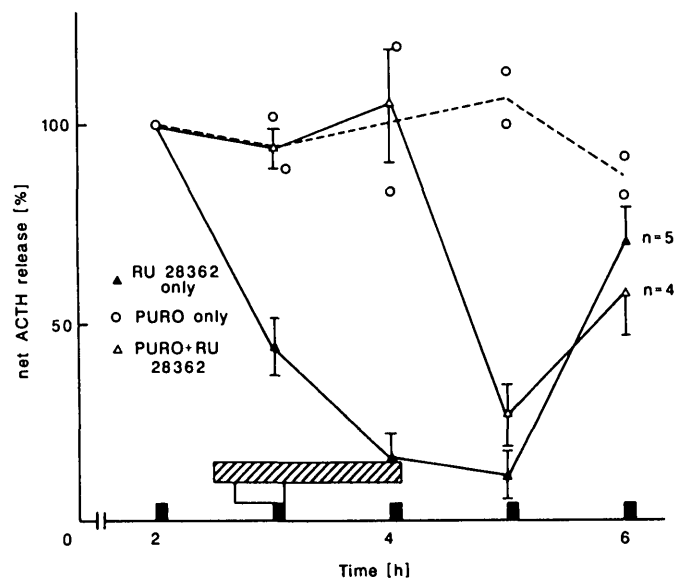


FIG. 7. Effect of puromycin on rapid and delayed feedback inhibition of stimulated ACTH release in perfused anterior pituitary cells. Data are expressed as percentage of the amount of ACTH released in response to the first CRF-41 stimulus. The points are means; the bars show the SE; in the case of the group receiving puromycin only, individual values from two columns are shown. ▨, Puromycin (10^{-4} M) for 95 min; □, RU28362 (10^{-7} M) for 25 min; ■, CRF-41 (10^{-9} M) for 5 min. The RU28362 group contains data presented in Fig. 5.

No rapid or delayed (1 h after steroid) inhibitory effect of RU28362 appeared in the presence of puromycin, an inhibitor of protein synthesis (Fig. 7). However, a marked suppression of ACTH release developed within 1 h after the removal of the drug (Fig. 7) indicating the presence of translatable mRNA. The hormonal response to CRF-41 returned toward normal by 3 h after steroid exposure in both RU28362- as well as RU28362- and puromycin-treated cell columns.

In experiments with cycloheximide (10^{-4} M), another blocker of protein synthesis, a partial blockade of rapid as well as delayed inhibition by RU28362 (10^{-7} M) was observed: At 20–25 min net ACTH release in cycloheximide-treated columns was 72 and 76% of control vs. 12–68% of which is the 99% confidence interval of the group treated with RU28362 only (presented in Fig. 7); at 80–85 min 53 and 65% vs. –11 to –16%, (after this stimulus cycloheximide was discontinued); at 140–145 min 20 and 22% vs. –5 to –23%; at 200–205 min 58 and 65% vs. 54–86%.

When the concentration of cycloheximide was increased to 5×10^{-4} M, the CRF-41-induced release of ACTH became highly variable, suggesting toxic side-effects (not shown).

Discussion

The present study shows that the glucocorticoid inhibition of CRF-41-stimulated ACTH secretion by adeno-hypophysial corticotropes is mediated via type II recep-

tors and requires newly synthesized mRNA and protein.

The complexities of the study of glucocorticoid feedback *in vivo* make it necessary to devise simpler *in vitro* models to analyze the cellular mechanism of action of glucocorticoid hormones in corticotrope cells. Previous work in this area has produced widely discrepant findings, *e.g.* the lag time of the onset of the steroid effect has been reported to be anywhere from virtually immediate (14) to nonapparent until 24 h (15). It is likely that the diversities of these reports are due to the use of different experimental models, ranging from AtT-20 tumor cells to hemipituitaries and a similarly wide variety of stimuli of ACTH secretion. Furthermore, as pointed out by Johnson *et al.* (16) "The magnitude and, in some cases, the direction of the glucocorticoid response are sensitive to the conditions to which the cells are exposed." It seems important to note in this context that under *in vivo* conditions glucocorticoid administration reduces the plasma ACTH response to CRF-41 within 10–15 min (17, 18).

Regarding the time course of the inhibitory effects of steroids on CRF-41-induced ACTH release, our data are in agreement with the majority of previous studies reporting lag times between 10–30 min for rapid feedback *in vitro* (8, 11, 19–22). In our hands corticosterone was effective within 30 min, RU28362 within 25 min. The somewhat longer lag time of corticosterone is possibly due to the fact that it is a substantially less potent activator of type II receptors than RU28362 (23, 24). If a smaller stimulus of ACTH release is used, *e.g.* CRF-41 at 0.1 nM, corticosterone is also effective within 25 min (Dayanithi, G., and F. A. Antoni, unpublished data).

It is of interest that RU28362, a steroid not bound by transcortin (23), also exhibited a significant lag time of action. Previously, it could be argued that the relatively slow nature of the onset of the corticosterone effect is due to intracellular buffering by transcortin-like binders (*cf.* Refs. 1–3).

The results with the antagonist RU38486 [type II/type I selectivity ~1000-fold (24)] and the agonist RU28362 [type II/type I selectivity > 3000-fold (23)] strongly suggest the exclusive involvement of type II glucocorticoid receptors in the rapid as well as delayed pituitary feedback actions of adrenal steroids. Regarding delayed feedback these findings are in agreement with previous preliminary reports (25, 26). In contrast, Abou-Samra *et al.* (8) concluded, on the basis of the differences in the EC₅₀ values for corticosterone at 40 min and 3 h after application in the incubation medium, that rapid and delayed effects are mediated by different receptor types. However, no selective receptor ligands were used in their studies.

On the whole, our findings strengthen the notion that the suppressive actions of glucocorticoids in stress are

mediated by the type II receptor or a receptor species closely related to it (7, 27).

While it is generally agreed that delayed glucocorticoid feedback requires mRNA synthesis, the issue whether it is important for rapid effects is controversial. Portanova and Sayers (28) reported that rapid steroid feedback (30 min) in normal pituitary cells is not blocked by actinomycin D, whereas that in cells from adrenalectomized rats is abolished by the drug. We find a clear inhibition of rapid as well as delayed feedback effects with actinomycin D in cells obtained from intact rats. Apart from the differences in experimental methods, *e.g.* a 20-fold higher concentration of actinomycin D was used by us, and perfused cells in Dulbecco's MEM-HEPES *vs.* static incubations in Krebs-Ringer solution *etc.*, we have no explanation for this difference.

Controversies in the literature are also evident regarding the need for ongoing protein synthesis in rapid feedback. In general, cycloheximide is not an effective inhibitor of the rapid effects of glucocorticoids despite an almost complete suppression of total pituitary protein synthesis (8, 19, 29). We found cycloheximide at 10⁻⁴ M to be only partially effective even in the case of delayed feedback, whereas a higher concentration impaired the secretory response to CRF-41. Brattin and Portanova (19) showed puromycin to be an effective inhibitor of rapid feedback in pituitary cells of adrenalectomized rats. We demonstrate here that puromycin abolishes both rapid and delayed feedback in cells of intact animals without any deleterious effects on stimulated ACTH release. Furthermore, delayed feedback can be demonstrated after the withdrawal of puromycin, indicating that steroid-induced mRNA is present and cellular functions are restored toward normal after the prolonged exposure to puromycin.

In summary, under the conditions used in the present study, the mechanism of rapid and delayed feedback appeared identical in terms of receptor pharmacology and sensitivity to inhibitors of protein and mRNA synthesis. It is only the degree of inhibition of ACTH release, increasing progressively with time, that sets apart rapid and delayed feedback in this system. The rapidity of the onset of the negative feedback mechanism is indicative of a primary induction of mRNA transcription that does not require the synthesis of additional transcription factor proteins (30).

We conclude that the early (0.5–3 h) feedback inhibition of pituitary ACTH release by glucocorticoids is mediated by a newly synthesized protein that suppresses hormone release induced by CRF-41.

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Pituitary Corticotrope Tumor (AtT20) Cells as a Model System for the Study of Early Inhibition by Glucocorticoids*

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ABSTRACT

The utility of the established ACTH secreting mouse pituitary tumor cell line AtT20 for investigating early glucocorticoid inhibition was examined. Three different strains of the cell line D1, D16v, and D16:16, respectively, were analyzed. In initial studies CRF and phorbol esters were used as secretagogues to examine the properties of hormone secretion. In a perfusion system (cells in suspension) D1 cells failed to respond to the secretagogues, whereas both D16v and D16:16 cells were responsive. However, hormone release declined upon repeated exposure to secretagogue in both D16v and D16:16 cells and similar data were obtained when cells adhering to cover slips were perfused. In static incubation D16:16 cells gave more consistent results especially with respect to inhibition by glucocorticoids and were used in all subsequent studies. Synthetic glucocorticoids acting through the type II receptor inhibited CRF-induced ACTH release within 45 min; at 120 min, stimulated release was strongly (80–90%) suppressed. In contrast, no consistent inhibition by corticosterone could be found. In the presence of glycyrrhetic acid, an inhibitor of 11 β -hydroxysteroid dehydroge-

nase, a high concentration of corticosterone (10 μ M) did produce a slight inhibition of ACTH release. Dexamethasone also inhibited ACTH release induced by the calcium channel activator compound (+)202–791. The accumulation of cAMP in response to CRF was not altered by dexamethasone. The inhibitory effect of synthetic glucocorticoids on ACTH release was prevented by blockers of messenger RNA (actinomycin D, dichlorobenzimidazole ribofuranoside) or protein (puromycin) biosynthesis, indicating the induction of new proteins. Immunoblotting for lipocortin I (annexin I) and chromogranin A revealed no induction by dexamethasone of any of these proteins in D16:16 cells. Messenger RNA encoding lipocortin I was not detectable and was not induced by treatment with dexamethasone in D16:16 cells. These data show that the AtT20 D16:16 strain is a useful model for early glucocorticoid action, which is mediated by type II receptors and involves the induction of new protein(s). Notably, induction of lipocortin I messenger RNA or protein could not be detected at a time when the inhibitory effect of glucocorticoids on stimulated hormone secretion was maximal. (*Endocrinology* 131: 2873–2880, 1992)

GLUCOCORTICOID hormones suppress the secretion of ACTH by anterior pituitary corticotropes *in vivo* and *in vitro* (see Ref. 1 for review). The action of glucocorticoids in the pituitary gland occurs in two main phases, which will be referred to as early (0.5–2 h) and slow (>6–8 h) feedback inhibition. Early glucocorticoid inhibition is mediated by type II glucocorticoid receptors and requires the induction of new protein(s) (2–4). The nature and function of the new protein(s) is not precisely known, although recent data suggest that early glucocorticoid inhibition is caused by the suppression of stimulus-evoked intracellular calcium signals (5, 6). Slow inhibition involves the repression of the biosynthesis of proopiomelanocortin (see Ref. 7 for review) and possibly the inhibition of cAMP generation by corticotropin-releasing factor (CRF) (8).

The low proportion of corticotropes in the adenohypophysis and the fact that virtually all cells of the gland are responsive to glucocorticoids make the biochemical charac-

terization of newly induced proteins and messenger RNAs (mRNA) in corticotropes extremely difficult. A more viable alternative would be the use of a nominally homogeneous population of cells such as the AtT20 cell line derived from a radiation-induced anterior pituitary tumor (9). This cell line currently has three strains: 1) D1 is the cell line first derived from the tumor and maintained as a suspension culture (10); 2) the D16v strain is a line derived from the D1 cells and grows as a monolayer (10, 11), and 3) the D16:16 strain subcloned from D16v cells on the basis of high intracellular β -endorphin content (12). The signalling pathways activated by secretagogues such as CRF and the mechanism of slow glucocorticoid inhibition (>24 h) have been extensively characterized (for reviews see Refs. 7, 13, 14). In contrast, only a few studies have addressed the mechanism of early glucocorticoid inhibition (12, 15, 16), and the findings reported are controversial on several counts. In particular, the time course of the inhibition, the requirement for protein induction, and the secretagogues used for eliciting ACTH release were all different in these experiments. The aim of the present study was to analyze early glucocorticoid inhibition of stimulated ACTH release in AtT20 cells in order to determine whether these cells could be a useful model for the characterization of proteins induced by glucocorticoids in anterior pituitary corticotropes.

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Materials and Methods

Reagents

All culture media were obtained from GIBCO BRL (Paisley, Scotland); sera were from GIBCO and Seralab (Crawley Down, Sussex). BSA (fraction V, pH 7) was from ICN (High Wycombe, Bucks, U.K.). Antibiotics and phorbol-dibutyrate ester (PdBu) were obtained from Sigma (Poole, Dorset, U.K.); ACTH and rat 41-residue corticotropin-releasing factor (CRF) from Bachem UK, (Saffron Walden, Essex, U.K.). The synthetic glucocorticoid type II receptor agonist RU28362 (17) was donated by Roussel Uclaf, Romainville, France; the L-type calcium channel activator (+)202-791 (18) was a gift from Sandoz (Basel, Switzerland), nimodipine, a dihydropyridine blocker of calcium-channels was from Semat (St Albans, Hertfordshire, U.K.). Mouse monoclonal antibody (MAB 105) against human recombinant lipocortin (annexin)I and recombinant protein were kindly provided by Biogen Inc. (Boston, Ma) (19). A polyclonal rabbit antiserum against bovine chromogranin A was kindly supplied by Dr. D. K. Apps, Department of Biochemistry, University of Edinburgh, Edinburgh, U.K. All other substances were of the highest analytical grade available.

Cell Culture

AtT20 D1 (passage unknown) cells were obtained from the ATCC cell line, through PHLS (Porton Down, Salisbury, U.K.), and maintained in suspension culture in Ham's F10 media supplemented with 10% fetal bovine serum (FBS) and grown in an atmosphere of 5% CO₂/95% air. Cells were passaged at a ratio of 1:4 every 7 days. AtT20 D16:16 (passage 13) cells were provided by Dr. S. L. Sabol, N.I.H., Bethesda, Md. (12); AtT20 D16v (passage 21) were from the laboratory of K. Docherty, University of Birmingham, Birmingham, U.K. These cultures were routinely maintained in monolayer in Dulbecco's modification of Eagle's medium with 25 mM glucose, supplemented with 10% FBS and grown in an atmosphere of 5% CO₂/95% air. Media changes were carried out every 4–5 days. D16 cells were passaged at a ratio of 1:4 every 7 days, using PBS (pH 7.4), containing EDTA (0.025%) and glucose (10 mM). All experiments were performed with cells of passage 35 or less. In order to optimize the performance of D16 cells in hormone secretion studies, various batches of serum were tested for rate of cell growth, ACTH secretory response to CRF, and its inhibition by dexamethasone. The batch of FBS used in this study contained 30 nM cortisol as measured by RIA.

Hormone secretion

Static incubation. For static ACTH release experiments, the cells were seeded onto polystyrene 24-well tissue culture plates at a density of 5×10^4 cells per well and grown for 6–7 days, with a medium change on day 3 or 5. Experiments were performed in Dulbecco's modification of Eagle's medium, buffered with HEPES (25 mM), pH 7.4, containing 0.25% BSA, and penicillin (100 U/ml)/streptomycin (100 U/ml) (medium A). Before experimental incubations, the cultures were washed free of serum-containing medium and equilibrated with medium A for 2 h at 37°C. Experimental incubations were carried out in a water-bath at 37°C, for the times and with concentrations of drugs indicated in the figure legends. Incubation was stopped by placing the culture plates on ice for 10–15 min. An aliquot of medium was taken from each well, centrifuged at 800 rpm for 10 min at 4°C, and the supernatants obtained stored at –40°C. Immunoreactive ACTH was measured by double antibody precipitation RIA (4) using a primary antiserum (AS846 courtesy of Dr. G. B. Makara, MTA KOKI, Budapest, Hungary) that weakly cross-reacts with α -MSH (13%) (20). Under the conditions employed, the assay detects 1 fmol/tube of human ACTH_{1–39} (courtesy of CIBA-Geigy Ltd, Basel, Switzerland), intraassay coefficient of variation is 11%, interassay coefficient of variation is 12%. For the measurement of monophosphate (cAMP), cells were incubated in the presence of 0.5 mM isobutylmethylxanthine (IBMX). The reaction was stopped by adding ice-cold 0.1 M HCl, and the cells were homogenized by ultrasound disruption. The levels of cAMP in the acidic extracts were measured by RIA using antiserum cAB4 (courtesy of K. J. Catt, NICHD, NIH, Bethesda, MD) (21).

Perfusion. Perfusion of AtT20 cells was performed as described for dispersed rat anterior pituitary cells (4). Briefly, 5×10^6 cells were mixed with Sephadex G-10 and perfused in 0.5 ml chambers with medium A at a flow rate of 200 μ l/min; fractions of the perfusate were collected on ice every 2.5 min. After a 2-h stabilization period, secretagogue pulses were applied as described under *Results*. Alternatively, D16:16 cells were grown on glass coverslips (19 mm diameter), treated with poly-L-lysine at 5 μ g/cm² (Sigma), and perfused at 200 μ l/min in a 0.5-ml perfusion chamber. The release of ACTH per secretagogue pulse was determined as previously described (4).

Preparation of crude pellet and supernatant total protein homogenates

Cells ($1\text{--}2 \times 10^7$) were homogenized by sonication (3×20 sec) on ice in 250 μ l 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, and 0.25 mM phenylmethylsulfonylfluoride and spun at $100 \times g$. The supernatant was centrifuged at $12,000 \times g$ for 30 min at 4°C and the pellet resuspended in 50 μ l of homogenization buffer; both supernatant and pellet fractions were stored at –40°C before use.

Isolation of Ca²⁺-binding proteins from AtT20 D16:16 cells

Calcium binding proteins were isolated using a modification of the calcium precipitation-elution method described by Woolgar *et al.* (22) for the isolation of annexins from mammalian brain. All subsequent steps were performed at 4°C. Cells (approximately 5×10^8) were homogenized by sonication (3×20 s) in 0.5 ml of 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 5 mM EGTA, and 0.25 mM phenylmethylsulfonylfluoride and centrifuged for 30 min at $40,000 \times g$. The supernatant was removed and CaCl₂ added to 1 mM excess (final concentration 6 mM); after 15 min, the sample was centrifuged at $40,000 \times g$ for 30 min. The pellet was washed sequentially with 2 vol of 10 mM HEPES, pH 7.4, 150 mM NaCl, and 1 mM CaCl₂ followed by 10 mM HEPES, pH 7.4, and 1 mM CaCl₂. The pellet was resuspended in 0.1 vol 10 mM HEPES, pH 7.4, 10 mM EGTA, and centrifuged for 30 min at $100,000 \times g$. The supernatant was collected and stored at –40°C before use. Protein concentration was determined using the method of Bradford (23).

Gel-electrophoresis and Western-blotting

Samples were boiled for 5 min in the presence of 2.5% sodium dodecyl sulphate and 5% β -mercaptoethanol and electrophoresed on 10–15% continuous gradient Phastgel sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels as described by the supplier (Pharmacia, Milton Keynes, U.K.). Proteins were transferred to Immobilon PVDF membranes (Millipore, Watford, Herts, U.K.) by electroblotting on the Phastsystem using the protocol supplied.

Membranes were blocked in 50 mM Tris-HCl, pH 7.6, containing 3% low fat milk powder (Marvel) for 1 h. After 5×5 min washes in Tris containing 0.1% Tween-20, blots were incubated overnight at 4°C with primary antibody. Blots were washed for 5×5 min at room temperature in Tris/Tween, incubated with a 1:200 dilution of the appropriate biotinylated antimouse or antirabbit immunoglobulin G antibodies to the primary antibody (Dakopatts, Denmark) in Tris for 1 h, washed 5×5 min Tris/Tween, and incubated with a 1:300 dilution of streptavidin biotinylperoxidase complex (Amersham, Aylesbury, Bucks) for 45 min. Blots were developed after a 3×5 min wash in Tris/Tween using diaminobenzidine tetrahydrochloride hydrate (Aldrich, Poole, Dorset) at a concentration of 0.75 mg/ml in Tris-HCl containing 0.02% H₂O₂. After 5–10 min at room temperature, the reaction was terminated by washing extensively in dH₂O.

RNA isolation and Northern blotting

Total cellular RNA was isolated using the acid guanidinium thiocyanate-phenol-chloroform method (24), mRNA was isolated by two rounds of poly-dT-cellulose chromatography (25). RNA samples and markers were separated by electrophoresis on a denaturing 1% agarose gel containing 6% formaldehyde using $1 \times$ MOPS [(0.04 M morpholin-

nopropane sulphonic acid), 10 mM sodium acetate, 1 mM EDTA, pH 8.0] as buffer. The RNA was transferred onto a Hybond N nylon membrane (Amersham, Bucks, UK) by capillary blotting overnight in 20× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0). RNA was bound to Hybond N by exposing to UV light for 5 min. Blots were prehybridized at 65 C for 15 min in hybridization buffer [10% dextran sulphate (mol wt, 500000, Sigma), 1 M NaCl, 1% SDS]. Hybridization was performed overnight at 65 C using fresh buffer containing 100 µg/ml denatured salmon sperm DNA and 1×10^6 dpm/ml of radiolabeled complementary DNA (cDNA). Blots were subsequently washed in 1× SSC at 65 C for 30 min. Blots were autoradiographed for 5 days for blots probed with the lipocortin I cDNA probe. Blots were stripped and reprobed for α -tubulin and autoradiographed for 5 h for RNA standardization.

cDNA probes

A 1376-base pair human lipocortin I cDNA (Biogen Inc, Boston, Ma) was cut from plasmid pUC 13 with *Eco*R1. The α -tubulin probe was a 1400-base pair human cDNA which was cut from the *Pst*I site of plasmid pSP64. The cDNA was separated from plasmid DNA by electrophoresis on a 1% low melting point agarose gel. Eluted cDNA was denatured by heating at 95 C for 5 min and labeled with 50 mCi of (α - ^{32} P)-dCTP (Amersham, Bucks, UK) by random primer elongation (Amersham) to a specific activity of $1-4 \times 10^8$ dpm/µg DNA.

Results

Perfusion

Initial studies were performed to verify the utility of the AtT20 cell strains in a perfusion model because this system is best suited for examining hormone secretion by normal rat corticotropes (for reviews see Refs. 26 and 27). Both D16v and D16:16 cells responded to 10 nM CRF and PdBu in perfusion using cells suspended in a matrix of Sephadex G-10 or cells maintained as a monolayer on glass coverslips. However, the secretory response declined on repeated application of secretagogue irrespective of the duration of stimulation (2.5–5 min) or interstimulus period (30–60 min) (data not shown). In contrast, D1 cells failed to respond to secretagogues altogether in the perfusion system (data not shown).

Static incubation

Secretagogue action. AtT20 D16:16 cells grown as monolayer on plastic 24-well tissue-culture trays consistently responded to CRF and PdBu (Fig. 1a). The time-course of CRF- and PdBu-stimulated ACTH release was linear over 5–30 min of incubation (data not shown). Stimulus to basal ratios of ACTH release at various time-points became constant after 20 min of continuous exposure to 10 nM CRF or 10 nM PdBu, typically giving a 2-fold increase of ACTH release above basal. The CRF response could be enhanced further by combining CRF with 0.25 mM BaCl₂ (CRF/Ba) (Fig. 1a), which causes depolarization by blocking Ca²⁺-activated K⁺ channels (28,29). The release of ACTH induced by CRF, CRF/Ba, or PdBu could be blocked by the dihydropyridine calcium channel antagonist nimodipine (Fig. 1b). The ACTH response of D16v cells to the secretagogue agents was considerably more variable than that of D16:16 cells; in some experiments, no significant increase of ACTH release could

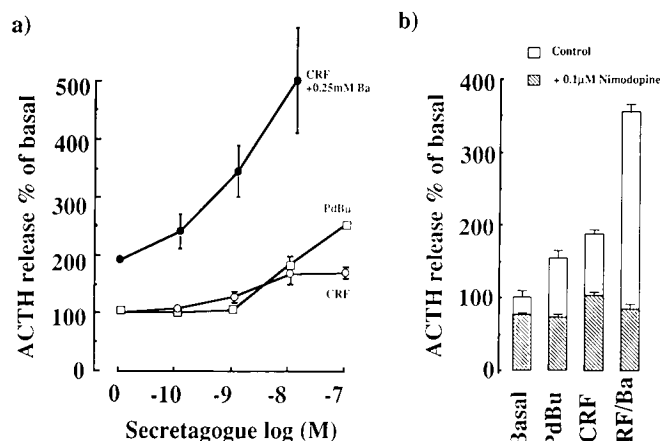


FIG. 1. a, Effects of CRF, CRF combined with 0.25 mM BaCl₂, and PdBu on ACTH release from AtT20 D16:16 cells in static incubation: b, blockage of CRF, CRF/Ba- and PdBu-stimulated ACTH release by the dihydropyridine calcium channel blocker nimodipine. Nimodipine (0.1 µM) was given for 30 min before the application of secretagogue, and was also present during the 20-min stimulus period. All data are expressed as the percentage of basal ACTH release which ranged between 0.7 and 1.4 pmol/well in these experiments. Data are mean $\pm$ SEM (n = 4–8 per datum point).

be demonstrated upon exposure to CRF. Attempts to improve reproducibility by serum-starving D16v cells for various periods (16) were not successful, and therefore all subsequent studies were performed in D16:16 cells.

Early glucocorticoid inhibition. Pretreatment with 10 nM dexamethasone significantly inhibited CRF (10 nM)-stimulated ACTH release within 45 min and increased further after 90 min of pretreatment: basal, 0.44 ± 0.02 ; CRF, 0.97 ± 0.03 ; 45 min dexamethasone+CRF, 0.76 ± 0.04 ; 90 min dexamethasone+CRF, 0.62 ± 0.04 ; data are ACTH pmol/well released in 20 min, means $\pm$ SEM, n = 6 per group. RU28362, a specific type II receptor agonist (17), inhibited CRF-stimulated ACTH release in a dose-dependent manner (Fig. 2). Dexamethasone also inhibited ACTH release evoked by the dihydropyridine L-channel activator (+)202–791: dexamethasone pretreatment (10 nM for 2 h) reduced (+)202–791 (1 µM for 20 min) stimulated ACTH release to $56.0 \pm 10.3\%$ of the control (+)202–791 stimulus. Dexamethasone had no detectable effect on CRF-induced cAMP accumulation (Table 1).

Overall, in 52 experiments, RU28362 or dexamethasone pretreatment (up to 2 h) had no significant inhibitory effect on basal ACTH release, although in some individual experiments a statistically significant inhibition (typically 20%) was found. Hormone release stimulated by CRF or CRF/Ba was invariably inhibited by these synthetic steroids in all experiments. In contrast, corticosterone only inhibited CRF-stimulated ACTH secretion when used at the high concentration of 10 µM and in conjunction with glycyrrhetic acid, an inhibitor of 11 β -hydroxysteroid dehydrogenase (Fig. 3).

Blockers of mRNA (actinomycin D, dichlorobenzimidazole ribofuranoside) or protein synthesis (puromycin) dose dependently prevented the action of RU28362 on CRF/Ba stimulated ACTH release suggesting that the induction of

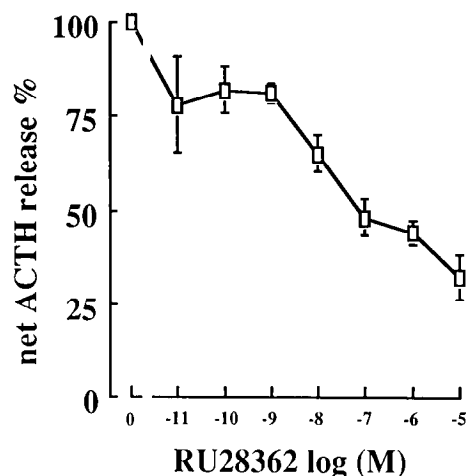


FIG. 2. Inhibition of stimulated ACTH release from AtT20 D16:16 cells by the specific type II glucocorticoid receptor agonist, RU28362. The control stimulus was 10 nM CRF and 0.25 mM BaCl₂ in combination; RU28362 was applied for 2 h before the stimulus at various concentrations. Data are expressed as the percentage of the increment in ACTH release (0.9 pmol/well) evoked by the control stimulus, basal release was 0.3 ± 0.03 pmol/well. Data are means $\pm$ SEM (n = 4 per group).

new mRNA and protein(s) is required for the inhibitory effect (Fig. 4). Similar data were obtained using dexamethasone to inhibit CRF-induced ACTH release (5).

Protein(s) involved in early glucocorticoid inhibition

Lipocortin I mRNA was constitutively expressed in AtT20 D1 cells; however, no induction of lipocortin I mRNA was observed after 24 h of dexamethasone treatment (Fig. 5a). In contrast, lipocortin I mRNA or protein were undetectable in AtT20 D16:16 cells, and no induction of mRNA or protein was evident after a 1.5 h exposure to 10 nM dexamethasone (Fig. 5c and 6), when inhibition of stimulated ACTH release was clearly evident. Chromogranin A (bands at about 74 and 33 kilodaltons) immunoreactive proteins were present in D16:16 cells; however, no increase of the intensity of immunostaining of these protein bands could be observed upon a 1.5-h treatment with dexamethasone (data not shown).

Discussion

These data suggest that the D16:16 subclone of AtT20 cells is a suitable model to study the early inhibitory effects

of glucocorticoids. The hallmarks of early glucocorticoid inhibition: onset within 2 h, mediation by type II receptors, requirement for mRNA and protein synthesis, could be all demonstrated in AtT20 D16:16 cells, which is in agreement with the properties of early glucocorticoid action in a variety of systems, including normal anterior pituitary cells (1–4, 30).

Secretory response

An important feature of early glucocorticoid inhibition in normal corticotropes is that it is restricted to stimulated ACTH secretion (2–4, 31). Thus, an adequate secretory response is important for the study of this effect of glucocorticoids. AtT-20 cells have relatively high rates of basal secretion which is probably due to the frequent spontaneous membrane depolarizations and neuron-like action potentials observed in electrophysiological studies (28, 32, 33), which are correlated, at least qualitatively, with large spontaneous excursions in the concentrations of intracellular free calcium (5, 33). Furthermore, blockage of dihydropyridine-sensitive calcium channels reduces basal ACTH secretion in these cells (34), suggesting that a proportion of basal hormone output occurs via the regulated secretory pathway and thus may be susceptible to inhibition by glucocorticoids. Recent studies in D16v cells using biochemical markers of the regulated and constitutive secretory pathways also show that approximately 60% of unstimulated ("basal") hormone release is derived from the regulated pathway (35). The portion of basal release derived from the regulated pathway may vary from experiment to experiment, which would explain the variability of the effect of glucocorticoids on basal secretion. Whether spontaneous electrical activity is also intrinsic to normal corticotropes remains controversial because the studies reported in the literature so far are discrepant on this point, two suggesting virtually no spontaneous fluctuations of intracellular free calcium (36, 37) and a third reporting rhythmic oscillations of the membrane-potential and temporally correlated large excursions of intracellular free calcium concentrations (38). Possibly because of the relatively high spontaneous activity, in AtT20 cells the size of the secretory response to CRF is rather small, maximally 3-fold in our hands, which is similar or smaller than that reported by others in D16:16 cells (39). One important difference is that in our studies the cells were incubated for only 20–30 min with secretagogue in order to stay within the time

TABLE 1. Effect of dexamethasone on CRF-stimulated accumulation of cAMP

Treatment	pmol cAMP per well					
	Time (min)					
	0'	2'	5'	10'	20'	30'
Basal (4)	3.16 $\pm$ 0.35	3.43 $\pm$ 0.08	3.43 $\pm$ 0.39	3.55 $\pm$ 0.47	3.78 $\pm$ 0.27	3.40 $\pm$ 0.39
10 nM CRF-41 (3)	3.47 $\pm$ 0.23	21.33 $\pm$ 1.05	16.34 $\pm$ 1.99	9.98 $\pm$ 0.74	8.46 $\pm$ 0.23	6.47 $\pm$ 0.23
10 nM CRF-41 + dexamethasone (3)	3.16 $\pm$ 0.35	19.50 $\pm$ 1.56	15.10 $\pm$ 0.78	9.67 $\pm$ 0.59	7.45 $\pm$ 0.66	7.41 $\pm$ 1.05

Data are expressed as pmol of cAMP generated per well for the respective time period. IBMX (0.5 mM) was applied for 15-min before time zero, fresh IBMX was applied with the stimulus at time zero. No accumulation of cAMP in response to CRF was observed in the absence of IBMX. Dexamethasone (10 nM) was applied for 1.5 h before the respective CRF stimulus and had no effect on basal cAMP levels at any time point. Data are means $\pm$ SEM (n = 3–4 per group).

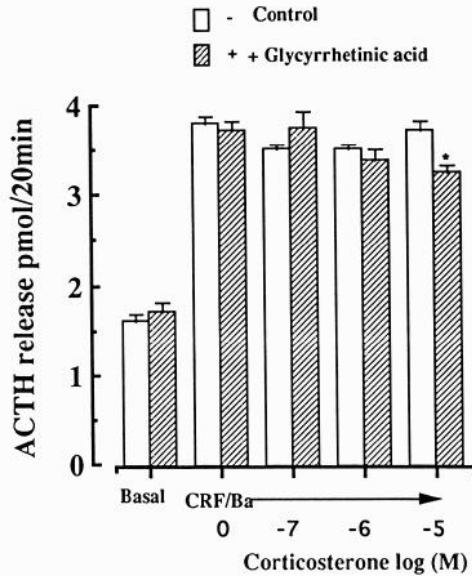


FIG. 3. Effect of corticosterone on CRF/Ba-stimulated ACTH release from AtT20 D16:16 cells. The control stimulus was a 20-min combination of 10 nM CRF and 0.25 mM BaCl₂; corticosterone was applied for 2 h before the stimulus at various concentrations with (shaded box) and without (open box) 10 μ M glycyrrhethinic acid, an inhibitor of 11 β -hydroxysteroid dehydrogenase. Data are means $\pm$ SEM from a representative experiment performed in sextuplicate; corticosterone had no effect on basal ACTH release. (*) $P < 0.05$ compared to 10 μ M corticosterone alone and control CRF/Ba stimulus (one-way analysis of variance, followed by Newman-Keuls test).

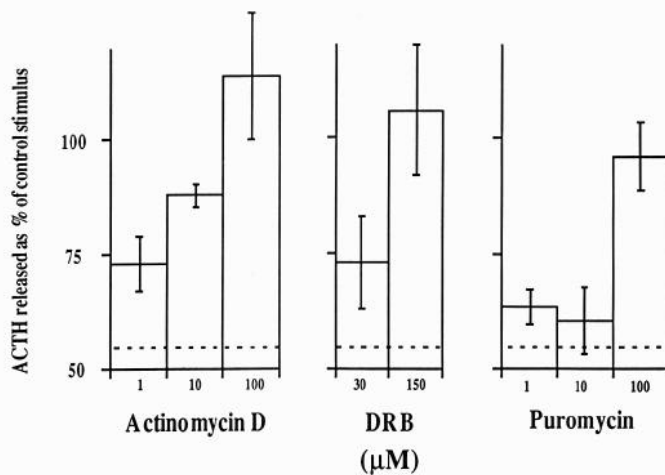


FIG. 4. Blockade of early glucocorticoid inhibition of stimulus-evoked ACTH secretion in AtT20 D16:16 cells by blockers of mRNA transcription, actinomycin D, and dichlorobenzimidazole ribofuranoside (DRB), or the protein synthesis blocker puromycin. The control stimulus was a combination of 10 nM CRF and 0.25 mM BaCl₂; the specific type II receptor agonist RU28362 (100 nM for 2 h) was used as the glucocorticoid. Data are means $\pm$ SEM ($n = 4-8$ per group), expressed as percentage of the amount of ACTH released by cells exposed to the secretagogue stimulus (2.7 pmol/well); the dashed line shows the inhibition achieved by 100 nmol/l RU28362; basal release was $33 \pm 2\%$ of the control stimulus. Blockers were applied at various concentrations for 20 min before RU28362 addition; DRB and puromycin were continued during the exposure to RU28362, whereas actinomycin D was removed after the first 20 min.

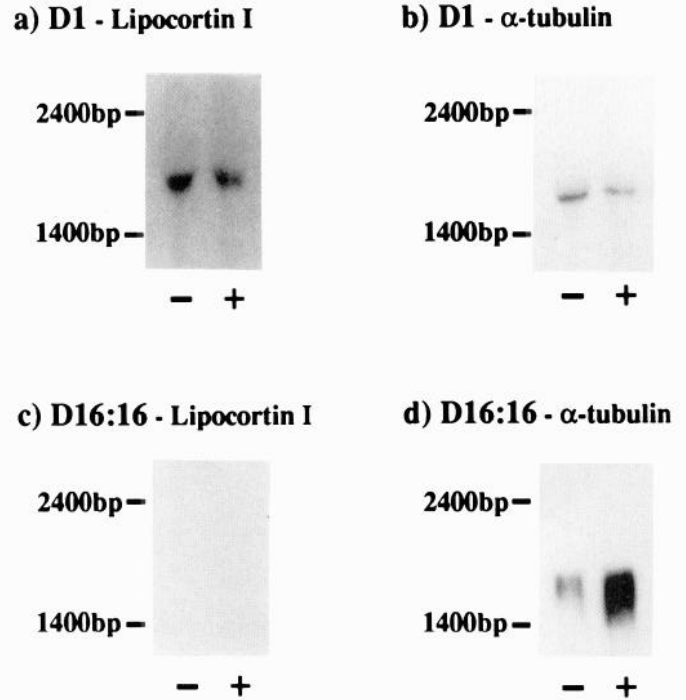


FIG. 5. Northern blotting for lipocortin I mRNA in AtT20 D1 and D16:16 cells. a, Autoradiograph of 20 μ g total RNA from AtT20 D1 cells hybridized with (α -³²P) dCTP labeled lipocortin I cDNA and exposed for 5 days. Cells were incubated for 24 h in the presence (+) or absence (-) of 1 μ M dexamethasone. b, Blot a) rehybridized with (α -³²P) dCTP labeled α -tubulin cDNA and exposed for 5 h. c, autoradiograph of 2 μ g poly(A⁺) mRNA from AtT20 D16:16 cells hybridized with (α -³²P) dCTP labeled lipocortin I cDNA and exposed for 5 days. Cells were incubated for 1.5 h in the presence (+) or absence (-) of 10 nM dexamethasone. d, Blot c) rehybridized with (α -³²P) dCTP labeled α -tubulin cDNA and exposed for 5 h.

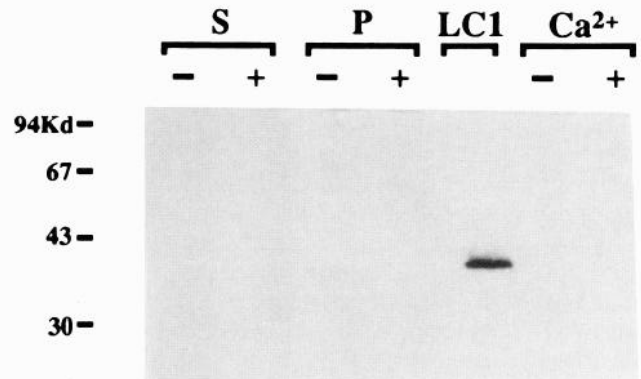


FIG. 6. Immunoblotting for lipocortin I protein in AtT20 D16:16 cells. Cells were incubated in the presence (+) or absence (-) of 10 nM dexamethasone for 1.5 h. Crude supernatant (S) and calcium precipitated (Ca²⁺) samples contained 2 μ g/lane, crude pellet samples (P) contained 1 μ g/lane, and cloned lipocortin I standard (LC 1) contained 25 ng/lane. Samples were electrophoresed on a 10-15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis Phastgel, electroblotted to Immobilon PVDF, and reacted for lipocortin I using monoclonal MAB 105 at 1:1000 dilution.

domain of early inhibition. Other workers usually incubate for longer, 1-3 h, periods in order to attain higher basal to stimulus ratios.

Although some reports have suggested that AtT20 cells respond to the other physiologically important ACTH secretagogue vasopressin (15, 40), most workers, including ourselves, have found no effect of vasopressin in this cell line (13, 26). However, the pathway of secretion operated by activation of protein kinase-C is present in these cells (see Ref. 14 for review) and is susceptible to early glucocorticoid inhibition as reported by Phillips and Tashjian for D16v cells (16).

Both CRF- and phorbol-ester stimulated ACTH release require the entry of calcium into the cells via dihydropyridine sensitive (L -type) calcium channels (34, 41). The combined stimulus CRF/Ba is similarly inhibited by nimodipine (present study); thus, AtT20 cells appear simpler than normal corticotropes (42, 43) with respect to calcium channel pharmacology.

Characteristics of glucocorticoid effect

Overall, the time-course and pharmacology of early inhibition in AtT20 cells are similar to those reported for normal anterior pituitary cells. The onset of inhibition is within 60 min, it is mediated by type II receptors, it requires the synthesis of new mRNA as well as protein (2–4), and finally, the site of inhibition is beyond cAMP generation (44, 45). The most striking discrepancy is the lack of a substantial inhibition by the physiological glucocorticoid, corticosterone. Previous studies have also reported this phenomenon with respect to slow inhibition of basal secretion in D1 cells and have shown that the hormone receptor complex is translocated less efficiently into the nucleus by corticosterone when compared with dexamethasone (46), although the correlation between nuclear translocation and biological action is by no means straightforward in this system (47). In our hands, a small, but significant inhibition by corticosterone could be demonstrated in the presence of glycyrrhetic acid which is a blocker of 11β -hydroxysteroid dehydrogenase (for review see Ref. 48) a major metabolizing enzyme of corticosterone. The possibility that this effect is due to activation of type I receptors by corticosterone or glycyrrhetic acid itself is highly unlikely, because AtT20 D16 (not specified whether 16:16 or 16v) as well as D1 cells only contain the type II receptor (49, 50). Furthermore, RU28362, a very highly selective type II receptor agonist is a powerful inhibitor of stimulus-evoked ACTH release in AtT20 cells showing that early inhibition does not require activation of type I receptors. Hence, it is reasonable to suggest that the low potency of corticosterone is at least partly due to metabolism by 11β -hydroxysteroid dehydrogenase.

A requirement for protein induction, but no change in lipocortin I protein mRNA

Inhibition of DNA transcription with two structurally and mechanistically distinct blockers prevented the early inhibitory effect of glucocorticoids in D16:16 cells. Furthermore, blockage of mRNA translation with puromycin was also effective in this regard, which is in agreement with the study of Phillips and Tashjian in D16v cells (16) as well as previous

work with normal pituitary cells (3, 4). With respect to the nature of the induced protein(s) it was of primary interest to examine the possible role of lipocortin I a purported mediator of early glucocorticoid action in macrophages and other systems (see Ref. 51 for review). Lipocortin I mRNA or protein could not be detected in the D16:16 subclone even after dexamethasone pretreatment. Furthermore, although lipocortin I is present in the anterior pituitary gland, D16v and D1 cells, no evidence for its induction by glucocorticoids has been found (52). Work on the induction of lipocortin I in other systems has also cast doubt on its suggested primary and generic function as a mediator of glucocorticoid action (for example, see Refs. 53 and 54). Among other candidate proteins, chromogranin A, a secretory vesicle associated protein (55, 56), has been implicated in slow (>24 h) glucocorticoid inhibition of ACTH release in AtT20 D16v cells (57), although no evidence for its regulation by glucocorticoids in normal corticotropes has been documented (58). We found no induction of chromogranin A protein in AtT20 D16:16 cells in the time-interval for early inhibition in this system.

In summary, these data show that most of the important properties of early glucocorticoid inhibition observed in normal corticotropes have been retained in AtT20 D16:16 cells. The single significant discrepancy is the low potency of the natural glucocorticoid corticosterone, which is possibly due to the inactivation of this steroid by 11β -hydroxysteroid dehydrogenase in AtT20 cells. Thus, AtT20 D16:16 cells appear to be a suitable model for the characterization of proteins involved in early glucocorticoid inhibition. Importantly, this study also shows that the purported glucocorticoid second messenger protein lipocortin I is not induced by glucocorticoids in AtT20 D16:16 cells at a time when a biologically relevant action of the steroids, the inhibition of stimulated ACTH release, is clearly manifested.

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Inactivation of Early Glucocorticoid Feedback by Corticotropin-Releasing Factor *in Vitro*

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ABSTRACT. We have investigated the interaction between hypothalamic ACTH secretagogues and adrenocortical glucocorticoids in rat anterior pituitary tissue using an *in vitro* perfusion system.

Repeated 5 min pulses of 41-residue CRF (CRF-41) or arginine vasopressin (AVP) were applied at 1 h intervals for up to 7 h. Administration of 0.1 μ M corticosterone 30 min before and during the 5 min 0.1 nM CRF-41 stimulus at 5 h resulted in a significant inhibition of CRF-41 stimulated ACTH release within 30 min. Inhibition of ACTH release also developed if no CRF-41 stimulus was applied in conjunction with steroid at 5 h. In contrast, if the exposure to corticosterone (0.1 μ M, 35 min total duration) was started simultaneously with the application of CRF-41 at 5 h, no inhibition of ACTH release ensued. Similarly, no inhibition of CRF-41-stimulated ACTH release was observed when corticosterone was started simultaneously with a

5 min pulse of cyclic 8-(4-Chlorophenylthio) AMP (8-CPT-cAMP), a cell membrane permeant analog of cAMP.

In contrast to CRF-41 and 8-CPT-cAMP, AVP failed to modify glucocorticoid-induced inhibition of AVP- or CRF-41-stimulated ACTH release. Moreover, CRF-41 did not prevent the glucocorticoid-induced inhibition of AVP-stimulated ACTH release.

In summary: 1) CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion, and this is mimicked by a cell membrane permeant analog of cAMP; 2) AVP does not inactivate glucocorticoid-induced inhibition of stimulated ACTH release; 3) the data point to an acute interaction between the cAMP/protein kinase A and glucocorticoid-responsive intracellular pathways. Such differential modulation of feedback inhibition by CRFs may be of functional importance *in vivo*. (*Endocrinology* 130: 2213–2218, 1992)

CORTICOTROPIN releasing factor-41 (CRF-41) and arginine vasopressin (AVP) are the major physiological hypothalamic regulators of ACTH secretion from anterior pituitary corticotrophs while the adrenocortical glucocorticoids, released in response to elevated ACTH levels, inhibit ACTH release over several time domains (for reviews see Refs. 1–4). At the pituitary level early-onset glucocorticoid inhibition decreases stimulated ACTH release within 30 min; the maximal effect is apparent within 1–2 h of glucocorticoid application. This action is mediated through type II glucocorticoid receptors and requires the synthesis of new messenger RNA as well as protein (5–8).

Using an *in vitro* perfusion model we have reported that the characteristics of early-onset glucocorticoid inhibition of stimulated ACTH release are dependent on both the nature of the secretagogue as well as the temporal relationship between secretagogue and glucocorticoid application. Notably, early-onset glucocorticoid in-

hibition of CRF-41 stimulated ACTH release did not develop if CRF-41 was applied at the start of corticosterone exposure. In contrast, AVP had no such action on early-onset inhibition of AVP-stimulated ACTH release (7).

In this study we have investigated whether CRF-41 blocks early-onset glucocorticoid inhibition and whether the action of CRF-41 is reproduced by its intracellular second messenger cAMP. Furthermore, we have investigated whether there is any interaction between CRF-41 and AVP with respect to the blockage of glucocorticoid-induced inhibition.

Materials and Methods

Animals

Female exbreeder Wistar rats (250–300 g body wt) were obtained from Charles River Ltd (Margate, Kent, UK) or Harlan-Olac (Bicester, Oxon, UK) and maintained four to a cage under controlled conditions of lighting (lights on 0500 h, off 1900 h) and temperature for at least 2 weeks before use with free access to standard lab chow and water.

Reagents

Rat CRF-41, AVP, and human ACTH_(1–39) were supplied by Bachem Inc. (Saffron Walden, Essex, UK); corticosterone was

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from Sigma Chemical Company Ltd (Poole, Dorset, UK). Cyclic 8-(4-Chlorophenylthio) AMP (8-CPT-cAMP) was from Boehringer Mannheim UK (Lewes, Sussex, UK). General chemicals were from BDH (Poole, Dorset, UK).

Perfusion

Perfusion of isolated rat pituitary segments was performed as previously described (7). Briefly, anterior pituitary glands were cut into eight segments and perfused at 200 μ l/min with Dulbecco's modified Eagle's medium buffered with 25 mM HEPES (Gibco-BRL, Paisley, Strathclyde, UK), pH 7.4, containing 0.25% BSA, 100 U/liter penicillin, and 100 U/liter streptomycin (Gibco-BRL, Paisley, Strathclyde, UK) at 37 C. Typically four perfusion columns were run in parallel with one pituitary equivalent of randomly distributed pituitary segments used per perfusion column. Column effluent was collected every 5 min on ice and stored at -40 C before assaying for ACTH immunoreactivity. After an initial preperfusion period of 3 h to allow basal ACTH release to stabilize, 5 min pulses of secretagogue were applied every 1 h for up to 7 h after the start of the experiment. Various treatments were applied as described in the figure subscripts.

RIA and data analysis

The RIA for immunoreactive ACTH in the column effluent was performed as previously described (9) using antiserum no.6 (courtesy G. B. Makara, MTA-KOKI, Budapest, Hungary).

Net ACTH release per 5 min exposure to secretagogue was determined for each stimulus as previously described (6). Net ACTH release is defined as $\{(V_1 + V_2 + \dots + V_n)/n\} - \{B\}$ where V_1 is the first 5 min fraction collected during the stimulus (100 sec transit time discarded) and V_2 to V_n are the next n consecutive fractions that are elevated with respect to basal ACTH release (B) immediately before the application of the stimulus. For 10 nM CRF-41 or AVP, $n = 5$. For 0.1 nM CRF-41, $n = 3$. Because of the variability in absolute ACTH release between experiments data were standardized by expressing the net ACTH release elicited by a treatment as a percentage of that elicited by CRF-41 (or AVP) alone. The net ACTH release at 4 h was defined as the 100% release value in all columns.

Statistical evaluation between different treatments at each time point was performed using the Kruskal-Wallis test with multiple comparisons for several independent samples, or the Mann-Whitney U-test for two independent samples (10).

Results

CRF-41 and 8-CPT-cAMP inactivate glucocorticoid inhibition of CRF-41-stimulated ACTH release

Corticosterone pretreatment (0.1 μ M for 35 min) significantly ($P < 0.05$) inhibited CRF-41-stimulated ACTH secretion within 30 min of corticosterone application (Fig. 1) before ACTH release returned toward control 2.5 h after steroid application. Corticosterone induced inhibition of CRF- and AVP-stimulated ACTH release developed with a similar time profile (data not shown).

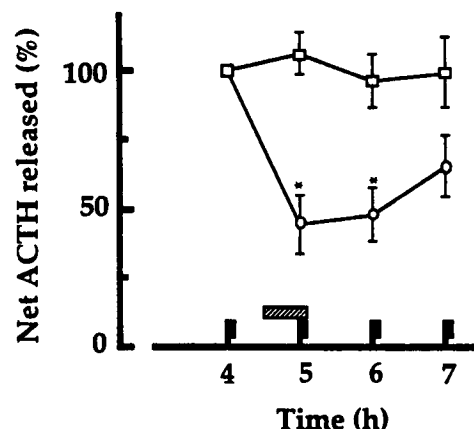


FIG. 1. Early-onset corticosterone-induced inhibition of CRF-41-stimulated ACTH secretion in perfused rat anterior pituitary gland segments. The control group ($\square$) received repeated 5 min pulses of 0.1 nM CRF-41 (solid vertical bars); the other group ($\circ$) received corticosterone (0.1 μ M, 35 min total exposure; horizontal hatched bar) 30 min before the start of the CRF-41 pulse at 5 h. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of CRF-41 applied at 4 h. Data are means $\pm$ SEM ($n = 4-7$ /group). *, $P < 0.05$ (nonparametric Mann-Whitney U-test) compared with the control group.

CRF-41 stimulated ACTH release was also significantly inhibited when no CRF-41 stimulus was applied during the exposure to corticosterone (Table 1). Omission of the CRF-41 stimulus at 5 h had no effect on the responses to subsequent pulses of CRF-41; thus these controls were pooled with controls receiving CRF-41 at 5 h. Early-onset glucocorticoid inhibition did not develop when CRF-41 was applied at the start of corticosterone exposure, suggesting that CRF-41 inactivates glucocorticoid-induced inhibition (Table 1). Furthermore, the cell membrane permeant cAMP analog, 8-CPT-cAMP [0.1 mM for 5 min (11)] also prevented the subsequent inhibition of CRF-41 induced ACTH release when applied at the start of corticosterone exposure (0.1 μ M, 35 min duration) at 5 h (Table 1). At this concentration a 5 min pulse of 8-CPT-cAMP elicited $26.0 \pm 5.1\%$ of the ACTH released by 0.1 nM CRF-41 and did not modify the ACTH release to subsequent CRF-41 pulses (Table 1).

To investigate whether the inactivation of glucocorticoid-induced inhibition was specific to the cAMP pathway, 10 nM AVP (that acts via the inositol phosphate/protein kinase C pathway) was applied for 5 min at 5 h. The release of ACTH in response to a 5 min pulse of 10 nM AVP at 5 h was $186.2 \pm 24.0\%$ ($n = 14$) of the 0.1 nM CRF-41 stimulus applied at 4 h. The CRF-41 stimulus at 6 h released similar amounts of ACTH compared to CRF-41 controls; however, down-regulation of the CRF-41 response at 7 h occurred in columns treated with AVP at 5 h (Table 1). Importantly, when AVP was applied at the start of corticosterone exposure, significant ($P < 0.01$) inhibition of CRF-41-stimulated ACTH release occurred at 6 h (Table 1). These data suggest that inac-

TABLE 1. Inactivation of corticosterone-induced inhibition of CRF-41-stimulated ACTH secretion by CRF-41 and the cAMP analog, 8-CPT-cAMP, in perfused rat anterior pituitary segments

Net ACTH released (%)			
4 h CRF-41 ■	5 h Treatment	6 h CRF-41 ■	7 h CRF-41 ■
100%	CRF-41 (7)	98.0 ± 8.4	99.0 ± 9.9
	■ Corticosterone (4)	61.5 ± 9.9 ^a	54.3 ± 9.3 ^a
	▨ Corticosterone + CRF-41 (5)	108.4 ± 4.3 ^b	111.2 ± 12.4 ^b
	▨ ■		
	8-CPT-cAMP (8)	103.5 ± 15.1	76.3 ± 12.2
	□ Corticosterone + 8- CPT-cAMP (6)	101.2 ± 13.2 ^b	106.0 ± 13.6 ^b
	▨ □		
	AVP (4)	82.5 ± 4.3	48.0 ± 15.6
	■ Corticosterone + AVP (4)	32.3 ± 18.7 ^c	50.0 ± 12.4
	▨ ■		

All groups received 5 min pulses of 0.1 nM CRF-41 at 4, 6, and 7 h. The various treatments applied, along with their temporal relationship of application, are shown in the 5 h treatment column. 8-CPT-cAMP was applied for 5 min at 0.1 mM. AVP for 5 min at 10 nM. Corticosterone (0.1 μM, 35 min total exposure) was started simultaneously with the 5 h stimulus. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of 0.1 nM CRF-41 applied at 4 h in all groups. Because omission of the 5 h CRF-41 stimulus did not affect subsequent stimulated ACTH release data from control columns receiving no CRF-41 at 5 h were pooled with the CRF-41 control group. Values are means ± SEM; the number of experiments in each treatment group are shown in parentheses.

^a $P < 0.05$ compared to CRF-41 control.

^b $P < 0.05$ compared to corticosterone alone.

^c $P < 0.05$ compared to AVP control using nonparametric Kruskal-Wallis test.

tivation is specific to the CRF-41/cAMP pathway.

The duration of corticosterone exposure is also important in determining the characteristics of early-onset inhibition. When CRF-41 was applied at the start of a continuous corticosterone infusion inhibition of CRF-41-stimulated ACTH release developed 2 h after the start of corticosterone treatment (Fig. 2).

Glucocorticoid inhibition of AVP-stimulated ACTH release is not modulated by CRF-41

CRF-41 (0.1 nM) applied simultaneously with the start of corticosterone exposure (35 min duration) at 5 h could not prevent the corticosterone-induced inhibition of AVP-stimulated ACTH release at 6 h (Table 2). Al-

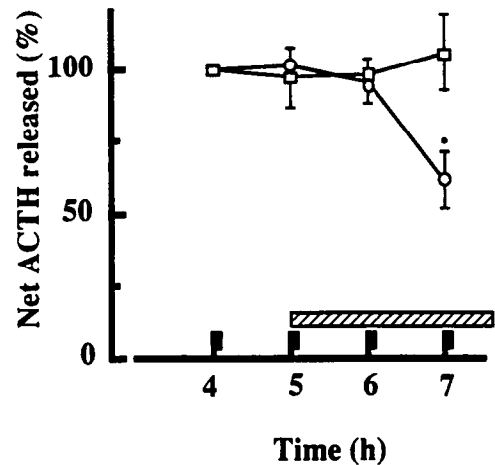


FIG. 2. Inhibition of CRF-41-stimulated ACTH secretion by continuous exposure to corticosterone started simultaneously with a 5 min CRF-41 stimulus in perfused rat anterior pituitary segments. The control groups (□) received repeated 5 min pulses of 10 nM CRF-41 (solid vertical bars) every 1 h. The other group (○) received 0.1 μM corticosterone (horizontal hatched bar) started simultaneously with the 5 h stimulus and maintained to the end of the experiment. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of CRF-41 applied at 4 h. Data are means ± SEM (n = 4–9/group). *, $P < 0.05$ (nonparametric Mann-Whitney U test) compared with the control group.

TABLE 2. CRF-41 does not inactivate corticosterone-induced inhibition of AVP-stimulated ACTH secretion in perfused rat anterior pituitary segments

Net ACTH released (%)			
4 h AVP ■	5 h Treatment	6 h AVP ■	7 h AVP ■
100%	AVP (9)	100.2 ± 9.7	87.1 ± 5.7
	■ Corticosterone + AVP (5)	60.4 ± 4.5 ^a	101.0 ± 18.6
	▨ ■		
	CRF-41 (5)	94.8 ± 9.5	65.6 ± 16.1
	■ Corticosterone + CRF-41 (5)	41.4 ± 12.0 ^b	63.8 ± 17.2
	▨ ■		

All groups received 5 min pulses of 10 nM AVP at 4, 6, and 7 h. The various treatments applied, along with their temporal relationship of application, are shown in the 5 h treatment column. Corticosterone (0.1 μM, 35 min total exposure) was started simultaneously with the 5 h stimulus; CRF-41 was applied for 5 min at 0.1 nM. Data are expressed as a percentage of the net ACTH released by a 5-min pulse of 10 nM AVP applied at 4 h in all groups. Values are means ± SEM; the numbers of experiments in each treatment group are shown in parentheses.

^a $P < 0.05$ compared to AVP control.

^b $P < 0.01$ compared to CRF-41 treated control using nonparametric Kruskal-Wallis test.

though CRF-41 appears to decrease the subsequent response to AVP at 7 h (Table 2) this effect is not statistically significant and is a consequence of one column out of the five in the group releasing a very low amount of ACTH at 7 h. Increasing the concentration of CRF-41 to 10 nM also failed to modify the inhibition by corticosterone (data not shown). Because these columns received AVP before the application of CRF-41 and corticosterone at 5 h, we examined whether this AVP pretreatment could prevent the CRF-inactivation of glucocorticoid inhibition. In order to test this, CRF-41 (0.1 nM) stimuli were applied at 3 h, 4 h, and 5 h, and 10 nM AVP applied at 6 and 7 h. Corticosterone (0.1 μ M, 35 min duration) started simultaneously with CRF-41 at 5 h resulted in significant ($P < 0.05$, using nonparametric Kruskal Wallis test) inhibition of AVP-stimulated ACTH release compared to control even under these conditions. The net ACTH release expressed as a percentage of the 4 h CRF-41 stimulus for control and corticosterone-treated groups, respectively, was: at 6 h $186.7 \pm 45.4\%$ vs. $67.8 \pm 16.6\%$ and at 7 h $214.7 \pm 48.0\%$ vs. $97.8 \pm 31.2\%$ ($n = 3-4/\text{group}$). Furthermore, CRF-41 prevented the corticosterone-induced inhibition of CRF-41-stimulated ACTH release after pretreatment of the segments with 10 nM AVP (data not shown) further suggesting that CRF-41 can only inactivate glucocorticoid-induced inhibition of CRF-41-stimulated ACTH release.

Discussion

The present study demonstrates that CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion from perfused rat anterior pituitary corticotrophs *in vitro*, and that this action is mediated by cAMP. Furthermore, the data suggest that glucocorticoids act via distinct mechanisms to inhibit CRF-41- and AVP-stimulated ACTH secretion.

CRF-41 inactivates early glucocorticoid inhibition of CRF-41-stimulated ACTH release

The data confirm our previous observations showing that the development of early glucocorticoid inhibition of CRF-41-stimulated ACTH secretion is dependent upon the relative timing of application of CRF-41 with steroid (7). However, our previous data did not resolve whether the timing phenomenon was a result of CRF-41 activating some step in the glucocorticoid-induced inhibition (for example posttranslational modification of the induced protein) when CRF-41 was applied after the start of corticosterone exposure, or alternatively, that CRF-41 inactivates glucocorticoid-induced inhibition, perhaps by preventing the protein induction, when applied simultaneously with the start of corticosterone

exposure.

CRF-41-stimulated ACTH release was significantly inhibited by corticosterone when no CRF-41 stimulus was applied during the exposure to corticosterone; similar results were also observed with the type II glucocorticoid receptor agonist RU28386 (M. J. Shipston and F. A. Antoni, unpublished data). Furthermore, application of CRF-41 or an analog of its intracellular messenger cAMP, simultaneously with the onset of corticosterone exposure, prevented the subsequent inhibition of CRF-41-stimulated ACTH release. Thus, it appears that CRF-41 actually prevents the action/induction of the glucocorticoid-induced protein(s) when applied at the start of glucocorticoid exposure. Interestingly, the duration of corticosterone exposure also plays a role in the characteristics of early glucocorticoid inhibition of CRF-41-stimulated ACTH release because if CRF-41 is applied simultaneously with the start of corticosterone then inhibition of CRF-41-stimulated ACTH release develops after 2 h of continuous steroid exposure.

The precise mechanism by which CRF-41 prevents glucocorticoid inhibition of ACTH release is not clear. However, because it can be reproduced with a cAMP analog but not with AVP (an activator of the inositol phosphate/protein kinase C pathway), it seems reasonable to suggest that the inactivation of glucocorticoid action is, in fact, mediated by cAMP and/or cAMP dependent phosphorylation. In turn, this may interfere with glucocorticoid action through modulation of the state of glucocorticoid receptor phosphorylation (12), and possibly preventing translocation of the hormone-receptor complex into the nucleus (13-15). Furthermore, transcription factors such as *c-fos* or cAMP response element binding protein (CREB) which are induced or activated by cAMP (16), have been shown to interfere with glucocorticoid-induced gene transcription (17-21). Alternatively, CRF-41 may induce some posttranslational modification in, or alter events downstream of, the glucocorticoid-induced protein to prevent the inhibition of CRF-41 stimulated ACTH secretion.

It is of interest that a reduction in glucocorticoid receptor number and glucocorticoid receptor mRNA levels develops 6 h after exposure to activators of cAMP synthesis in AtT-20 corticotroph tumor cells (22). Thus, CRF-41 acting via cAMP exerts both short- (present findings) and long-term (22) control over glucocorticoid action at the pituitary level. In turn, reciprocally opposing actions are provided by early- and late-onset glucocorticoid inhibition of CRF-41 action on ACTH release and biosynthesis (*cf* Refs. 3 and 23 for reviews).

CRF-41 does not inactivate glucocorticoid-induced inhibition of AVP-stimulated ACTH release.

Intriguingly, CRF-41 failed to prevent glucocorticoid induced-inhibition of AVP-stimulated ACTH secretion.

From among the several possible explanations to this phenomenon, on the basis of the present data we can exclude that the priming of the tissue segments with AVP inhibits the action of a subsequent CRF-41 pulse to prevent glucocorticoid inhibition, or that priming with CRF-41 is required for this effect of CRF-41. Thus, it appears that glucocorticoids suppress CRF-41- and AVP-induced ACTH secretion through different mechanisms. Indeed, previous evidence indicates that distinct pools of ACTH are mobilized by these neurohormones: CRF-41 releases a stored, slow-turnover pool of ACTH, whereas AVP-induced secretion is derived from a rapid-turnover pool of hormone (7, 24). A further possibility, (this in fact does not exclude the previous one) is that distinct populations of corticotrophs are involved (*cf* Refs. 24 and 25) in which cAMP phosphorylation targets are expressed in a cell-specific manner.

When CRF-41 and AVP were used in combination as secretagogue at concentrations approximating levels recorded in rat hypophysial portal blood (0.1 nM and 0.2 nM, respectively, *cf* Ref. 1), early-onset glucocorticoid inhibition can be prevented by a secretagogue pulse started at the onset of the exposure to glucocorticoids (26), *i.e.* in this respect the 'physiological' CRF-41/AVP stimulus resembles CRF-41 alone. This is in agreement with the AVP-induced enhancement of CRF-41-stimulated accumulation of cAMP (*cf* Ref. 23).

The implications of these findings for the control of ACTH release *in vivo* are that glucocorticoid feedback at the pituitary level is modulated in a differential manner by secretagogue neurohormones. In general, stimuli involving CRF-41 may be relatively resistant to inhibition by glucocorticoids, and those involving primarily AVP are invariably suppressed. For instance, it may be predicted that a prolonged increase in ACTH release such as that found in autoimmune inflammation (*cf* Ref. 27) rely more on CRF-41 while during acute stimulation the AVP component is more dominant and consequently readily suppressed by glucocorticoids. Finally, it is of importance to determine whether cAMP-mobilizing stimuli have similar actions on glucocorticoid-mediated inhibition at central nervous sites.

Acknowledgments

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Depolarization counteracts glucocorticoid inhibition of adeno-hypophyseal corticotroph cells*

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1 In AtT20 mouse corticotroph tumour cells large conductance Ca^{2+} -activated K^{+} -channels (BK-channels) have an essential role in the early glucocorticoid inhibition of adrenocorticotrophin (ACTH) secretion evoked by corticotrophin-releasing factor. The present study examined whether or not BK-channels are also pivotal to glucocorticoid inhibition of normal rat anterior pituitary cells.

2 A membrane-permeant, non-metabolizable cyclic AMP analogue, 8-(4-Chlorophenylthio)adenosine-3',5'-cyclic-monophosphate (CPT-cAMP) was used as the primary secretagogue stimulus, as this mimics the increase of intracellular cyclic AMP caused by corticotrophin-releasing factor, but is not subject to the complex Ca^{2+} -dependent regulation of cyclic AMP metabolism that is evident in corticotroph cells.

3 Experiments in AtT20 cells showed that ACTH secretion stimulated by 1 mM CPT-cAMP was suppressed to $34 \pm 1.5\%$ ($n=12$) of the control stimulus by a maximal dose of 100 nM dexamethasone. The ACTH secretion evoked by the combination of 1 mM CPT-cAMP with either 5 μM (–)BayK8644 (L-type Ca^{2+} -channel activator) or 5 mM TEA (K^{+} -channel blocker) was respectively $69.1 \pm 7.6\%$ and $69.3 \pm 11.8\%$ of control after 2 h preincubation with 100 nM dexamethasone ($P < 0.05$ vs CPT-cAMP). The ACTH response elicited by 5 μM (–)BayK8644 and 5 mM TEA given together was completely resistant to inhibition by 100 nM dexamethasone. Furthermore, TEA and (–)BayK8644 given together synergistically stimulated ACTH release in combination with 0.1 mM or 1 mM CPT-cAMP, and these ACTH responses were not inhibited by 100 nM dexamethasone.

4 In primary cultures of rat anterior pituitary cells, TEA (up to 20 mM), charybdotoxin (30 nM) or apamin (100 nM) failed to modify the glucocorticoid inhibition of 0.1 mM CPT-cAMP-induced ACTH release. The combination of 5 mM TEA and 5 μM (–)BayK8644 elicited a small but significant increase in ACTH secretion but did not modify the inhibition of 0.3 mM CPT-cAMP-induced ACTH secretion by 100 nM dexamethasone.

5 In primary cultures of rat anterior pituitary cells, depolarization of the membrane potential with 40 mM KCl enhanced the ACTH response to CPT-cAMP and markedly reduced the maximal inhibitory effect of dexamethasone to $55 \pm 1.2\%$ as well as that of corticosterone to $33 \pm 2.1\%$ vs $100 \pm 2.5\%$ and $100 \pm 1.9\%$ inhibition respectively, when 0.1 mM CPT-cAMP was used alone. Introduction of 5 μM (–)BayK8644 with 40 mM KCl in this system had no additional effect on glucocorticoid inhibition.

6 No glucocorticoid inhibition of ACTH release to any of the stimuli applied was observed in cells pretreated with the mRNA synthesis inhibitor, 5,6-dichloro-furanosyl-benzimidazole riboside (DRB) (0.1 mM) or the protein synthesis blocker, puromycin (0.1 mM).

7 In summary, early glucocorticoid inhibition of stimulated ACTH release by cultured rat anterior pituitary cells was dependent on the synthesis of new mRNA and protein. Depolarization of the membrane potential potentiated CPT-cAMP-induced ACTH secretion in AtT20 cells as well as cultured rat corticotrophs and this was associated with a resistance to the early inhibitory effect of glucocorticoids. Glucocorticoid inhibition in rat anterior pituitary corticotrophs was unaltered by TEA, charybdotoxin as well as apamin, and hence it is unlikely to involve predominantly BK-or SK-type Ca^{2+} -activated K^{+} -channels.

8 These results support the thesis that a prime target of glucocorticoid feedback inhibition in anterior pituitary corticotrophs is the membrane potential and indicate that glucocorticoid-induced proteins regulate the activities of several distinct plasma membrane ion channels.

Keywords: Pituitary gland; potassium channels; dexamethasone; corticosterone; AtT20 cells; stress

Introduction

Adrenal corticosteroids are powerful regulators of gene expression in several organ systems of the body. The plasma levels of these steroids are controlled through a neuroendocrine feedback loop which ensures that corticosteroid levels are optimal for homeostatic adaptation (Dallman *et al.*, 1992). Major sites of corticosteroid feedback action include the

anterior pituitary gland, the hypothalamus and further sites in the central nervous system (Sapolsky *et al.*, 1990).

The cellular mechanisms underlying corticosteroid feedback of the hypothalamic-pituitary-adrenocortical system are not well understood. The anterior pituitary corticotroph has served as a useful model to examine this problem (Antoni, 1996; Buckingham, 1996). In corticotroph cells corticosteroid inhibition typically occurs in two main phases, designated early (within 2 h) and late (2–24 h) (Antoni, 1996; Buckingham, 1996). Late inhibition appears to be mediated by negative regulatory elements in DNA and leads to the suppression of

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the proopiomelanocortin (ACTH precursor) (Lundblad & Roberts, 1988), the type 1 corticotrophin releasing-factor (CRF) receptor (Pozzoli *et al.*, 1996) and possibly other genes. In contrast, the early component of inhibition is brought about by glucocorticoid-induced protein(s), the identity of which is unclear at present (Shipston, 1995; Antoni, 1996; Buckingham, 1996).

Recent studies in the mouse corticotroph tumour cell line AtT20 (Shipston *et al.*, 1996) have shown that high conductance Ca^{2+} -activated K^{+} -channels (BK-channels) play an essential role in the inhibitory effect of dexamethasone on CRF-induced ACTH secretion. Briefly, activation of adenyl cyclase by CRF produces an increase of cyclic AMP-dependent phosphorylation and triggers Ca^{2+} -dependent action potentials which lead to the exocytotic release of ACTH (see Antoni, 1993). An important element of this stimulatory effect is the inhibition of BK-channels by cyclic AMP-dependent protein kinase (Shipston *et al.*, 1996). Treatment with dexamethasone for 90 min prevented the action of CRF and cyclic AMP analogues on BK-channels through a process requiring the synthesis of mRNA and protein (Shipston *et al.*, 1996). Accordingly, the blockade of BK-channels by charybdotoxin (Antoni, 1996) or iberiotoxin (Shipston *et al.*, 1996) obliterated the inhibitory effect of dexamethasone on stimulated ACTH release.

The aim of the present study was to examine whether BK-channels are also essential for corticosteroid feedback inhibition in non-tumoural corticotroph cells. Experiments were carried out *in vitro*, using AtT20 cells and primary cultures of rat anterior pituitary cells. A cell-permeant cyclic AMP analogue resistant to hydrolysis by cyclic nucleotide phosphodiesterases (PDEs) was used as the stimulus in combination with various compounds that cause membrane depolarization. The rationale for this design was that increases of intracellular cyclic AMP induced by agonists are strongly suppressed by intracellular Ca^{2+} in both AtT20 and normal rat corticotrophs (Antoni *et al.*, 1995; Antoni, 1997), hence changes of cyclic AMP metabolism induced by corticosteroids could mask effects downstream of cyclic AMP formation.

The results clearly show that in contrast to AtT20 cells, blockage of BK-channels fails to counteract early glucocorticoid inhibition in cultures of rat anterior pituitary cells. Importantly, however, corticosteroid inhibition in the rat pituitary culture system was markedly reduced upon depolarization with high extracellular KCl, in which respect its behaviour is analogous to AtT20 cells. Thus, the findings support the notion that early inhibition by glucocorticoids involves control of the membrane potential by rapidly induced proteins.

Methods

Reagents

Unless otherwise indicated all reagents were from Sigma U.K. (Poole, Dorset), and of the highest grade available. The sources of other materials were as follows: CRF (human), ACTH (human) synthetic charybdotoxin (Bachem U.K. Ltd., Saffron Walden); corticosterone (Roussel-UCLAF, Romainville, France); 8-(4-chlorophenylthio)adenosine-3',5'-cyclic-monophosphate (CPT-cAMP, BIOLOG, Bremen, Germany); tetraethyl ammonium hydrochloride (TEA, Aldrich, Gillingham, Dorset); (–)BayK8644, (RBI, Sigma, U.K.); (+)202-791 (Novartis, U.K.); TRL-3 trypsin (Worthington, Lorne Laboratories, Reading, U.K.); Sheep anti-ACTH serum was

generously provided by Prof P.J. Lowry, University of Reading; anti-sheep/goat IgG donkey serum was generously provided by the Scottish Antibody Production Unit, Carluke, Scotland.

Cell culture

Male Wistar rats (120–200 g) were caged singly with free access to pelleted food and tap water in a light- and temperature-controlled environment for 24 h, and sacrificed by decapitation before 10.30 a.m. Precautions were taken to avoid any stressful stimulation of the animals. The anterior pituitary glands were collected and placed in a plastic Petri dish so that the ventral surfaces of the glands contacted the bottom of the dish. The glands were chopped twice (the second time after 90° rotation of the specimen platform) on a hand-operated McIlwain tissue-chopper setting 0.5 mm and lowest blade force. The tissue segments were incubated in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS, GIBCO, Life Technologies, Paisley) supplemented with 25 mM HEPES pH 7.4, 0.15 mM CaCl_2 , 0.15 mM MgSO_4 , 0.5 mg ml^{–1} trypsin, 0.01 µg ml^{–1} DNase I (Sigma D-4527) and 0.25% bovine serum albumin (BSA, Sigma A-7906) for 30 min at 37°C under constant shaking at 250 cycles/min, on a IKA Vibrax orbital shaker. Following trypsinization, 200 µl of 10⁴ kallikrein unit/ml Trasylol® (Bayer U.K.) was added and the tissue segments were triturated with a 1 ml Gilson pipette tip attached to the end of a 5 ml polystyrene pipette for 10 min. The suspension was then filtered through 70 µm nylon mesh and centrifuged at 200 g for 10 min. The pelleted cells were resuspended in 5 ml HBSS supplemented with 25 mM HEPES pH 7.4, 0.15 mM CaCl_2 , 0.15 mM MgSO_4 , 0.01 µg ml^{–1} DNase I, 0.25 mg ml^{–1} soybean trypsin inhibitor (Sigma T-6522), recentrifuged and suspended in Dulbecco's Modified Eagle's Medium (DMEM, containing 4.5 g glucose/L) supplemented with 2.5% foetal calf serum (Harlan Seralab, Sussex) and 7.5% horse serum (Sigma H-1138). Cell viability as assessed by Trypan Blue exclusion was over 95%. Approximately 5 × 10⁴ cells/well were plated in 24-well plates and cultured in a humidified incubator with 5% CO₂/95% air at 37°C for 4 days.

AtT20 cells were maintained in DMEM (containing 4.5 g/L glucose) supplemented with 10% foetal calf serum as previously described (Woods *et al.*, 1992). All cells used were of passage 30 or less.

Assay of ACTH secretion

The experimental procedures for ACTH secretion were identical for AtT20 cells and primary cultures. The cells were first washed free of serum with 2 × 0.5 ml of incubation medium (DMEM, GIBCO 31600-026, without Na₂HCO₃, supplemented with 25 mM HEPES pH 7.4 and 0.25% BSA (Sigma, A-4378) and incubated in this medium at 37°C for 1 h, after which steroids and inhibitors of mRNA or protein synthesis were introduced, and incubation was continued for a further 2 h. Steroids were dissolved at 10^{–2} M in dimethylsulphoxide and diluted with incubation medium. Corresponding vehicle controls contained the requisite amounts of dimethylsulphoxide throughout.

In the case of primary cultures, K⁺ channel blockers and Ca^{2+} -channel activators were added during the last 30 min of the 2 h preincubation period because normal corticotrophs appear to be relatively resistant to depolarization. After 2 h of preincubation, the wells were washed once more with

incubation medium and the secretagogues and requisite inhibitors were added in 0.5 ml of fresh incubation medium. In some experiments, high KCl medium which was composed of two parts incubation medium and one part 120 mM KCl, 1 mM MgSO_4 , 1.8 mM CaCl_2 , 25 mM HEPES 0.25% BSA, Ca^{2+} -free medium (120 mM NaCl, 2.4 mM KCl, 1.2 mM K_2HPO_4 , 1.2 mM MgSO_4 , 4.5 g/L D-glucose, 0.2 mM EGTA, 25 mM HEPES, 0.25% BSA, Minimum Essential Medium vitamins and amino acids) or Ca^{2+} -free high KCl medium (two parts of the Ca^{2+} -free medium mixed with one part of 120 mM KCl, 1.2 mM MgSO_4 , 0.2 mM EGTA, 25 mM HEPES, 0.25% BSA, vitamins and amino acids) were used. The test incubation period was 60 min for primary cultures and 30 min for AtT20 cells after which the tissue culture trays were placed on ice. Any cells detached from the bottom of the wells were pelleted by centrifugation at 200 g for 10 min at 4°C after which aliquots of the supernatant were collected and assayed for ACTH content by radioimmunoassay. Sheep anti ACTH serum used in the assay was generously provided by Prof P.J. Lowry, University of Reading and the donkey antisheep IgG serum was obtained from the Scottish Antibody Production Unit, Carlisle, Scotland.

Analysis of results

Each experiment was carried out at least twice with *n* replicates for each treatment within each experiment. All data are presented as mean $\pm$ s.e.mean and were analysed by 2- or 1-way analysis of variance followed by Dunnett's test, Newman Keuls' test or contrast of means where appropriate. Results presented as percentage of control ACTH release were derived from the raw data as follows: $(X - B)/(C - B) \times 100\%$, where B refers to the basal ACTH secretion, C refers to ACTH secretion elicited by the control stimulus and X refers to ACTH secretion elicited by the control stimulus in the presence of dexamethasone or corticosterone.

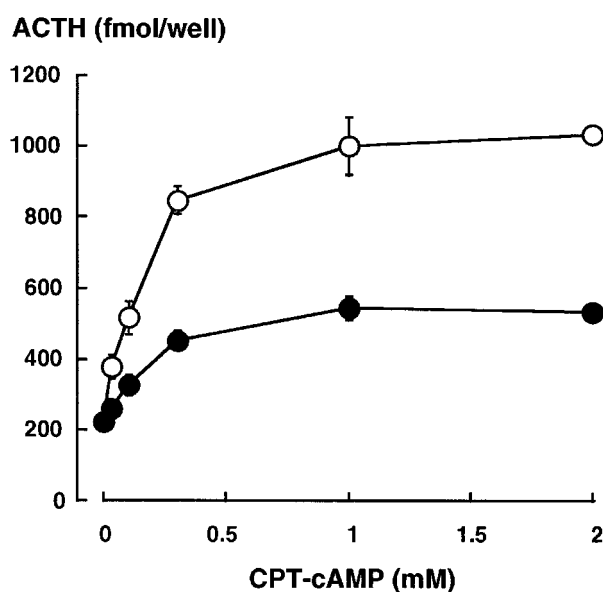


Figure 1 The effect of CPT-cAMP on ACTH secretion by AtT20 cells pretreated for 2 h with vehicle (○) or 100 nM dexamethasone (●). Means, the bars indicate s.e.mean, *n* = 5/group. Where error bars are not visible, the s.e.mean bar is less than the space required for the symbol. Results shown are representative of four identical experiments.

Results

Experiments with AtT20 cells

Response to CPT-cAMP and dexamethasone Concentration-dependent stimulation of ACTH release was evoked by CPT-cAMP (Figure 1) and the response plateaued at 1 mM CPT-cAMP. Pretreatment with 100 nM dexamethasone significantly reduced the increase in CPT-cAMP-stimulated ACTH secretion, basal hormone output remained unaltered (Figure 1).

Effects of depolarizing agents The ACTH response to 1 mM CPT-cAMP could be enhanced further by the application of

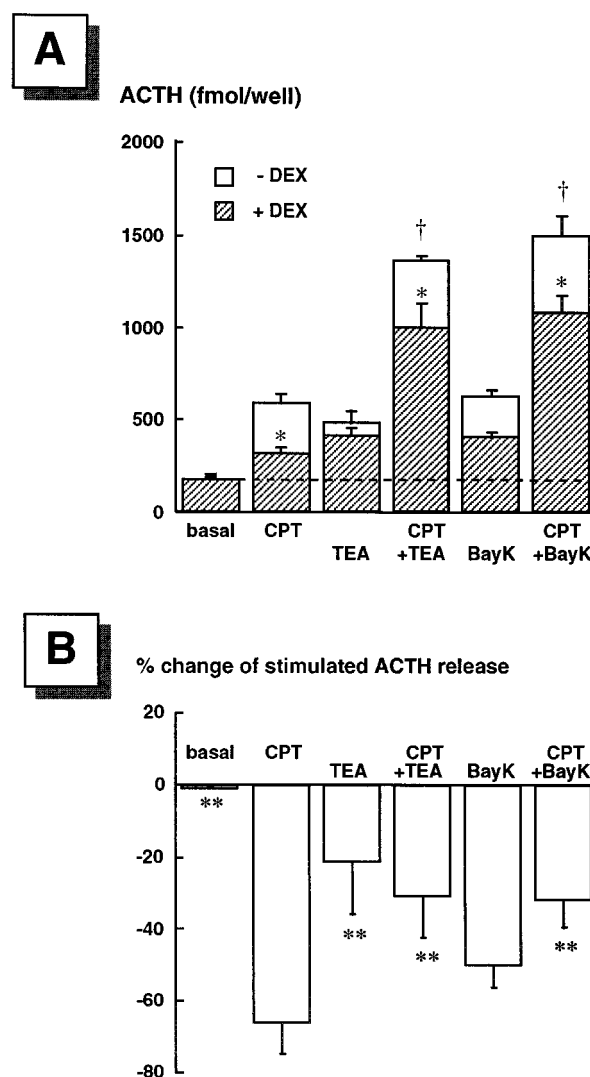


Figure 2 Effect of membrane depolarizing agents, 5 μM (—) BayK8644 (BayK) and 5 mM tetraethylammonium (TEA) on ACTH secretion elicited by 1 mM CPT-cAMP (CPT) after 2 h preincubation with vehicle or 100 nM dexamethasone in AtT20 cells. Data are means, bars indicate s.e.mean, *n* = 4/group. (A) The empty columns show ACTH release by vehicle treated cells and the hatched columns show release after treatment with dexamethasone. †*P* < 0.02 for the interaction between CPT alone and the respective depolarizing agents alone, 2-way ANOVA. **P* < 0.05 when compared with corresponding vehicle-treated release, 1-way ANOVA followed by contrast of means. (B) data are expressed as % change in the amount of ACTH secretion caused by dexamethasone when compared with the respective control groups shown in A. ***P* < 0.05 when compared with CPT alone, 1-way-ANOVA followed by Dunnett's test. Results shown are representative of two identical experiments.

5 mM tetraethylammonium (TEA) or the Ca^{2+} -channel activator (–)BayK8644 at 5 μM (Figure 2A). Both of these compounds stimulated ACTH release when given alone and exhibited synergistic interaction with CPT-cAMP. Similar data were obtained with another L-channel activator (+)202–791 at 5 μM (not shown).

The release of ACTH evoked by 1 mM CPT-cAMP was inhibited by $65.8 \pm 8.9\%$ ($n=5$) by 100 nM dexamethasone, while basal secretion was not significantly altered (Figure 2A and B). The inhibition of ACTH secretion stimulated by a combination of 1 mM CPT-cAMP and 5 mM TEA or 1 mM CPT-cAMP and 5 μM (–)BayK8644 was significantly attenuated to $30.7 \pm 11.8\%$ and $31.9 \pm 7.6\%$ ($P < 0.05$, $n=5$), respectively (Figure 2A and B). The combination of 1 mM CPT-cAMP with 5 μM (+)202–791 also significantly reduced the inhibition of the stimulated ACTH secretion by 100 nM dexamethasone to $27 \pm 1.3\%$ ($P < 0.05$, $n=4/\text{group}$, 1-way-ANOVA, Dunnett's test).

As both (–)BayK8644 and TEA partially reversed dexamethasone inhibition of CPT-cAMP stimulated ACTH secretion, the combination of these compounds with CPT-cAMP was tested subsequently, using a submaximal concentration (0.1 mM) of CPT-cAMP as well as 1 mM CPT-cAMP. The ACTH secretion elicited by 0.1 mM CPT-cAMP was significantly potentiated by 5 μM (–)BayK8644 and 5 mM TEA given together (Table 1). CPT-cAMP-induced ACTH secretion was inhibited by dexamethasone (1 nM to 1 μM) in a concentration dependent manner by up to 84% (Table 1). The combination of 5 μM (–)BayK8644 and 5 mM TEA elicited a 3-fold increase in ACTH secretion over basal levels and this response was not inhibited by dexamethasone (Table 1). The ACTH response elicited by the combination of 0.1 mM CPT-cAMP, 5 μM (–)BayK8644 and 5 mM TEA was also fully resistant to inhibition by 1–1000 nM dexamethasone (Table 1).

Similar results were obtained by combining 1 mM CPT-cAMP with 5 mM TEA and 5 μM (–)BayK8644. A summary of these data (Figure 3) shows that the extent of resistance to glucocorticoid inhibition was independent of the amplitude of stimulus-evoked ACTH secretion. For instance, although the ACTH response elicited by 5 mM TEA or 1 mM CPT-cAMP alone were similar, the ACTH response to 5 mM TEA was much more resistant to dexamethasone inhibition than the ACTH response to 1 mM CPT-cAMP.

Taken together, these results are in agreement with previous studies of CRF-induced ACTH release in AtT20 cells, which

have shown that early glucocorticoid inhibition is effectively antagonized by depolarization of the membrane potential (Woods *et al.*, 1994) and that TEA sensitive K^+ -channels, particularly BK-type channels underlie the inhibitory action of corticosteroids (Shipston *et al.*, 1996).

Experiments with primary cultures of rat anterior pituitary cells

Response to CPT-cAMP The time-course of the ACTH response to CPT-cAMP was close to linear over 4 h (Figure 4A). The concentration-response relationship between CPT-cAMP and ACTH release was similar at 60, 120 and 240 min, in that 0.1 mM CPT-cAMP elicited close to maximal

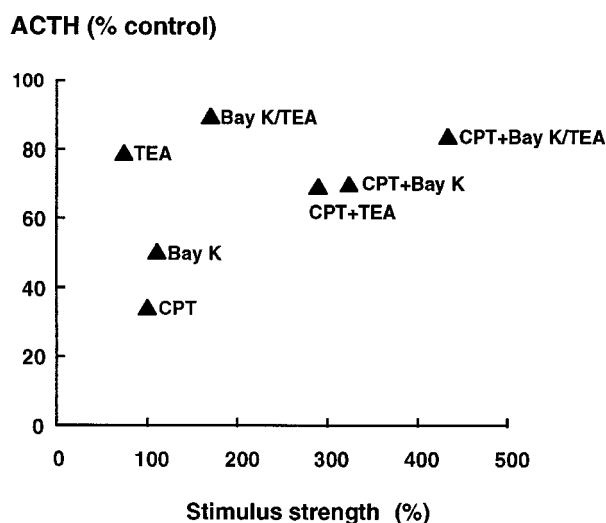


Figure 3 Size of the ACTH secretory response fails to predict the degree of dexamethasone inhibition in AtT20 cells. Data were plotted as stimulus strength vs ACTH response, where stimulus strength is the size of the evoked release of ACTH expressed as % of the response to 1 mM CPT-cAMP run in the same experiment, and ACTH response is the size of the ACTH response to the respective stimulus in cells preincubated with 100 nM dexamethasone, expressed as percentage of the control (i.e. no dexamethasone) response. Data are means $n=4/\text{group}$. Symbols: CPT, 1 mM CPT-cAMP; BayK, 5 μM (–)BayK8644; TEA, 5 mM tetraethylammonium; BayK/TEA, combination of 5 μM (–)BayK8644 and 5 mM tetraethylammonium; + sign indicates that two stimuli were applied together. Results shown are representative of two identical experiments.

Table 1 Characterization of the ACTH secretory response to CPT-cAMP, TEA and (–)BayK8644 in the presence of increasing dexamethasone concentrations in AtT20 cells

	Dexamethasone (nM)				
	0	3	10	100	1000
Vehicle	608 ± 63				
0.1 mM CPT	1122 ± 136 (100 ± 26%)	860 ± 94 (49 ± 18%)	795 ± 57 (36 ± 11%)	734 ± 19 (25 ± 4%)	690 ± 22 (16 ± 4%)
5 mM TEA + 5 μM BayK	1883 ± 65 (100 ± 5%)	1973 ± 125 (107 ± 10%)†	1767 ± 57 (91 ± 4%)†	1783 ± 84 (92 ± 7%)†	1743 ± 167 (89 ± 13%)†
0.1 mM CPT + 5 mM TEA + 5 μM BayK	2919 ± 93* (100 ± 4%)	2912 ± 126 (100 ± 5%)†	2593 ± 170 (86 ± 7%)†	2626 ± 62 (87 ± 3%)†	3016 ± 161 (104 ± 7%)†

Data show ACTH release expressed in fmol per well and represent means $\pm$ s.e.mean. of four replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean $\pm$ s.e.mean), as defined in Methods. * $P < 0.02$ for the interaction between CPT and TEA/BayK in the absence of dexamethasone pretreatment, 2-way ANOVA. Statistical analysis of the effect of dexamethasone was carried out on the data expressed as percentages (values shown in parentheses) of the respective stimulus-evoked release, † $P < 0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by Dunnett's test. Results shown are representative of two identical experiments.

stimulation of ACTH release (Figure 4B). This was not the case at 30 min, where 1 mM CPT-cAMP was significantly more effective than 0.1 mM CPT-cAMP. All subsequent experiments were terminated at the 60 min time-point.

Lack of an effect of K^+ -channel blockers and BayK8644 on corticosteroid inhibition of ACTH release Corticosterone (100 nM) reduced the ACTH response to 0.1 mM CPT-cAMP by $74.8 \pm 10.8\%$ ($n=5$) and this was not modified in the presence of TEA (5–20 mM) or (–)BayK8644 (5 μ M). Similar findings were obtained with 30 nM dexamethasone. Combination of 5 mM TEA and (–)BayK8644 (5 μ M) produced a significant enhancement of the ACTH releasing effect of CPT-cAMP (0.3 mM) (Basal: 34 ± 6 fmol/well; CPT-cAMP: 125 ± 5 fmol/well, $P < 0.0001$, $n=6$; TEA + BayK: 60 ± 6 fmol/well,

$P < 0.0002$, $n=5$; CPT-cAMP + TEA + BayK: 204 ± 14 fmol/well, $P < 0.03$, interaction, $n=7$, 2-way-ANOVA) but failed to modify the inhibition by 100 nM dexamethasone (% inhibition of CPT-cAMP vs CPT-cAMP + TEA + BayK: $63 \pm 7\%$ vs $66 \pm 6\%$, $n=4$). Finally, preincubation with charybdotoxin (30 nM) or apamin (100 nM) and the combination of these agents with 0.1 mM CPT-cAMP also failed to modify corticosterone inhibition of stimulated ACTH secretion (not shown).

These data indicate that in contrast to AtT20 cells, TEA-sensitive ion-channels are not fundamentally important for corticosteroid inhibition of ACTH secretion in primary cultures of rat anterior pituitary cells.

Depolarization with 40 mM KCl counteracts glucocorticoid effect on ACTH The ACTH responses to 0.1 mM CPT-cAMP and 40 mM KCl are shown in (Table 2). KCl (40 mM) synergized with 0.1 mM CPT-cAMP to stimulate ACTH release, furthermore, it produced a marked reduction in the inhibitory effect of dexamethasone (Table 2). Omission of Ca^{2+} from the incubation medium completely blocked ACTH release induced by CPT-cAMP alone or CPT-cAMP and KCl (not shown). Dexamethasone (10 nM) inhibited the ACTH response to 0.1 mM CPT-cAMP and 40 mM KCl by only $44 \pm 12\%$ ($n=4$), while that evoked by 0.1 mM CPT-cAMP was completely blocked. Similar results were also obtained using the naturally occurring corticosteroid corticosterone (Table 2).

The data above demonstrate that depolarization of the membrane potential markedly reduces the early inhibitory effect of glucocorticoids. However, the lack of a complete block of steroid action by 40 mM KCl raises the question of whether or not the inhibitory effect may be completely accounted for by the rapid induction of mRNA and protein by glucocorticoids.

Blockers of mRNA and protein synthesis abolish glucocorticoid inhibition The inhibition of 0.1 mM CPT-cAMP stimulated ACTH secretion by 10 nM dexamethasone was eliminated in the presence of 5,6-dichloro-furanosyl-benzimidazole riboside (DRB), an adenosine analogue and inhibitor of heteronuclear RNA synthesis (Egyházi *et al.*, 1982) (Figure 5A). In the presence 40 mM KCl, DRB suppressed ACTH secretion elicited by 0.1 mM CPT-cAMP but no further change was observed after treatment with dexamethasone (Figure 5B). Therefore, the residual inhibition by corticosteroids observed in the presence of 40 mM KCl and 0.1 mM CPT-cAMP is likely to be due to glucocorticoid blockade of this DRB sensitive mechanism. Similar results were obtained using 0.1 mM puromycin, an inhibitor of protein synthesis (not shown).

Discussion

The data presented here show that control of the membrane potential is essential for glucocorticoid inhibition of stimulated ACTH release in both AtT20 cells and primary cultures of rat anterior pituitary cells. However, in contrast to tumoural AtT20 cells where BK-channels are pivotal targets of glucocorticoid action (Shipston *et al.*, 1996), blockage of BK- and other TEA-sensitive K^+ -channels failed to modify the inhibitory action of glucocorticoids in normal rat pituitary cells. Thus the data conform with the general hypothesis that glucocorticoids modulate the function of plasma membrane ion channels to reduce cellular responses of excitable cells (Joëls & de Kloet, 1994), and indicate that glucocorticoid-

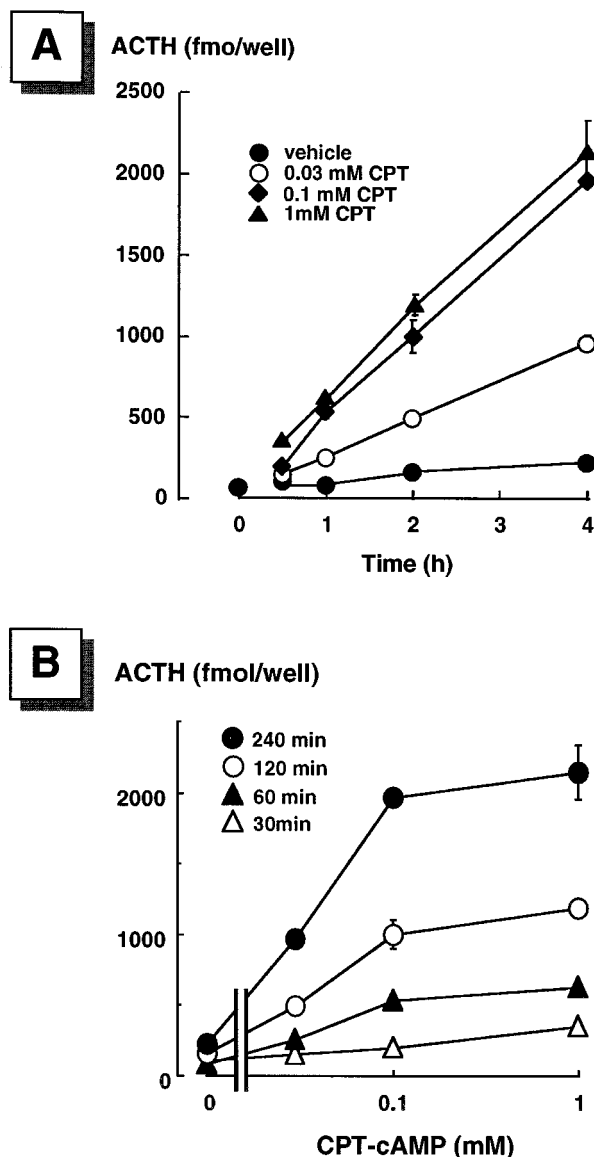


Figure 4 Effect of CPT-cAMP on ACTH secretion by primary cultures of rat anterior pituitary cells. (A) Time-course of hormone release in cells treated with vehicle (●), 30 μ M CPT-cAMP (○), 0.1 mM CPT-cAMP (◆) or 1 mM CPT-cAMP (▲). (B) Concentration-dependent stimulation of ACTH secretion by CPT-cAMP at 30 min (△), 1 h (▲), 2 h (○) and 4 h (●) of incubation. Means, bars indicate s.e.mean, $n=4$ /group. Where error bars are not visible, the s.e.mean bar is less than the space required for the symbol. Results shown are representative of two identical experiments.

Table 2 Counteraction of glucocorticoid inhibition of the ACTH secretory response by 40 mM KCl in primary cultures of rat anterior pituitary cells

	Corticosterone (nM)				
	0	3	10	100	1000
Vehicle	100.4 ± 11.0				
0.1 mM CPT	163.8 ± 6.9 (100 ± 11%)	150.8 ± 8.1 (80 ± 13%)	132.6 ± 15.2 (51 ± 24%)	86.4 ± 2.1 (-22 ± 3%)	80.3 ± 3.0 (-31 ± 5%)
40 mM KCl	125.5 ± 2.9				
0.1 mM	284.8 ± 4.1*	269.8 ± 16.3	260.8 ± 7.6	221.9 ± 23.1	216.3 ± 22.7
CPT + 40 mM KCl	(100 ± 2%)	(92 ± 9%)	(87 ± 4%)†	(66 ± 13%)†	(63 ± 12%)
	Dexamethasone (nM)				
	0	3	10	100	
Vehicle	66.1 ± 7.0				
0.1 mM CPT	112.8 ± 5.3 (100 ± 11%)	62.8 ± 2.5 (-7 ± 5%)	54.5 ± 3.3 (-25 ± 7%)	52.0 ± 3.1 (-30 ± 7%)	
40 mM KCl	127.6 ± 14.4				
0.1 mM	280.6 ± 27.2**	192 ± 31.8	187.2 ± 26.1	165.2 ± 11.8	
CPT + 40 mM KCl	(100 ± 13%)	(59 ± 15%)†	(56 ± 12%)†	(46 ± 6%)†	

Data show ACTH release expressed in fmol per well and represent means ± s.e.mean. of four replicates per treatment group. Values in parentheses represent percentages of control ACTH release (mean ± s.e.mean), as defined in Methods. * $P < 0.0001$ and ** $P < 0.004$ for the interaction between CPT-cAMP and KCl in the absence of glucocorticoid pretreatment, 2-way ANOVA. Statistical analysis of the effect of glucocorticoids was carried out on the data expressed as percentages (values shown in the parentheses) of the respective stimulus-evoked release, † $P < 0.05$ when compared to the corresponding CPT group, 1-way ANOVA followed by contrast of means. Results shown are representative of two and three identical experiments for the corticosterone and dexamethasone dose responses respectively.

induced proteins may regulate the function of multiple plasma membrane ion channels.

Characteristics of the primary culture system as a model for secretagogue-glucocorticoid interaction and the use of CPT-cAMP

Previous studies have established that the main hallmarks of early glucocorticoid inhibition are: (i) selectivity for stimulated ACTH release, (ii) mediation through Type II glucocorticoid receptors, (iii) manifestation within 2 h, and (iv) a requirement for new mRNA and protein synthesis reviewed in (Shipston, 1995). These features have been largely retained in the AtT20 D16:16 cell line (Antoni, 1996), and also characterize the rat anterior pituitary primary culture system (Abou-Samra *et al.*, 1986, and present study).

A further important property of the primary culture system is its responsiveness to physiological concentrations of CRF and AVP (Vale *et al.*, 1983; Oki *et al.*, (1990)). A somewhat surprising feature is the low response to depolarizing stimuli such as 40 mM KCl and BayK8644 given alone. These stimuli have been reported as inducers of robust ACTH release by acutely prepared pituitary cells (Gillies & Lowry, 1978; Antoni & Dayanithi, 1990b), tissue segments (Taylor *et al.*, 1993) as well as AtT20 cells (Guild & Reisine, 1987). A plausible explanation of this finding is that the aforementioned preparations possess spontaneous electrical activity, which may be due to endogenous pacemaker potentials (e.g. AtT20 cells), or is derived from factors released upon tissue injury due to dissection in pituitary segments (Taylor *et al.*, 1993), or is a remnant of the trophic actions of CRF *in vivo* (Ixart *et al.*, 1991). Spontaneous electrical activity will facilitate the effects of the usage-dependent L-channel activator (-)BayK8644 (Sanguinetti & Kass, 1984). Furthermore, the secretagogue potencies of (-)BayK8644 as well as high KCl are likely to depend on tonic cyclic AMP-dependent phosphorylation which is required for the optimal functioning of L-type Ca^{2+} channels (Armstrong & Eckert, 1987) and exocytotic processes (Morgan *et al.*, 1993).

With respect to the requirement for cyclic AMP, it is known that acutely dispersed cells produce much more reliable responses to short pulses CRF if 'primed' with several pulses of the peptide (Antoni *et al.*, 1990b). Moreover, it has been reported that cyclic AMP and Ca^{2+} synergize to trigger ACTH release from permeabilized AtT20 cells (Guild, 1991), further indicating that cyclic AMP-dependent processes acting in parallel to the mobilization of Ca^{2+} are important for the secretory response of corticotroph cells. It is of note that while depolarization with 40 mM KCl was not a reliable stimulus of ACTH release on its own, it consistently produced a synergistic enhancement of the CPT-cAMP induced ACTH response. As the main result of KCl depolarization is Ca^{2+} influx through voltage-operated channels (Meier *et al.*, 1988), the synergy of CPT-cAMP and KCl depolarization appears analogous to the Ca^{2+} potentiation of cAMP-induced ACTH release in AtT20 cells. Similar results have been obtained using physiological concentrations of CRF (Lim *et al.*, unpublished).

A cell-membrane permeant, non-metabolizable analogue of cyclic AMP, CPT-cAMP, was used as the secretagogue stimulus in this study in order to produce a sustained increase in cyclic AMP-dependent phosphorylation. As the physiological ACTH secretagogues, CRF or CRF and AVP in combination, activate cyclic AMP-dependent phosphorylation in corticotrophs (see Antoni, 1993), CPT-cAMP will mimic this aspect of agonist action. It is also clear that CRF-induced cyclic AMP responses are subject to negative feedback regulation by intracellular free Ca^{2+} at the level of adenylyl cyclase and cyclic AMP hydrolysis in AtT20 cells (Antoni, 1996) as well as rat anterior pituitary cells (Antoni, 1997). CPT-cAMP is largely resistant to Ca^{2+} -feedback and this property makes it a potentially useful tool for studying processes of ACTH release downstream of cyclic AMP formation. It is of note, however, that CRF elicits oscillations of intracellular free Ca^{2+} in AtT20 cells as well as normal rat corticotrophs, (see Antoni, 1993 for review), which may be accompanied by oscillations of intracellular cyclic AMP levels (Cooper *et al.*, 1998). Thus, clamping the intracellular cyclic AMP through CPT-cAMP, which leads to sustained increases of intracellular free Ca^{2+} as opposed to oscillations (Mollard

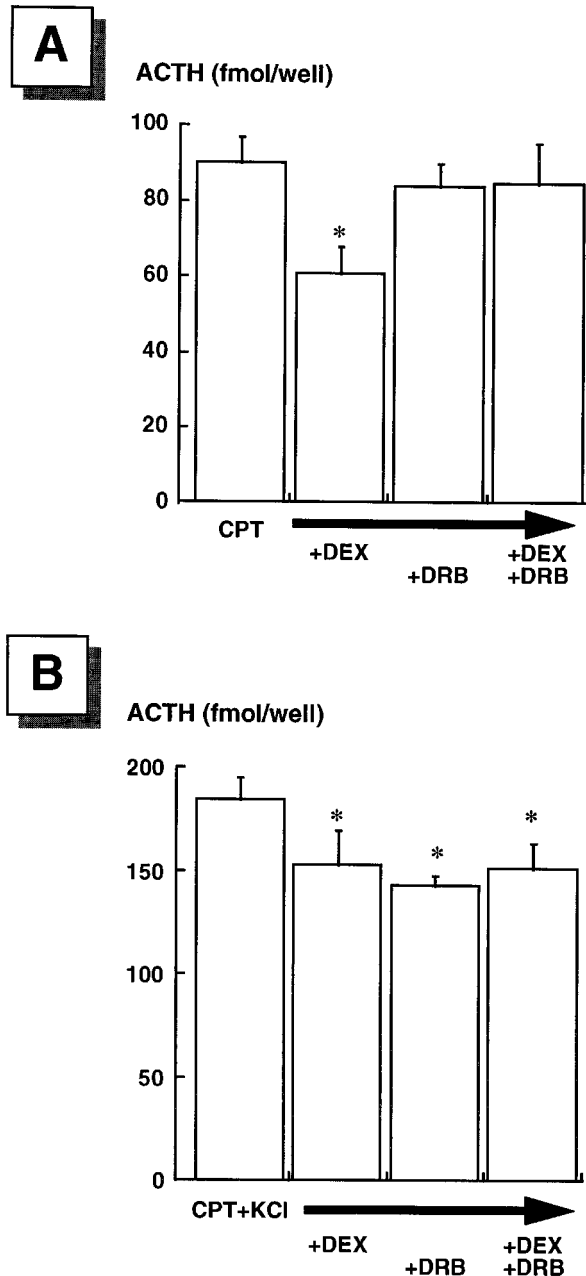


Figure 5 Effect of the RNA synthesis inhibitor 5,6-dichloro-furanosyl-benzimidazole riboside (DRB) on ACTH secretion in primary cultures of rat anterior pituitary cells. 100 μ M DRB and 100 nM dexamethasone (DEX) were applied 2 h before challenging the cells with (A) 0.1 mM CPT-cAMP or (B) 0.1 mM CPT-cAMP and 40 mM KCl for 60 min. Basal ACTH release was 44 ± 3 fmol/well and was not altered by DRB or dexamethasone. Data are means, bars indicate s.e.mean, $n = 5$ /group. * $P < 0.05$ when compared with control group receiving vehicle only. 1-way-ANOVA followed by Newman-Keuls test. Results shown are representative of four identical experiments.

et al., 1992), may potentially alter some properties of the ACTH response when compared with physiological agonists. Indeed, inhibition of mRNA or protein synthesis significantly attenuated the release of ACTH by rat pituitary corticotrophs to CPT-cAMP-based stimuli, whereas such manipulations were without effect on CRF-induced ACTH secretion (Dayanithi & Antoni, 1989; Taylor *et al.*, 1993 and Lim *et al.*, unpublished). In other respects, however, CPT-cAMP-stimulated ACTH release was largely dependent on extracellular

Ca^{2+} and blocked by glucocorticoids similarly to ACTH secretion evoked by CRF.

Properties of early glucocorticoid inhibition in AtT20 cells

The results with AtT20 cells extend previous work from this laboratory examining CRF-induced ACTH release in these cells. Overall, ACTH release induced by CPT-cAMP alone was potentially inhibited by dexamethasone. By contrast, responses to stimuli that cause depolarization such as TEA, (–)BayK8644 or (+)202–791 were resistant to glucocorticoid inhibition. It was also clear that the nature of the stimulus rather than the size of the evoked response predicted the degree of glucocorticoid inhibition in AtT20 cells. Thus, these data are in full agreement with earlier findings from this laboratory indicating that the principal target of corticosteroid inhibition is the membrane potential (Pennington *et al.*, 1994; Shipston *et al.*, 1996) rather than the secretory apparatus as proposed by others (Castellino *et al.*, 1992). Further, the data are in agreement with the findings that BK-channels (Shipston *et al.*, 1996) are pivotal for glucocorticoid inhibition in AtT20 cells.

Corticosterone action in primary cultures: deviation from and convergence to AtT20 model

Importantly, the involvement of plasma membrane ion-channels in glucocorticoid action has not been previously addressed in normal rat corticotrophs. A report from another laboratory has shown (Halili-Manabat *et al.*, 1995) that ACTH secretion stimulated by the depolarizing agent veratridine, an opener of tetrodotoxin sensitive Na^{+} -channels is resistant to inhibition by dexamethasone, and our group has reported similar findings in preliminary form using combinations of CRF and veratridine (Antoni & Woods, 1992). However, no stimuli physiological to corticotrophs have been identified so far that operate through Na^{+} -channels and thus counteract glucocorticoid feedback inhibition (Antoni, 1993; Halili-Manabat *et al.*, 1995).

In sharp contrast to AtT20 cells, concentrations of TEA as high as 20 mM failed to dent significantly the early inhibitory action of corticosterone or dexamethasone in primary cultures of rat anterior pituitary cells. Also of note is that apamin and charybdotoxin, blockers of SK- and BK-type Ca^{2+} -activated K^{+} -channels were also without effect. In contrast to the channel blockers tested so far, 40 mM KCl synergized with CPT-cAMP to stimulate the release of ACTH, while having relatively little effect on its own. Furthermore, the early inhibitory effects of dexamethasone as well as corticosterone were drastically reduced with CPT-cAMP and KCl as the stimulus. The release of ACTH under these conditions was fully dependent on extracellular Ca^{2+} , hence indicating that the mechanism of hormone release is not fundamentally altered by the addition of 40 mM KCl.

Overall, these findings are surprising, as channels sensitive to the K^{+} -channel blockers applied in the present study, and functionally relevant for the regulation of ACTH release have been previously reported in various preparations of corticotroph cells (Marchetti *et al.*, 1987; Antoni & Dayanithi, 1990a). The possibility that the K^{+} -channel profile of corticotrophs is altered by culture conditions cannot be discounted at present. A recent report (Lee & Tse, 1997) suggests that CRF-induced activation of cultured rat pituitary corticotrophs is the result of the inhibition of a K^{+} -current that is insensitive to TEA as well as 4-aminopyridine. However, as noted above, the role of K^{+}

Table 3 Comparison of early corticosteroid inhibition of stimulated ACTH release in AtT20 cells and primary cultures of rat anterior pituitary cells

Common features:
Onset within 2 h
Mediated by Type II glucocorticoid receptor
Requirement of new mRNA and protein synthesis
Specific for agonist-induced release
Opposed by depolarization of the membrane potential
Specific features:
AtT20 cells
Mediated by BK-type potassium channels
Primary cultures
Mediated by unidentified ion channel
May involve transcriptional block of protein expression required for sustained release of ACTH

channels in glucocorticoid feedback has not been previously examined in non-tumoural corticotrophs.

The nature of the residual inhibitory effect of glucocorticoids on KCl/CPT-cAMP stimulated ACTH release was revealed in experiments with inhibitors of protein and mRNA synthesis. As some reports suggested that early corticosteroid inhibition does not require gene induction (Taylor *et al.*, 1993) we have re-examined this issue in the primary culture system. Similarly to actinomycin D (Arimura *et al.*, 1969; Portanova & Sayers, 1974; Dayanithi *et al.*, 1989) DRB blocked the early corticosteroid inhibition of ACTH release, indicating a requirement for mRNA synthesis. Moreover, DRB as well as puromycin caused similar reductions in ACTH release induced by 40 mM KCl/0.1 mM CPT-cAMP in the presence of 100 nM

dexamethasone, which had no additional effect in the presence of these agents. The findings indicate that in case of relatively high (>4 fold basal) ACTH responses elicited by CPT-cAMP-based stimuli the ACTH secreted is derived through the synthesis of new mRNA and protein, plausibly POMC, which is blocked by glucocorticoids. In contrast to CPT-cAMP-based stimuli, there was no apparent requirement for mRNA or protein synthesis when CRF was used to stimulate ACTH release (Abou-Samra *et al.*, 1986; Dayanithi *et al.*, 1989; Taylor *et al.*, 1993). Thus oscillating Ca^{2+} /cAMP signals may be more efficient in mobilizing/processing ACTH without a requirement for *de novo* protein synthesis than CPT-cAMP which elicits prolonged elevations of intracellular Ca^{2+} lacking an oscillatory pattern (Mollard *et al.*, 1992).

In summary (Table 3), it is intriguing that the same physiologically relevant phenotype of early corticosteroid inhibition is found in AtT20 cells and rat corticotrophs, including counteraction by membrane depolarization, but the underlying membrane-control mechanisms have strikingly distinct pharmacology. Further studies should clarify whether glucocorticoids are capable of inducing different families of ion-channel subunits depending on the cell system under study (Attardi *et al.*, 1993; Levitan *et al.*, 1996) or evoke the synthesis of proteins that regulate the activity of several distinct ion channels through generic mechanisms such as protein phosphorylation (Wang *et al.*, 1997). Data from our laboratories (Shipston *et al.*, 1996) favour the latter possibility in the case of adeno-hypophysial corticotrophs.

We thank Prof P.J. Lowry (University of Reading) for the generous provision of sheep anti-ACTH serum, the Scottish Antibody Production Unit for anti-sheep and anti-rabbit IgG serum, Novartis, U.K. for (+)202–791.

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Glucocorticoids Block Protein Kinase A Inhibition of Calcium-activated Potassium Channels*

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Adrenal corticosteroids have well known and profound effects on neurons and neuroendocrine cells, but the underlying cellular mechanisms are poorly understood. The present study analyzed membrane currents and ACTH release in AtT20 mouse pituitary corticotrope tumor cells. Patch-clamp analysis revealed a significant and selective inhibition of calcium-activated (BK-type) potassium channels upon activation of protein kinase A by corticotropin-releasing factor or 8-chlorophenylthio-cAMP. The synthetic glucocorticoid dexamethasone had no effect on potassium currents evoked by depolarization but prevented the inhibitory effect of protein kinase A activators. The action of dexamethasone had the hallmarks of protein induction, i.e. a lag time and sensitivity to inhibitors of DNA transcription and mRNA translation. In parallel, the specific BK channel blocker iberiotoxin abolished early glucocorticoid inhibition of corticotropin-releasing factor-stimulated ACTH secretion. In summary, the present data show that glucocorticoid-induced proteins render BK-type channels resistant to inhibition by protein kinase A and that this action of the steroid is pivotal for its early inhibitory effect on the secretion of ACTH.

Stressors provoke a cohort of homeostatic defense mechanisms by the central nervous, the immune, and the metabolic control systems of the body (1). Adrenal corticosteroids released during the stress response terminate many of these adaptive responses through the rapid induction of proteins, the nature and mechanism of which are not understood (1–4).

A common motif of corticosteroid action in hippocampal neurons (5, 6) and pituitary somatotrope cells (7) is the regulation of potassium channels that are important determinants of cellular excitability. In hippocampal neurons, the target of glucocorticoid action has not been defined. In somatotrope cells, glucocorticoid induction of Kv1.5 voltage-regulated potassium channel subunits has been demonstrated (7).

A physiologically important early action of glucocorticoids is

the suppression of CRF¹-induced ACTH secretion from anterior pituitary corticotropes (for reviews see Refs. 8 and 9). CRF stimulates ACTH release through activation of protein kinase A and the enhancement of calcium influx through voltage-operated calcium channels (10–12). In turn, glucocorticoids rapidly (within 2 h) inhibit CRF-stimulated ACTH secretion through the induction of new protein(s) (2, 8, 10, 13, 14), while basal secretion remains unchanged. The early inhibition of stimulated ACTH release in AtT20 corticotropes is antagonized by membrane depolarization (13, 15), calcium channel activator drugs (11, 16), and potassium channel blockers (15, 16), collectively suggesting that the actions of glucocorticoids may involve potassium channels. However, the well established glucocorticoid induction of the Kv1.5 channel protein does not occur in AtT20 corticotropes (17).

In endocrine cells and neurons large conductance calcium- and voltage-activated potassium (BK) channels have been proposed as important negative feedback regulators of voltage-dependent calcium influx (18). Such channels have been previously identified in AtT20 corticotrope tumor cells (19, 20).

This report describes a correlated study of the regulation of BK channels and ACTH release by CRF and glucocorticoids in the mouse anterior pituitary corticotrope cell line AtT20. The data demonstrate that glucocorticoid-induced proteins prevent protein kinase A-dependent inhibition of BK channels and that this is pivotal to early glucocorticoid inhibition of CRF-induced ACTH secretion.

MATERIALS AND METHODS

AtT20 D16:16 Cell Culture—Clonal mouse anterior pituitary (AtT20 D16:16, passage 19–30) cells were maintained as described previously (14) except that cells were passaged by brief trypsinization 1 week before replating on glass coverslips. Cells (3–7 days postplating) were treated with dexamethasone or vehicle (0.01% Me₂SO) for 2 h at 37 °C in serum-free HEPES-buffered Dulbecco's modified Eagle's medium. Cells were then transferred to physiological saline containing (in mM): 140 NaCl, 5 KCl, 25 HEPES, 0.8 MgCl₂, 2 CaCl₂, 30 glucose, and 0.001 tetrodotoxin (TTX), pH 7.4, at room temperature (19–24 °C) for electrophysiological recording. Regulation of currents in control and dexamethasone-treated cells was performed in parallel on the same passage of cells to avoid intrapassage variations.

Electrophysiology—Whole cell currents were recorded under voltage clamp using the amphotericin B (200 µg/ml) perforated patch configuration of the whole cell patch clamp recording technique (21). Data acquisition and voltage protocols were controlled by an Axopatch 200 amplifier and pCLAMP software (Axon Instruments Inc., Foster City, CA). All traces are leak-subtracted records from cells with compensated series resistance of <15 megaohms. Pipettes were manufactured from Garner 7052 glass with resistances of 1.5–3 megaohms in physiological saline after fire polishing.

For determination of outward potassium currents cells were voltage-clamped at –60 mV in physiological saline containing (in mM): 140 NaCl, 5 KCl, 25 HEPES, 0.8 MgCl₂, 2 CaCl₂, 30 glucose, and 0.001 TTX, pH 7.4. The patch pipette contained (in mM): 95 KCH₃SO₃, 55 KCl, 10 HEPES, 2 MgCl₂, 0.1 CaCl₂, and 200 µg/ml amphotericin B, pH 7.35. Outward potassium currents were evoked by 100-ms step depolarization (–30 to +50 mV), and the average steady-state current amplitude between 90 and 100 ms was determined at each potential. Outward currents were stable for >1 h in this configuration. Single BK channel

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¹ The abbreviations used are: CRF, corticotropin-releasing factor; ACTH, adrenocorticotropin; Me₂SO, dimethyl sulfoxide; TTX, tetrodotoxin; IbTx, iberiotoxin; BK, large conductance calcium- and voltage-activated potassium channel; TEA, tetraethylammonium; 8-CPT-cAMP, 8-chlorophenylthio-cAMP; Rp-cAMPS, adenosine 3',5'-cyclic monophosphothioate-Rp; 4-AP, 4-aminopyridine; pF, picofarads.

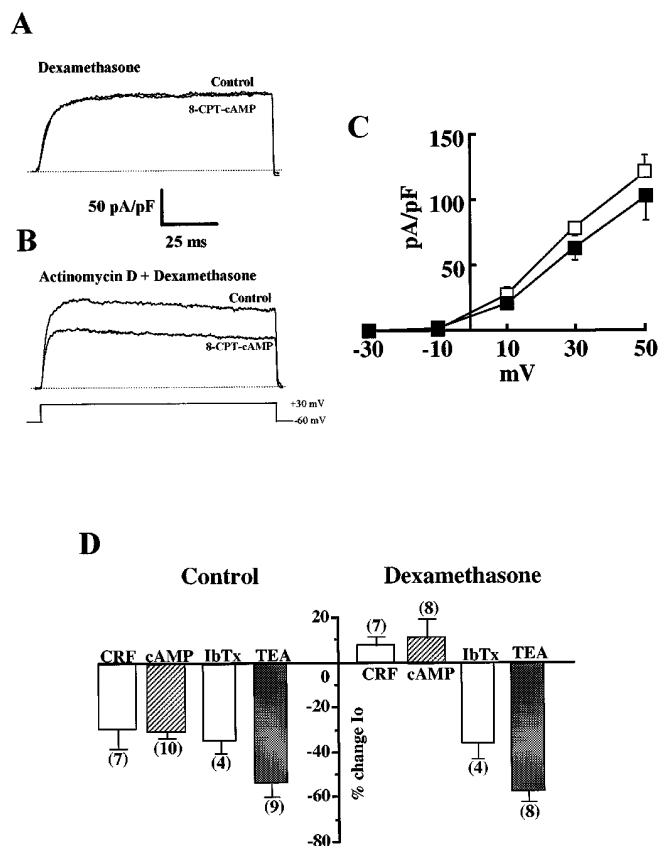


FIG. 2. Dexamethasone prevents protein kinase A-mediated inhibition of BK currents in intact AtT20 D16:16 cells. A, representative (1 of 8 cells) leak-subtracted traces of voltage-activated outward current (at +30 mV) before and after exposure to 8-CPT-cAMP (0.1 mM, 10 min) in AtT20 cells pretreated for 2 h with the glucocorticoid agonist, dexamethasone (1 μ M). B, 8-CPT-cAMP inhibits outward steady-state current in dexamethasone-treated cells pretreated with the mRNA transcription inhibitor, actinomycin D (0.1 mM applied 15 min before and during dexamethasone treatment). A representative trace at +30 mV from 1 of 3 cells is shown. C, current/voltage relationship of steady-state outward current density from control (open squares, $n = 43$) and dexamethasone-treated (filled squares, $n = 29$) cells. Outward steady-state current at each potential was normalized to membrane capacitance to compensate for variations in cell size and expressed as mean current density, pA/pF. D, summary of effect of bath application of CRF (100 nM, 5 min), 8-CPT-cAMP (5 min, 0.1 mM), IbTx (20 min, 100 nM), or TEA (2 min, 1 mM) on outward steady-state potassium current determined at +30 mV. Data are expressed as the percentage change (% change I_o) in outward steady-state current compared with pretreated control current amplitude (I_o) as described in Fig. 1C. Cells were voltage-clamped at -60 mV as described in Fig. 1 and under "Materials and Methods." The number in parentheses indicates number of cells per group. Means $\pm$ S.E. are shown.

and sustained calcium influx through voltage-sensitive L-type calcium channels (19, 23, 24); in parallel, inhibition of protein kinase A by various methods also blocks the hormone secretory response to CRF in AtT20 cells (25, 26).

The Target of Protein Kinase A Action Is the BK Potassium Channel—Both iberiotoxin (100 nM), a highly specific blocker of BK type channels (27) and TEA (1 mM), a broader spectrum blocker, inhibited the steady-state outward current by $34.0 \pm 6.5\%$ ($n = 4$, Fig. 1D) and $52.9 \pm 6.3\%$ ($n = 9$), respectively. No significant inhibition of the residual outward current by CRF (or 8-CPT-cAMP) occurred in cells pretreated with 100 nM iberiotoxin (mean inhibition, $6.2 \pm 5.6\%$, $n = 4$, Fig. 1D) or 1 mM TEA (mean inhibition, $5.1 \pm 7.2\%$, $n = 4$). Similar results were obtained with 100 nM charybdotoxin ($n = 2$).

Bath application of 100 nM CRF significantly ($p < 0.05$) reduced the average open probability of single large conduct-

ance (~ 120 pS) BK channels in 4/6 patches in cell-attached patch recordings (Fig. 1, E and F). The time of onset (~ 2 min) of the effect of CRF was identical to that seen for the macroscopic current (compare Fig. 1, C and F).

These results clearly show that CRF and cAMP suppress the activity of BK channels in AtT20 cells under conditions when these channels are exposed to elevated intracellular free calcium levels that enhance their open probability (20). As BK channels are thought to act as immediate negative feedback inhibitors of voltage-dependent calcium influx, inhibition of these channels is a key element for optimal activation of calcium channels involved in hormone secretion (18).

Previous evidence shows that BK channels may be up- or down-regulated by reversible cAMP-dependent phosphorylation (28). However, in pituitary cells only protein kinase A-dependent inhibition has been found so far (29, 30), and the present study is the first example of inhibition of BK channels by a physiologically relevant cAMP-mobilizing hypothalamic peptide, CRF.

Dexamethasone Prevents Inhibition of Potassium Current by Protein Kinase A—Pretreatment with 1 μ M dexamethasone for 2 h, which produces maximal inhibition of CRF-stimulated ACTH release (14), blocked the inhibition of outward steady-state potassium current by 8-CPT-cAMP (Fig. 2, A and D) or CRF (Fig. 2D). This effect of dexamethasone was prevented with actinomycin D (0.1 mM, Fig. 2B) or puromycin (2 μ M, $n = 2$), indicating a requirement for *de novo* RNA and protein synthesis, a defining feature of early inhibition (8, 14). In addition an acute (10 min) exposure to dexamethasone did not block CRF inhibition of the steady-state current (not shown).

In the absence of protein kinase A activation, dexamethasone had no significant effect on mean current density, threshold of activation, or sensitivity to 100 nM IbTx or 1 mM TEA of the steady-state outward current (Fig. 2, C and D), in agreement with a previous study (16).

Similar to the findings with the macroscopic current, no significant inhibition of single BK channels was observed by CRF in cell-attached patch recordings (0/5 patches) from dexamethasone-treated cells.

These observations show that dexamethasone action is mediated by newly induced proteins and is selective for BK channels inhibited by protein kinase A. Glucocorticoids have also been reported to enhance a 4-aminopyridine (4-AP)-sensitive potassium current in AtT20 cells (16). In functional secretion assays 4-AP affects ACTH release; however, 4-AP alone does not reverse glucocorticoid inhibition of CRF-stimulated ACTH release (16). In contrast, blockade of BK channels alone with IbTx (Fig. 3) completely reverses the inhibitory effect of dexamethasone suggesting BK channels are a primary target for glucocorticoid action in AtT20 cells.

The early inhibitory effect of glucocorticoids on CRF-stimulated ACTH release also requires mRNA and protein synthesis (2, 8, 10, 14), and in this respect the characteristics of the action of dexamethasone on BK channels and ACTH release are identical. The selectivity of the effect of dexamethasone on protein kinase A-inhibited BK channels also helps to explain why early glucocorticoid inhibition suppresses CRF-stimulated ACTH release but does not affect basal output of hormone (2, 8, 13, 14).

Effects of Dexamethasone Are Not Secondary to Changes in Calcium Currents—As intracellular calcium ions are potent activators of BK channels and glucocorticoids reportedly (31) enhance calcium entry to stimulate potassium currents in hippocampal neurons, the actions of CRF and dexamethasone on calcium currents were also analyzed.

As shown by others previously (12) CRF enhanced peak pharmacologically isolated high voltage-activated calcium cur-

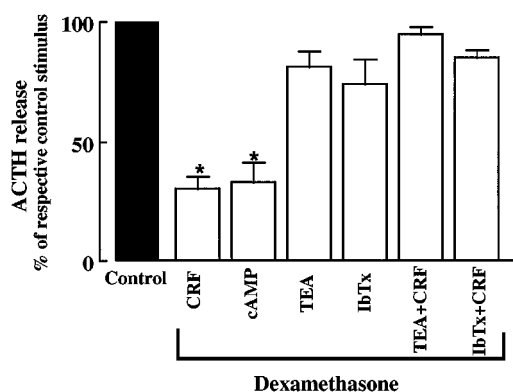


FIG. 3. IbTx prevents early glucocorticoid inhibition of CRF-stimulated ACTH secretion. A, static incubation assays of CRF-stimulated ACTH secretion were performed at room temperature (24 °C) under the conditions used to monitor outward currents as described under "Materials and Methods." CRF (100 nM) was applied for 30 min. TEA (1 mM) was applied during the CRF exposure, and IbTx (100 nM) was applied >15 min before and during CRF exposure. Cells were treated for 2 h with 1 μ M dexamethasone or vehicle (0.01% Me₂SO) as appropriate. ACTH release for each treatment in dexamethasone-treated cells (open box) is expressed as the percent of the respective control (absence of dexamethasone, filled box) stimulus (100%). In these studies CRF (100 nM) stimulated ACTH release to $157 \pm 6\%$ ($n = 6$) of basal levels. IbTx (100 nM) or TEA (1 mM) alone elicited a small but significant ($p < 0.05$) ACTH response ($114 \pm 2\%$, $n = 3$ and $127 \pm 7\%$, $n = 5$ of basal, respectively). IbTx had no significant effect on CRF-stimulated ACTH secretion ($155 \pm 7.9\%$, $n = 6$ of basal release). TEA (1 mM) significantly ($p < 0.05$, non-parametric Kruskal-Wallis test) enhanced CRF-stimulated ACTH release to $185 \pm 10.5\%$, ($n = 5$) of basal. Means $\pm$ S.E. are shown; *, $p < 0.01$ compared with control ACTH release (non-parametric Kruskal-Wallis test).

rents in control (mean increase at +20 mV was $44 \pm 15\%$, $n = 4$) and dexamethasone-treated ($33 \pm 5\%$, $n = 5$) cells. Similar enhancement was also seen with 8-CPT-cAMP ($n = 2$). Dexamethasone pretreatment (2 h, 1 μ M) had no significant effect on peak calcium current density (measured at +20 mV: control, 8.9 ± 1.5 pA/pF ($n = 8$); dexamethasone, 9.2 ± 1.4 pA/pF ($n = 7$)).

On the basis of these data it seems reasonable to suggest that direct modulation of calcium influx through high voltage-activated calcium channels is not responsible for the effects of dexamethasone on BK currents. Moreover, previous work indicates a reduction (11, 32) or no significant change (10) in the average levels of intracellular free calcium after dexamethasone treatment in AtT20 cells, thus excluding increases in intracellular free calcium levels derived from intracellular sources as mediators of the effect of the steroid.

IbTirotoxin-sensitive Channels Are Pivotal for Early Glucocorticoid Inhibition of CRF-stimulated ACTH Release—Under the same conditions that were used to monitor outward potassium currents, CRF (100 nM) stimulated ACTH release to $157 \pm 6\%$ ($n = 6$) of basal levels. IbTx (100 nM) or TEA (1 mM) alone elicited a small but statistically significant ($p < 0.05$ non-parametric Kruskal-Wallis test) ACTH response ($114 \pm 2\%$, $n = 3$ and $127 \pm 7\%$, $n = 5$ of basal, respectively). IbTx had no significant effect on CRF-stimulated ACTH secretion ($155 \pm 7.9\%$, $n = 6$ of basal release). TEA (1 mM) significantly ($p < 0.05$, Kruskal-Wallis test) enhanced CRF-stimulated ACTH release to $185 \pm 10.5\%$ ($n = 5$) of basal. AtT20 cells contain multiple voltage-activated potassium conductances (16), and in this system 1 mM TEA blocks a greater proportion of outward, steady-state, voltage-activated current than 100 nM IbTx (Fig.

2D). The effects of these inhibitors are not additive (not shown), indicating that TEA blocks ibTirotoxin-sensitive BK channels as well as other potassium conductances in this system.

The IC₅₀ of dexamethasone to block CRF-induced ACTH release was 7.7 ± 1.9 nM ($n = 6$). Importantly, IbTx (100 nM) as well as TEA (1 mM) completely blocked the inhibitory effect of 1 μ M dexamethasone on CRF-stimulated ACTH secretion (Fig. 3). Furthermore, dexamethasone (1 μ M) had no significant inhibitory effect on ACTH secretion stimulated by IbTx or TEA alone.

Final Comment—Taken together with previous results (8, 12) the present data suggest that concerted cAMP-dependent phosphorylations of L-type calcium channels as well as BK-type potassium channels are required for triggering the ACTH secretory response to CRF. Inhibition of BK channels by cAMP-dependent phosphorylation prevents the calcium-induced feedback hyperpolarization mediated by these channels and thus enhances voltage-activated calcium entry (18). The critical role of this process in CRF-stimulated ACTH secretion is shown by the observation that dexamethasone prevents protein kinase A inhibition of BK channels and in turn that selective blockage of BK channels abolished the early inhibition of ACTH release.

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dc 385 12 Molecular Components of Large Conductance Calcium-Activated Potassium (BK) Channels in Mouse Pituitary Corticotropes

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Large-conductance calcium- and voltage- activated potassium (BK) channels play a fundamental role in the signaling pathways regulating mouse anterior pituitary corticotrope function. Here we describe the cloning and functional characterization of the components of mouse corticotrope BK channels. RT-PCR cloning and splice variant analysis of mouse AtT20 D16:16 corticotropes revealed robust expression of *mslo* transcripts encoding pore-forming α -subunits containing the mouse homolog of the 59-amino acid STREX-1 exon at splice site 2. RT-PCR and functional analysis, using the triterpenoid glycoside, DHS-1, revealed that native corticotrope BK channels are not functionally coupled to β -subunits *in vivo*. Functional expression of the STREX-1 containing α -subunit in HEK 293 cells resulted in BK channels with calcium sensitivity, single-channel conductance, and inhibition by protein kinase A identical to that of native mouse corticotrope BK channels. This report represents the first corticotrope ion channel to be characterized at the molecular level and demonstrates that mouse corticotrope BK channels are composed of α -subunits expressing the mouse STREX-1 exon. (Molecular Endocrinology 13: 1728–1737, 1999)

INTRODUCTION

The excitability of anterior pituitary cells is tightly controlled by G-protein-linked receptor-mediated regulation of multiple ion channels in the plasma membrane of target cells (1–3). Although pharmacological and electrophysiological analysis has revealed the nature of some of these ion channels, molecular components

of anterior pituitary ion channels are poorly characterized.

In mouse AtT20 corticotropes large conductance calcium- and voltage- activated potassium (BK) channels play a pivotal role in the regulation of ACTH secretion (4, 5). In this system the stimulatory neuropeptide, CRH, potently inhibits BK channels through protein kinase A (PKA)-mediated phosphorylation of the BK channel complex (5, 6). Importantly, glucocorticoids prevent PKA inhibition of BK channels, and this action of the steroid is pivotal to early glucocorticoid inhibition of ACTH secretion in this system (4–6).

In contrast to the multiple gene families of other voltage-dependent cation channels, the pore-forming α -subunits of BK channels are encoded by a single gene, *Slo* (7, 8), that undergoes extensive, hormonally regulated, alternative RNA splicing (9, 10). These splice variants give rise to channels with different functional properties, including calcium sensitivity, single-channel conductance, and regulation by intracellular signaling pathways (11–14). In addition, association of α -subunits with accessory subunits may result in differential channel properties or modify their cellular distribution (15–18).

Mouse corticotrope BK channels display considerably higher calcium sensitivity (6) than previously identified α -subunits cloned from mouse brain. Furthermore, in common with native BK channels from several cell types of the anterior pituitary (5, 19, 20), mouse corticotrope BK channels are inhibited by PKA-mediated phosphorylation, whereas the majority of brain BK channels are activated by PKA (21, 22).

Elucidation of the molecular components of BK channels in specific cell types is beginning to reveal the molecular basis for their functional role in fundamental physiological processes (23, 24). To further address the functional role, and molecular regulation, of BK channels in mouse corticotrope cell function, we

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have 1) characterized and cloned the BK channel α -subunit splice variants expressed in mouse AtT20 D16:16 corticotropes, and 2) directly compared the functional characteristics (calcium sensitivity and regulation by PKA) of these identified subunits expressed in HEK 293 cells with native AtT20 D16:16 BK channels.

This report represents the first corticotrope ion channel to be functionally characterized at the molecular level. We demonstrate that mouse corticotrope BK channels are composed of α -subunits expressing the mouse homolog of the previously described cysteine-rich STREX-1 exon (10, 25). Furthermore, the STREX-1 α -subunit variants expressed in HEK 293 cells are inhibited by PKA-mediated phosphorylation.

RESULTS AND DISCUSSION

Cloning and Splice Variant Analysis of Mouse Corticotrope BK Channel α -Subunits

Cloned RT-PCR products of the entire open reading frame (ORF) of BK channel α -subunits from AtT20 D16:16 corticotropes had sequence identical to previously published mouse brain *mslo* BK channels (8, 26) with variations at known mammalian splice sites (25) (Fig. 1A). The majority of full-length cDNA clones, as well as RT-PCR products spanning α -subunit splice sites expressed in mouse AtT20 D16:16 corticotropes, contained the mouse variant of the previously described cysteine-rich, 59-amino acid insert at splice

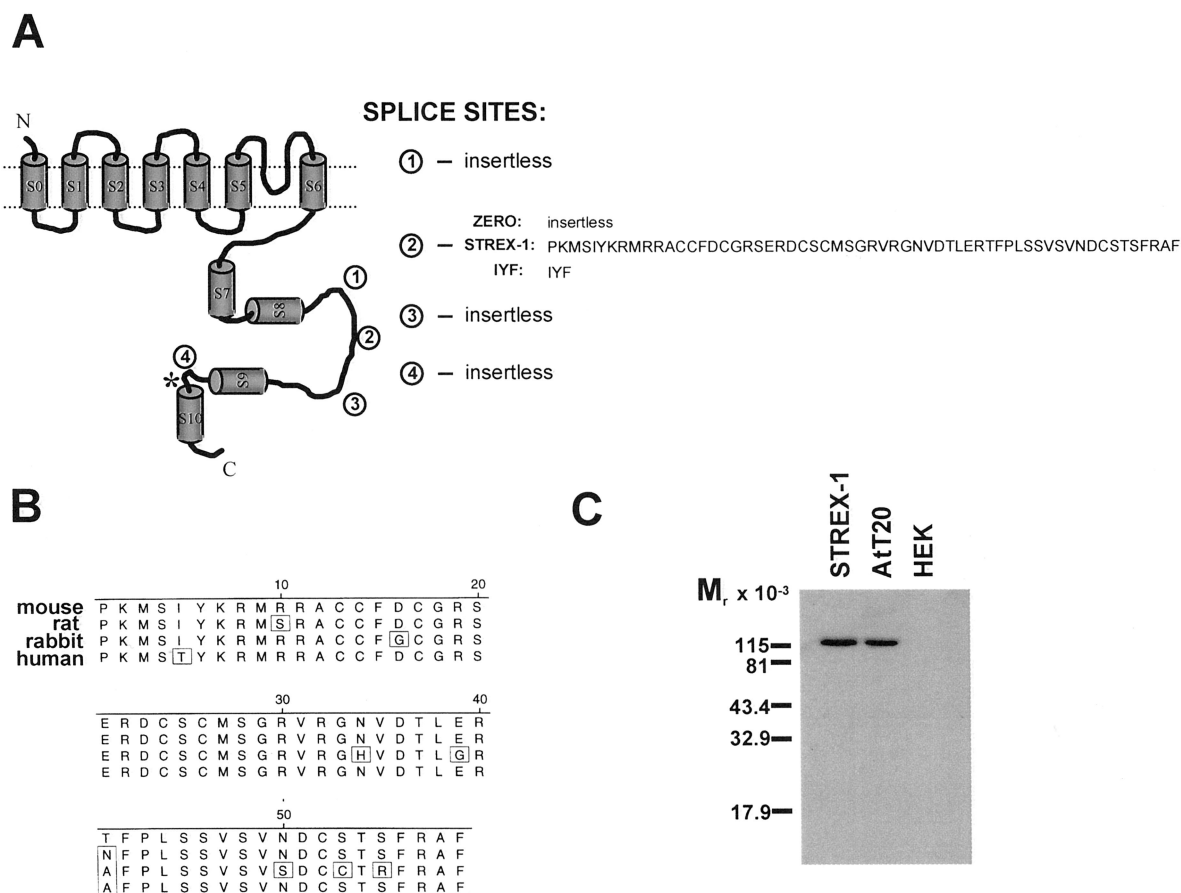


Fig. 1. BK Channel α -Subunit Splice Variants Expressed in Mouse AtT20 Corticotropes

A, Schematic of mammalian splice site inserts characterized in mouse AtT20 D16:16 corticotropes by RT-PCR analysis (see *Materials and Methods*) with predicted inserts represented by single amino acid codes. B, Amino acid sequence alignment of mammalian STREX-1 inserts characterized to date: mouse (AtT20 D16:16 cell and normal mouse pituitary, this study); rat chromaffin cells (25) and rat anterior pituitary (this study); rabbit kidney tubules (29); and human pancreatic islets (27). Boxed amino acids indicate differences from the mouse STREX-1 insert. Note: the STREX-2 insert (10) that contains additional amino acids at the N terminus of the STREX-1 exon have not been included in the alignment. Alignment positions are relevant to the start proline (P) amino acid of the respective splice insert. C, Western blot analysis of BK channel α -subunit expression in partially purified membrane fractions using the C-terminally directed α slo₍₉₁₃₋₉₂₆₎ antibody as described in *Materials and Methods*. This antibody recognizes the VNDTNVQFLDQDDD motif in the C-terminal tail (indicated by * in panel A) conserved in all *mslo* variants (28). Membranes were isolated from: HEK 293 cells stably expressing the mouse AtT20 corticotrope STREX-1 variant (10 μ g/lane, STREX-1); native AtT20 cells (40 μ g/lane, AtT20); and mock transfected HEK 293 cells (40 μ g/lane, HEK).

site 2, termed STREX-1 (10, 25). In approximately 25% of clones, either the IYF motif or null (ZERO) insert was expressed at splice site 2 (Fig. 1A). No evidence for inserts at splice sites 1, 3, or 4 was found (Fig. 1A). A STREX-1 splice site 2 insert identical to that characterized in AtT20 D16:16 corticotropes was also expressed in adult mouse anterior pituitary.

The mouse corticotrope splice site 2 insert (STREX-1) was similar, at the amino acid level, to recently identified STREX-1 exons in other mammalian endocrine cells including rat chromaffin cells (25) and human pancreatic islets (27) (Fig. 1B). Western blot analysis was performed using the C-terminally directed α -subunit antibody α slo₍₉₁₃₋₉₂₆₎ (28) that recognizes all *mslo* variants. Immunoblotting of partially purified plasma membranes from native mouse AtT20 corticotropes and HEK 293 cells stably expressing the entire coding region of the cloned STREX-1 α -subunit revealed expression of an approximately 125-kDa immunoreactive protein band that was not detectable in

mock transfected HEK 293 cells: the predicted molecular mass of the STREX-1 variant from translation of the ORF is 131 kDa.

Functional Characterization of Mouse Corticotrope α -Subunits Expressed in HEK 293 Cells

Expression of the STREX-1 and ZERO variants of mouse AtT20 corticotrope BK channel α -subunits in HEK 293 cells resulted in large outward macropatch voltage- and calcium-activated potassium currents (Fig. 2). In mock transfected HEK 293, under identical recording conditions, outward macropatch calcium- or voltage-activated potassium currents were not observed (Fig. 2A). Furthermore, RT-PCR (not shown) and Western blot (Fig. 1D) analysis did not reveal endogenous BK channel expression in this cell line. Transfection of cloned mouse AtT20 corticotrope BK channel α -subunits lacking the proposed initiator me-

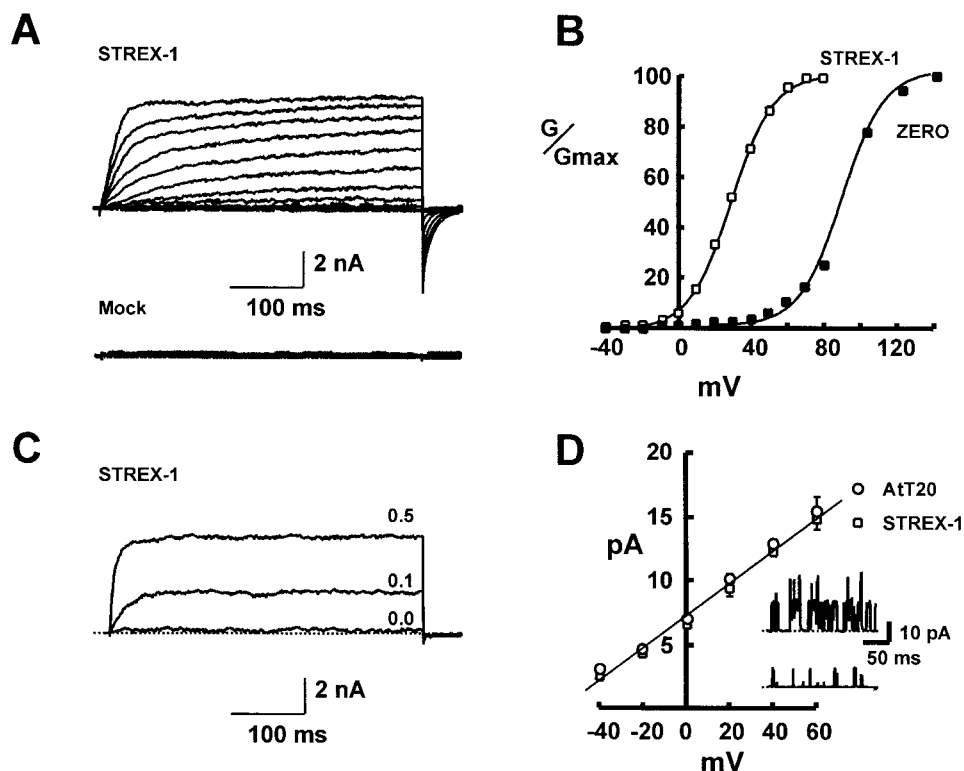


Fig. 2. Functional Characterization of STREX-1 and ZERO α -Subunits Expressed in HEK 293 Cells

A, Representative isolated inside-out macropatch recordings from mock transfected HEK 293 cells and cells expressing the mouse AtT20 D16:16 corticotrope STREX-1 α -subunit. The intracellular face of patches was exposed to 0.1 μ M $[Ca^{2+}]_i$, as described in *Materials and Methods* in the absence of ATP under physiological potassium gradients. Patches were held at -50 mV and depolarized for 500 msec to the respective potential (-40 to +80 mV). B, Relative conductance-voltage plots for the STREX-1 (open square, $n = 9$) and ZERO (closed square, $n = 4$) variants determined from macropatch recordings under the conditions in panel A. The lines show the best fit of a single Boltzmann function (see *Materials and Methods*). C, Representative current recordings at +40 mV demonstrating the effect of $[Ca^{2+}]_i$ (<10 nM (0), 0.1 μ M, and 0.5 μ M) on outward STREX-1 macropatch currents under the conditions in panel A with 1 mM ATP in the intracellular solution. D, Plot of unitary current amplitude as a function of activation voltage determined at 0.1 μ M $[Ca^{2+}]_i$ as in panel A for the STREX-1 (open square, $n = 8$) variant expressed in HEK 293 cells and native AtT20 BK channels (open circle, $n = 8$). The inset shows representative unitary current records from patches expressing the STREX-1 variant in HEK 293 cells at +40 mV (top trace) and 0 mV (bottom trace), respectively. All data are means $\pm$ SEM with error bars within the symbol size unless otherwise indicated.

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thionine (8, 26) in HEK 293 cells did not result in functional BK channel expression (M. J. Shipston, R. Duncan, and L. Tian, unpublished data).

The half-maximal activation voltage (V_{50}) for the STREX-1 variant exposed to $0.1 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ in physiological potassium gradients was 30.7 ± 1.6 mV ($n = 9$) compared with 96.1 ± 3.8 mV ($n = 4$) for the ZERO variant (Fig. 2B). Exposure of the intracellular face of ZERO channels to $10 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ resulted in a V_{50} of 15.3 ± 2.4 mV ($n = 3$). The V_{50} for the STREX-1 variant is similar to that observed for the majority ($> 90\%$) of native mouse AtT20 BK channels (26.3 ± 5.8 mV, $n = 11$, not shown) under identical recording conditions. Furthermore, the negative shift in V_{50} is similar in magnitude to that observed between other mammalian STREX-1 and corresponding ZERO BK α -subunit variants expressed in mammalian cell lines (25, 29). Increasing $[\text{Ca}^{2+}]_i$ at the intracellular face of patches expressing the STREX-1 variant resulted in outward macropatch currents with faster activation kinetics and larger steady-state current amplitude, demonstrating the calcium sensitivity of the channel (Fig. 2C). It is likely that native AtT20 BK channels ($< 10\%$) with low calcium sensitivity are composed of ZERO α -subunit variants.

The slope conductance of homomeric unitary STREX-1 channels [127.2 ± 4.2 picosiemens (pS), $n = 8$] in physiological potassium gradients was not significantly different from the slope conductance of native mouse AtT20 corticotrope channels recorded under identical conditions (125.2 ± 3.6 pS, $n = 8$, Fig. 2D). In contrast, the slope conductance of the homomeric ZERO channel variant was significantly greater (145.3 ± 3.1 pS, $n = 7$) than either STREX-1 or native mouse AtT20 corticotrope channels (not shown).

β -Subunits Are Not Integral Components of BK Channels in Mouse AtT20 Corticotropes

Taken together, these data suggest that native mouse AtT20 corticotrope BK channels are largely composed of the α -subunit STREX-1 variant. However, in some systems, including vascular smooth muscle cells (24), BK channel α -subunits functionally associate with regulatory β -subunits that confer enhanced calcium sensitivity compared with the α -subunit expressed alone (16, 30). To establish whether the enhanced calcium sensitivity of native mouse AtT20 corticotrope is a result of expression of the STREX-1 exon rather than association of ZERO variants with β_1 - or β_2 -subunits, we assayed the sensitivity of native mouse AtT20 BK channels for the triterpenoid glycoside, DHS-1 (16, 30). Previous studies have shown that submicromolar concentrations of DHS-1 activates BK channels only when the β_1 (or β_2)-subunit is functionally coupled to α -subunits (16, 25, 30). Coexpression of the ZERO α -subunit variant with β_1 -subunit in HEK 293 cells resulted in a leftward shift of the half-maximal activation potential by approximately 60 mV to 35.3 ± 7.4 mV, ($n = 3$) at $0.1 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ compared with the ZERO

variant alone (see Fig. 2B). Application of 100 nM DHS-1 to the intracellular face of patches containing ZERO + β_1 -subunit resulted in robust activation ($259.0 \pm 86.7\%$, $n = 4$) of channel activity in all patches tested (Fig. 3, A and B) that reversed to control upon washout. In contrast, under identical recording conditions, application of 100–500 nM DHS-1 to the intracellular face of patches from native mouse AtT20 BK channels or HEK 293 cells expressing the STREX-1 α -subunit variant or ZERO subunits alone (Fig. 3B) had no significant effect on BK channel activity. Previous studies (25) have reported that β_1 -subunits increase the sensitivity of the STREX-1 variant to $[\text{Ca}^{2+}]_i$ and DHS-1. Furthermore, RT-PCR analysis using degenerate primers based on the published mouse, human, and bovine β_1 -subunit sequences (16, 31) support our functional data that native mouse AtT20 corticotropes do not express endogenous β_1 -subunits (Fig. 3C). As a positive control for the RT-PCR screen, RNA from HEK 293 cells cotransfected with the ZERO α -subunit and β_1 -subunit showed robust expression (Fig. 3C). Thus, as DHS-1 has no effect on endogenous BK channel activity, β_1 -subunit transcripts are not expressed, and native AtT20 BK channels do not inactivate (5, 6, 32), our data suggests that the previously described β_1 - or β_2 -subunits are not integral components of native AtT20 corticotrope BK channels (16, 30).

STREX-1 α -Subunits Are Inhibited by cAMP-Mediated Protein Phosphorylation

BK channels from mouse AtT20 corticotropes are potentially inhibited by cAMP-dependent PKA activity intimately associated with the channel protein (5, 6). BK channels in other anterior pituitary cell types are also inhibited by PKA-dependent phosphorylation (19, 20). Previous studies have shown that expression of the human BK channel α -subunit homolog of the ZERO variant in *Xenopus* oocytes results in channel activation by PKA (33). Thus, to investigate whether the mouse STREX-1 variant could be the functional target for PKA-mediated inhibition in mouse AtT20 corticotropes, we examined the effect of activating endogenous PKA activity in excised inside-out patches from HEK 293 cells expressing the STREX-1 variant.

Application of 1 mM cAMP to the intracellular face of patches, in the presence of Mg-ATP, resulted in a robust inhibition of channel activity at both the macropatch and unitary current level (Figs. 4 and 5) in all patches tested. In macropatch recordings maximal inhibition (mean percentage change in pretreatment steady-state outward current, I_o , was $-47.5 \pm 3.6\%$, $n = 4$, $P < 0.01$ determined at +40 mV) was observed 10 min after application of cAMP to the intracellular face of the patch at all potentials examined (Fig. 4, A–C). The inhibitory effect of cAMP was completely blocked by the specific PKA inhibitor peptide, PKI_(5–24) (mean percentage change in I_o was $5.5 \pm 13.2\%$, $n = 3$). Furthermore, in the absence of ATP,

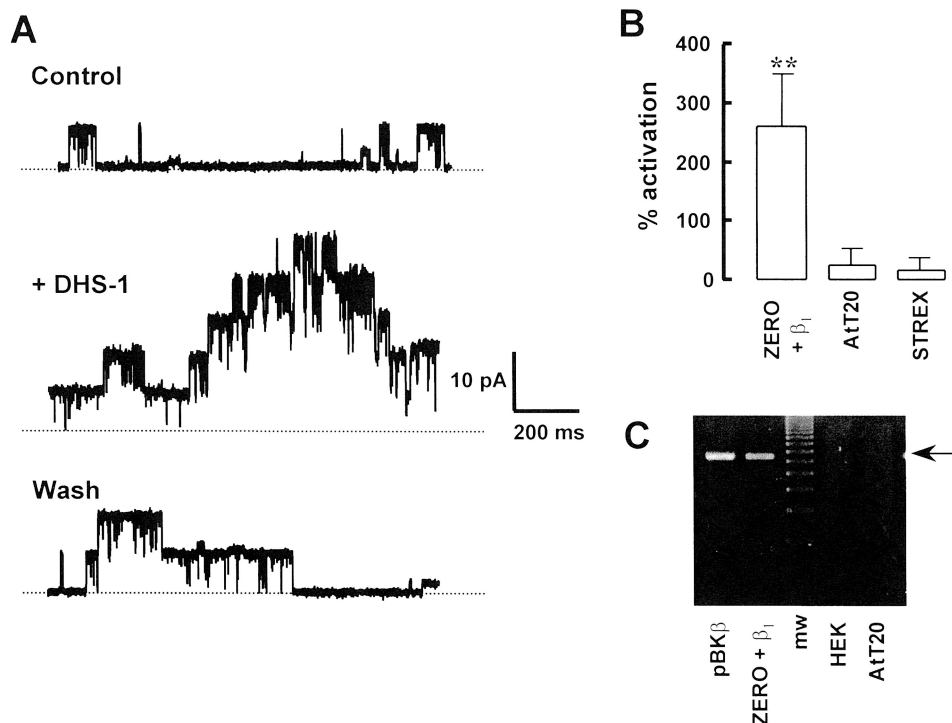


Fig. 3. Mouse Corticotrope BK Channel α -Subunits Are Not Functionally Coupled to β -Subunits

A, Representative traces of the effect of 100 nM DHS-1 applied to the intracellular face of a patch excised from HEK 293 cells coexpressing the ZERO mouse AtT20 corticotrope BK channel α -subunit and bovine β_1 -subunit (β_1) in HEK 293 cells. Traces from the same patch are for control (Control), 5 min after DHS-1 application (+ DHS-1), and 5 min after DHS-1 washout (wash). B, Summary of effect of DHS-1 on coexpressed channels as in A (ZERO + β_1 , $n = 4$, 100 nM DHS-1); native mouse AtT20 corticotrope BK channels (AtT20, $n = 5$, 100–500 nM DHS-1); and mouse AtT20 corticotrope STREX-1 subunits expressed alone in HEK 293 cells (STREX-1, $n = 3$, 100–500 nM DHS-1). Data are expressed as the mean % activation (percentage increase in channel NPo 5 min after DHS-1 application relative to pretreatment control) $\pm$ SEM. **, $P < 0.01$ (ANOVA) compared with STREX-1 or AtT20 group. C, Representative 1% agarose gel of ethidium bromide-stained PCR products generated as described in *Materials and Methods* from: pcDNA3 plasmid containing bovine β_1 subunit construct (pBK β); reverse transcription (RT) product from HEK 293 cells transiently coexpressing the mouse AtT20 ZERO corticotrope BK α -subunit variant and bovine β_1 subunit (ZERO + β_1); RT product from mock transfected HEK 293 cells (HEK); and RT product from native mouse AtT20 corticotropes (AtT20). Molecular weight markers (mw: 100-bp ladder) are shown; arrow indicates position of full-length β_1 -subunit transcript.

cAMP alone had no significant inhibitory effect. Indeed, removal of ATP resulted in a small, but significant, increase in steady-state outward current by $24.6 \pm 11.9\%$, $n = 4$, $P < 0.05$ after 10 min (not shown). In an inside-out patch containing a single STREX-1 BK channel, application of cAMP to the intracellular face of the patch resulted in a robust inhibition of single-channel mean open probability (P_o) with a time course similar to that observed for macro-patch records (Fig. 5, A–C). Furthermore, in three other patches in which unitary currents could be resolved, cAMP significantly inhibited NP_o ($P < 0.01$), an effect that was blocked by PKI_(5–24) ($n = 2$ patches; not shown).

Taken together, these results demonstrate that cAMP-dependent activation of PKA closely associated with the STREX-1 BK channel complex inhibits STREX-1 channel activity expressed in HEK 293 cells. As native BK channels in AtT20 mouse corticotrope cells are potently inhibited by PKA closely associated with the channel complex (6), our data suggest that the

STREX-1 α -subunit, or a protein that functionally associates with the STREX-1 α -subunit in corticotropes and HEK 293 cells, is the functional target for PKA-mediated inhibition.

Mouse Corticotrope BK Channels Are Predominantly Composed of the STREX-1 α -Subunit Variant

The data presented in this report provide the first molecular and functional characterization of an ion channel expressed in anterior pituitary corticotropes. Several lines of evidence suggest that mouse AtT20 corticotrope BK channels are composed of α -subunits containing the mouse homolog of the previously described 59-amino acid, cysteine-rich, STREX-1 exon at splice site 2 (10, 25).

First, cDNA cloning and RT-PCR splice site analysis revealed robust expression of STREX-1 containing α -subunits in mouse AtT20 corticotropes. Importantly, an identical STREX-1 exon is expressed

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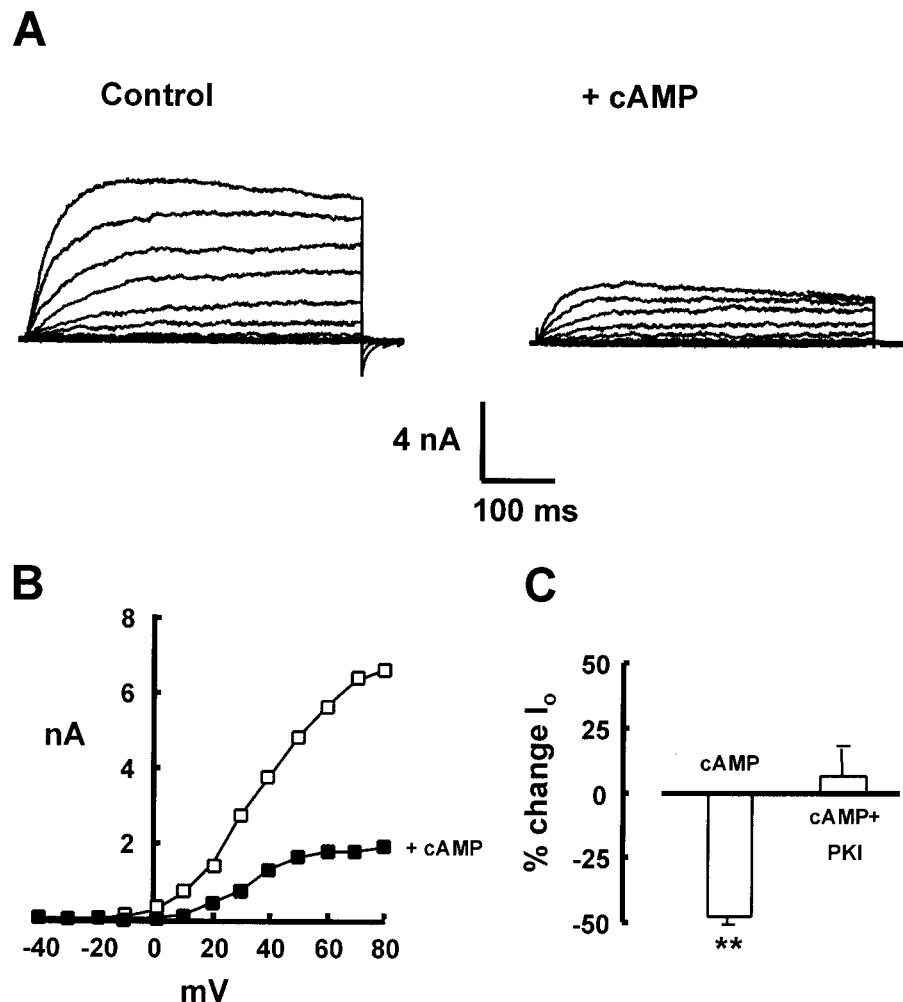


Fig. 4. Activation of PKA Inhibits Macropatch STREX-1 BK Currents

A, Representative outward macropatch currents from HEK 293 cells stably transfected with the STREX-1 variant before (control) and 10 min after application of 1 mM cAMP to the intracellular face of the patch. Macropatch currents were determined as in Fig. 2 by depolarization from -50 mV to the respective potential (-40 to $+80$ mV) with $0.1 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP. B, Current-voltage relationship for the traces shown in panel A above. Peak steady-state current (I_o) was determined 450 msec into the pulse and plotted as a function of membrane potential. C, Effect of cAMP on I_o 10 min after application of 1 mM cAMP to the intracellular face of macropatches (cAMP, $n = 4$) or after preincubation with the specific PKA inhibitor, PKI₍₆₋₂₄₎, and subsequent 10 min application of cAMP (cAMP + PKI, $n = 3$). Data were determined as in panel A with percentage change in I_o determined at $+40$ mV and expressed as mean $\pm$ SEM; $P < 0.01$ (t test) compared with cAMP + PKI group

in normal adult mouse anterior pituitary gland and a highly homologous exon (2 amino acid differences out of 59) is expressed in rat anterior pituitary cells. Second, functional expression of the STREX-1 α -subunit in HEK 293 cells revealed channels with almost identical calcium sensitivity, unitary conductance, and inhibition by PKA as for the majority ($> 90\%$) of native AtT20 D16:16 BK channels under identical recording conditions. In contrast, expression of the ZERO α -subunit variant resulted in channels with higher single-channel slope conductance and significantly lower calcium sensitivity compared with the STREX-1 α -subunit or native AtT20 BK channels. Finally, RT-PCR analysis and functional

assays using the triterpenoid glycoside, DHS-1, revealed that the high calcium sensitivity of native mouse AtT20 corticotrope BK channels is not a result of association of α -subunits with previously described β -subunits (16, 30).

Importantly, the STREX-1 α -subunit variant, as for endogenous BK channels in mouse AtT20 corticotropes (6), is inhibited by PKA-dependent protein phosphorylation. Indeed, several putative PKA consensus phosphorylation sites can be assigned from primary sequence data that are distributed across the C-terminal intracellular domain. However, we cannot exclude that the STREX-1 α -subunit associates with unidentified regulatory subunits, in native AtT20

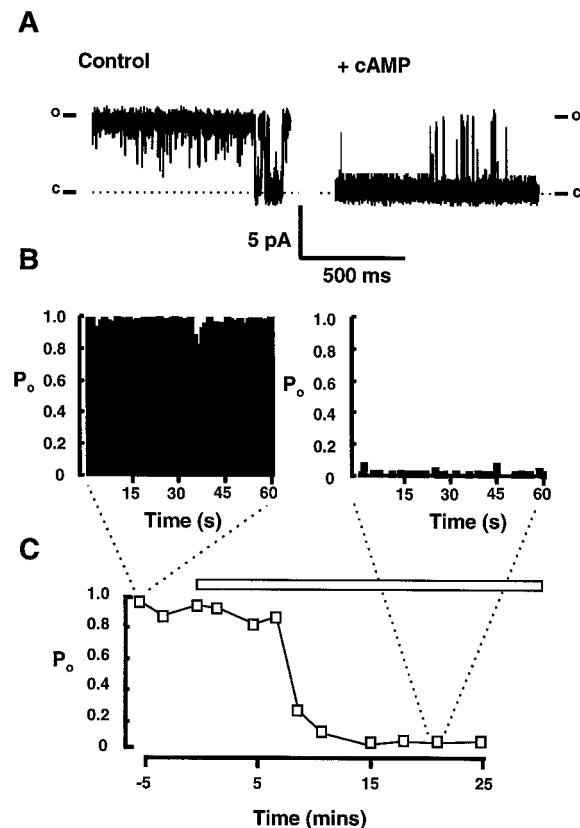


Fig. 5. Closely Associated PKA Inhibits Single STREX-1 BK Channels

A, Representative unitary current records from an isolated inside-out patch containing a single BK channel from HEK 293 cells stably expressing the STREX-1 variant before (Control) and 10 min after (+ cAMP) application of 1 mM cAMP to the intracellular face of the patch. Patches were exposed to $0.5 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP and depolarized to 0 mV for 60 sec at the respective time point. B, Diary plots of mean single-channel open probability (P_o) determined every second (vertical solid bars) 3 min before and 10 min after the application of cAMP. C, Mean P_o determined from 60 sec of continuous recording and plotted as a function of time for the above. cAMP (1 mM) was applied at 0 min for the duration of the experiment (horizontal open bar).

D16:16 corticotropes or HEK 293 cells, that confer sensitivity to PKA inhibition.

Increasing evidence suggests that the STREX-1 or STREX-2 (STREX) exon (10, 25) is widely expressed in neuroendocrine cells including pancreatic β -cells and adrenal chromaffin cells (25, 27) as well as anterior pituitary corticotropes (this study). Thus, the STREX exon may be a common feature of neuroendocrine cells in which BK channel α -subunits retain a high sensitivity to calcium in the absence of functional interaction with β -subunits, as described for vascular smooth muscle cells (24). Intriguingly, the rat STREX exons expressed in adrenal chromaffin cells is hormonally regulated by the stress axis and has been proposed to be expressed in excitable cells associated with enhanced repetitive action potential firing

(10, 23). Thus, the STREX variants in other components of the stress axis, including anterior pituitary cells, may also be under dynamic long-term regulation as well as involved in the short-term regulation of corticotrope function by CRH and other signaling pathways (5, 6, 32). Elucidation of the molecular components of mouse corticotrope BK channels reported in this study should allow us to define further the functional role, and molecular regulation, of these important multiple coincidence detectors in anterior pituitary corticotropes.

MATERIALS AND METHODS

Cell Culture

AtT20 D16:16 cells were maintained as previously described (5). Briefly, cells were maintained in DMEM containing 10% FCS (Harlan Seralab, Crawley Down, UK) in a humidified atmosphere of 95% air-5% CO_2 at 37°C. Cells were routinely passaged every 7 days using 0.25% trypsin in HBSS containing 0.1% EDTA after reaching 80% confluency. HEK 293 cells, a generous gift from David N. Sheppard, University of Edinburgh, were maintained as for AtT20 D16:16 cells except cells were passaged every 3–4 days.

Isolation of cDNAs Encoding Mouse Corticotrope BK Channels

An RT-PCR strategy was used to amplify cDNAs encoding the entire ORF of mouse anterior pituitary AtT20 D16:16 corticotrope BK channel α -subunits. Briefly, total cellular RNA from approximately 10^7 mouse AtT20 D16:16 corticotropes was isolated using standard techniques (34) and mRNA purified using poly (A) quik columns (Stratagene, La Jolla, CA) according to the manufacturer's instructions. One microgram of this RNA was used as template in a first strand cDNA synthesis directed from an anchored deoxyoligo d(T) primer (5'-TTCTAGAATTGAGCGGCCGC(T)₃₀N₁N₂), using Superscript II reverse transcriptase (Life Technologies, Inc., Paisley, UK). The resultant cDNA was diluted and used in a PCR to amplify the entire ORF using forward (spanning initiator methionine: 5'-GAT GGA TGC GCT CAT CAT MCC G) and reverse (spanning stop codon: 5'-CTG GGA TAG GCA TTA TCC GGC TCA) deoxynucleotides, with Expand polymerase (Roche Diagnostics Ltd, Lewes, UK). PCR product(s) were ligated to a T/A vector (pCR2.1, Invitrogen, San Diego, CA) and completely sequenced on both strands (OSWEL DNA Services, Southampton, UK). The STREX-1 variant sequence has been deposited in Genbank, accession number: AF156674.

Splice Variant Analysis

First-strand cDNA from mRNA isolated from AtT20 D16:16 cells and adult mouse anterior pituitary glands was generated as described above. Analysis of splice sites was performed using primers encompassing splice sites 1–3: forward (5'-CAG AGT CAA GAT AGA GTC AGC); reverse (5'-AAG TGG CAT CAC CAG GTT CCG) and for splice site 4: forward (5'-GAT ACT TCG CTT CAG GAC AAG G); reverse (5'-AAT GTC TGC GGA GTG CTG TAG C). In some experiments a nested PCR approach, with external primers that spanned the pore region and splice sites 1–4 in the first round amplification, followed by a second round amplification with the above internal primer sets, was used to confirm splice vari-

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ants (not shown). PCR products were characterized by gel electrophoresis and restriction digestion, and products of interest were ligated into the T/A vector pCR2.1 (Invitrogen) and sequenced on both strands.

For RT-PCR analysis of β_1 -subunit expression, degenerate primers were designed against the published bovine, human, and mouse β_1 -subunits (16, 31). Forward primer: 5'-ATG GKR AAG AAG CTG GTG ATG GCC; reverse primer: 5'-TCT GRG CCG CCA GGA TGG; and PCR products analyzed as above.

Construction of Expression Plasmids and Expression in HEK 293 Cells

Subcloning of the entire ORF of AtT20 BK channel subunits into pcDNA3.1 resulted in low expression of channels in HEK 293 cells compared with channel constructs containing additional 5'- and 3'-untranslated region (UTR) (14). To improve channel expression *HindIII-NheI* restriction fragment(s) from AtT20 BK channel clones, spanning splice sites 1–3, were ligated into the *HindIII-NheI* site of the mbr5 *mslo* (8) variant (Genbank accession number: L16912). The *HindIII-NheI* sites are conserved in all *mslo* variants, and constructs were generated from the mbr5-BSMxt plasmid construct, a generous gift from Dr Lawrence Salkoff (Washington University, St. Louis, MO) (8). The mbr5 clone is identical in sequence to characterized mouse corticotrope clones except that splice sites 1–4 do not contain inserts and mbr5 contains additional 5'- and 3'-UTR. Thus mbr5 is identical to the ZERO variant (see *Results*) isolated from AtT20 D16:16 corticotropes. The *KpnI-XbaI* fragment of mbr5 encompassing the entire ORF and additional 5'- and 3'-UTR and containing either the AtT20 null splice site 2 (ZERO, see *Results*) or containing the AtT20 STREX-1 splice site 2 insert (STREX-1) were subcloned into the mammalian expression vector pcDNA3.1+ or pcDNA3.1+ zeo respectively (Invitrogen BV, Leek, The Netherlands) for expression in HEK 293 cells.

The bovine tracheal smooth muscle cell BK channel β_1 -subunit (β_1) cDNA was kindly provided by Dr. Reid J. Leonard (Merck Research Laboratories, Rahway, NJ) (16). The *EcoRI-NotI* fragment encoding the entire ORF was subcloned into the mammalian expression vector pcDNA3.1+ zeo (Invitrogen BV).

For transient transfections, HEK 293 cells were seeded onto glass coverslips 24 h before transfection at 40% confluency and transfected with 1 μ g of the respective expression plasmid using lipofectamine (Life Technologies, Inc.) essentially as described by the manufacturer. For cotransfections, β_1 -subunit DNA was transfected at a 5-fold excess over the α -subunit. Cells were used 24–72 h after the start of transfection at between 40–80% confluency. Stable cell lines were created as above by seeding in 24-well cluster dishes (Costar, Cambridge, MA), and stable transformants were selected for zeocin resistance using 0.2 mg/ml zeocin (Invitrogen).

Electrophysiology

BK channels in AtT20 D16:16 corticotropes or cloned channels expressed in HEK 293 cells were analyzed under voltage-clamp in the excised inside-out configuration of the patch clamp technique at room temperature (20–24 °C) using physiological potassium gradients essentially as previously described (6, 14). The pipette solution (extracellular) contained (in millimolar concentration): 140 NaCl, 5 KCl, 0.1 CaCl₂, 5 MgCl₂, 20 glucose, 10 HEPES, pH 7.4. The bath solution (intracellular) contained (in millimolar concentration): 140 KCl, 5 NaCl, 2 MgCl₂, 1 or 5 (1,2-bis-O-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid (BAPTA), 30 glucose, 10 HEPES, pH 7.35, with free calcium [Ca²⁺], buffered to the concentration indicated in the respective figure legend and calculated as previously described (35). For [Ca²⁺], greater

than, or equal to, 1 μ M, dibromo-BAPTA was used as the calcium buffer.

Data acquisition and voltage protocols were controlled by an Axopatch 200 A or B amplifier and pCLAMP6 software (Axon Instruments Inc., Foster City, CA). All recordings were sampled at 10 kHz and filtered at 2 kHz. For patches in which unitary currents could be resolved, channels were voltage clamped at the potential indicated in the respective figure legend. Mean steady-state single-channel open probability (P_o) was determined from at least 30 sec of continuous recording under each experimental condition. For macro-patch recordings, outward BK currents were evoked by 500-msec step depolarizations (–50 to +80 mV), and the steady-state current amplitude, averaged from five consecutive depolarizations 450 msec into the pulse, was determined at each potential. For macro-patch recordings with seal resistances > 10 G Ω leak subtraction was not routinely applied. Pipettes were manufactured from Garner no. 7052 glass, coated with sylgard, and had typical resistances of between 2–10 M Ω in bath solution after fire polishing. Patches containing a single BK channel (verified at +80 mV and 10 μ M [Ca²⁺]) were extremely rare (<0.5% of total patches) even with pipette resistance values > 10 M Ω .

Additional analysis and curve fitting was performed using Igor Pro3.1 (Wavemetrics Inc., Lake Oswego, OR). Conductance values (G) were calculated from peak current (I) measured at 450 msec into the voltage pulse using the relationship $G = I/(V - E_k)$ where V is the activation potential and E_k is the calculated potassium reversal potential. Conductance-voltage curves were fit with a single Boltzmann function, $G(V) = G_{\max}/(1 + \exp(V_{50} - V)/k)$, where $G_{\max}$ corresponds to the maximal conductance, V_{50} is the voltage for half-maximal activation, V is the activation potential, and k is the slope factor reflecting the voltage dependence of conductance.

Western Blotting

Partially purified membrane homogenates from AtT20 D16:16 cells were prepared by homogenizing approximately 10⁷ cells on ice in the following (in millimolar concentration): 150 KCl, 5 EGTA, 2 MgCl₂, pH 10.6, containing 12 U/ml aprotinin, 5 μ g/ml leupeptin, 6 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, and 4 mM Pepstatin A followed by two freeze thaw cycles. After centrifugation for 5 min 1,000 $\times g$ at 4 °C, the resultant supernatant was pelleted at 40,000 $\times g$ to give the crude membrane fraction. Protein samples (10–40 μ g) were separated on a 10% SDS gel and electroblotted to Immobilon polyvinylidene fluoride membranes. Membranes were blocked for 2 h at room temperature with PBS containing 0.1 mM EDTA, 0.1% Triton X-100, pH 7.4 (PBS-TE), and 5% (wt/vol) low-fat milk (Marvel). Blots were incubated overnight at 4 °C with a 1:2000 dilution of the affinity-purified antibody α slo_(913–926) [directed toward residues 913–926 of the pore-forming α -subunit of mouse brain BK channels (28)] in PBS-TE containing 1% wt/vol Marvel. Blots were washed five times with PBS-TE and incubated for 45 min at room temperature with horseradish peroxidase-conjugated antirabbit IgG (Amersham Pharmacia Biotech, Piscataway, NJ; 1:5000 final dilution) in PBS-TE containing 5% (wt/vol) Marvel. After five washes in PBS-TE, blots were incubated with enhanced chemiluminescence (ECL) reagents (Amersham Pharmacia Biotech) according to the manufacturer's protocol and blots were exposed to ECL film in the linear response range (Amersham Pharmacia Biotech).

Reagents

ATP magnesium or potassium salt, was from Sigma-Aldrich Co. (St. Louis, MO) and stored as buffered 1 M stock solutions at –20 °C before use. ATP and cAMP were buffered in bath solution to pH 7.35 and applied to the intracellular patch by gravity perfusion with 10 volumes of bath solution at a flow

rate of 1–2 ml/min. PKI_{5–24}, BAPTA, dibromo-BAPTA, and Fura-2 were obtained from Calbiochem (Nottingham, UK). DHS-1 was a generous gift of Dr. Owen McManus (Merck Research Laboratories, Rahway, NJ). The C-terminal α slo_(913–926) antibody was a generous gift of Dr. Hans-Guenther Knaus (University of Innsbruck, Innsbruck, Austria). All other reagents, unless otherwise stated, were of the highest analytical grade available from Sigma-Aldrich Co. Ltd., Poole, UK, or Merck Ltd., Poole, UK.

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Alternative splicing determines sensitivity of murine calcium-activated potassium channels to glucocorticoids

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1. Large-conductance Ca^{2+} - and voltage-activated potassium (BK) channels are important regulators of cellular excitability. Here, we present a patch-clamp electrophysiological analysis of splice-variant-specific regulation by the synthetic glucocorticoid dexamethasone (DEX) of BK channels consisting of cloned STREX or ZERO α -subunit variants expressed in human embryonic kidney (HEK 293) cells.
2. STREX channels in isolated membrane patches were inhibited by protein kinase A (PKA) and this was blocked on pre-treatment of intact cells with DEX (100 nM) for 2 h.
3. The effect of DEX required the synthesis of new mRNA and protein. Furthermore, it required protein phosphatase 2A (PP2A)-like activity intimately associated with the channels, as it was blocked by 10 nM okadaic acid but not by the specific protein phosphatase-1 inhibitor peptide PPI-2.
4. ZERO variant channels that lack the STREX insert were activated by PKA but were not influenced by DEX. ZERO channels containing a mutant STREX domain ($\text{S4}_{\text{STREX}}\text{A}$) were also activated by PKA. Importantly, DEX blocked PKA activation of $\text{S4}_{\text{STREX}}\text{A}$ channels in a PP2A-dependent manner.
5. Taken together, the STREX domain is crucial for glucocorticoid regulation of BK channels through a PP2A-type enzyme. Moreover, glucocorticoids appear to induce a generic set of proteins in different types of cells, the actions of which depend on the expression of cell-specific targets.

Adrenal glucocorticoids are pivotal for adaptation to environmental challenge in a wide range of tissues including the central nervous and endocrine systems (Sapolsky *et al.* 2000). These hormones can potently modify the electrical excitability of target cells through regulation of ion channel activity that requires the rapid induction of new proteins. Increasing evidence suggests that in neurones and endocrine cells, potassium channels are important targets for glucocorticoid action. For example glucocorticoids rapidly induce the expression of potassium channel subunits in some systems (Levitan *et al.* 1991; Attali *et al.* 1995), but in both hippocampal CA1 neurones and neuroendocrine anterior pituitary corticotroph (AtT20) cells (Joels & de Kloet, 1989; Shipston *et al.* 1996; Tian *et al.* 1998), the activity of Ca^{2+} -activated potassium channels is modulated by glucocorticoid-induced signalling pathways. In both of these systems glucocorticoid action is mediated by type II glucocorticoid receptors, occurs within 2 h, and requires *de*

novo synthesis of mRNA as well as protein. However, the underlying mechanism(s) are poorly understood.

Large-conductance Ca^{2+} -activated potassium channels (BK channels) are pivotal targets of glucocorticoids in AtT20 cells (Shipston *et al.* 1996; Tian *et al.* 1998). In these neuroendocrine cells, cAMP-generating agonists inhibit BK channels through cAMP-dependent protein kinase (PKA). Proteins rapidly induced by the synthetic glucocorticoid dexamethasone block the inhibition of BK channels by PKA, and this is crucial for the suppression of stimulated ACTH release by glucocorticoids (Shipston *et al.* 1996). The effect of glucocorticoids on BK channels is blocked by low nanomolar concentrations of okadaic acid (Tian *et al.* 1998), suggesting an involvement of serine/threonine protein phosphatases, for example protein phosphatase (PP) 1 or 2A in glucocorticoid action.

AtT20 cells express two major BK channel splice variants, ZERO and STREX (Shipston *et al.* 1999). Unlike many

other voltage-gated potassium channels, the pore-forming BK channel subunits are derived from a single gene that undergoes extensive hormonally regulated alternative splicing (Adelman *et al.* 1992; Butler *et al.* 1993; Pallanck & Ganetzky, 1994; Tseng-Crank *et al.* 1994; Xie & McCobb, 1998; Xie & Black, 2001). The STREX variant is identical to ZERO except for a 59 amino acid insert at site 2, which is one of at least five different splice sites in the C terminal portion of the BK channel α -subunit protein encoded by the mammalian *Slo* gene (Saito *et al.* 1997; Shipston *et al.* 1999). The STREX insert confers enhanced calcium sensitivity to BK channels (Saito *et al.* 1997; Shipston *et al.* 1999). Furthermore, STREX channels are inhibited, whereas ZERO channels are activated, by PKA closely associated with the channel complex (Tian *et al.* 2001).

In this report we have examined whether the pore-forming subunits of BK channels are targets for glucocorticoid regulation and whether such modulation is dependent upon the BK channel splice variant expressed. To address these issues we have investigated the regulation of the cloned BK channel splice variants, STREX and ZERO, heterologously expressed in human embryonic kidney 293 (HEK 293) cells. We demonstrate that the STREX insert confers glucocorticoid sensitivity on BK channels by targeting a glucocorticoid-induced signalling pathway, involving serine/threonine protein phosphatases, to potassium channels. Differential modulation of BK channel splice variants by glucocorticoids would provide a powerful mechanism to tune the excitability of glucocorticoid-sensitive target cells.

METHODS

Construction of BK channel splice variant and mutant cDNA constructs

The cloning and sub-cloning of the mouse BK channel splice variants and respective site directed mutants into the mammalian expression vector pcDNA3 or pcDNA3.1+ (Invitrogen, Leek, The Netherlands) have been described previously (Clark *et al.* 1999; Shipston *et al.* 1999; Tian *et al.* 2001). Briefly, the ZERO variant lacks inserts at mammalian splice sites 1–5 and STREX channels are identical except for a 59 amino acid cysteine-rich insert at mammalian splice site 2 that increase the apparent calcium sensitivity of the channel (Saito *et al.* 1997; Shipston *et al.* 1999). The ZERO variant is activated by PKA, requiring a functional serine residue at position 869 within a putative PKA consensus motif present in the C-termini of all mammalian BK channel variants. The STREX variant is inhibited by PKA, dependent upon a conserved serine residue (S_4) within the STREX insert itself. The $S_{4\text{STREX}}A$ mutant has this serine residue mutated to alanine. The STREX-S869A mutant has a single point mutation of the conserved serine residue at position 869 to alanine. Site-directed mutagenesis was performed using Quik-change (Stratagene) and verified by DNA sequencing. The constructs used in these studies are shown schematically in Fig. 6B.

HEK 293 and AtT20 D16:16 cell culture and transfection

HEK 293 and AtT20 D16:16 cells were subcultured as previously described (Shipston *et al.* 1996, 1999). Briefly, cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS) in a humidified atmosphere of 95% air–5% CO₂ at

37°C. Cells were routinely passaged every 3–7 days using 0.25% trypsin in Hanks' buffered salt solution (HBSS) containing 0.1% EDTA. For immunoblotting studies, cells were grown to 70–80% confluence in 75 cm² flasks. For electrophysiological assays, cells were plated on glass coverslips in six-well cluster dishes.

For transient transfections of BK channels, HEK 293 cells were seeded onto glass coverslips in six-well cluster dishes at a density to allow cells to reach 40–60% confluence after 24 h. Cells were then transfected with 1 μ g of the respective cDNA using lipofectamine (Life Technologies) in DMEM, essentially as described by the manufacturer. After 5 h, medium was supplemented with 10% FCS, replaced after 24 h and electrophysiological recordings made 24–72 h post-transfection. Stable cell lines containing different BK variants were also created from these transfections and the transformants were then selected and maintained for neomycin or zeocin resistance using 0.2 mg ml⁻¹ zeocin (Invitrogen) or 0.8 mg ml⁻¹ geneticin (Life Technologies) as appropriate. The HEK 293 cells do not express endogenous BK channels at the mRNA (by RT-PCR), protein (Western blotting) or functional electrophysiology level.

For luciferase assays, HEK 293 cells were plated directly into the six-well cluster dishes and transfected as above except that transfections and subsequent maintenance of cells was carried out in steroid-free medium (DMEM supplemented with insulin–transferrin–selenium (ITS, Sigma-Aldrich) supplement. The mammary tumour virus long terminal repeat (MMTV-LTR)–luciferase reporter construct (Leckie *et al.* 1995) was a generous gift from Dr K. Chapman (University of Edinburgh). Similar results were obtained with other artificial serum replacements and supra-maximal stimulation was identical in 10% FCS. However, the majority of FCS batches contain low to sub-nanomolar concentrations of cortisol that preclude accurate determination of the full dose–response range of MMTV-LTR–luciferase reporter activity to the synthetic glucocorticoid agonist dexamethasone.

Western blot analysis of glucocorticoid receptors

AtT20 and HEK 293 whole cell extracts (from $\sim 10^7$ cells) were prepared by resuspending cells in lysis buffer containing (mM): 20 Tris-HCl, 2 EDTA, 150 NaCl, 0.5% Triton X-100, pH 7.4, and supplemented with a protease inhibitor cocktail (Boehringer Mannheim). Cells were sheared through a 26G needle and incubated on ice for approximately 1 h. The lysate was immediately spun for 10 min at 10 000 *g* and 4°C and the supernatant used for subsequent SDS-PAGE and Western blot analysis. Total protein content was calculated using the Bradford protein assay (BioRad). Whole cell lysates (10 μ g protein from AtT20 cells and 50 μ g from HEK 293 cells) were resolved on 8% SDS-polyacrylamide gels and transferred under semi-dry conditions onto polyvinylidene difluoride (PVDF) membrane. Membranes were blocked using phosphate buffered saline containing 0.05% Tween 20 (0.05%) (PBS-T) and 4% non-fat dry milk (Marvel) for 1.5 h at room temperature (or overnight at 4°C). All washing stages were carried out using PBS-T. Blots were incubated for 1 h at room temperature with a 1:2000 dilution of anti-GR (M-20) rabbit primary antibody (Autogen BioClear UK, Calne, Wiltshire, UK), and probed with a 1:4000 dilution of donkey anti-rabbit IgG horseradish peroxidase conjugate (Amersham Life Sciences) under the same conditions. Visualisation and processing of the blots was by enhanced chemiluminescence (ECL) essentially as described by the manufacturer.

Luciferase reporter assay

HEK 293 cells transiently transfected with the MMTV-LTR luciferase reporter construct were used 24–72 h post-transfection. Cells were treated with varying concentrations of the synthetic glucocorticoid agonist dexamethasone or vehicle (DMSO) for 2 h at 37°C. Cells were quickly washed in phosphate buffered saline, lysed

by the addition of 0.4 ml lysis buffer mix (25 mM Tris phosphate, pH 7.8, 2 mM DTT, 1% Triton X-100 and 10% glycerol, containing 1.25 mg ml⁻¹ lysozyme and 2.5 mg ml⁻¹ BSA) scraped and briefly centrifuged at 12000 *g*. Duplicate (20 µl) aliquots of the resulting supernatants were assayed for luciferase activity using Promega luciferase assay reagent (Promega, Madison, WI, USA) and emitted light detected with a LKB (Wallac) 1250 luminometer. Light emission was averaged over 10 s with a 2 s delay after substrate addition to allow the signal to stabilise. All values were calculated as a percentage of maximum light production with cells treated with 1000 nM of dexamethasone as 100% after background (vehicle alone) subtraction. The apparent K_D was calculated according to the equation:

$$Y = Y_{\max}/(1 + ([\text{DEX}]/K_D)^h),$$

where Y is luciferase activity at a given dexamethasone concentration [DEX], $Y_{\max}$ is maximal luciferase activity, [DEX] is concentration of dexamethasone and h is the Hill coefficient.

Patch clamp electrophysiology

All experiments were performed in the inside-out configuration of the patch clamp technique at room temperature (20–24 °C) using physiological potassium gradients essentially as described previously (Clark *et al.* 1999; Shipston *et al.* 1999; Tian *et al.* 2001). The pipette solution (extracellular) contained (mM): 140 NaCl, 5 KCl, 0.1 CaCl₂, 2 MgCl₂, 20 glucose, 10 Hepes, pH 7.4. The bath solution (intracellular) contained (mM): 140 KCl, 5 NaCl, 2 MgCl₂, 1 or 5 BAPTA, 30 glucose, 10 Hepes, 1 mM ATP, pH 7.3 with free calcium ([Ca²⁺]_i) buffered to 0.2 µM, unless indicated otherwise. Data acquisition and voltage protocols were controlled by an Axopatch 200A or -B amplifier and pCLAMP 6 software (Axon Instruments, Foster City, USA). All recordings were sampled at 10 kHz and filtered at 2 kHz. Following patch excision, channel activity was allowed to stabilise for at least 10 min (typically 10–15 min after excision) and stability plot experiments demonstrated that BK channel activity was stable for > 1 h under the recording conditions used (data not shown) in the absence of channel modulators. Application of cAMP or other reagents to the intracellular face of patches was by gravity-driven perfusion (10 volumes of the recording bath solution (bath volume, 0.5 ml) at a flow rate of 1–2 ml min⁻¹) or direct application to the bath. In all experiments, 1 mM cAMP was used to activate endogenous PKA; similar results were also observed using 0.1 mM cAMP (not shown). Patches were held at 0 mV and channel activity was determined during voltage steps to +40 mV. To determine mean percentage change in channel activity after a treatment, in patches with low to moderate levels of channel expression, mean NP_o (number of functional channels × open probability of channel) was averaged from 60 s of recording at +40 mV immediately before and 10 min after the respective drug treatment. Mean change in activity was expressed as a percentage of the pre-treatment control ± S.E.M. with analysis undertaken using pCLAMP 6 and IGOR Pro 3.1 (Wavemetrics, Lake Oswego, OR, USA). In the respective figure legends and text a positive percentage change in activity reflects activation whereas a negative percentage change reflects channel inhibition.

The single channel slope conductance and half-maximal voltage for activation ($V_{0.5}$) for each construct under the recording conditions detailed above (except that the assays were performed in the absence of ATP) were as follows: STREX, 126.3 ± 4.1 pS, 26.8 ± 4.2 mV, $n = 4$; ZERO, 138.0 ± 5.1 pS, 76.1 ± 5.2 mV, $n = 4$; STREX-S869A, 125.2 ± 3.2 pS, 38.1 ± 7.3 mV, $n = 5$; S4_{STREX}A, 124.9 ± 3.7 pS, 32.7 ± 6.5 mV, $n = 4$.

Chemicals and materials

ATP magnesium salt, adenosine 5'-O-(3-thiotriphosphate) (ATPγS) tetralithium salt, adenosine 3',5'-cyclic monophosphate (cAMP),

dexamethasone, puromycin and 5,6-dichloro-1-β-D-ribofluranosylbenzimidazole (DRB) were all purchased from Sigma/Aldrich (Poole, UK). Nucleotides were stored as buffered 1 M or 0.1 M stock solutions at -20 °C prior to use. Dexamethasone was stored at -20 °C at 10 mM in Me₂SO. Purified protein phosphatase 2A catalytic subunit was from Promega. Okadaic acid was from LC Laboratories (Alexis Laboratories, Nottingham, UK). The specific protein kinase A inhibitor peptide (PKI₅₋₂₄) and protein phosphatase 1 inhibitor (PPI-2) were from Calbiochem-Novabiochem (Nottingham, UK). Polyvinylidene difluoride membranes and reagents for SDS-polyacrylamide gel electrophoresis and Western blotting were from Bio-Rad Laboratories (Hemel Hempstead, UK). All other reagents were from Sigma or BDH-Merck (Poole, UK).

Statistical analysis

Data are expressed as means ± S.E.M unless otherwise stated. Statistical significance was defined at $P < 0.05$ using a non-parametric Kruskal–Wallis or Mann–Whitney U test as appropriate.

RESULTS

HEK 293 cells express functional glucocorticoid receptors

In whole cell lysates of HEK 293 cells, Western blot analysis, using a rabbit polyclonal antibody that recognises both the GRα and GRβ isoforms (Hollenberg *et al.* 1985; Bamberger *et al.* 1995), revealed specific immunoreactivity at a molecular mass consistent with that for the GR isoforms (~95 kDa, Fig. 1A). No immunoreactivity was observed in the absence of primary antibody (not shown) and similar immunoreactive bands were present in whole cell lysates from mouse anterior pituitary AtT20 D16:16 corticotrophs, a cell line that expresses functional GR at a high level (Woods *et al.* 1992).

To examine whether HEK 293 cells contain functional GRα receptors, the mouse mammary tumour virus long terminal repeat (MMTV-LTR)–luciferase reporter plasmid, which responds to activated GR by enhancing luciferase expression (Leckie *et al.* 1995), was transiently transfected into the cells. Indeed, in this system DEX produced a concentration-dependent stimulation of luciferase activity with a K_D of 4.5 ± 3.6 nM with maximal activity at 100 nM.

Dexamethasone pre-treatment blocks PKA-mediated inhibition of STREX BK channels

In inside-out patches from HEK 293 cells expressing cloned BK channel splice variants, single BK channel events were characterised by their unitary slope conductance and sensitivity to voltage and calcium as previously described (data not shown; Shipston *et al.* 1999). Pre-treatment of cells with a maximally effective concentration (0.1–1 µM) of the synthetic glucocorticoid agonist dexamethasone (Woods *et al.* 1992; Shipston *et al.* 1996) had no significant effects on intrinsic BK channel properties.

Application of cAMP (0.1–1 mM) to the intracellular face of isolated inside-out patches from HEK 293 cells expressing STREX or the site directed mutant STREX-

S869A in the presence of 1 mM Mg-ATP and $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ resulted in a significant inhibition of BK channel activity (expressed as the percentage change of pre-treatment BK channel activity: for STREX, $-57.5 \pm 8.7\%$, $n = 13$, $P < 0.01$; for STREX-S869A, $-58.1 \pm 12.6\%$, $n = 11$, $P < 0.01$; determined 10 min after cAMP application compared with pre-treatment BK channel activity, Fig. 2A and B). As the effect of cAMP was identical in STREX and STREX-S869A channels, further analysis was largely carried out on STREX-S869A channels with results identical to those in STREX channels (not shown). The cAMP inhibition was dependent upon protein kinase A associated with the channels as the specific protein kinase A inhibitor peptide PKI₅₋₂₄ blocked the inhibitory effect of cAMP (the change was $-2.2 \pm 7.8\%$, $P < 0.01$, compared to $-58.1 \pm 12.6\%$ with cAMP alone, Fig. 2B). In parallel experiments, pre-treatment of HEK 293 cells with maximally effective concentrations of dexamethasone (0.1 – $1 \mu\text{M}$ for 2 h) abolished the inhibitory action of cAMP in excised inside-out patches containing STREX or STREX-S869A channels compared to the inhibition observed in patches from control cells (Fig. 2B). The percentage change in BK channel activity was $0.24 \pm 7.7\%$ in DEX-treated cells ($n = 11$).

Dexamethasone action is dependent upon *de novo* mRNA and protein synthesis

Early (within 2 h) glucocorticoid action on BK channel regulation and hormone secretion in corticotrophs is dependent upon activation of intracellular GR and induction of new mRNA and protein synthesis (Woods *et al.*

1992; Shipston, 1995; Shipston *et al.* 1996). To address whether such a genomic mechanism is responsible for the action of dexamethasone, HEK 293 cells were pre-treated with inhibitors of mRNA transcription (5,6-dichloro-1- β -D-ribofluranosylbenzimidazole (DRB), 50 nM, 15 min before and during DEX treatment) or protein translation (puromycin, 100 μM (Woods *et al.* 1992; Tian *et al.* 1999), 20 min before and during DEX treatment). Treatment of HEK 293 cells expressing STREX-S869A channels with either DRB or puromycin prior to and during exposure to dexamethasone blocked the action of dexamethasone, i.e. cAMP-mediated inhibition of BK channel activity returned to near control (non-dexamethasone-treated) levels (Fig. 2A and B). The cAMP-mediated percentage inhibition of BK channel activity in DRB and puromycin pre-treated cells was $-39.8 \pm 11.4\%$ ($P < 0.01$, $n = 3$) and $-39.7 \pm 2.0\%$ ($P < 0.01$, $n = 4$), respectively. These data suggest that dexamethasone attenuates PKA-mediated inhibition of BK channel activity via a genomic mechanism. Dexamethasone had no effect on BK channel transcript or protein expression levels as determined by RT-PCR and Western blotting, respectively, over the time course (2 h) of these studies (not shown).

Glucocorticoid action is mediated via protein phosphatase activity intimately associated with the channel complex

Using ATP γ S as the phosphate donor in place of ATP in excised inside-out patches from dexamethasone-treated STREX-S869A cells restored cAMP-mediated inhibition of BK channel activity to almost the same extent as that

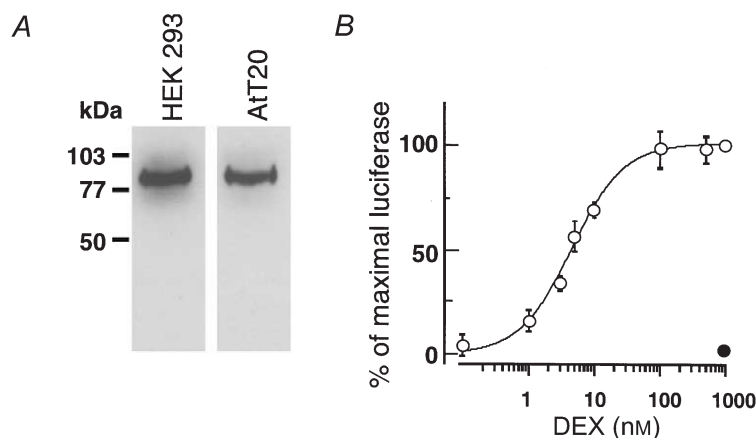


Figure 1. HEK 293 cells express functional glucocorticoid receptors

A, whole cell lysates (10 μg protein from AtT20 cells as positive control and 50 μg from HEK 293 cells) were separated by SDS-PAGE and transferred to PVDF membranes. Blots were probed as described in methods with an anti-GR (M20) rabbit primary antibody that recognises both GR α and GR β isoforms with detection by ECL; ~ 95 kDa immunoreactive bands were detectable in both AtT20 and HEK 293 cell lysates. B, functional assay of glucocorticoid receptor activation in HEK 293 cells using a MMTV-luciferase reporter assay. HEK 293 cells were transiently transfected with 1 μg of MMTV-luc plasmid (○) or pcDNA3 vector alone (●) and treated for 2 h with various concentrations of the synthetic glucocorticoid receptor agonist dexamethasone or vehicle before assay for luciferase activity as described in Methods. Data are expressed as a percentage of maximal (with 1 μM dexamethasone) luciferase activity after vehicle background subtraction. Each data point represents the mean $\pm$ S.E.M ($n = 4$ –8).

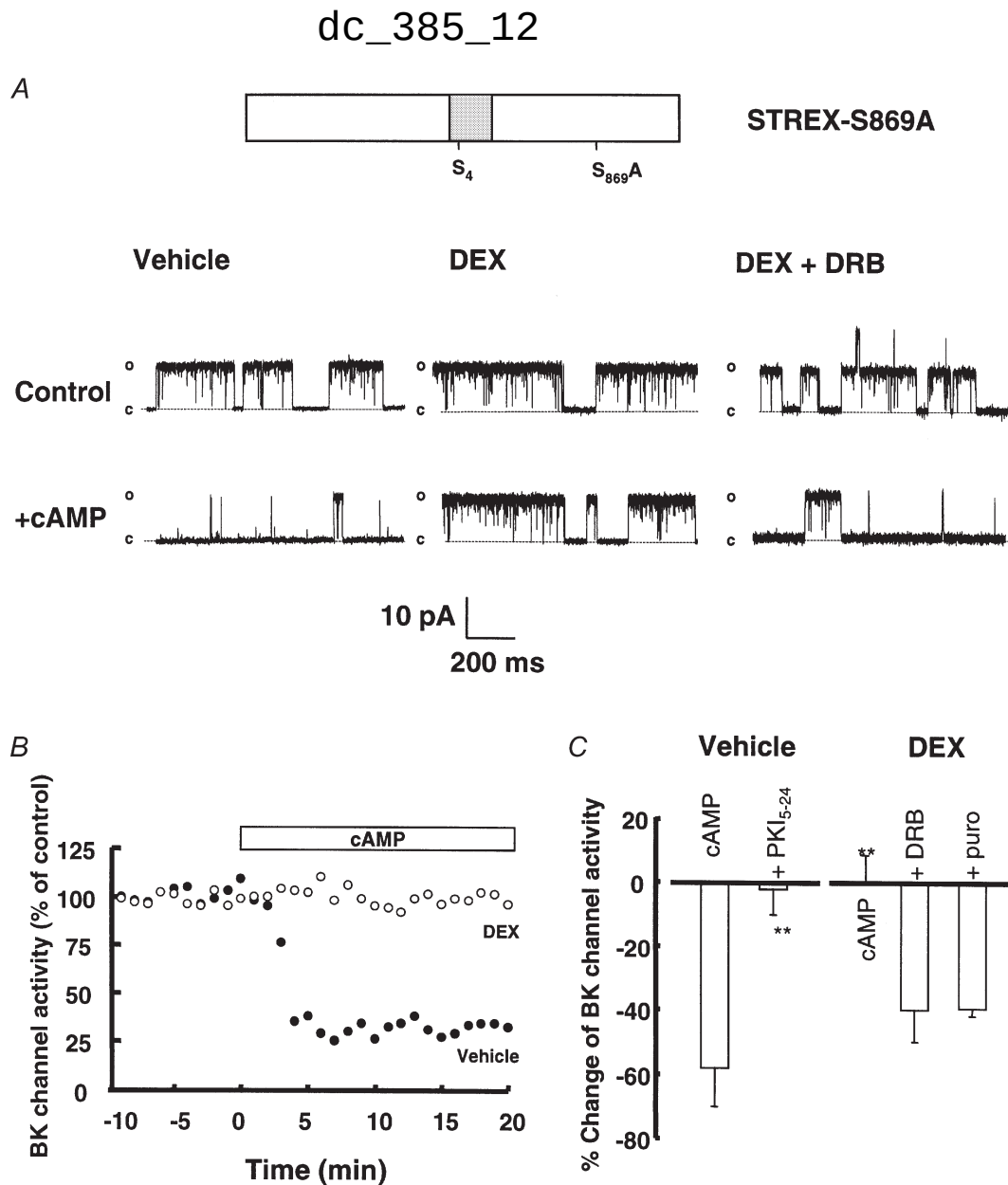


Figure 2. Dexamethasone blocks PKA-mediated inhibition of STREX BK channels via a genomic mechanism

A, representative single channel traces from isolated inside-out patches from vehicle, dexamethasone and dexamethasone + DRB pre-treated HEK 293 cells expressing STREX-S869A channels before and 10 min after application of 1 mM cAMP to the intracellular face of the patch. Patches containing a single STREX BK channel were exposed to $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP in physiological potassium gradients and depolarised to +40 mV as described in Methods. *B*, representative time course of the effect cAMP on BK channel activity in patches from vehicle-treated (Vehicle, ●) and dexamethasone-treated (DEX, ○) cells. Activity is expressed as a percentage of the activity at time -10 min before cAMP application and averaged over 1 min for each data point. The open horizontal bar indicates the period of cAMP application. *C*, summary of the effect of cAMP application to the intracellular face of patches from STREX-S869A-expressing HEK 293 cells pre-treated for 2 h with vehicle ($n = 11$) or $1 \mu\text{M}$ dexamethasone ($n = 11$) prior to patch excision. Inhibitors of transcription (DRB, 0.1 mM, $n = 4$) and translation (puromycin, 0.1 mM, $n = 4$) were applied 15 min before and during dexamethasone treatment of intact HEK 293 cells. The specific PKA inhibitor peptide PKI₅₋₂₄ ($n = 4$) was applied to the intracellular face of inside-out patches 5 min before application of cAMP. All data are expressed as the percentage change of pre-treatment BK channel activity measured at +40 mV in the presence of $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP as described in Methods. The bars show means and S.E.M. ** $P < 0.01$ compared with vehicle-treated cAMP group.

observed in control cells (percentage change of BK channel activity: $-33.9 \pm 5.2\%$, $n = 5$, Fig. 3*B*). This effect required PKA activity as it was abolished by PKI₅₋₂₄ (not shown). ATP γ S can be used by protein kinases to phosphorylate target proteins. However, thio-phosphorylated amino acid residues are largely resistant to dephosphorylation by protein phosphatases. Thus these data suggest a closely associated protein phosphatase is responsible for the attenuation of PKA-mediated inhibition of BK channel activity in isolated patches from dexamethasone-treated STREX-S869A cells. Identical results were obtained using the STREX channel under the same experimental conditions ($n = 3$, data not shown).

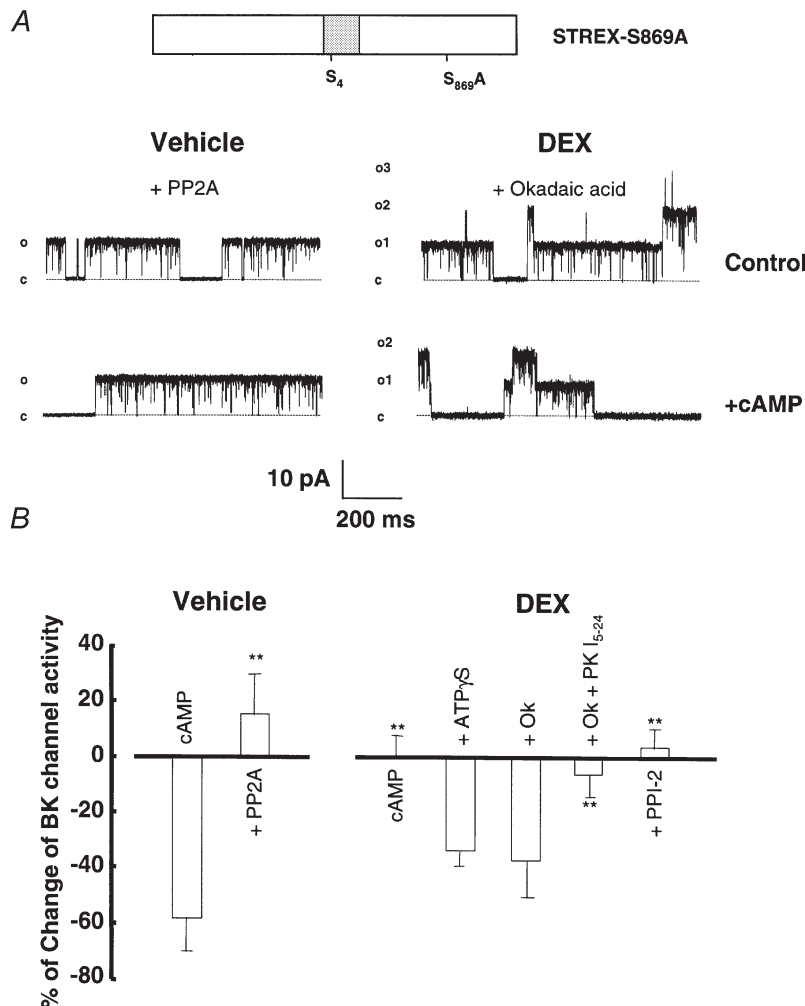


Figure 3. Glucocorticoid action is mediated via protein phosphatase activity associated with the patch

A, representative single channel traces from isolated inside-out patches from a vehicle-treated cell with patch exposed to exogenous PP2A (left) and a dexamethasone pre-treated cell with patch exposed to 10 nM okadaic acid before (control) and after (cAMP) application of cAMP to the intracellular face of the patch. Patches containing STREX-S869A BK channels were exposed to $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP in physiological potassium gradients and depolarised to +40 mV as described in Methods. *B*, summary of the effect of manipulating serine/threonine protein phosphatase activity in isolated inside-out patches. Vehicle: effect of 1 mM cAMP on BK channel activity in the absence ($n = 11$) and presence (PP2A, $n = 4$) of exogenous catalytic subunit of PP2A (1 unit ml^{-1}) applied to the intracellular face of isolated patches from vehicle-treated HEK 293 cells expressing STREX-S869A channels. DEX: effect of 1 mM cAMP on STREX-S869A channel activity in the presence of ATP ($n = 11$) or ATP γ S (0.1 mM , $n = 5$) as phosphate donor or on co-application with the serine/threonine phosphatase inhibitor, okadaic acid (Ok, 10–100 nM, $n = 5$) or the specific protein phosphatase 1 (PP1) inhibitor peptide (PPI-2, 20 nM) in the presence of ATP. The PKA inhibitor peptide PKI₅₋₂₄ ($0.45 \mu\text{M}$) blocked the effects of cAMP in the presence of okadaic acid ($n = 6$). All data are expressed as the percentage change of pre-treatment BK channel activity measured at +40 mV in the presence of $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP as described in Methods. The bars show means and S.E.M. ** $P < 0.01$ compared with vehicle-treated cAMP group.

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In the presence of 10–100 nM okadaic acid, a blocker of serine/threonine phosphatases, cAMP significantly inhibited BK channel activity in isolated patches from dexamethasone-treated HEK 293 cells expressing STREX-S869A channels (Fig. 3*A* and *B*). The percentage change of BK channel activity in the presence of okadaic acid was $-37.3 \pm 12.9\%$ ($n = 5$), similar to that seen in control (non-dexamethasone-treated cells). Importantly, the effect of cAMP was blocked by PKI_{5–24} (Fig. 3*B*). The percentage change of BK channel activity in the presence of okadaic acid and PKI_{5–24} was $-6.1 \pm 12.6\%$ ($P < 0.01$, $n = 6$). In contrast, application of a specific peptide inhibitor of protein phosphatase 1 (PP1), PPI-2 (20 nM), failed to restore cAMP-mediated inhibition of BK channels in patches from dexamethasone-treated HEK 293 cells expressing STREX-S869A channels (percentage change of BK channel activity after cAMP application in the presence of PPI-2 was $3.8 \pm 7.8\%$, $n = 5$, Fig. 3*B*). The PPI-2 was functionally active in other electrophysiological and biochemical assays of PP1 action (not shown). Thus the action of glucocorticoids on BK channel function does not involve PP1 activity and is likely to be

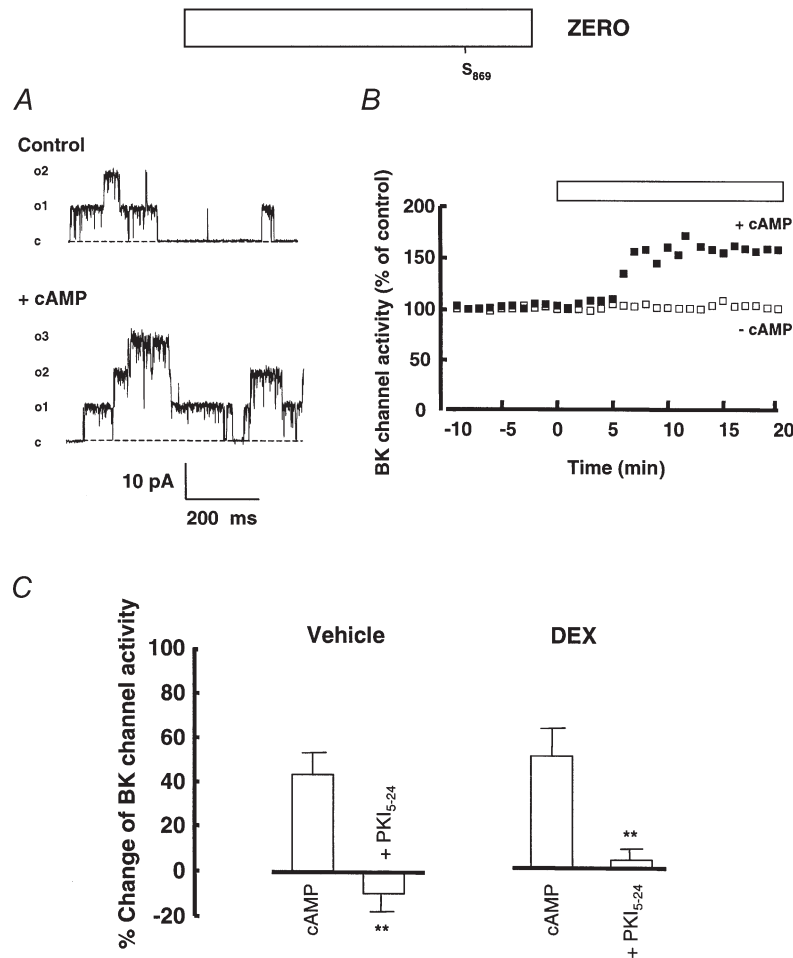


Figure 4. The ZERO variant is not regulated by glucocorticoids

A, representative single channel traces from isolated inside-out patches from dexamethasone pre-treated HEK 293 cells expressing ZERO variant channels before and 10 min after application of 1 mM cAMP to the intracellular face of the patch. Patches containing ZERO channels were exposed to $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP in physiological potassium gradients and depolarised to +40 mV as described in Methods. *B*, representative time course of ZERO channel activity from vehicle-treated cells in the presence (+cAMP, ■) or absence (–cAMP, □) of cAMP. Activity is expressed as a percentage of the activity at time –10 min before cAMP application and averaged over 1 min for each data point. Open horizontal bar indicates the period of cAMP application for the +cAMP patch. *C*, summary of the effect of cAMP application to the intracellular face of patches from ZERO-expressing HEK 293 cells pre-treated for 2 h with vehicle ($n = 8$) or $1 \mu\text{M}$ dexamethasone ($n = 8$) prior to patch excision. The specific PKA inhibitor peptide PKI_{5–24} was applied to the intracellular face of inside-out patches 5 min before application of cAMP in vehicle ($n = 4$) or dexamethasone-treated ($n = 6$) cells. All data are expressed as the percentage change of pre-treatment BK channel activity measured at +40 mV in the presence of $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP as described in Methods. The bars show means and S.E.M. ** $P < 0.01$ compared with vehicle-treated cAMP group.

mediated via PP2A-like phosphatase activity associated with the channel.

To test this hypothesis the purified catalytic subunit of PP2A was applied to the intracellular face of patches containing STREX-S869A channels from vehicle-treated HEK 293 cells. In the presence of 1 U ml^{-1} PP2A, cAMP failed to inhibit BK channel activity (Fig. 3*A* and *B*): the percentage change of BK channel activity 10 min after application of cAMP in the presence of PP2A was $15.3 \pm 15.0\%$ ($P < 0.01$, $n = 4$).

Overall, these results suggest that the glucocorticoid blockade of PKA-mediated inhibition of BK channel activity is mediated via a PP2A-like phosphatase closely associated with the STREX variant channel complex.

ZERO channels are stimulated by PKA and are not modulated by dexamethasone

Application of cAMP (1 mM) to the intracellular face of isolated inside-out patches from vehicle-treated HEK 293 cells expressing ZERO BK channels (identical to STREX except they lack the STREX insert) results in significant stimulation of ZERO BK channel activity (Fig. 4*A* and *B* (see also Nara *et al.* 1998; Tian *et al.* 2001); the mean percentage activation was $43.0 \pm 10.0\%$, $n = 8$, $P < 0.01$). This stimulation is entirely dependent upon activation of PKA associated with the patch as the protein kinase A-specific inhibitor peptide, PKI₅₋₂₄, prevents channel activation (the mean percentage change in activity after cAMP in the presence of PKI₅₋₂₄ was $-9.3 \pm 8.2\%$, $n = 6$).

In direct contrast to the effect of dexamethasone on PKA-mediated regulation of BK channels containing the STREX insert, dexamethasone pre-treatment did not significantly modify PKA-mediated stimulation of ZERO BK channels. The percentage change of BK channel activity 10 min after cAMP application to the

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intracellular face of patches from dexamethasone-treated HEK 293 cells expressing ZERO channels was $49.8 \pm 13.0\%$, $n = 8$ (Fig. 4*B*). Importantly, this activation was dependent upon PKA activation as the specific protein kinase A inhibitor peptide (PKI₅₋₂₄, Fig. 4*B*) blocked activation by cAMP (mean percentage change in activity was $3.6 \pm 4.8\%$, $n = 4$).

S4_{STREX} domain confers glucocorticoid sensitivity to ZERO channels

To examine whether the lack of glucocorticoid regulation of ZERO channels is a result of the absence of the STREX insert *per se* or a consequence of ZERO channels being activated by PKA (dependent upon a functional S869; Nara *et al.* 1998; Tian *et al.* 2001) compared to the inhibition observed with channels containing the STREX insert, we examined the glucocorticoid regulation of the site directed mutant channel S4_{STREX}A. This construct is identical to STREX channels except that the serine residue (S4_{STREX}) within a PKA consensus site of the STREX insert (required for PKA-inhibition; Tian *et al.* 2001) is mutated to alanine. This channel is activated by PKA as for ZERO and this activation is dependent upon a functional S869 residue within the C-terminal consensus PKA site (Fig. 5; see also Tian *et al.* 2001). S4_{STREX}A channel activity was stimulated to the same extent as ZERO channels (by $45.9 \pm 8.9\%$, $P < 0.01$, $n = 8$) upon cAMP application to the intracellular face of the channel and this effect was abolished by PKI₅₋₂₄ (mean percentage change in activity was $-0.9 \pm 11.9\%$, $n = 4$, $P < 0.01$).

However, in contrast to ZERO channels, dexamethasone pre-treatment abolished PKA-mediated activation of S4_{STREX}A channels. The percentage change in BK channel activity after cAMP application to patches from dexamethasone-treated S4_{STREX}A was $-6.8 \pm 8.6\%$ ($n = 8$, $P < 0.01$, Fig. 5). Importantly, PKA-mediated activation of S4_{STREX}A channels could be restored in patches from

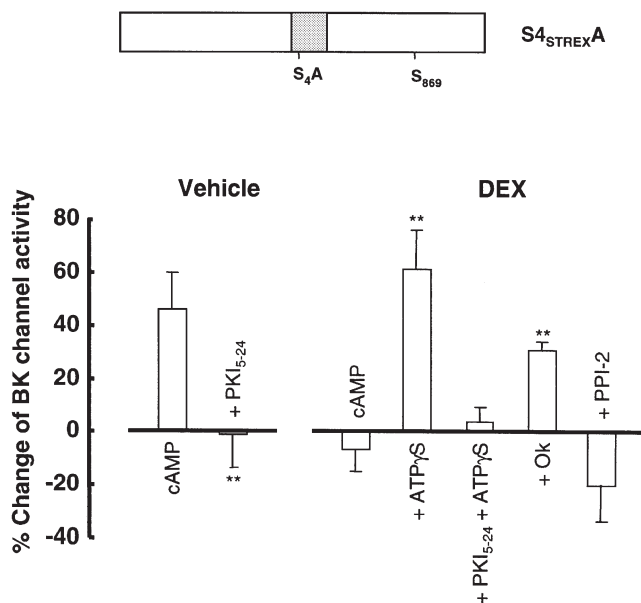


Figure 5. S4_{STREX}A is activated by PKA and is modulated by DEX

Summary of the effect of cAMP application to the intracellular face of patches from HEK 293 cells expressing S4_{STREX}A channels pre-treated for 2 h with vehicle ($n = 8$) or $1 \mu\text{M}$ dexamethasone ($n = 8$) prior to patch excision. The specific PKA inhibitor peptide PKI₅₋₂₄ was applied to the intracellular face of inside-out patches 5 min before application of cAMP in vehicle ($n = 4$) or dexamethasone-treated ($n = 4$) cells. ATP γ S, in the presence ($n = 4$) or absence ($n = 6$) of PKI₅₋₂₄, and okadaic acid (Ok, 2 nM) ($n = 3$) were applied as in Fig. 3. All data are expressed as the percentage change of pre-treatment BK channel activity measured at +40 mV in the presence of $0.2 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ and 1 mM ATP as described in Methods. The bars show means and S.E.M. ** $P < 0.01$ compared with vehicle-treated cAMP group.

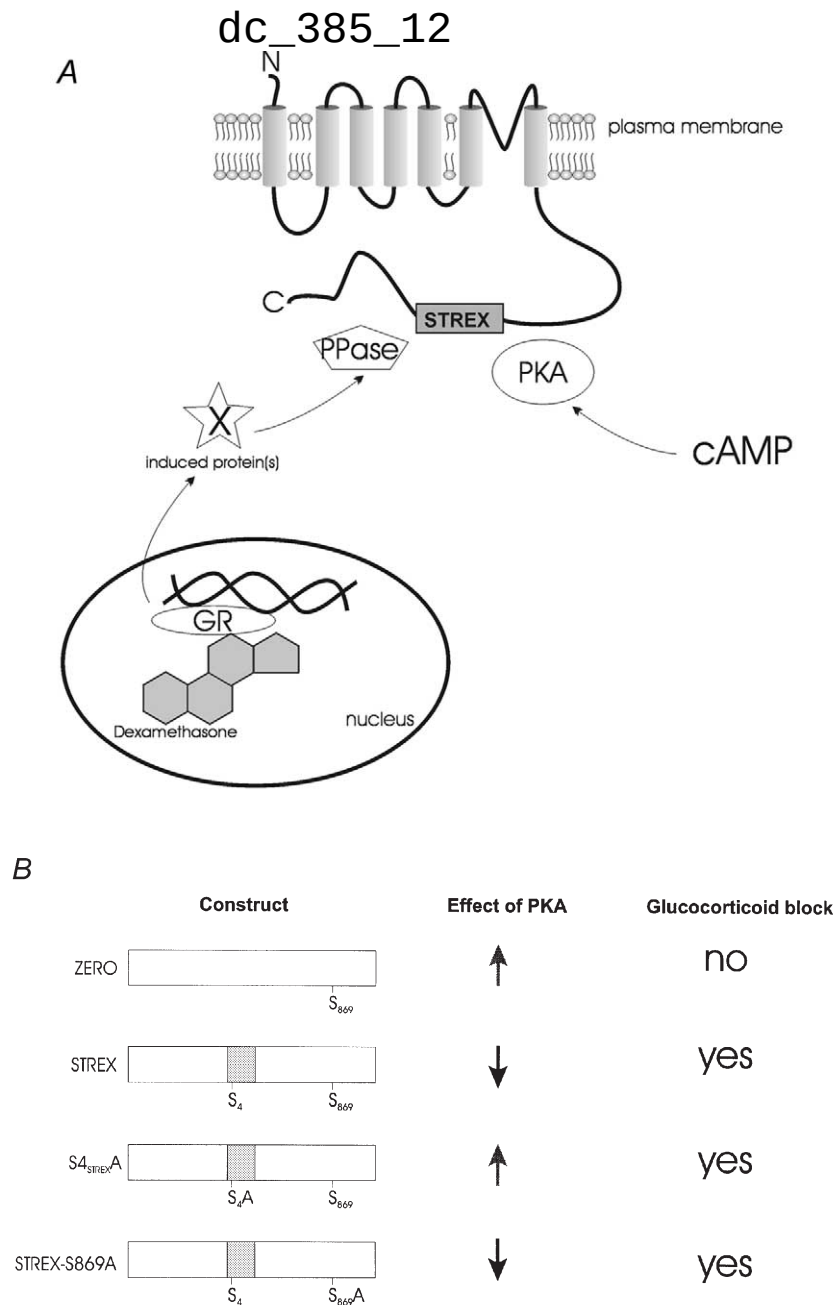


Figure 6. STREX is required for glucocorticoid regulation of BK channels

A, schematic illustrating that BK channels containing the STREX insert are targets for both protein kinase A (PKA) and glucocorticoid signalling pathways. PKA closely associated with membrane patches is activated by cyclic 3',5'-adenosine monophosphate (cAMP). Steroids, such as the synthetic glucocorticoid dexamethasone, activate intracellular glucocorticoid receptors (GR) to regulate gene transcription. GR induced *de novo* synthesis of unidentified (X) proteins that block PKA-dependent regulation of STREX-containing channels through modification of protein phosphatase 2A-like (PPase) activity closely associated with the membrane patch in dexamethasone-treated cells. *B*, schematic summarising the effect of PKA and glucocorticoids on the different BK channel splice variant constructs used in this study. The effect of PKA is indicated by activation (up arrow) or inhibition (down arrow) of channel activity. The ability of dexamethasone to block the PKA-induced regulation of BK channel activity is indicated. The STREX insert is essential for glucocorticoid-mediated regulation of BK channel activity irrespective of the functional effect of PKA on channel activity (activation or inhibition) for the different constructs. Serine residues essential for PKA-mediated activation of channel activity (conserved S₈₆₉ in C-terminal tail of channel) and PKA-mediated inhibition (S₄ in the STREX insert) are indicated.

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dexamethasone-treated HEK 293 cells when ATP γ S was used as the phosphate donor (mean percentage PKA activation of BK channel activity was $60.9 \pm 14.9\%$, $n = 6$, Fig. 5) and this cAMP-dependent stimulation was blocked by PKI $_{5-24}$ (mean percentage PKA activation of BK channel activity was $3.9 \pm 5.3\%$, $n = 4$, $P < 0.01$ Fig. 5). This suggested protein phosphatase activity is required for the action of glucocorticoid. Indeed, inhibition of PP2A-like activity with 2 nM okadaic acid, but not PP1 activity when inhibited by PPI-2 (Fig. 5), prevented the glucocorticoid block of S4_{STREX}A channel regulation by PKA (mean percentage PKA activation of BK channel activity in the presence of okadaic acid was $30.7 \pm 3.1\%$, $n = 3$, Fig. 5).

DISCUSSION

We have established in HEK 293 cells a model for the analysis of the modulation of ion channels by glucocorticoids. In this system, novel proteins rapidly induced by dexamethasone differentially modulate the regulation of BK channels by PKA. The presence of the 59 amino acid STREX insert at splice site 2 of the BK channel α -subunit is sufficient for modulation by glucocorticoid-induced proteins through a serine/threonine protein phosphatase, irrespective of the direction of the PKA effect on the channel (Fig. 6).

Glucocorticoids differentially modulate the activity of BK channel splice variants

In order to address whether the pore-forming subunits of BK channels are the targets for glucocorticoids, the regulation of cloned BK channel splice variants was analysed in HEK 293 cells as a heterologous mammalian expression system. HEK 293 cells do not endogenously express BK channel subunits and show the pivotal features of the glucocorticoid response previously reported in anterior pituitary corticotroph cells and CA1 pyramidal neurones (Joels & de Kloet, 1989; Shipston *et al.* 1996). Thus HEK 293 cells are a suitable model for the analysis of glucocorticoid regulation of BK, and possibly other, ion channels.

BK channels that express the wild-type STREX insert at site 2 (STREX and the site directed mutant STREX-S869A) are potently inhibited by cAMP-dependent protein kinase (PKA) activity intimately associated with the channel complex in HEK 293 cells (Shipston *et al.* 1999; Tian *et al.* 2001). This inhibition requires a PKA consensus site in the STREX insert (S₄) but is not dependent upon the conserved PKA site at serine residue 869 of the C-terminus (Tian *et al.* 2001). Glucocorticoid pre-treatment blocked the PKA-mediated inhibition of STREX, or STREX-S869A, BK channels expressed in HEK 293 cells. Glucocorticoid action was manifest within 2 h of exposure, was dependent upon *de novo* mRNA and protein synthesis and was mediated by okadaic acid-sensitive (PP2A-like) protein phosphatase activity intimately associated with the channel.

However, glucocorticoid regulation was not evident in the ZERO BK channel splice variant that does not contain the STREX insert. ZERO channels are activated by PKA, dependent upon a functional conserved C-terminal PKA consensus phosphorylation site serine residue, S869 (Nara *et al.* 1998; Tian *et al.* 2001). The difference in responsiveness of the STREX and ZERO variants to glucocorticoids could be a reflection of the opposite action of PKA on these channels. To address this issue, we used a STREX site directed mutant (S4_{STREX}A) that lacks the STREX PKA consensus site but retains a functional C-terminal site S869. S4_{STREX}A is activated by PKA and this activation was blocked by glucocorticoids, which indicates that the STREX domain confers glucocorticoid modulation. The exact mode of the STREX-dependent modulation remains to be clarified.

Evidence for short- and long-term regulation of cellular excitability through glucocorticoid modulation of BK channels

Previous studies have shown that endogenously expressed BK channels from a variety of tissues may be activated or inhibited by PKA-dependent phosphorylation (Reinhart *et al.* 1991, 1995; White *et al.* 1991) and that the direction of this regulation is influenced by circulating hormones such as adrenal corticosteroids (Perez & Toro, 1994; Shipston *et al.* 1996; Zhou *et al.* 2000). The diversity and plasticity of modulation is, at least in part, a result of the opposing effects of PKA phosphorylation on distinct BK channel splice variants. Yet a further level of control is the ability of proteins rapidly induced by glucocorticoids to switch the functional outcome of PKA activation targeted to BK channels. Importantly, STREX subunits are widely expressed in glucocorticoid-sensitive endocrine cells and neurones (Tseng-Crank *et al.* 1994; Ferrer *et al.* 1996; Xie & McCobb, 1998; Shipston *et al.* 1999) where cAMP signalling is pivotal for functional activity. Thus glucocorticoid modulation of the phosphorylation of BK channels could provide a short-term switch of cellular excitability in these systems.

In a longer time domain (> 6 h) the regulation of the expression of STREX variant subunits, along with other BK channel splice variants, could also contribute to the specification of the control of cellular excitability. Indeed, the expression of STREX channels is regulated by stress hormones in adrenal medullary chromaffin cells (Xie & McCobb, 1998) and by depolarisation in GH3 pituitary tumour cells (Xie & Black, 2001), and the STREX mRNA profile changes during pregnancy in adult myometrium (Benkusky *et al.* 2000). Although the impact of STREX α -subunit expression on heteromultimeric BK channel properties has not been explored, previous evidence suggests that the resultant channel properties would reflect the functional characteristics of the STREX subunit (Shen *et al.* 1994; Lovell & McCobb, 2001).

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Dex-ras1 and Serum- and Glucocorticoid-inducible Protein Kinase 1: Regulation of Expression by Dexamethasone in HEK293 Cells

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Abstract The molecular and cellular basis of the psychotropic actions of adrenal corticosteroids is poorly understood. Previously, we reported that modulation of large conductance Ca^{2+} -activated potassium channel (BK-channel) function by glucocorticoids can be recapitulated in human embryonic kidney293 (HEK293) cells (J Physiol 537:57, 2001). In the present paper, we examined the effect of dexamethasone on the expression of candidate mediator proteins of glucocorticoid action, dex-ras1 and serum and glucocorticoid inducible protein kinase 1 (SGK), in HEK293 cells. Dex-ras1 mRNA was readily detectable under basal conditions however, no changes of dex-ras1 mRNA expression occurred upon exposure to 100 nM of dexamethasone for 2 h. In contrast, a 2.5-fold increase of SGK mRNA was found under similar conditions. Total levels of cellular SGK protein were unaltered upon exposure to dexamethasone, but a marked increase of SGK in a Triton-X100 insoluble fraction was observed. BK-channel α -subunits could not be co-immunoprecipitated with SGK. In summary, SGK, but not dex-ras1, mRNA is rapidly induced by glucocorticoid stimulation in HEK293 cells. However, there appears to be no direct protein-protein interaction between SGK and BK-channel α -subunits.

Introduction

Adrenal corticosteroids have been implicated in the pathoetiology of mood disorders for a long time cf. [1, 2]. An important facet of corticosteroid action on the brain that appears relevant for disorders of mood is the feedback inhibition of the neuroendocrine stress axis [3]. Despite the established physiological significance of corticosteroid modulation of neuronal function, precious little is known about the cellular and molecular actions of corticosteroids in the brain [4, 5]. The vast majority of neurons and glial cells are equipped with nuclear receptors for corticosteroids. However, the steps beyond receptor activation and the biological outcomes are poorly characterised. With respect to the neuroendocrine feedback action of corticosteroids, pyramidal cells in the CA1 region of the hippocampus and anterior pituitary corticotroph cells have been analysed in some detail [4, 5]. Overall, these studies provide a convergent picture indicating that corticosteroids influence the membrane potential by modifying Ca^{2+} -activated K^{+} currents. Furthermore, there is broad agreement that rapid induction of gene transcription and protein synthesis via Type II glucocorticoid receptors is required for this effect of corticosteroids. However, the mechanisms beyond Type II receptor activation remain to be resolved. In the murine corticotroph tumour cell line AtT20, cAMP-mediated regulation of BK channels is modified by glucocorticoid induced proteins [6, 7], which is essential for the inhibition of corticotrophin releasing-factor stimulated hormone secretion [6]. The modulation of BK-channel function by glucocorticoids can be fully recapitulated in HEK293 cells transfected with the STREX variant α -subunit of BK-channels [8] suggesting that the complement of glucocorticoid induced genes is similar in AtT20 and HEK293 cells. A candidate mediator of glucocorticoid action in AtT20 cells is the Ras related protein

Presented to mark the 70th birthday of Professor George Fink.

Special issue article in honor of George Fink.

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dex-ras1 [9, 10]. The mRNA for dex-ras1 is rapidly induced by dexamethasone in AtT20 cells, furthermore overexpression of dex-ras1 inhibited cAMP induced secretion of a reporter hormone in this system [11]. In the present study we examined whether or not dex-ras1 was a glucocorticoid regulated gene in HEK293 cells and thus a potential candidate for the modulation of BK channel function in this system.

Materials and methods

Cell culture

Wild type HEK293 cells and HEK293 cells stably overexpressing the STREX variant of the BK-channel α -subunit [8] were maintained as previously described. Transient transfections were carried out using Lipofectamine 2000 (Invitrogen, Paisley, U.K.) as per the manufacturer's instructions.

Treatment with dexamethasone and luciferase reporter gene assays

In order to minimize the effect of steroids contained in serum, the cells were maintained in DMEM supplemented with insulin, transferrin and selenium, ($1 \times$ ITS, Sigma, cat no13146) for 24 h in a humidified incubator containing 5% CO₂ at 37°C. Prior to experimental manipulation the cells were incubated with DMEM supplemented with 25 mM HEPES pH 7.4 and 0.25% w/v bovine serum albumin (DMEM–BSA) for 2 h at 37°C and then treated with dexamethasone or vehicle (0.001% DMSO) in DMEM–BSA at 37°C for the time indicated in the respective figure legends. Reporter gene assays were carried out on wild type HEK293 cells transiently transfected with a mouse mammary tumor virus long terminal repeat luciferase reporter construct (MMTV-Luc) [12] as previously described [8].

RT-PCR and Northern analysis of mRNA RT-PCR was carried out as previously described [13]. For Northern analysis, 20 μ g of total RNA prepared by extraction with Trizol (Invitrogen, Paisley, UK) was electrophoresed under denaturing conditions and transferred to nylon membranes (Hybond-N, GE Life Sciences, Aylesbury, UK). The probes used for hybridization were a 207 bp fragment of human dex-ras1 (nucleotide 639–846, NCBI accession number AF172846), a 238 bp (nucleotide 108–346, NCBI accession number: CAA71138) fragment of human SGK1 and a 170 bp (nucleotides 295–465) fragment of cyclophilin A produced by RT-PCR from HEK293 cell mRNA and labelled with Ready To-Go beads and α -³²P-dCTP (GE

Life Sciences, Aylesbury, UK) as per the manufacturer's instructions.

The transferred membranes were incubated at 55°C with hybridization buffer for 1.5 h in a sealed plastic box and then the membranes were incubated with denatured-probes (incubation of labelled probes at 60°C for 10 min) in hybridization buffer at 55°C for 16 h. Autoradiography was by exposure on a Cyclone storage phosphor screen, (Canberra-Packard, Pangbourne, U.K.) for 0.5–1 days. Radioactive bands were outlined and the optical density (O.D) in each band was determined by the OptiQuant (Canberra-Packard, Pangbourne, U.K.) image analysis software.

Analysis of SGK protein levels

Cells in 75 cm² flasks at 80–90% confluence were washed 3 times with cold phosphate buffered saline (PBS) and scraped into 0.5 ml of 150 mM NaCl, 50 mM Tris–HCl, pH 7.4, 1% (v/v) Triton X-100 (Triton lysis buffer) supplemented with 1/100 of protease inhibitor cocktail (P 8340 Sigma, Poole, Dorset, U.K.). The cells were sonicated on ice with five sonication cycles, each consisting of 10 s pulses at 10 s intervals with an ultrasonic Cole-Parmer sonicator (TORBEO, 36800) at setting 3. Subsequently, the lysate was centrifuged at 1000g for 5 min at 4°C to pellet large cell debris. The resultant supernatant was the total lysate, which was spun at 20,000g for 30 min at 4°C to obtain the Triton insoluble and soluble fractions, respectively. Aliquots of the lysate and the fractions were analysed in SDS-PAGE followed by transblotting to PVDF membranes and ECL based detection of SGK immunoreactive bands using anti-SGK1 rabbit antibodies (Upstate Biologicals, Dundee, U.K.). Cyclophilin A staining was performed after membrane stripping (50°C for 30 min 2% w/v SDS, 100 mM mercaptoethanol, 62.5 mM Tris, pH 6.7, followed by 3 washes in PBS/0.3% Tween 20) to correct for sample loading in total lysates as well as the Triton soluble fractions. The blots of the insoluble fraction were initially stained with Coomassie blue and photographed. Subsequently, the dye was removed and the blots were reacted for SGK. A prominent Coomassie Blue stained band was chosen to standardize for gel loading in these samples. Densitometry was carried out using Opti-Quant Image analysis software (Packard Bioscience, Pangbourne, Berkshire, UK).

Immunoprecipitation of BK channels

Total cell lysates adjusted to 2.5 mg protein in 500 μ l were pre-cleared by incubation with 10 μ l of 50% (v/v)

washed protein-G-sepharose (Sigma p3296) in 1% Triton X-100 lysis buffer and agitated for 1 h at 4°C, and the beads were removed by brief centrifugation. Subsequently, 5 µl each of antisera raised in sheep against residues 628–641 and 919–933, respectively, of the STREX variant of the mouse BK channel α -subunit were added to the pre-cleared cell lysates and incubated overnight at 4°C under gentle agitation. Subsequently, 16.6 µl of 50% Protein-G Sepharose were added for 2 h at 4°C. The beads were pelleted at 10,000g for 1 min, and processed for SDS-PAGE/immunoblotting [14], with primary antibodies against the HA-tag (Zymed) to detect the BK channel α -subunit and anti-SGK antibodies to detect SGK, respectively.

Results

Exposure to dexamethasone produced a time- and concentration-dependent increase of MMTV-Luc activity in transiently transfected wild type HEK293 cells. The time-dependence (Fig. 1) appeared biphasic with a significant increase at 30 min, the degree of which only changed at 90 min and plateaued at 2 h. Concentration-dependence was examined for the 2 h time-point: the maximal response to was obtained with 100 nM dexamethasone, EC_{50} was around 5 nM (not shown). In subsequent experiments dexamethasone was used at 100 nM in cells stably expressing the STREX variant α -subunit of the BK-channel.

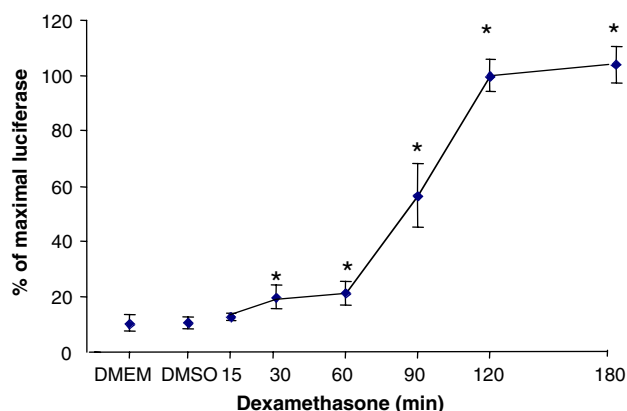


Fig. 1 Time-course of dexamethasone induction of MMTV-Luc reporter gene in transiently transfected HEK293 cells. Cells incubated overnight in DMEM supplemented with insulin, selenium and transferrin were exposed to DMEM alone (DMEM) or 0.001% v/v dimethylsulfoxide vehicle (DMSO) for 120 min or to 100 nM dexamethasone for various time periods. Data are expressed as percentage of the maximal light intensity at 180 min. means $\pm$ SEM $n = 3$ /group, representative of two separate studies with similar results

A 5 Kbp mRNA species was readily detectable with the dex-ras1 anti-sense probe in HEK293 cells under 'resting' conditions (Fig. 2). The levels of this transcript were unaltered by 100 nM dexamethasone at all time-points examined. In contrast, a clear induction of SGK mRNA was already apparent at 15 min after the application of dexamethasone (Fig. 3) and persisted up to 120 min. Despite the increase of SGK mRNA levels, the concentration of SGK protein was not changed in the total TritonX-100 lysate prepared from dexamethasone treated

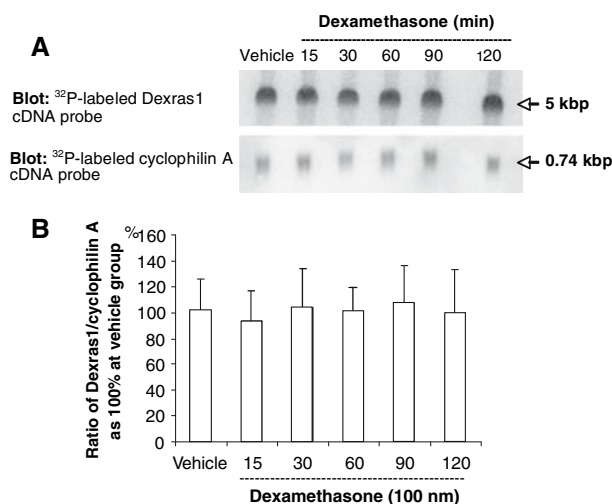


Fig. 2 Lack of an enhancement of dex-ras1 mRNA in HEK293 cells stably expressing the STREX variant α -subunit of the BK-channel. Optical densities of the bands hybridising with the dex-ras1 probe were divided with those of the housekeeping gene cyclophilin A. The cyclophilin A probe was applied after stripping the dex-ras1 probe from the blot. Data are means $\pm$ SEM, $n = 3$. Similar data were generated using wild type HEK293 cells

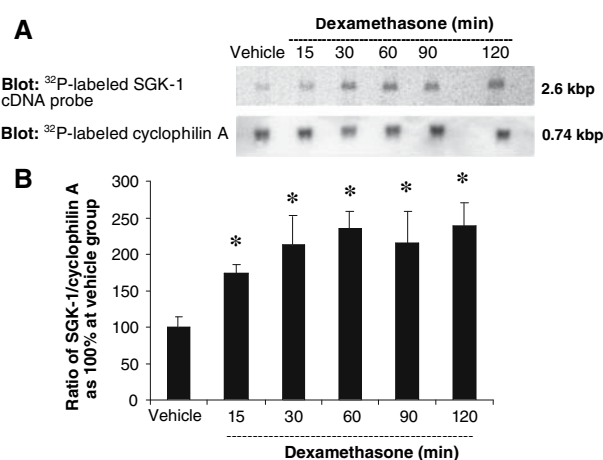


Fig. 3 Increase of SGK mRNA induced by dexamethasone in HEK293 cells stably expressing the STREX variant α -subunit of the BK-channel. Cells were treated and analysed as per Fig. 2 except a probe hybridising to SGK mRNA as used. Data are means $\pm$ SEM, $n = 3$. Similar data were generated using wild type HEK293 cells

cells. However, there appeared to be a substantial dexamethasone-induced rise of SGK1 immunoreactive material in a Triton insoluble fraction of the cells (Fig. 4). In order to probe the possibility that BK-channels are physically associated with SGK, immunoprecipitates prepared with anti-BK sera from vehicle and dexamethasone treated cells were probed for the presence of SGK. No evidence for co-immunoprecipitation was found (Fig. 5).

Discussion

The present study is in concordance with previous work ([8] and references therein) showing that glucocorticoid preferring nuclear receptors (GR), are expressed and are functionally active in HEK293 cells. Activation of endogenous GR in HEK293 cells triggers a process dependent on RNA and protein synthesis and results in a marked change in the control of STREX type BK-channels by cAMP-dependent protein kinase. As dex-ras1 (also known as AGS-1) is a potential modulator of cAMP signalling [15, 16] and its expression is induced by dexamethasone in AtT20 cells [9], we analysed cellular dex-ras1 mRNA levels in HEK293 cells. An mRNA

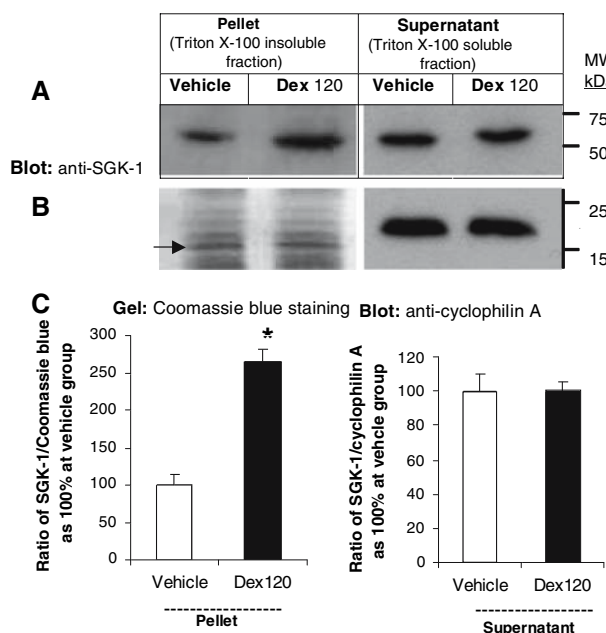


Fig. 4 Selective increase of SGK immunoreactive protein in a Triton-X100 insoluble fraction upon exposure of HEK293 cells to dexamethasone. HEK293 cells stably expressing the STREX variant BK channel α -subunit were exposed to 0.1% DMSO or 100 nM dexamethasone for 120 min and Triton soluble (Supernatant) and insoluble (Pellet) fractions were prepared and analysed for SGK content using SDS-PAGE. Gel loading was standardised by anti cyclophilin for the soluble fractions and by selecting a prominent protein band stained by Coomassie Blue in the Triton insoluble fraction which contained no detectable levels of cyclophilin A

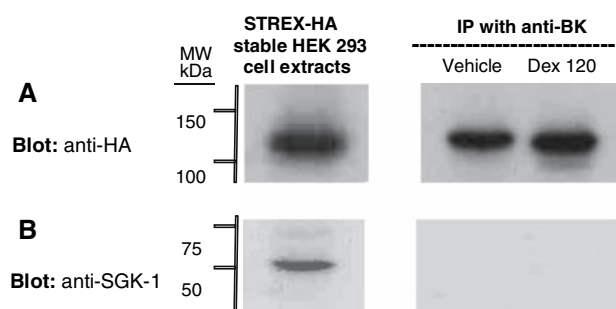


Fig. 5 Lack of co-immunoprecipitation of SGK with the STREX variant BK channel α -subunit protein. Homogenates of HEK293 cells stably expressing the STREX variant BK channel α -subunit and previously exposed to 0.1% DMSO or 100 nM dexamethasone for 120 min were homogenised and solubilised with 1% Triton-X100. The lysate was used to carry out an immunoprecipitation reaction with sheep antibodies against the BK-channel α -subunit. The immunoprecipitate was analysed for the presence of the HA-tagged BK channel α -subunit as well as for SGK by Western blotting

species of the expected sized was readily detectable by Northern analysis under “resting” conditions which involved incubation of the cells in serum free medium containing insulin, transferrin and selenium. There was no effect of dexamethasone on the levels of dex-ras1 mRNA in HEK293 cells, while both the MMTV-Luc data as well as the analysis of SGK mRNA indicated that the steroid treatment regime was biologically effective. The regulation of gene expression by glucocorticoids is complex and it is entirely conceivable that dex-ras1 is glucocorticoid inducible in a different cellular context [17]. Furthermore, the lack of an induction by steroids in HEK293 cells does not exclude the possible involvement of dex-ras1 in glucocorticoid feedback inhibition, as dex-ras1 mRNA was already abundantly expressed. Other approaches such as RNA interference could shed light on this issue.

SGK1 has been implicated in the action of aldosterone in the urinary tract [18]. The subcellular distribution of SGK protein in cells is controversial, and plausibly cell-specific [19]. Most reports concur that the bulk of SGK1 is found in the particulate fraction of cells indicative of predominant association to membranes [19–21]. Indeed association of SGK1 to the plasma membrane [20] as well as mitochondria [21] has been reported. Furthermore, the cellular levels of the protein appear to be controlled by polyubiquitylation and proteosomal degradation [20]. In our hands, application of proteasome inhibitors failed to alter the lack of an effect of dexamethasone to total cellular SGK1 protein concentration (data not shown). However, a significant increase of SGK protein was found in a TritonX-100 insoluble fraction the nature of which requires further study—e.g. using markers for the putative lipid raft fraction of the plasma membrane [22] or mitochondria [21]. An immunoprecipitation approach failed to provide

evidence of a physical interaction between BK channel α -subunit protein and SGK. However, work on SGK with respect to sodium transport in kidney cells indicated a spatial segregation of sodium transport proteins and SGK [19]. Thus a direct protein–protein interaction between the kinase and its target may not be required for SGK-mediated regulation of ion channels and transporter proteins.

In summary, our data raise the possibility that SGK1 is involved in the control of BK channels expressed in HEK293 cells whereas dex-ras1 is not a glucocorticoid responsive gene in this system.

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Functional plasticity of cyclic AMP hydrolysis in rat adenohypophyseal corticotroph cells

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Abstract

Characterisation of cyclic nucleotide-hydrolysing phosphodiesterases (PDEs) in recombinant systems has highlighted regulatory properties indicative of distinct physiological roles for these enzymes. The present study investigated the role of PDEs in the adenosine 3′5′-monophosphate (cAMP) response to the hypothalamic neuropeptides corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) in acutely dispersed rat adenohypophyseal cells. Ca^{2+} -activated PDE (PDE1) and Ca^{2+} -independent, rolipram-sensitive PDE (PDE4) accounted for close to 90% of cAMP-hydrolysing activity in the adenohypophysis. Messenger RNA transcripts of PDE1 (isotypes 1A and 1C) and PDE4 (isotypes B and D3) were detected by RT-PCR. The PDE blockers rolipram and IBMX enhanced cAMP accumulation induced by CRF or CRF and AVP. Vinpocetine, an inhibitor of low K_m PDE1 isotypes, did not alter the response to CRF but enhanced the effect of the combined CRF/AVP stimulus. Thus, PDE4s terminate the cAMP response to moderate stimulation, while low-affinity PDE1 becomes important when the concentrations of CRF and AVP are characteristic of exposure to intensive stress. Crown Copyright © 2002 Elsevier Science Inc. All rights reserved.

1. Introduction

Corticotrophin-releasing factor (CRF) [1] is a major mediator of anxiety and stress in mammals [2]. Arginine vasopressin (AVP), another physiological regulator of the neuroendocrine stress response [3], exerts its effects on anterior pituitary corticotrophs by augmenting the adenosine 3′5′-monophosphate (cAMP) response to CRF. This effect may involve the enhancement of cAMP biosynthesis as well as the inhibition of cAMP hydrolysis [4].

Concepts on the role of cAMP hydrolysis in intracellular signalling have evolved from viewing cyclic nucleotide-hydrolysing phosphodiesterases (PDEs) as passive termina-

tors of the cAMP signal to the present level of understanding where PDEs are established targets of complex intracellular regulatory pathways [5–7]. At least 11 different gene families of PDE have been identified in mammals [5–7]. These enzymes have differential tissue and subcellular distributions and exhibit distinct but functionally concerted modes of regulation at the levels of mRNA transcription and posttranslational modification [5–7].

Previous biochemical characterisation of PDEs from the anterior pituitary gland suggested that the bulk of PDE activity is Ca^{2+} /calmodulin (CaM) dependent, i.e. attributable to PDE1 [8,9]. There are currently three families of PDE1 enzymes: A, B and C, respectively. These hydrolyse cAMP as well as guanosine 3′5′-monophosphate (cGMP) and have a wide range of K_m 's—30 μM for PDE1A and 1 μM for PDE1C isotypes [5,6]. Sensitivity towards Ca^{2+} ions is also variable. Moreover, PDE1 enzymes are subject to isotype-specific regulation by protein phosphorylation [10,11]. Despite the reported preponderance of PDE1 in the adenohypophysis, the highly selective PDE4 inhibitor rolipram is known to activate the hypothalamic–pituitary–adrenocortical system at the pituitary level [12,13], implicating PDE4 in the control of corticotroph cell function. PDE4 enzymes are relatively selective for cAMP and are

Abbreviations: AVP, arginine vasopressin; cAMP, adenosine 3′5′-monophosphate; cGMP, guanosine 3′5′-monophosphate; CRF, 41-residue corticotrophin-releasing factor; CaM, Calmodulin; DMCPX, dimethyl-8-cyclopentyl-xanthine; PDE, cyclic nucleotide-hydrolysing phosphodiesterase

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regulated by a wide variety of mechanisms including intracellular targeting and protein phosphorylation [14]. Given the wide range of physiological stimuli [3,15–17] that regulate the anterior pituitary corticotroph cell, we tested the hypothesis that multiple PDEs are involved in shaping the cAMP response to CRF and AVP. The data indicated that (1) cAMP hydrolysis in the adenohypophysis proceeds through high-affinity, low-capacity as well as low-affinity, high-capacity PDEs, (2) PDE1A constitutes the bulk of PDE activity in the adenohypophysis and (3) PDE1A and PDE4 are key cAMP-metabolising enzymes in rat corticotrophs—the cAMP response to moderate stimulation is terminated by PDE4, whereas PDE1A becomes important when high levels of CRF and AVP characteristic of intensive or chronic stress are present.

2. Materials and methods

2.1. Reagents

Unless otherwise indicated, all reagents were of the highest grade available and obtained from Sigma–Aldrich (Poole, Dorset, UK). Rolipram (Schering, Berlin, F.R.G.) and vinpocetine (Gedeon Richter, Budapest, Hungary) were provided by the manufacturers. Human CRF, human GRF and AVP were from Bachem, UK. [2,8,5-³H]adenosine, [2-³H]5'-AMP and [2,8-³H]3',5'-cAMP were from Dupont, Stevenage, UK or Amersham, Little Chalfont, UK. 1,3-dimethyl-8-cyclopentyl-xanthine (DMCPX) and 1,3-dipropyl-8-cyclopentyl-xanthine (DPCPX) were from RBI, Natick, MA.

2.2. Preparation and incubation of rat anterior pituitary cells

Male Wistar rats (150–200 g BW) were housed in individual cages for 24–48 h under controlled lighting and air conditioning, with free access to tap water and food pellets. Animals were decapitated between 8:30 and 9:00 AM, taking precautions to avoid stressful stimulation. The anterior pituitary glands (usually from five animals) were removed and dispersed cells were prepared by digestion in 1 ml/gland of 0.025% trypsin (TRL-3, Worthington), 10 µg/ml DNase I and 0.25% BSA (Sigma A-7906) in Hank's balanced salt solution without Ca²⁺ and Mg²⁺ (HBSS, GIBCO–Life Technologies, Paisley, UK) supplemented with 25-mM HEPES, pH 7.4, 0.15-mM CaCl₂ and 0.15-mM MgSO₄, for 30 min at 37 °C under constant shaking at 250 cycles/min on an IKA Vibrax orbital shaker. The digested tissue was processed further to obtain dispersed cells as previously described [18]. The cell suspension was preincubated for 2–3 h in a sealed tube in HBSS supplemented with HEPES, pH 7.4, 2-mM CaCl₂, 1-mM MgSO₄ and 0.25% BSA by end-over-end rotation in a total volume of 10 ml, 70 rpm at 37 °C in order to allow the cells to adapt

to the in vitro metabolic conditions. After preincubation, the cells were sedimented by centrifugation, resuspended, counted in a hematocytometer, diluted and distributed (3–4 × 10⁴ cells/50 µl) into conical polypropylene microvials in 96-well format and incubated in a water bath at 37 °C. After 20 min, PDE blockers were added, and 5 min later, agonists were introduced (total volume 200 µl). The incubation was continued for 8 min after which 200 µl of 0.2-M HCl were rapidly added. Total cAMP content was measured by RIA after acetylation [19]. Under these conditions, over 90% of the immunoreactive cAMP was associated with the cells.

2.3. Assay of PDE activity

Rat anterior pituitary glands were homogenised in Teflon/glass homogenisers in five volumes of 20-mM Tris–HCl, pH 7.5, 1-mM EDTA, 0.2-mM EGTA, 50-mM sodium fluoride, 10-mM sodium pyrophosphate, 50-mM benzamidine, 0.5-µg/ml leupeptin, 0.7-µg/ml pepstatin, 4-µg/ml aprotinin, 10-µg/ml soybean trypsin inhibitor, 2-mM phenylmethylsulfonyl fluoride and 1-mM dithiothreitol. The homogenate was centrifuged at 200 × g for 10 min at 4 °C. The resultant supernatant was recentrifuged at 30,000 × g for 20 min at 4 °C, and the supernatant and the pellet were used as the cytosolic and the particulate fractions, respectively. Protein content was measured by the Coomassie blue method with BSA as standard [20]. PDE activity was measured by minor modifications of a radiometric assay [21] based on the coprecipitation of the cAMP hydrolysis product 5'-AMP with BaSO₄. The assay buffer contained 100-mM Tris–HCl, pH 7.5, 5-mM MgSO₄ and cell extract (1–10 µg protein) in a total volume of 150 µl. The reaction was initiated by the addition of 50-µl substrate to give a final concentration of 1-µM cAMP and 0.2-µCi/ml [2,8-³H] cAMP and the tubes were incubated at 30 °C for 5–30 min. The reaction was terminated by the addition of 100 µl of 21.5-mM ZnSO₄ followed by 100 µl of 17.5-mM Ba(OH)₂ to precipitate the 5'-AMP formed. The suspension was filtered over Whatman GF/C filters on a vacuum manifold and washed twice with 3 ml of 1-mM NaOH/100-mM NaCl. Radioactivity retained on the filters was measured by liquid scintillation counting. Blanks were either with homogenisation buffer in place of cell extracts or heat-inactivated (90 °C, 5 min) cell extracts, which gave similar results. Under these conditions, cAMP hydrolysis was linear with respect to time and the amount of protein added, provided substrate depletion was below 30% of total. Total PDE activity is defined as that measured in the presence of 100-µM Ca²⁺ and 100-nM CaM (Sigma P2277), while Ca²⁺/CaM-independent activity was assayed in the presence of 0.5-mM EGTA and 1-µM calmidazolium. Ca²⁺/CaM-dependent (PDE1) activity was taken as the difference between the two activities. PDE4 activity is defined as Ca²⁺/CaM-independent activity inhibited by 10-µM rolipram.

The coprecipitation assay was controlled for the metabolism of 5'-AMP to adenosine. Firstly, most of the experiments

Table 1

Detection of cAMP-hydrolysing phosphodiesterase activities in the rat adenohypophysis with 1- μ M cAMP as substrate

Cell fraction	Ca ²⁺ /CaM activated (PDE1)	Rolipram-inhibitable activity (PDE4)	Other Ca ²⁺ -independent activity
Homogenate	71 $\pm$ 3	19 $\pm$ 2	9 $\pm$ 0.5
Cytosol	81 $\pm$ 2	11 $\pm$ 1	8 $\pm$ 0.5

Total PDE activity was measured in the presence of 100- μ M free Ca²⁺, 100-nM CaM, while Ca²⁺-independent activity was measured in the presence of 0.5-mM EGTA, and 1- μ M calmidazolium, no Ca²⁺ or CaM was added. Total PDE activity was 537 $\pm$ 20-pmol/min/mg protein in the whole homogenate and 555 $\pm$ 17-pmol/min/mg protein the soluble fraction. Data are mean $\pm$ S.E.M. expressed as percent of the respective total activity. $n=4$ /group.

were carried out with cytosolic fractions where 5'-nucleotidase activity is low. Secondly, under the conditions of the filtration assay, 97 $\pm$ 0.1% of ³H-AMP ($n=24$), 0.6 $\pm$ 0.01% of ³H-cAMP ($n=12$) and 1.7 $\pm$ 0.01% of ³H-adenosine ($n=8$) were precipitated with BaSO₄ in the presence of up to 3 μ M of the respective unlabelled nucleotides (values are means $\pm$ S.E.M. of n separate determinations). Thirdly, parallel time courses of PDE and 5'-nucleotidase activity in cytosol extracts indicated that less than 0.3% of ³H-AMP was converted to adenosine by 30 min, while up to 30% of ³H-cAMP was hydrolysed. Finally, the time course of total PDE activity in the cytosol measured by the coprecipitation assay or with the QAE-Sephadex column elution method [22] was near identical.

2.4. Chromatographic separation of PDE activities

Whatman DEAE cellulose (DE-52) was used to pack a 9 $\times$ 15-cm column (Pharmacia K9/15 column). The column was equilibrated with the starting buffer containing 50-mM sodium acetate, pH 6.5, 0.1-mM EGTA, 5-mM β -mercaptoethanol, 10-mM sodium fluoride, 1-mM EDTA, 0.5-mg/l leupeptin, 0.7-mg/l pepstatin and 0.2-mM PMSF. Typically, 2 ml of the cytosolic fraction (approximately 2-mg protein) was loaded and the column was washed with two-column volumes of starting buffer. The column was then eluted with a linear 0.05–1-M sodium acetate gradient. Six-minute (about 2 ml) fractions were collected and 10 or 20 μ l of each fraction was assayed for PDE activity. All chromatographic steps were carried out at 4 $^{\circ}$ C.

2.5. RT-PCR amplification

Total RNA was prepared from adult male rat or mouse anterior pituitary glands using TRIzol reagent (GIBCO–Life Sciences, Paisley, UK) according to the manufacturer's instructions. First-strand cDNA was synthesised from total RNA using the SUPERScript preamplification system (GIBCO). Briefly, 5 μ g of total RNA was heated with 2.5- μ M random hexamers to 70 $^{\circ}$ C for 10 min and quickly chilled on ice. Ten units of SUPERScript II reverse transcriptase were added to start first-strand cDNA synthesis at 42 $^{\circ}$ C for 50 min in a 20- μ l reaction volume containing 1 $\times$ first-strand buffer, 10-mM DTT and 0.5-mM dNTP mix. The reaction was terminated by heating at 70 $^{\circ}$ C for 15 min and aliquots of the cDNA were stored at –40 $^{\circ}$ C. PCR was carried out in a 50- μ l

reaction volume containing 1 μ l of cDNA, 0.1–0.5- μ M primers, 20-mM Tris–HCl, pH 8.0, 50-mM KCl, 2-mM MgCl₂ and 0.2-mM dNTP mix. After heating the mixture to 95 $^{\circ}$ C for 5 min, the PCR was initiated by the addition of 2.5 units *Taq* DNA polymerase (Life Technologies) at 80 $^{\circ}$ C followed by denaturation at 95 $^{\circ}$ C for 1 min, annealing at 60 $^{\circ}$ C for 1 min, extension at 72 $^{\circ}$ C for 3 min for 35 cycles and a final 10 min of extension at 72 $^{\circ}$ C. PCR primers were tailed at the 3'-end in order to optimise for adenylation [23] and thus facilitate T/A ligation. PCR products that corresponded to the expected size were purified and ligated into pGEM-T or pGEM-T Easy vectors (Promega) according to the manufacturer's instructions and transfected into JM109 cells. Plasmid DNA was isolated and sequenced in both directions using

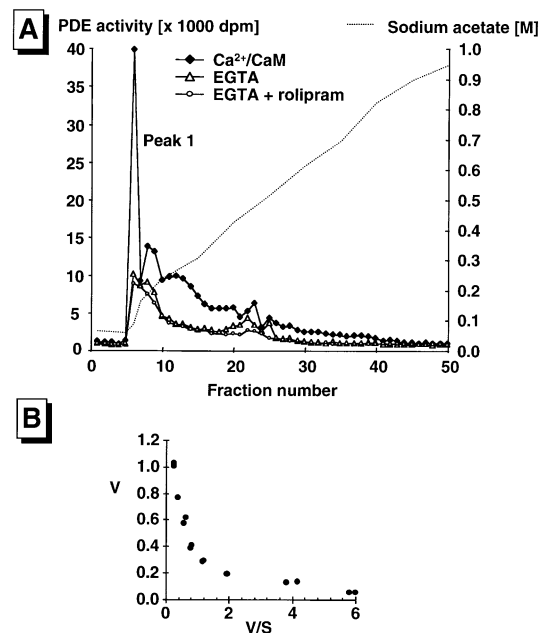


Figure 1. DEAE chromatography of rat adenohypophyseal cytosol. (A) A peak of 100- μ M Ca²⁺/100-nM CaM-stimulated PDE activity at 0.05–0.1 M as well as a shoulder at 0.1–0.4-M sodium acetate were observed. (B) Substrate saturation analysis of the PDE activity eluting between 0.05- and 0.1-M sodium acetate indicated the presence of high and low K_m PDE1. K_m values in this particular experiment determined by nonlinear regression using a two-site binding model (LIGAND) were 0.65 and 63 μ M, with V_{max} values of 0.78- and 21-nmol/min/mg protein, respectively. V =velocity, S =concentration of substrate. Data are individual points from a representative of three separate experiments.

Table 2

Analysis of PDE mRNA expression in rat anterior pituitary gland by RT-PCR

Sense	Antisense	Results
<i>Analysis of PDE1 mRNA expression</i>		
pde1af GGGGATGATGAAAAAGA AGCCTGAAG	pde1r GTTGGCTCCACWATGA ARTCAATGAAAC	Product obtained with mouse anterior pituitary, no product detected with rat tissue. Sequence of mouse RT-PCR product matched the conserved region of mouse PDE1A variants. No RT-PCR product detected in rat anterior pituitary.
pde1bf GCAGTCCACAACCTGTCT CAAGAACCTG	pde1r	PCR product obtained from rat tissue sequence matched conserved region of mouse and rat PDE1Cs. No PCR product.
pde1Cf(2) CCTCCTTTATAAGAC AGGAGTAGCAAAC	pde1cr GTTCTCTGTCAACCCT GTCTAAAGAACTC	RT-PCR product obtained from mouse tissue which matched mouse PDE1C4/5 splice variants [L76946/7]. No product with rat pituitary.
pde1c2f GTCATGACGGACACCA GCCACAAGAAG	pde1cr(2) GTTTTCTTTGACTCA TTTCCGGTGTGGAG	
pde1c145f GTTCTGGATGATGT TTTATCCGTAGTCTCC	pde1c45r GTTCTGGATGATGT TTTATCCGTAGTCTCC	
<i>Analysis of PDE4 mRNA expression</i>		
pde4A5f GACCCCGATCCGCATC CAGCAACG	pde4r MCGRTTMAGCATCCT YTTGAAC	No PCR product.
pde4Bf GTTTATCACAACAGCCT GCACGCTGCTGA	pde4br GTTATGGGAGTACCCA GCCACGTTG	Sequence obtained matched generic core sequence of rat PDE4Bs.
pde4D3Bf CATTCTGGATATGT TTCGATGTGGAC	pde4dr GTTGCCAGACAGCTCC GCTATTCGG	Sequence obtained matched with rat PDE4D3 (U09547).

Primer pairs (5' → 3') used to detect the presence of mRNAs of PDE1 and PDE4 isozymes/splice variants are tabulated. Where PCR products were obtained, they were of the expected size. Where relevant, the database accession numbers are shown in brackets.

the Applied Biosystems Taq DyeDeoxy terminator cycle kit in a model 373 DNA sequencer.

2.6. Data analysis

Data were evaluated by Student's *t* test and one-way ANOVA followed by Newman–Keuls' or Dunnett's two-tailed test as appropriate. Values of $P < 0.05$ were considered statistically significant. K_m and V_{max} values of substrate saturation studies were determined by nonlinear regression analysis [24].

3. Results

3.1. Evidence for multiple cAMP PDEs in the adenohypophysis

In crude homogenates of rat adenohypophysis, Ca^{2+} /CaM-dependent (PDE1) as well as Ca^{2+} /CaM-independent PDE activities were detected with 1- μ M cAMP as substrate. Over 90% of the PDE activity was in the soluble fraction, and the initial characterisation of this activity is shown in Table 1. It was clear that PDE1 constituted the bulk ($\sim 70\%$) of adenohypophysial PDE activity. Importantly, $67 \pm 2\%$ (mean $\pm$ S.E.M., $n = 3$) of the Ca^{2+} /CaM-independent PDE

activity was inhibited by 10- μ M rolipram in the whole homogenate, i.e. about 19% of total adenohypophysial PDE activity was attributable to PDE4. Thus, in rat adenohypophysis, PDE1 and PDE4 type enzymes accounted for approximately 90% of cAMP-hydrolysing activity.

DEAE cellulose chromatography of the cytosol gave a sharp peak of PDE activity eluting at 0.05–0.1-M sodium acetate, followed by a broad shoulder (0.1–0.4-M sodium acetate) which were stimulated 4- and 2.5-fold, respectively, by 100- μ M Ca^{2+} /100-nM CaM (Fig. 1A). These fractions are known to contain mainly PDE1 type activity and PDE1 enzymes have substantial cAMP-hydrolysing activity even

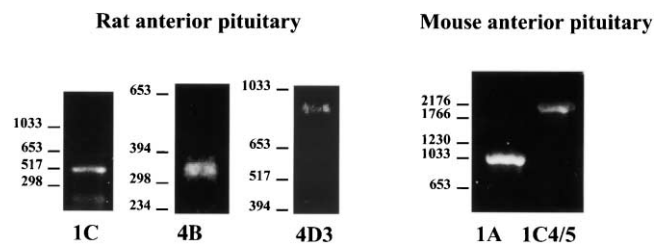


Fig. 2. Analysis of PDE expression in anterior pituitary tissue by RT-PCR. Reverse transcriptase reactions and Taq polymerase-based PCR was carried out on total RNA isolated from rat and mouse anterior pituitary glands. Primers are summarised in Table 1. The expected products for PDEs 1C, 4B and 4D3 and PDEs 1A and 1C4/5 from rat and mouse adenohypophysial RNA, respectively, were purified, subcloned and sequenced.

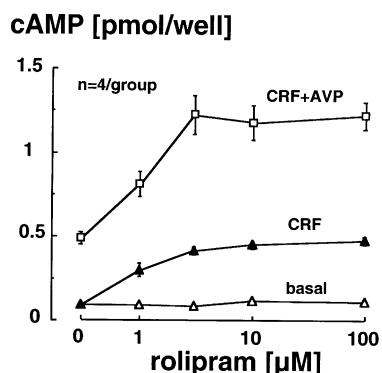


Fig. 3. Effect of rolipram on CRF (0.3 nM)- and CRF/AVP (0.3/2 nM)-induced cAMP responses in dispersed rat anterior pituitary cells. Cells were pretreated for 5 min with vehicle (0.1% dimethylsulfoxide) and various concentrations of rolipram for 10 min after which CRF or CRF/AVP was added and the incubation carried on for 8 min. Data are mean $\pm$ S.E.M. ($n = 4$ /group). The effect of rolipram on CRF- and CRF/AVP-induced cAMP levels were significant between 1 and 100 μ M ($P < .05$, one-way ANOVA followed by Dunnett's test). Representative of two identical experiments.

in the absence of $\text{Ca}^{2+}/\text{CaM}$ [11]. Substrate saturation analysis of the fractions eluting at 0.05–0.1-M sodium acetate with cAMP indicated the presence of low as well as high K_m PDE1 activities (Fig. 1B). The mean K_m values from three separate experiments determined by nonlinear regression analysis using a two-site model were 0.36 ± 0.11 and 53 ± 12 μ M with $V_{\max}$ values of 0.56 ± 0.12 and 20 ± 8 nmol/min/mg, respectively (data are means $\pm$ S.E.M., $n = 3$), i.e. indicated the presence of low K_m , low capacity as well as high K_m and high-capacity PDE1 activities.

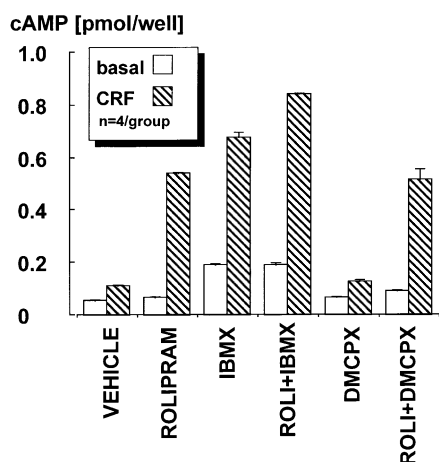


Fig. 4. Specificity of the action of IBMX as an inhibitor of PDE. Isolated rat anterior pituitary cells were treated with various combinations of the PDE blockers rolipram (ROLI, 0.1 mM), IBMX (1 mM) or the adenosine antagonist DMCPX (1 μ M) 5 min before the addition of medium alone or 0.3-nM CRF dissolved in medium. IBMX significantly ($P < .05$, one-way ANOVA followed by Newman–Keuls' test) increased cAMP production alone or in the presence of rolipram, while DMCPX had no such effects. In addition, IBMX enhanced CRF-induced cAMP in the presence of a maximally effective concentration of rolipram. Data are means $\pm$ S.E.M. of quadruplicates from a representative of two experiments.

RT-PCR analysis of rat anterior pituitary total RNA showed the expression of PDE1C, PDE4B and PDE4D3 (Table 2 and Fig. 2). The analysis was restricted to three PDE4 isotypes as parallel studies on AtT20 cells [25] indicated that these were expressed in corticotroph cells. Finally, RT-PCR of mouse adenohypophysial RNA revealed the expression of PDE1A and PDE1C4/5.

3.2. Pharmacological analysis of the role of PDEs in rat anterior pituitary cells

Corticotrophs constitute maximally 10% of the total cell population in the rat adenohypophysis. However, the cAMP response to CRF or CRF and AVP is a selective indicator of the activation of these cells. Upon exposure to high physiological concentrations of CRF (≤ 0.3 nM), cAMP levels in isolated anterior pituitary cells peaked at 2 min and returned towards basal by 8 min [26], indicating the activation of cAMP hydrolysis. In the presence of PDE blockers, steady state was apparent at 8 min. Hence, all measurements reported here were carried out at the 8-min time point.

Rolipram, an inhibitor highly specific for PDE4, markedly enhanced the CRF-evoked cAMP response with an apparent EC_{50} of 1 μ M (Fig. 3). Combination of a max-

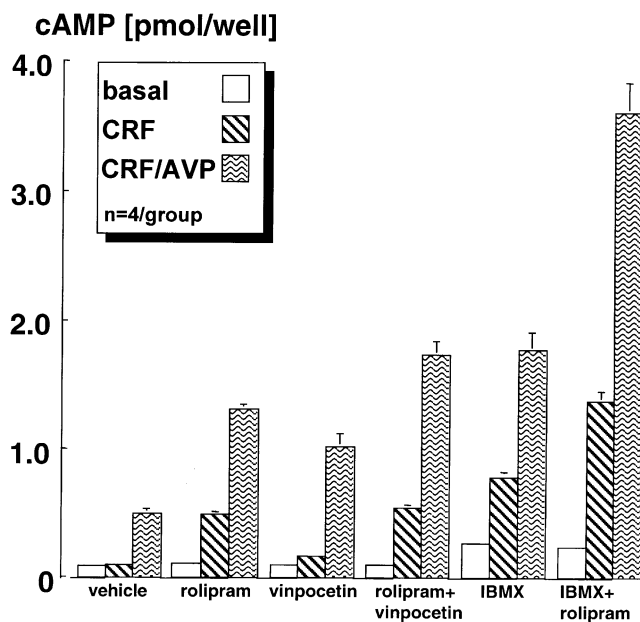


Fig. 5. Effect of prototype PDE inhibitors on cAMP levels in dispersed rat anterior pituitary cells. Isolated rat anterior pituitary cells were treated with vehicle (0.2% DMSO/0.1% acetone) and various combinations of the PDE blockers rolipram (0.1 mM), vinpocetine (0.1 mM) and IBMX (1 mM) 5 min before the addition of medium alone (empty columns), 0.3-nM CRF (hatched column) or 0.3-nM CRF and 2-nM AVP (wavy columns). Data are means $\pm$ S.E.M. of quadruplicates from a representative of two experiments ($P < .05$, one-way ANOVA followed by Newman–Keuls' test). All inhibitor concentrations, except vinpocetine alone, significantly enhanced the cAMP response to CRF ($P < .05$, one-way ANOVA followed by Newman–Keuls' test) and only IBMX or IBMX and rolipram in combination had a significant ($P < .05$, one-way ANOVA followed by Newman–Keuls' test) effect on basal cAMP levels.

imally effective concentration of rolipram with 1-mM IBMX, an inhibitor of most PDE isotypes, caused significant further augmentation of the cAMP response when compared with rolipram alone (Fig. 4). The effect of IBMX was not due antagonism of adenosine receptors [27] as known antagonists of these receptors such as DMCPX (Fig. 4) and 1,3-dipropyl-8-cyclopentyl-xanthine (not shown) caused no change in the effect of rolipram or rolipram and IBMX in combination.

Vinopocetine, a potent blocker of low K_m PDE1 enzymes [6], had no significant effect on cAMP responses evoked by CRF but markedly increased the cAMP response to CRF and AVP in combination (Fig. 5). Moreover, the effects of vinopocetine and rolipram on CRF/AVP-induced cAMP formation were additive (Fig. 5).

Finally, it was examined whether rolipram had any effect on other pituitary cells. The cAMP response to 1-nM GRF was significantly enhanced by rolipram (30 μ M) (*basal*: 10 ± 1 , *basal + rolipram*: 10 ± 1.8 , *GRF* 330 ± 30 , *GRF + rolipram*: 575 ± 43 , data are fmol/well, means $\pm$ S.E.M., $n = 4$ /group), a selective activator of adenylyl cyclase in GH-secreting cells of the adenohypophysis, thus indicating that type 4 PDEs are not restricted to corticotrophs.

4. Discussion

The studies reported here indicated that the cAMP response to ACTH secretagogues is dissipated by multiple species of PDE. Above all, a shift of cAMP hydrolysis from type 4 PDE to type 1A PDE appears to take place in the presence of high physiological levels of AVP. Evidence for the adenohypophysial expression for multiple PDEs that could correspond to the observed pharmacological profiles was found by biochemical as well as RT-PCR analysis of adenohypophysial tissue.

Examination of adenohypophysial cAMP hydrolysis showed that close to 90% of PDE activity is due to type 1 and 4 PDEs. Furthermore, the presence of high and low K_m Ca^{2+} -activated PDE1 was evident. At the mRNA level, the expression low (PDE1C4/5) and high (PDE1A) K_m PDE1 as well as that of two PDE4 isoenzymes (PDE4B, PDE4D3) was detected. While these latter findings correspond reasonably well with the pharmacological and biochemical data, some issues deserve comment. PDE1A mRNA could be only successfully amplified from mouse adenohypophysial RNA from which the primers were designed. Examination of a recent entry of rat PDE1A cDNA sequence (accession no. AF327838) in the database, which was not available at the time of these studies, revealed that there are significant differences between the mouse and rat cDNA sequences in the region of the primers. By contrast, this was not the case for PDE1B, where the primers were suitable for amplifying PDE1B from other tissues (Ang and Antoni, unpublished data) but gave no PCR product in the adenohypophysis. Therefore, it seems reasonable to conclude that a PDE1A

type enzyme is the low-affinity, high-capacity PDE in rat and mouse anterior pituitary gland. This enzyme is reportedly inhibited by cAMP-dependent phosphorylation [10,11], which is opposed by Ca^{2+} -mediated dephosphorylation through protein phosphatase 2B (calcineurin) and Ca^{2+} -mediated activation of catalytic activity through CaM [10]. Thus, PDE1A is an integrator of intracellular cAMP and Ca^{2+} signalling.

Previously, Lobban et al. [28] found no evidence for PDE4 expression in rat pituitary gland. On the basis of the high pharmacological selectivity of rolipram [6] and the present RT-PCR analysis, it is justified to conclude that PDE4 is present in the adenohypophysis and is functionally important in corticotroph cells. However, the relatively low abundance of PDE4 in the rat adenohypophysis is unlikely to reflect a restricted localisation to corticotrophs as rolipram enhanced the cAMP response evoked by GRF, which specifically targets somatotroph cells that represent approximately 30% of the total pituitary cell population. In summary, the RT-PCR analysis of PDE gene expression in rat and mouse adenohypophysis demonstrated the presence of PDE1 and PDE4 isotypes, the characteristics of which conformed with the biochemical properties of cAMP hydrolysis. While this RT-PCR may not be exhaustive, it clearly underscores the potential for functional plasticity of cAMP hydrolysis in anterior pituitary cells.

In order to gain insight into the operation of PDEs in corticotrophs, prototype PDE blockers were applied to assess the role of PDE1 and PDE4 isotypes in the cAMP response generated by CRF and AVP. Assuming that pituitary cells in suspension are spherical with a radius of ~ 15 μ m, the average unstimulated level of cAMP in the cells was between 0.2 and 0.5 μ M. Rolipram or vinopocetine had no appreciable effect on basal cAMP levels, while IBMX caused a marked increase. The lack of an effect of rolipram under these conditions is contradictory to previous reports that it increased ACTH secretion by anterior pituitary tissue in vitro [12,13]. However, the different experimental preparations used in the present (isolated cells) and the aforementioned (pituitary segments) studies may explain this discrepancy. Furthermore, as in other systems [29], a small and selective rise of cAMP in corticotroph cells could be sufficient to activate protein kinase A and trigger ACTH release but may not be discernible by measurement of total cAMP levels in the entire population of pituitary cells. The effect of IBMX on basal cAMP levels is consistent with the finding that low K_m and IBMX-sensitive PDEs, namely PDE1C (present study) and PDE type 11 [30], are prominently expressed in the pituitary gland and thus could be involved in maintaining basal cAMP levels in the majority of pituitary cells. Interestingly, PDE1C is a major PDE in AtT20 corticotroph tumour cells [25]. Furthermore, both AtT20 cells [31,32] and rat anterior pituitary corticotroph cells [33] generate spontaneous Ca^{2+} spikes reaching 0.5–1- μ M intracellular free Ca^{2+} , which is sufficient to activate PDE1C [34].

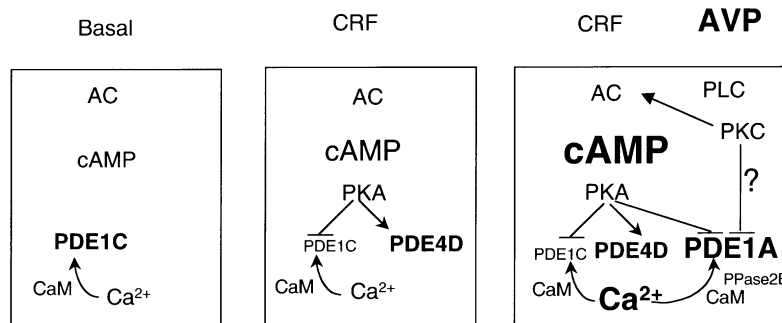


Fig. 6. Schematic diagram of cAMP hydrolysis in multiple functional states of the adenohypophyseal corticotroph cell. Abbreviations: AC, adenylyl cyclase; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PPase2B, protein phosphatase 2B (alias calcineurin). Arrows indicate facilitation, while T-bars indicate inhibition. The size and thickness of the lettering is proportional to the amplitude/activity of the denoted entity.

Assuming that $\sim 10\%$ of the total population of cells responds to CRF, peak intracellular cAMP concentrations in response to 0.3-nM CRF at 2 min [26] may be estimated to be around $7\text{ }\mu\text{M}$. This is sufficient to activate PKA, which would inhibit PDE1C and activate PDE4D3, but is substantially below the K_m of PDE1A. Accordingly, rolipram markedly increased cAMP levels under these conditions, while the prototype blocker of low K_m PDE1 vinpocetine had no effect. In contrast, in the presence of 2-nM AVP when CRF-induced cAMP levels were close to $50\text{ }\mu\text{M}$, both rolipram and vinpocetine enhanced cAMP levels and their effects were additive, indicating distinct targets of action. However, the size of the cAMP response may not be the only determinant of the effectiveness of vinpocetine. As shown in Fig. 5, in the presence of CRF and rolipram, cAMP levels were similar to those seen with CRF and AVP in combination, yet vinpocetine had no significant effect. Thus, it appears that the effect of vinpocetine is only manifest in the presence of AVP. The action of AVP in corticotroph cells is mediated by protein kinase C [35] and reportedly involves inhibition of cAMP hydrolysis [4]. Activation of protein kinase C also reduced low-affinity PDE1 activity in rat kidney [36].

In summary, the data presented indicate remarkable functional plasticity of cAMP hydrolysis in pituitary corticotroph cells (Fig. 6). Note that the concentrations of CRF and AVP applied were within the physiological range, characteristic of intensive surgical stress [37]. Under unstimulated conditions, slow spontaneous Ca^{2+} spikes would delimit cAMP accumulation at the level of the low K_m PDE1C enzyme. Moderate stimulation of the cAMP system by concentrations of secretagogue expected during emotional stress [38,39] would tend to shift PDE hydrolysis to PDE4 isotypes, conceivably PDE4D3, which is activated by cAMP-dependent protein kinase (reviewed in Ref. [5]), while the activity of PDE1C would be simultaneously reduced by cAMP-dependent phosphorylation [25]. Upon more intensive stimulation, i.e. that seen during physical stress [38,39], higher levels of AVP would induce more marked increases of cAMP and intracellular Ca^{2+} by enhancing CRF-induced adenylyl cyclase activity through

protein kinase C [4] and inhibition of PDE1A through protein kinase A and possibly protein kinase C[4] (Fig. 6). The levels of cAMP ($\sim 50\text{ }\mu\text{M}$) thus achieved would require the high capacity of PDE1A to be effectively controlled. Hence, in the case of intensive agonist stimulation, the regulation of PDE1A would be a pivotal determinant of the ensuing intracellular patterns of cAMP and free Ca^{2+} . The three states of the corticotroph cell outlined above are likely to generate different spatiotemporal patterns of Ca^{2+} and cAMP, which could be decoded by effector systems located in various cellular compartments and serve to operate the cellular machinery as required by graded changes in the extracellular stimulus environment.

Acknowledgments

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Short-Term Plasticity of ~~Cyclic~~ Adenosine 3',5'-Monophosphate Signaling in Anterior Pituitary Corticotrope Cells: The Role of Adenylyl Cyclase Isotypes

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Anterior pituitary corticotropes show a wide repertory of responses to hypothalamic neuropeptides and adrenal corticosteroids. The hypothesis that plasticity of the cAMP signaling system underlies this adaptive versatility was investigated. In dispersed rat anterior pituitary cells, depletion of intracellular Ca^{2+} stores with thapsigargin combined with ryanodine or caffeine enhanced the corticotropin releasing-factor (CRF)-evoked cAMP response by 4-fold, whereas reduction of Ca^{2+} entry alone had no effect. CRF-induced cAMP was amplified 15-fold by arginine-vasopressin (AVP) or phorbol-dibutyrate ester. In the presence of inhibitors of cyclic nucleotide phosphodiesterases and phorbol-dibutyrate ester, the depletion of Ca^{2+} stores had no further effect on CRF-induced cAMP

accumulation. Adenohypophysial expression of mRNAs for the Ca^{2+} -inhibited adenylyl cyclases (ACs) VI and IX, and the protein kinase C-stimulated ACs II and VII was demonstrated. ACIX was detected in corticotropes by immunocytochemistry, whereas ACII and ACVI were not present. The data show negative feedback regulation of CRF-induced cAMP levels by Ca^{2+} derived from ryanodine receptor-operated intracellular stores. Stimulation of protein kinase C by AVP enhances Ca^{2+} -independent cAMP synthesis, thus changing the characteristics of intracellular Ca^{2+} feedback. It is proposed that the modulation of intracellular Ca^{2+} feedback in corticotropes by AVP is an important element of physiological control. (*Molecular Endocrinology* 17: 692-703, 2003)

FORTY-ONE AMINO ACID residue corticotropin-releasing factor (CRF) and arginine vasopressin (AVP) are the main hypothalamic mediators of the stress response (1) that stimulate the secretion of ACTH by adenohypophysial corticotrope cells. Adrenal corticosteroids are mobilized in response to ACTH and provide a negative feedback signal in the brain and the anterior pituitary gland to prevent an overshoot of the stress response (2, 3). Additional hypothalamic inhibitory factors also contribute to the regulation of corticotropes (1, 4). Corticotrope cells show remarkable functional plasticity, e.g. glucocorticoid inhibition of pituitary ACTH secretion is diminished in autoimmune disease models (5, 6), while it is very pronounced in psychological stress (7).

The currently identified receptors for CRF all activate adenylyl cyclase (AC) through the stimulatory G

protein G_s (8). In corticotropes, activation of $\text{CRF}_{1\alpha}$ receptors enhances cAMP levels, which leads to increased firing of action potentials and oscillations of intracellular free Ca^{2+} ($[\text{Ca}^{2+}]_i$) (1, 9). In contrast to CRF, AVP activates $V_{1\beta}$ receptors coupled to phospholipase C_β through G_q in corticotrope cells (10). When given alone at physiological concentrations (≤ 2 nM), AVP has little or no effect on ACTH secretion. However, it markedly augments ACTH release induced by CRF (11) by amplifying the CRF-evoked cAMP response (12, 13). These effects are mediated by protein kinase C (14).

Studies in the AtT20 corticotrope tumor cell line have shown that CRF-induced cAMP accumulation as well as membrane depolarization are under feedback inhibition by $[\text{Ca}^{2+}]_i$ (15, 16). Disruption of the Ca^{2+} negative feedback in AtT20 cells leads to enhanced cAMP levels and a marked reduction of glucocorticoid inhibition of stimulated ACTH release (17). Previous work in cultured rat anterior pituitary cells (18) suggested that intracellular Ca^{2+} is an obligatory cofactor for CRF-induced cAMP production. This contradicts the findings in AtT20 cells (15) and the more generalized Ca^{2+} -feedback hypothesis of glucocorticoid action (17, 19).

Therefore, the aim of the present study was to analyze the effects of Ca^{2+} on cAMP levels in cortico-

Abbreviations: AC, Adenylyl cyclase; AVP, arginine vasopressin; BAPTA-AM, 1,2-bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid tetra-(acetoxymethyl)-ester; BCECF-AM, 2',7'-bis-(carboxymethyl)-5(6'-carboxyfluorescein acetoxymethyl ester; $[\text{Ca}^{2+}]_i$, intracellular free Ca^{2+} ; CRF, corticotropin-releasing factor; FURA2-AM, 1-[2-(5-carboxyoxazole-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid pentaacetoxymethyl ester; HBSS, Hanks' balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; PdBu, phorbol-dibutyrate ester; PDE, phosphodiesterase; RNase, ribonuclease; RyR, ryanodine receptor.

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trope cells exposed to CRF and AVP. The results demonstrate Ca^{2+} negative feedback control of CRF-induced cAMP levels in rat pituitary corticotropes by $[\text{Ca}^{2+}]_i$ derived from a ryanodine receptor (RyR)-operated intracellular source. AVP markedly enhanced the cAMP response to CRF and amplified by 10-fold the dynamic range of the modulation of cAMP levels by $[\text{Ca}^{2+}]_i$. This change was in part due to the enhancement of Ca^{2+} -independent cAMP synthesis. Analysis of AC expression indicated that ACIX and ACVII are likely to mediate the actions of CRF and AVP in corticotrope cells. It is proposed that the reduction of Ca^{2+} feedback inhibition of cAMP synthesis underlies the ability of AVP to reduce glucocorticoid feedback inhibition of ACTH release.

RESULTS

Ca^{2+} Feedback Inhibition of CRF-Induced cAMP Synthesis

The time course of the cAMP response to a high physiological concentration of CRF (0.3 nM) is shown in Fig. 1A. Levels of cAMP peaked at 2 min, declined sharply by 5 min, and returned to baseline at 20 min. As CRF is highly specific to corticotropes and because corticotrope cells represent, at best, 10% of the total number of cells in this preparation, 2- to 3-fold increases of total cAMP may correspond to approximately 20- to 30-fold increases of intracellular cAMP in corticotropes.

Depletion of Ca^{2+} pools by means of 2 mM EGTA, 30 μM ryanodine, and 2 μM thapsigargin before the application of CRF increased the basal levels of cAMP in the cell suspension. The amplitude of the cAMP response to CRF was similar to that observed in medium containing 2 mM Ca^{2+} (Fig. 1B); however, it was sustained: steady state was reached within 5 min and maintained up to 20 min.

The decline of cAMP levels after 2 min in 2 mM Ca^{2+} medium indicated the involvement of cyclic nucleotide phosphodiesterases (PDEs), while the steady state seen in Ca^{2+} -depleted cells suggested regulation by Ca^{2+} of PDE and/or AC. Indeed, the combination of the PDE inhibitors 3-isobutyl-1-methylxanthine (IBMX) and rolipram markedly enhanced the cAMP response to 0.3 nM CRF without the involvement of adenosine receptors (20). In the presence of 1 mM IBMX and 0.1 mM rolipram (PDE-I), application of 3 μM nifedipine or 2 mM CoCl_2 or acute depletion of extracellular Ca^{2+} by resuspension of the cells in low Ca^{2+} medium failed to alter the cAMP response to 0.3 nM CRF (not shown). Thus, reduction of Ca^{2+} entry mechanisms had no apparent effect on the cAMP response to CRF. Attempts to use intracellular chelators of Ca^{2+} failed to yield useful data because of the apparently nonspecific effects of all acetoxymethyl esters tested [1-[2-(5-carboxyoxazol-2-yl)-6-aminobenzofuran-5-oxy]-2-(2'-amino-5'-methylphenoxy)-ethane-*N,N,N',N'*-tetraacetic acid pentaacetoxymethyl

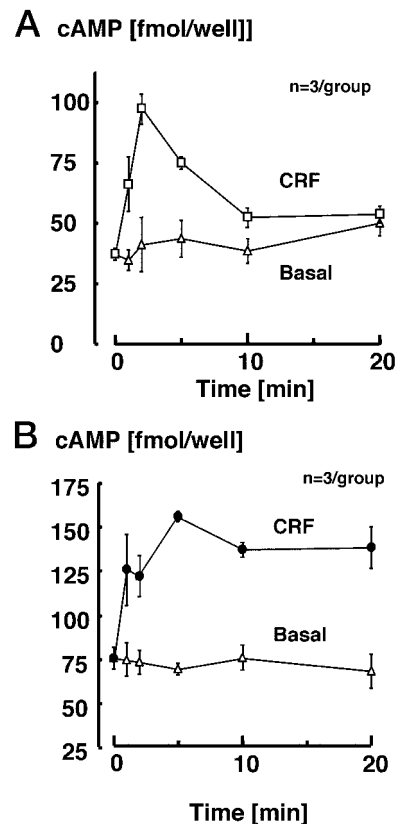


Fig. 1. Time Course of cAMP Accumulation in Isolated Rat Anterior Pituitary Cells [Vehicle (Basal) and 0.3 nM CRF-Treated (CRF) Cells]

A, Incubations in medium containing 2 mM Ca^{2+} . B, Incubations in low Ca^{2+} medium containing 30 μM ryanodine and 2 μM thapsigargin. Data are means $\pm$ SEM of triplicates, from a representative of two separate experiments.

ester (FURA2/AM), 1, 2-bis-(*o*-aminophenoxy)-ethane-*N,N,N',N'*-tetraacetic acid tetra-(acetoxymethyl)-ester (BAPTA/AM) as well as 2',7'-bis-(carboxyethyl)-5(6')-carboxyfluorescein acetoxymethyl ester (BCECF/AM)] to enhance cAMP accumulation in this system (data not shown). Therefore, drugs that deplete or block intracellular Ca^{2+} stores were used. Application of 10 mM caffeine significantly increased basal cAMP levels, but failed to enhance the response to 0.3 nM CRF (Table 1). Thapsigargin at 2 μM had no effect; however, in combination with caffeine it produced a significant enhancement of basal as well as CRF-induced cAMP formation (Table 1). Preincubation in low Ca^{2+} medium containing 2 mM EGTA for 30 min augmented basal and CRF-induced cAMP (Fig. 2A). The application of 2 mM EGTA with 10 mM caffeine and 2 μM thapsigargin gave the highest response to CRF (Fig. 2A).

The time course of the CRF-induced increment of cAMP in Ca^{2+} -depleted cells treated with PDE-I is shown in Fig. 2B. Note that the rate of cAMP accumulation was close to linear up to 8 min, with a marked reduction afterward. In medium containing 2 mM Ca^{2+}

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Table 1. Effect of Depletion of Intracellular Ca^{2+} Pools on 0.3 nM CRF-Induced cAMP Accumulation in the Presence of 1 mM IBMX and 0.1 mM Rolipram

Treatment	Basal	CRF-Induced Increment
Vehicle	108 $\pm$ 12	556 $\pm$ 37
Caffeine	220 $\pm$ 24 ^a	764 $\pm$ 103
Thapsigargin	144 $\pm$ 16	652 $\pm$ 84.4
Caffeine + Thapsigargin	218 $\pm$ 16 ^a	1068 $\pm$ 56 ^a

Cells were preincubated for 30 min in medium supplemented with 0.1% ethanol (vehicle), 10 mM caffeine, or 2 μM thapsigargin or the combination of the two drugs. Data are cAMP (fmol/well), means $\pm$ SEM, $n = 4$, representative of two experiments.

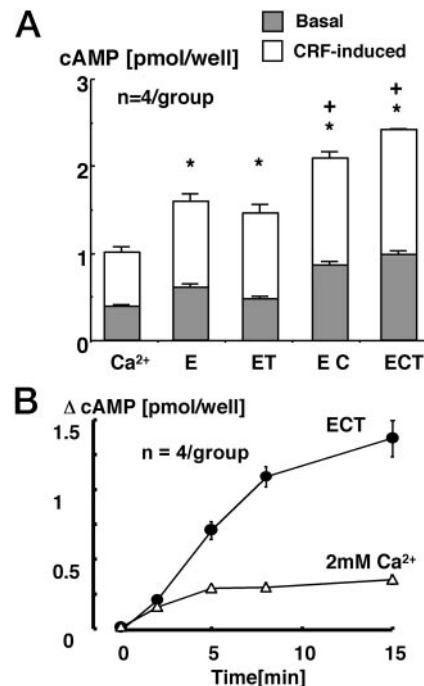
^a $P < 0.05$ when compared with the respective vehicle group. One-way ANOVA, followed by Dunnett's test.

the response reached steady state as early as 5 min. At 8 min, the cAMP increment in Ca^{2+} -depleted cells was 4.0 ± 0.22 -fold higher (mean $\pm$ SEM, $n = 5$, each in quadruplicate) than in 2 mM Ca^{2+} . Taken together, these data indicated that CRF-induced cAMP synthesis was inhibited by Ca^{2+} derived from RyR-operated Ca^{2+} stores.

Concentration-response curves to CRF in the absence (Fig. 3A) or the presence of PDE-I (Fig. 3B) revealed that the Ca^{2+} -regulated component of cAMP synthesis, i.e. the difference between the CRF-induced increments in Ca^{2+} -depleted conditions and in 2 mM Ca^{2+} , approached maximum between 0.3 and 1 nM CRF [Fig. 3, A and B (inset)], which is the upper limit of the physiological range. Importantly, cellular cAMP continued to rise in response to concentrations of CRF up to 100-fold above this level (Fig. 3, A and B).

Activation of Protein Kinase C Reduces the Ca^{2+} -Inhibited Component of cAMP Synthesis

Physiological concentrations of AVP (0.3 and 2 nM) strongly enhanced the cAMP response to 0.3 nM CRF (Fig. 4A), whereas there was no detectable change in cAMP levels in the presence of up to 100 nM AVP alone (not shown). Pretreatment with the protein kinase inhibitor bis-indolyl maleimide I (1 μM) blocked approximately 90% of the effect of 2 nM AVP, suggesting its mediation by protein kinase C (data not shown). Combination of 0.3 nM CRF with 2 nM AVP (CRF/AVP) augmented the cAMP response to 14.5 ± 2 - and 15.9 ± 2.3 -fold (mean $\pm$ SEM; $n = 3$, each carried out in quadruplicate) of the responses to 0.3 nM CRF in 2 mM Ca^{2+} and 0.3 nM CRF in 2 mM EGTA, 30 μM ryanodine, and 2 μM thapsigargin, respectively. Thus, the effect of AVP was largely independent of Ca^{2+} status and, as a result, the dynamic range of Ca^{2+} control, i.e. the difference in the cAMP response in 2 mM Ca^{2+} vs. ERT-medium, increased almost 10-fold. The cAMP response to CRF/AVP was not maximal as application of supraphysiological concentrations of AVP revealed the ability of the system to produce greater than 100-fold increases of cAMP (Fig. 4A).

**Fig. 2.** Analysis of the Effects of Ca^{2+} on cAMP Synthesis Induced by CRF in Isolated Rat Anterior Pituitary Cells

A, Depletion of intracellular Ca^{2+} pools leads to enhanced synthesis of cAMP in the presence of PDE blockers (1 mM IBMX, 0.1 mM rolipram). Cells were preincubated in 2 mM Ca^{2+} (Ca^{2+}), or various combinations of 2 mM EGTA (E), 2 μM thapsigargin (T), and 10 mM caffeine (C). Data are means $\pm$ SEM of quadruplicates from a representative of four experiments. Note marked increases in basal (hatched columns) cAMP levels; however, these were not proportional to the enhancement of the response to CRF: two-way ANOVA gave a significant interaction ($P < 0.05$) between the effects of Ca^{2+} depletion and CRF. The increments of cAMP (empty portion of columns) were analyzed by one-way ANOVA and Newman-Keuls test. *, $P < 0.05$ when compared with the increment of cAMP in 2 mM Ca^{2+} group; +, $P < 0.05$ when compared with the increment of the EGTA group. B, Time course of 0.3 nM CRF-stimulated cAMP accumulation in the presence of blockers of PDE in control cells (2 mM Ca^{2+}) and in Ca^{2+} -depleted cells in 2 mM EGTA, 10 mM caffeine, and 2 μM thapsigargin (ECT). Increments above basal cAMP levels are shown; the basal level of cAMP in 2 mM Ca^{2+} was 0.11 ± 0.014 pmol/well. Data are means $\pm$ SEM of quadruplicates from a representative of two experiments.

In the presence of PDE-I, AVP produced significant, concentration-dependent potentiation of the cAMP response to 0.3 nM CRF (Fig. 4B). At 2 nM AVP the amplification was 3.5 ± 0.7 -fold (mean $\pm$ SEM; $n = 5$, each carried out in quadruplicate) of the control CRF response, which was substantially less than the 15-fold increase in the absence of PDE-I. Furthermore, Ca^{2+} -depletion had a significantly smaller effect on the cAMP response to CRF/AVP in the presence of PDE inhibitors [Vehicle (4.1 ± 0.7) vs. PDE-I (2.2 ± 0.4)-fold increase of the response in 2 mM Ca^{2+} medium, mean $\pm$ SEM, $n = 3$ and $n = 5$, respectively, each

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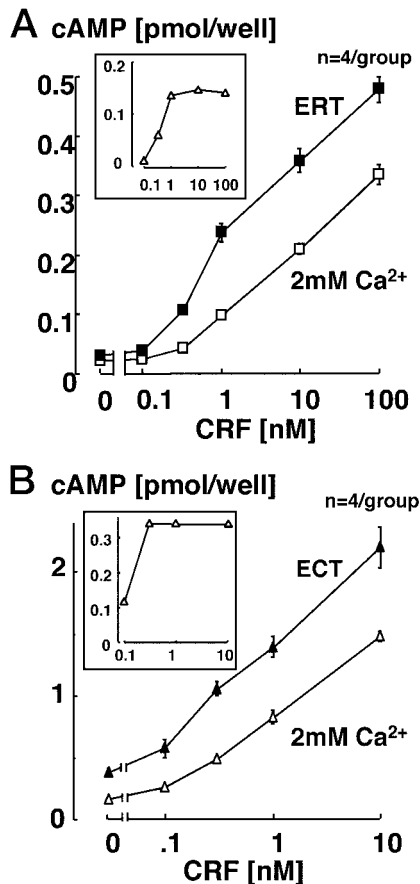


Fig. 3. Enhancement of the CRF-Induced cAMP Response by Ca²⁺ Depletion in Isolated Rat Anterior Pituitary Cells

A, Cells in medium containing 2 mM Ca²⁺ or low Ca²⁺ medium with 30 μ M ryanodine and 2 μ M thapsigargin (ERT); the *inset* shows the difference between the CRF-induced increments of cAMP in the ERT and 2 mM Ca²⁺ groups for each concentration of CRF. B, Cells treated with 1 mM IBMX and 0.1 mM rolipram, incubated in medium containing 2 mM Ca²⁺ or low Ca²⁺ medium with 10 mM caffeine and 2 μ M thapsigargin (ECT); means $\pm$ SEM of quadruplicates representative of two experiments. The *inset* is as in panel A.

in quadruplicate, $P < 0.05$ compared by Student's *t* test]. At a high, supraphysiological AVP concentration (50 nM), no enhancement of the cAMP response was observed upon Ca²⁺ depletion (Fig. 4B). Similarly, increasing concentrations of the protein kinase C activator PdBu (phorbol-dibutyrate ester) progressively diminished the Ca²⁺ modulation of CRF-induced cAMP production (Fig. 4C).

In sum, both AVP-induced protein kinase C activation and the depletion of Ca²⁺ pools enhanced the cAMP response to CRF in the presence as well as the absence of PDE-I. In the absence of PDE-I there was clear synergy between the effects of AVP and Ca²⁺ depletion, whereas in the presence of PDE-I the effects were additive and much smaller in amplitude. In the presence of PDE-I, intensive stimulation of the protein kinase C pathway eventually enhanced the

cAMP response to levels at which the effects of Ca²⁺ depletion were not discernible.

Specificity of Ca²⁺ Inhibition of Stimulus-Induced cAMP

Introduction of Ca/EGTA into the medium suppressed the CRF-induced increment of cAMP levels to that seen in cells incubated with 2 mM Ca²⁺ throughout in the presence (Fig. 5A) as well as in the absence of PDE-I (not shown). This was also the case for cells stimulated with CRF/AVP (Fig. 5A); moreover, inclusion of 1 μ M of the Ca²⁺ ionophore ionomycin which supports Ca²⁺-dependent ACTH secretion in this system (21) failed to augment the inhibitory effect of 2 mM extracellular Ca²⁺ on CRF- or CRF/AVP-induced cAMP responses (data not shown).

Specificity of the action of Ca²⁺ was analyzed in cells incubated in 0.2 mM EGTA, 10 mM caffeine, and 2 μ M thapsigargin to avoid displacement of significant amounts of Ca²⁺ by Co²⁺ and Ba²⁺ which have much higher affinities for EGTA. In the presence of PDE-I the inhibitory effect of Ca²⁺ was mimicked by Co²⁺ but not by Ba²⁺ (Fig. 5B).

AC Isotype Expression and Localization in Rat Adenohypophysis

The data presented so far indicated the involvement of Ca²⁺-inhibited and protein kinase C-stimulated ACs in the response to CRF and AVP. RT-PCR with primers that amplify ACV and ACVI yielded the expected 1095-bp product, which was cleaved by restriction enzymes in a pattern that indicated the presence of ACVI but not of ACV in rat adenohypophysis (Fig. 6). The expression of ACVII was also observed with RT-PCR (Fig. 6; GenBank accession no. AF542508).

In situ hybridization revealed diffuse positive labeling for ACII (Fig. 7), as well as ACIX (Fig. 7), in the adenohypophysis. Ribonuclease (RNase) protection assays showed a strong signal for both ACII and ACIX (Fig. 7). Immunohistochemical staining showed that ACIX was widely expressed in the adenohypophysis and was found in all ACTH-immunopositive cells (Fig. 8). In contrast, ACII (Fig. 8), as well as ACVI, were completely segregated from ACTH. No discernible positive signal could be obtained with the antibody against ACVII in pituitary or brain tissue (data not shown).

DISCUSSION

These data show that in rat anterior pituitary corticotropes the CRF-induced cAMP response is under feedback inhibition by intracellular free Ca²⁺ primarily derived from intracellular stores sensitive to ryanodine and caffeine. AVP, the physiologically relevant hormone of CRF, modulates Ca²⁺ feedback by shifting

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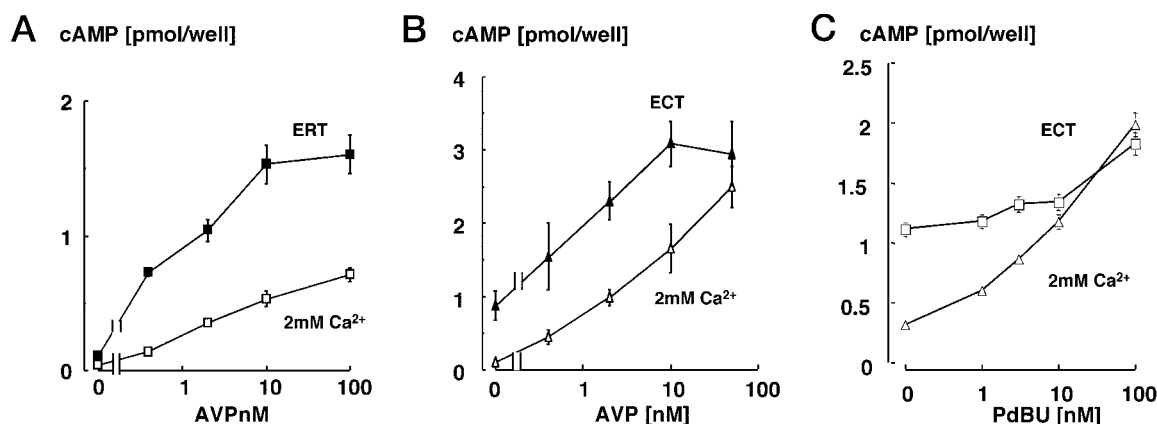


Fig. 4. Interaction of Protein Kinase C Activation and Ca^{2+} Depletion on cAMP Accumulation Evoked by 0.3 nM CRF

A, Cells in medium containing 2 mM Ca^{2+} or low Ca^{2+} medium with 30 μM ryanodine and 2 μM thapsigargin (ERT). The basal level of cAMP in 2 mM Ca^{2+} was 0.04 ± 0.006 pmol/well. B and C, Cells treated with 1 mM IBMX and 0.1 mM rolipram, incubated in medium containing 2 mM Ca^{2+} or low Ca^{2+} medium with 10 mM caffeine and 2 μM thapsigargin (ECT). Data are means $\pm$ SEM of quadruplicates representative of three separate experiments. The basal level of cAMP in 2 mM Ca^{2+} was 0.10 ± 0.02 in panel B and 0.12 ± 0.02 pmol/well in panel C.

cAMP synthesis toward a Ca^{2+} -independent pathway, thereby markedly enhancing the cAMP response.

Nature of the Ca^{2+} Feedback Signal

CRF triggers an increase of $[\text{Ca}^{2+}]_i$ through cAMP in corticotropes (1, 9). Hence, the inhibition of cAMP accumulation by $[\text{Ca}^{2+}]_i$ constitutes a negative feedback effect. In contrast, previous work carried out in cultured rat anterior pituitary cells suggested that Ca^{2+} is a necessary cofactor for CRF-induced cAMP synthesis (18). However, this conclusion was derived from cells extensively treated with the ionophore A23187, which may lead to mitochondrial Ca^{2+} leakage and thus impairment of oxidative metabolism (e.g. see Ref. 22).

Acute reduction of extracellular Ca^{2+} entry produced no change in CRF-induced cAMP responses, while treatment with 2 mM EGTA, caffeine, and thapsigargin had marked effects. Previously, Abou-Samra et al. (18) reported that intracellular Ca^{2+} stores contributed to the secretagogue action of CRF, but at the time these pools were not identified. An inositol-1,3,5-triphosphate receptor-operated intracellular Ca^{2+} store is functional in pituitary corticotropes (reviewed in Refs. 1 and 10). A RyR-operated store has not been previously reported, although caffeine is a well established stimulus for ACTH release (23). Moreover, mRNAs for RyR type 2 and type 3 are abundant in rat adenohypophysis (24). In smooth muscle cells, the RyR system is known to play a prominent role in the regulation of Ca^{2+} -activated membrane proteins such as ion channels (25), while the control of RyR stores in nonmuscle cells is not well characterized. All RyR isoforms are capable of Ca^{2+} -induced Ca^{2+} release. Furthermore, the type 2 receptor is sensitized to Ca^{2+} by cAMP-dependent phosphorylation (reviewed in Ref. 26). The present data suggest that the stimulation of

protein kinase A by CRF may be sufficient to activate the RyR-operated store, e.g. by amplification of spontaneous Ca^{2+} sparks (25). Another possibility, that of activation of RyR through dihydropyridine-sensitive channels (26), seems unlikely as nifedipine had no significant effect on cAMP production evoked by CRF.

The Ca^{2+} control of CRF-induced cAMP appeared redundant: upon the depletion of intracellular Ca^{2+} stores, CRF- as well as CRF/AVP-induced cAMP was effectively suppressed by Ca^{2+} added to the extracellular fluid. The mode of entry of Ca^{2+} that controls cAMP synthesis was not addressed in detail, as this was beyond the power of resolution of the experimental system. Previous work suggests that voltage-regulated (15, 27, 28) as well as capacitative Ca^{2+} entry (29, 30), i.e. that evoked by the depletion of intracellular Ca^{2+} stores, may control cAMP synthesis in a variety of systems.

The action of Ca^{2+} to inhibit cAMP synthesis appeared specific. Co^{2+} , which is known to be taken up by cells via capacitative Ca^{2+} entry mechanisms (31), and to mimic some of the biological actions of Ca^{2+} (32, 33) suppressed CRF-induced cAMP synthesis. In contrast, Ba^{2+} was ineffective, which is consistent with the general view that Ba^{2+} does not readily replace Ca^{2+} as an effector in biological systems, including the inhibition of ACs (34).

In sum, the primary source of Ca^{2+} for the regulation of cAMP levels appears to be a RyR-operated intracellular Ca^{2+} pool. If this pool is depleted, Ca^{2+} entry mechanisms may also provide a negative feedback signal to secretagogue-induced cAMP synthesis.

Targets of Ca^{2+} Feedback in Cells Exposed to Physiological Concentrations of CRF

The effects of Ca^{2+} on CRF-induced cAMP accumulation were prominent in the presence of PDE block-

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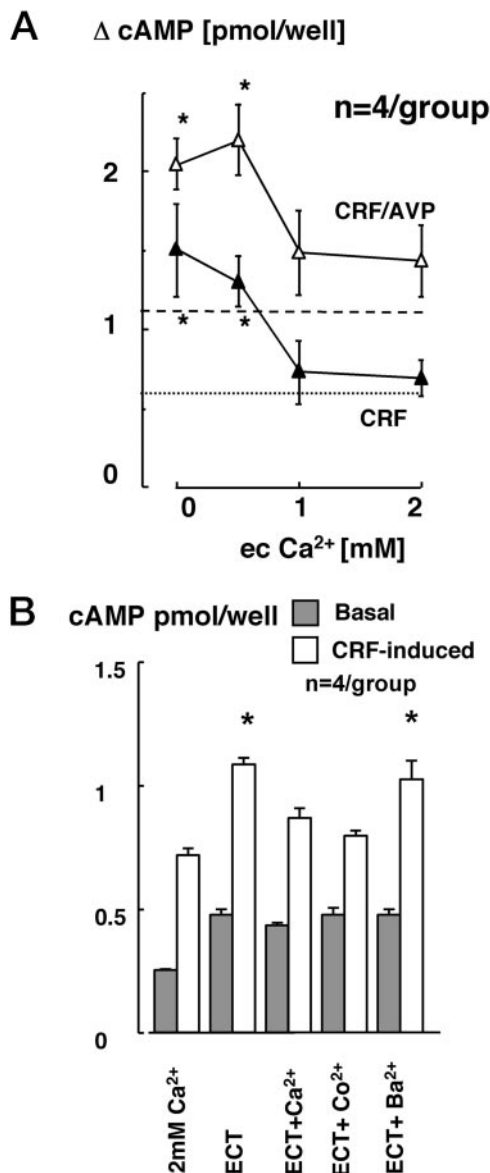


Fig. 5. Introduction of Ca^{2+} into the Extracellular Fluid Suppresses cAMP Responses to CRF or CRF and AVP in Combination

Experiments carried out in the presence of PDE blockers, 0.1 mM rolipram and 1 mM IBMX. A, Increments of cAMP above respective basals; data are means $\pm$ SEM from quadruplicates representative of four experiments. *, $P < 0.05$ when compared with 0 Ca^{2+} group, but not different from response of cells incubated in 2 mM Ca^{2+} throughout (dashed line, CRF 0.3 nM/AVP 2 nM; dotted line, CRF 0.3 nM); one-way ANOVA, Newman-Keuls test. B, Specificity of the inhibitory effect of Ca^{2+} on CRF-induced cAMP synthesis. Cells incubated in 2 mM Ca^{2+} throughout, or depleted of Ca^{2+} with 0.2 EGTA, 10 mM caffeine, 2 μM thapsigargin (ECT) before the addition of 0.3 nM CRF in medium containing Ca^{2+} , Co^{2+} , and Ba^{2+} to reach 2 mM final concentration. Data are means $\pm$ SEM from quadruplicates representative of two experiments. *, $P < 0.05$ when compared with the increment of cAMP above the respective basal in the 2 mM Ca^{2+} group, one-way ANOVA followed by Dunnett's test.

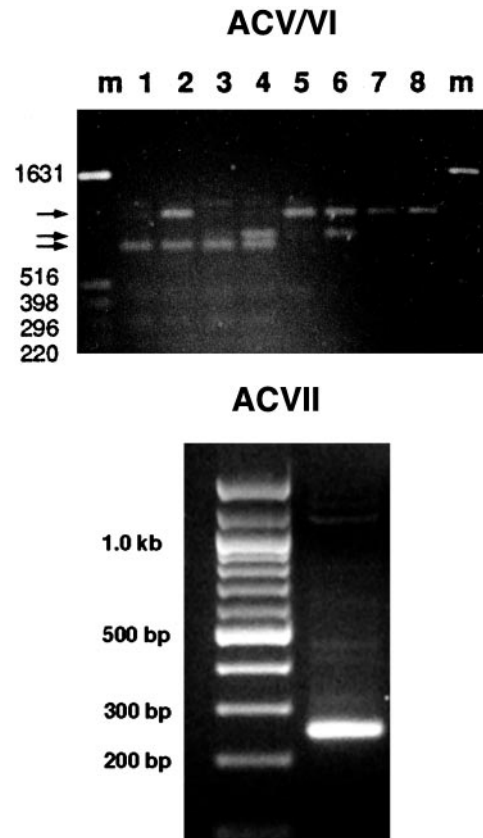


Fig. 6. Analysis of AC mRNA Expression in Rat Adenohypophysis by RT-PCR

A, RT-PCRs with common primers for ACV and ACVI and restriction digests of the PCR products were carried out as described by Premont *et al.* (55). Agarose gel chromatogram of PCR/restriction digest products from rat adenohypophysis (lanes 1, 3, 5, and 7) and brain (lanes 2, 4, 6, and 8). PCR products were digested with *Xho*I (lanes 1 and 2), *Sac*I (lanes 5 and 6), or both enzymes (lanes 3 and 4). Undigested PCR products are shown in lanes 7 and 8. The positions of standards (bp) are shown in the extreme left and right lanes (m). Upper arrow indicates 1095-bp product derived from ACV mRNA digested by *Sac*I to yield fragments of 220 bp and 875 bp (middle arrow); 1095-bp ACVI product remains undigested. Digestion with *Xho*I yields fragments of 300 bp and 795 bp (lower arrow) from the ACVI-derived PCR product but does not cut that derived from ACV. B, Agarose gel chromatogram of the PCR product for ACVII using unique sequence-specific primers; the amplified segment was subcloned, sequenced, and verified as ACVII.

ers, indicating that Ca^{2+} inhibits CRF-activated AC in corticotrope cells. Currently, there are no examples of Ca^{2+} inhibition of CRF- G_s -AC coupling, whereas inhibition of ACs by Ca^{2+} is well established (29, 35). Here we have demonstrated the expression of mRNAs for the Ca^{2+} -inhibited ACs, ACVI and ACIX in the rat anterior pituitary gland, also reported in sheep (36). Furthermore, immunocytochemical analysis indicated the presence of ACIX but not ACVI in corticotropes. Others (37) have reported that the activity of AC in rat anterior pituitary membranes is strongly inhibited by

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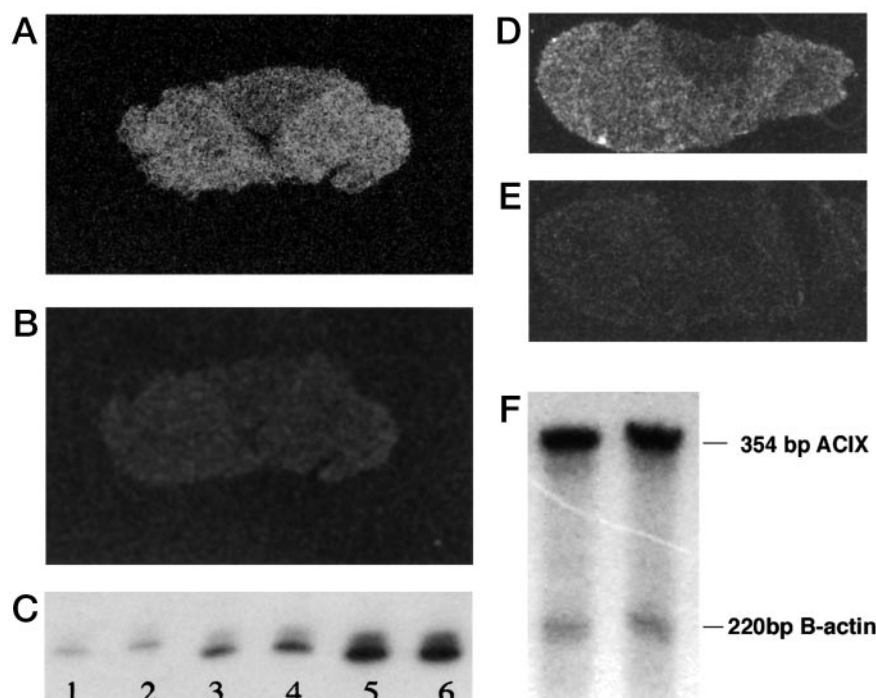


Fig. 7. Expression of ACII and ACIX mRNAs in Rat Adenohypophysis

In situ hybridization with ^{35}S -labeled antisense (A) and sense (B) riboprobes for ACII indicating specific mRNA labeling in the anterior lobe. C, RNase protection assay with ^{32}P -labeled ACII probe (330 bp). Lanes 1 and 2, skeletal muscle; lanes 3 and 4, adenohypophysis; lanes 5 and 6, hypothalamus. D, *In situ* hybridization with ^{35}F -labeled antisense and (E) sense riboprobes for ACIX indicating specific mRNA labeling in the anterior lobe. F, RNase protection assay with ^{32}P -labeled AC9 probe (356 bp) and a 220-bp β -actin probe in both lanes. Each probe produced a single protected radiolabeled band.

submicromolar Ca^{2+} . Both ACVI (29) and ACIX (38) are inhibited by $0.1\text{--}1\ \mu\text{M}$ Ca^{2+} and $[\text{Ca}^{2+}]_i$ levels in CRF-stimulated corticotropes are within this range (39, 40).

A further possible mode of Ca^{2+} negative feedback action on cAMP is the stimulation of cAMP hydrolysis. Approximately 70% of the total PDE activity in the adenohypophysis is Ca^{2+} /calmodulin activated (20, 41) and consists of both low K_m ($\sim 1.5\ \mu\text{M}$) and high K_m ($\sim 50\ \mu\text{M}$) species. Based on studies with various inhibitors of PDE (20), it would appear that high K_m , Ca^{2+} -activated PDE is active only during stimulation by CRF and AVP. Work in GH3 pituitary tumor cells indicates that low K_m Ca^{2+} -activated PDEs (PDE1C) may be important for basal cAMP levels (42). These enzymes are expressed in the rat adenohypophysis (20); however, there is no compelling evidence for a major involvement of Ca^{2+} -activated PDEs in the response to physiological levels of CRF.

Taken together, the main target of Ca^{2+} feedback inhibition upon activation by physiological concentrations of CRF appears to be ACIX (Fig. 9A).

Modulation of Ca^{2+} Feedback Inhibition of cAMP Synthesis by AVP

AVP augmented CRF-induced cAMP accumulation in the presence of PDE blockers and had no detectable effects on basal cAMP in the presence or absence of

PDE blockers. This confirms previous reports (12, 14) showing that, in corticotrope cells, AC is a coincidence detector for the simultaneous activation of $G_{s\alpha}$ and protein kinase C by CRF and AVP, respectively. Indeed, mRNA for both ACII and ACVII, ACs that are stimulated by protein kinase C in the context of activation by $G_{s\alpha}$, is expressed in rat adenohypophysis (Refs. 36 and 43 and present study). The potentiation of the cAMP response by AVP and PdBu was prominent in Ca^{2+} -depleted cells, suggesting the involvement of a Ca^{2+} -independent and phorbol ester-activated form of protein kinase C, such as ϵ or δ , which are prominently expressed in rat adenohypophysis (44, 45). Human erythroid progenitor cells, which express predominantly ACVII, show a similar potentiation of cAMP synthesis by Ca^{2+} -independent, phorbol ester-activated protein kinase C (46). As ACII protein was not found in corticotrope cells, it is reasonable to suggest that ACVII is the AC activated by the combination of physiological concentrations CRF and AVP, and by supraphysiological ($>1\ \text{nM}$) concentrations of CRF.

The reduction of the effect of AVP on CRF-induced cAMP formation in the presence of PDE blockers observed in the present study is consistent with the inhibition of high K_m PDE activity by AVP observed previously (12). The cAMP concentration in cortico-

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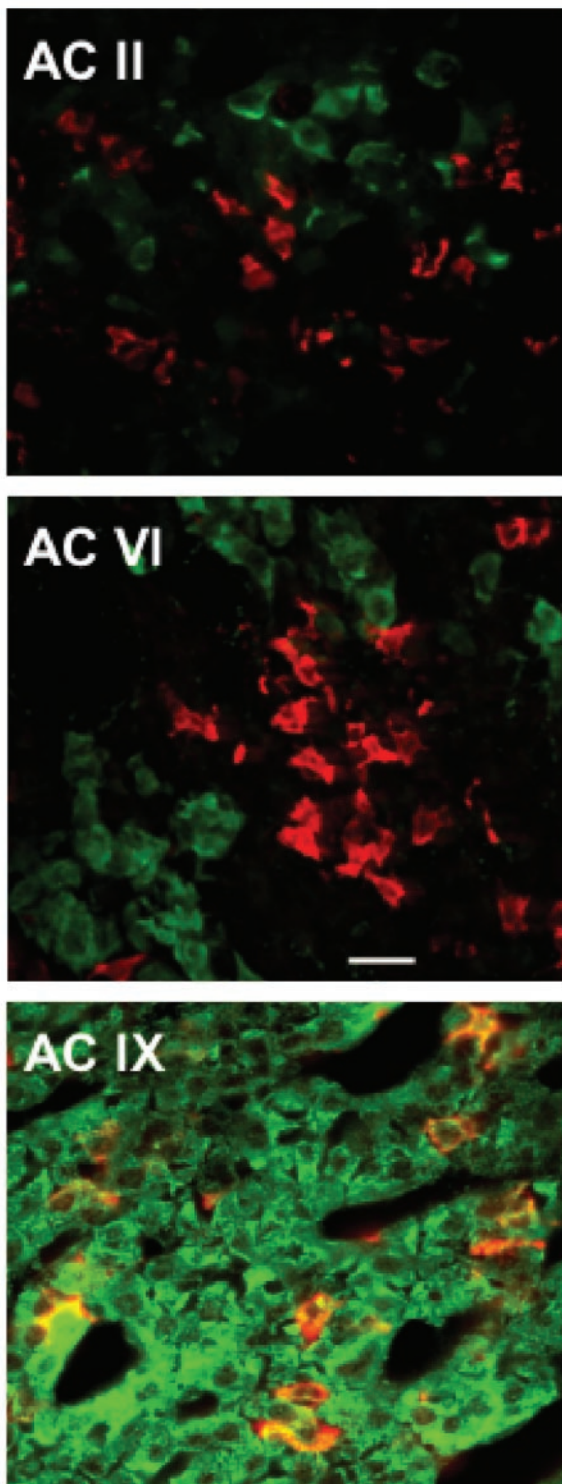


Fig. 8. Localization of AC Expression in Corticotrope Cells by Indirect Immunofluorescence

Affinity-purified antibodies against ACII, V/VI, and IX were reacted with free-floating sections of rat adenohypophysis. The reaction for ACs was detected with secondary antibodies conjugated to Alexa 488 dye, ACTH was visualized with Alexa 546 conjugates. The bar represents 20 μm . Note widespread expression of ACIX and its colocalization with ACTH. No colocalization with ACTH was apparent for ACII or ACV/VI.

trope cells exposed to high physiological levels of CRF/AVP was approximately 100–200 μM , which is in the range of high K_m , high-capacity, Ca^{2+} -activated PDEs (47). Indeed, this type of PDE constitutes close to 70% of cAMP-hydrolyzing activity in the adenohypophysis (41) and is likely to be PDE1A or a closely related enzyme (20). In the presence of PDE blockers, Ca^{2+} control of cAMP was blocked by PdBu activation of protein kinase C or supraphysiological AVP concentrations. In the absence of PDE inhibitors, Ca^{2+} depletion still markedly enhanced the cAMP response at all AVP concentrations tested. Hence, the data indicate that the predominant mode of operation of Ca^{2+} feedback is switched by AVP from the inhibition of ACIX to the activation of high K_m PDE (Fig. 9, A and B). Intriguingly, AVP also appears to suppress the activity of the same PDE, leading to a functionally concerted and highly effective augmentation of intracellular cAMP levels. Whether or not there are intracellular gradients of cAMP (48, 49) in corticotropes, and what the physiological target(s) of 100 μM cAMP may be, remains to be investigated.

Implications for Physiological Control

The present data conform to a model incorporating Ca^{2+} -inhibited ACIX, protein kinase C-activated ACVII, and high- K_m , high-capacity PDE (Fig. 9, A and B). The Ca^{2+} -inhibited component of the CRF-induced cAMP response appeared to saturate between 0.3 nM and 1 nM CRF. Thus, it is reasonable to hypothesize that at physiological concentrations (*i.e.* ≤ 0.3 nM) of CRF, ACIX is predominant and ACVII is only weakly activated (Fig. 9A). Under these conditions, PDE4 is important for cAMP hydrolysis and no evidence for the involvement of high- K_m , Ca^{2+} -activated PDE was apparent (20). Activation of protein kinase C stimulates ACVII; thus, in the presence of AVP, ACVII would emerge as the dominant AC (Fig. 9B). A further mechanism that would facilitate the switch to Ca^{2+} -independent cAMP synthesis through ACVII is that activation of protein kinase C inhibits the activation of ACIX by G_s -coupled receptors (50).

The concentrations of CRF (0.3 nM) and AVP (0.3–2 nM) used in the present study approximate the physiological levels in the hypophyseal portal blood of animals subjected to various forms of stress (reviewed in Refs. 1 and 51). The intracellular cAMP response is a major determinant of the efficiency of glucocorticoid inhibition at the pituitary gland (52), which is the main site of action of the synthetic glucocorticoid dexamethasone (53) and is also significant for physiological control by endogenous corticosteroids. The present study shows that functionally concerted actions of AVP through protein kinase C produce a rapid switch in the cAMP biosynthetic pathway. An escape of cAMP biosynthesis from Ca^{2+} -negative feedback inhibition ensues, which is made possible by the molecular diversity of cAMP signaling proteins expressed in corticotrope

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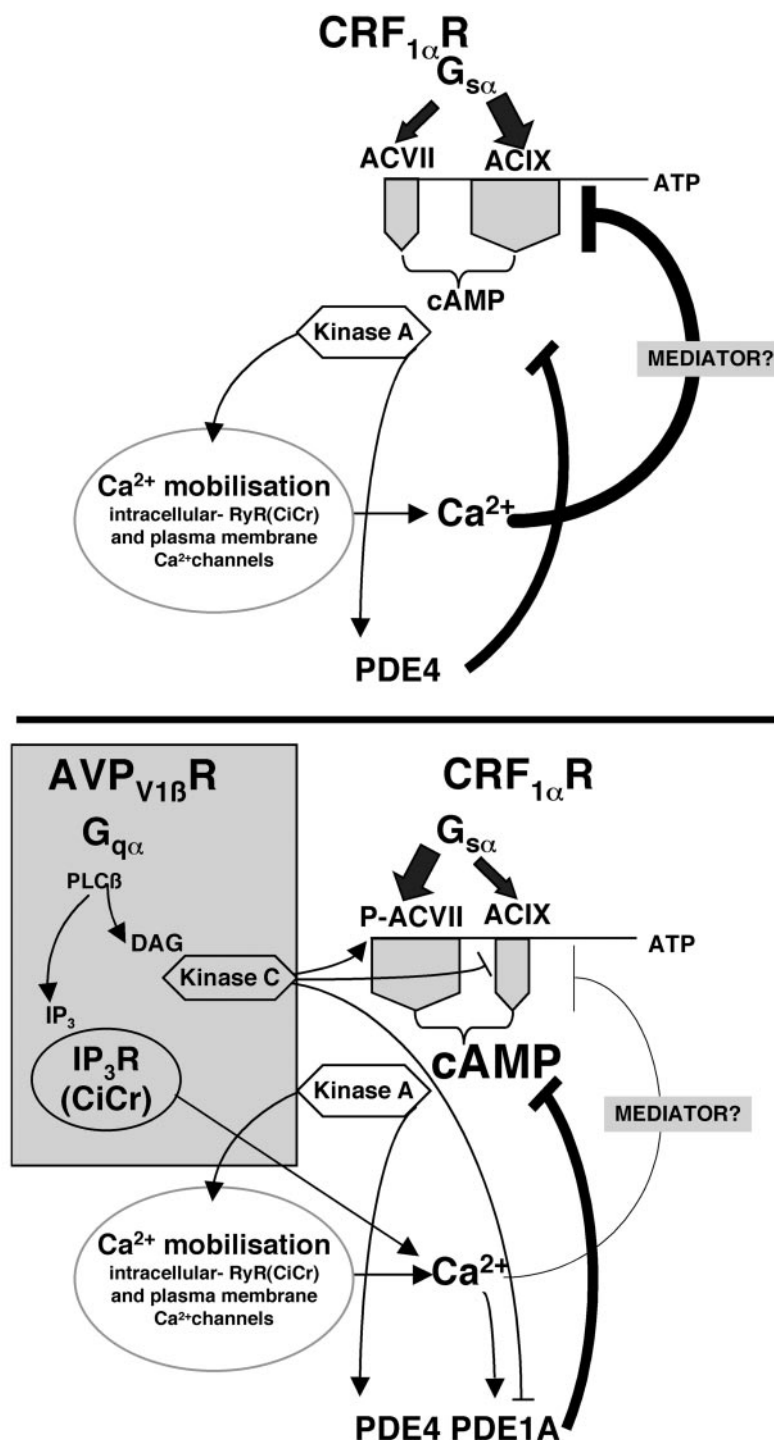


Fig. 9. Schematic Summary of the Effects of AVP and Ca^{2+} on CRF-Induced cAMP Responses in Adenohypophysial Corticotrope Cells

A, CRF signaling at physiological concentrations of the neurohormone. Note that the main mode of Ca^{2+} feedback (in *thick line*) is the inhibition of AC. Whether this is a direct action of Ca^{2+} on the enzyme or is mediated by other factors remains to be investigated. B, Switch of Ca^{2+} feedback by AVP. Note functionally coordinated effects of AVP to activate ACVII and to suppress the Ca^{2+} -inhibited AC as well as the Ca^{2+} -activated PDE. As a result, the rate of cAMP synthesis is increased, and the main avenue of Ca^{2+} feedback is through Ca^{2+} -activated PDE (PDE1A). CRF $_{1\alpha}$ R, CRF $_{1\alpha}$ type receptor; AVP $_{V1\beta}$ R, AVP $_{V1\beta}$ receptor; PLC β , Phospholipase C β_3 ; P-ACVII, phosphorylated adenylyl cyclase VII; IP $_3$, inositol 1,3,5 trisphosphate; ACIX, adenylyl cyclase IX; PDE1, type 1, Ca^{2+} /calmodulin-activated cyclic nucleotide phosphodiesterase; PDE4, type 4, cAMP-specific cyclic nucleotide phosphodiesterase; IP $_3$ R, IP $_3$ receptor; Kinase A, cAMP-dependent protein kinase; Kinase C, Ca^{2+} -independent, phorbol-ester activated protein kinase C; CiCr, Ca^{2+} -induced Ca^{2+} release from intracellular stores.

cells. The main physiological consequence is the resistance of ACTH release to glucocorticoid feedback inhibition (52).

MATERIALS AND METHODS

Reagents

Unless stated otherwise, all reagents were of the highest analytical grade available. Rat/hCRF and AVP were from Bachem (Saffron Walden, UK); bis-indolyl maleimide I, PdBu, thapsigargin, and ryanodine (technical grade 96+%) were supplied by LC Laboratories, Alexis Corp., Nottingham, UK; IBMX and caffeine were from Sigma-Aldrich Corp. (Poole, Dorset, UK); and rolipram was provided courtesy of Schering AG (Berlin, Germany). BAPTA/AM, BCECF/AM, and FURA2/AM were from Calbiochem-Novabiochem (Nottingham, UK).

Rat Anterior Pituitary Cells

Isolated cells were prepared by tryptic digestion from the anterior pituitary glands of male Wistar rats (150–200 g body weight) and preconditioned for 2–3 h in Hanks' balanced salt solution (HBSS) without Mg^{2+} and Ca^{2+} (HBSS⁺, Life Technologies, Inc., Paisley, UK) supplemented with HEPES (pH 7.4), 2 mM $CaCl_2$, and 1 mM $MgSO_4$ 0.25% BSA as previously described (20). Subsequently the cells were sedimented by centrifugation, resuspended, counted in a hemacytometer, and diluted 10-fold or greater with low Ca^{2+} medium [HBSS with 2 mM EGTA, 1 mM $MgSO_4$, 25 mM HEPES (pH 7.4), 0.25% wt/vol BSA] or the same medium containing 4 mM $CaCl_2$ as required by the experiment. Aliquots (50 μ l) of the cell suspension ($0.6\text{--}1 \times 10^6$ cells/ml) were distributed into conical polypropylene RIA microvials (Sarstedt, Leicester, UK) in 96-well format and incubated in a water bath at 37 C.

Ca^{2+} Depletion Protocols and the Measurement of cAMP in Intact Cells

Depletors/blockers of intracellular Ca^{2+} stores or acetoxymethyl-esters (BAPTA-AM, FURA2-AM, and BCECF-AM) were added in 50 μ l at the start of preincubation, as indicated in the text and figure legends. The drugs used to modify intracellular Ca^{2+} handling were caffeine, an activator of RyR, ryanodine, a blocker of RyRs, and thapsigargin, an inhibitor of intracellular Ca^{2+} -ATPases that refill RyR and inositol-1,3,5-triphosphate receptor-operated Ca^{2+} stores. The two latter drugs were dissolved in ethanol; hence, vehicle controls were used as appropriate (0.1–0.3% vol/vol ethanol). Caffeine, because it has PDE inhibitor activity, was applied only in the presence of blockers of cyclic nucleotide PDEs. Overall, when combined with 2 mM EGTA and 2 μ M thapsigargin in the presence of PDE blockers, caffeine or ryanodine produced similar changes of cAMP levels.

Aliquoted cells were preincubated in 100 μ l of medium for 30 min. Unless indicated otherwise, blockers of PDE (1 mM IBMX and 0.1 mM rolipram) were introduced after 20 min of preincubation in a volume of 10 μ l. The only exception to this pattern was when the effects of acutely applied modified low Ca^{2+} medium containing 0.2 instead of 2 mM EGTA were assessed, in which case the cells received PDE blockers immediately upon resuspension in the incubation medium.

After 30 min of preincubation, agonists dissolved in 2 mM or low Ca^{2+} medium were added in 50 μ l volume. The pH of this solution was 7.65 to minimize the change of pH upon mixing with 2 mM EGTA; a pH shift from 7.40–7.29 occurred upon achieving a free Ca^{2+} concentration of 2 mM. This

protocol was modified when the effects of Ba^{2+} and Co^{2+} were compared with those of Ca^{2+} , as these divalent cations potentially displace Ca^{2+} from EGTA. Hence, cells were incubated for 20 min under Ca^{2+} -depleting conditions and subsequently pelleted by centrifugation at $200 \times g$ for 5 min. Resuspension and all other additions were in modified low Ca^{2+} medium with 30 μ M ryanodine and 2 μ M thapsigargin. Ca^{2+} and other divalent cations were added assuming that 0.2 mM EGTA was present.

Incubation with agonists continued for 8 min unless indicated otherwise. An equal volume (160 μ l) of 0.2 M HCl was added to terminate the reaction. Total cAMP levels were measured by RIA upon freeze-thawing and acetylation (15). More than 90% of the cAMP content was associated with the cells under these conditions, both in the presence and the absence of PDE blockers.

RNAse Protection Assay

Total tissue RNA was prepared using Trizol reagent (Life Technologies, Inc., Paisley, UK) according to the manufacturer's instructions. The general solution hybridization method and reagents were as provided in kit form by Ambion, Inc. (Austin, TX). ACIX mRNA was detected using a probe transcribed from rat ACIX cDNA (Paterson, J. M., unpublished) corresponding to nucleotides 1107–1450 of the mouse ACIX cDNA (GenBank accession no. Z50190) and subcloned into pcDNA3. The riboprobe for ACII was transcribed from cDNA corresponding to nucleotides 1755–2106 of the rat ACII cDNA (GenBank M80550, courtesy of Dr. R. R. Reed, Johns Hopkins University, Baltimore, MD), which was subcloned into pcDNA3 (Invitrogen, San Diego, CA) by standard procedures (54). ^{32}P -labeled sense and antisense riboprobes were synthesized using the requisite RNA polymerases. ^{32}P -labeled β -actin (220 bp) cRNA probe was also prepared and used as control for sample loading according to the manufacturer's instructions.

In Situ Hybridization

Plasmids described above for RNAse protection assay were transcribed with the requisite RNA polymerases to generate ^{35}S -UTP-labeled sense and antisense strand riboprobes according to standard procedures (54). The radiolabeled riboprobes were used for *in situ* hybridization histochemistry on 15- μ m sections of rat pituitary gland as previously described (38). The sections were autoradiographed on Kodak x-ray film (Eastman Kodak Co., Rochester, NY) for 5–18 d at -70 C.

RT-PCR

Total RNA was prepared from brains and pituitary glands of male Wistar rats and reverse transcribed as previously reported (15). The PCR reaction and restriction enzyme analysis of the products for ACV and ACVI were as described by Premont *et al.* (55). Primers for rat ACVII were: forward, GAAGAAGTTCAGAAGGAGC; and reverse, AATCACTC-CAGCAATCACAGGC. PCR was carried out as previously reported (15) and the product was subcloned into pGEM-T and sequenced in an automated DNA sequencer (Microsynth A.G., Balgach, Switzerland).

Immunocytochemistry

Male Wistar rats (200–300 g body weight) were perfused transcardially with 4% freshly depolymerized paraformaldehyde in 0.1 M sodium phosphate, pH 7.4. The pituitary gland was removed from the skull and postfixed overnight at 4 C. Subsequently the tissue was placed in 15% and then 30%

sucrose in PBS for cryoprotection. Transverse, 60- to 90- μ m-thick sections were cut on a cryostat, collected, and processed for immunocytochemistry as free floating sections (56). Primary antisera were as follows: chicken antibodies directed against the C terminus of ACIX (38) were purified from egg-yolk extracts on antigen peptide coupled to Sepharose 4B by differential desorption (57) and used at 1:200 to 1:500 dilution. Affinity-purified rabbit antibodies against rat ACII (lots J105 and L227, used at 1:300), human ACVII (lot K070 used up to 1:50), and rat ACV/VI (lot I 110, 1:400) and their respective antigen peptides were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). C-terminally directed mouse anti-ACTH monoclonal antibody (clone CBL56) was purchased from Cymbus Biotech (Chandlers Ford, Hants, UK) and used at 1:1000. Affinity-purified secondary antibodies raised against rabbit or mouse IgG in goat, and coupled to Alexa 488 and 598 fluorescent dyes, respectively, were from Molecular Probes, Inc. (Eugene, OR) and used at 1:300-fold dilution. Sections were mounted on glass slides coated with polylysine (Merck & Co., Inc.), covered with Permafluor (Beckman Coulter, Inc., High Wycombe, Buckinghamshire, UK) and viewed in a E600FM Eclipse microscope (Nikon, Melville, NY) equipped with a Radiance 2000 confocal headstage (Bio-Rad Laboratories, Inc., Hercules, CA) at 10 $\times$ and 40 $\times$ (oil immersion) magnifications. Image acquisition was adjusted by the Lasersharp software (Bio-Rad Laboratories, Inc.), so that no cross-talk between the green and red detection channels was apparent. The specificity of each of the primary sera was verified by preabsorption with the respective peptide antigens at 5 μ M for commercial antibodies and 10 μ M for chick anti-ACIX for 16 h at 4 C before application to the sections; secondary antibody specificities were also verified in reactions where one or both of the primary antibodies were omitted.

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GUANOSINE 3':5'CYCLIC MONOPHOSPHATE AND ACTIVATORS OF GUANYLATE CYCLASE
INHIBIT SECRETAGOGUE-INDUCED CORTICOTROPIN RELEASE BY RAT
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The secretion of corticotropin by perfused rat anterior pituitary cell columns was studied. Forty-one residue corticotropin releasing factor, vasopressin and high extracellular KCl all stimulated the secretion of corticotropin. The hormonal response to corticotropin-releasing factor (10^{-10} mol/l), vasopressin (10^{-9} mol/l) as well as KCl (48 mmol/l) was reduced by membrane permeant analogs of cGMP, such as 8-BrcGMP and dibutyryl-cGMP. The 8-BrcGMP analog (10^{-5} mol/l) inhibited corticotropin release in response to corticotropin-releasing factor by 30%, that to vasopressin by 70%, and that to KCl by 50%. Atriopeptin₁₋₂₈ (10^{-8} and 10^{-7} mol/l), a peptide known to activate membrane-bound guanylate cyclase in the anterior pituitary gland, decreased the release of corticotropin induced by vasopressin to about 30% of control. Similarly, activators of soluble guanylate cyclase, such as glyceryltrinitrate and sodium nitroprusside (10^{-5} mol/l) inhibited vasopressin-stimulated corticotropin release by 60%.

In conclusion, the data show that purported activators of particulate and soluble guanylate cyclase, as well as derivatives of cGMP itself are strong inhibitors of secretagogue-induced corticotropin release by corticotroph cells of the anterior pituitary gland. © 1989 Academic Press, Inc.

The secretion of ACTH by the anterior pituitary gland is regulated by hypothalamic neurons that release their neurohormones into hypophyseal portal blood. Forty-one residue corticotropin releasing factor and vasopressin are physiologically relevant mediators of the hypothalamic control of ACTH release [1-3]. Furthermore, a cohort of other hypothalamic neuropeptides have a direct stimulatory effect on adenohypophyseal corticotroph cells [1-3]. In contrast, the only known functionally significant inhibitors of ACTH release are adrenocortical steroid hormones with glucocorticoid activity [3,4].

Abbreviations used: 41-residue corticotropin-releasing factor (CRF-41), guanosine 3':5'cyclic monophosphate (cGMP), 8-bromoguanosine 3':5'-cyclic monophosphate (8-BrcGMP) and N⁶,2'-O-Dibutyrylguanosine 3':5'-cyclic monophosphate (di-BucGMP).

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Several laboratories have investigated the effects of atriopeptin on ACTH secretion. The findings of these studies are highly controversial, minor stimulation [5] or inhibition [6] and no change [7-10], all have been reported. Atriopeptin activates membrane-bound guanylate cyclase in a number of tissues, including the adenohypophysis [7,10], moreover, some of its effects on cellular activity are mimicked by membrane permeant cGMP derivatives [cf. 11]. In the present study we examined whether such cGMP derivatives, atriopeptin and nitroso activators of soluble guanylate cyclase can influence the secretion of ACTH.

MATERIALS AND METHODS

If not otherwise indicated all chemicals were of the highest analytical standard available. The peptides used in this study were as follows: CRF-41 (Peninsula, St Helens, U.K.), 8-arginine-vasopressin and rat atriopeptin₁₋₂₈ (Bachem, Saffron Walden, U.K.) Another batch of atriopeptin₁₋₂₈, the cGMP analogs 8-BrGMP and di-BucGMP, as well as sodium nitroprusside were from Sigma, Poole, Dorset U.K. Glyceryltrinitrate was obtained as 1mg/ml solution (Nitrocline, Sanol Schwarz Pharmaceuticals).

Dispersed anterior pituitary cells were prepared from the anterior pituitary glands of 8-10 randomly cycling female Wistar albino rats (body weight 250-350 g, supplier Harlan Olac, Bicester, U.K., by digestion with trypsin (Code TRL, Worthington Biologicals) following previously published procedures [12]. The cells (1 ml) were resuspended in Dulbecco's MEM (GIBCO), buffered with HEPES at pH 7.4, containing bovine serum albumin (Miles) 1g/l and mixed with 1 ml of preswollen Sephadex G-10 (Pharmacia) in the same medium. Aliquots of the gel-cell slurry were then transferred to the cell columns [13], the chamber volume was adjusted to 0.6 ml and perfusion was started with a multichannel peristaltic pump at a rate of 1ml/5min. Fractions of the effluent were collected every 5 min on ice. The transit-time from the medium reservoir to the fraction collector was 1.5 min. After an equilibration period of 2h, various treatments were applied every hour for 5 min. Fifty μ l aliquots of the effluent were assayed for immunoreactive ACTH content using antiserum No. 6 (courtesy of G.B. Makara, Budapest, Hungary) as previously described [14]. The assay standard ACTH₁₋₃₉ was kindly provided by CIBA-Geigy A.G., Basel, Switzerland. The sensitivity of the assay is 0.5 fmol/tube ACTH, and no interference by 8-BrGMP is apparent.

Upon a 5 min exposure to secretagogues the concentration of ACTH in the column effluent increased in three consecutive 5 min fractions. Net ACTH release is therefore defined as $(X+Y+Z)/3(B)$, where X is the fraction collected during the presence of stimulus (1.5 min transit-time effluent discarded), Y and Z are the next consecutive 5 min fractions, and B is the basal value immediately before the application of a stimulus.

The experiments were performed in a balanced complete block design. Because of the significant variation in the output of ACTH between experiments and the low number of observations per treatment (usually 4-8) data were analysed by non-parametric statistics with correction for repeated measures. In particular, Wilcoxon's matched pairs, signed ranks test, and Quade's two-way analysis of variance with subsequent multiple comparisons were used as described in ref 15. For the ease of comparison data from a pool of columns are expressed after division with the amount of ACTH released in response to the control AVP, CRF or KCl stimulus applied in the respective column.

RESULTS

Basal ACTH release was usually around the limit of detection of the radioimmunoassay (10 fmol/5 min). Five minute exposure to increasing concen-

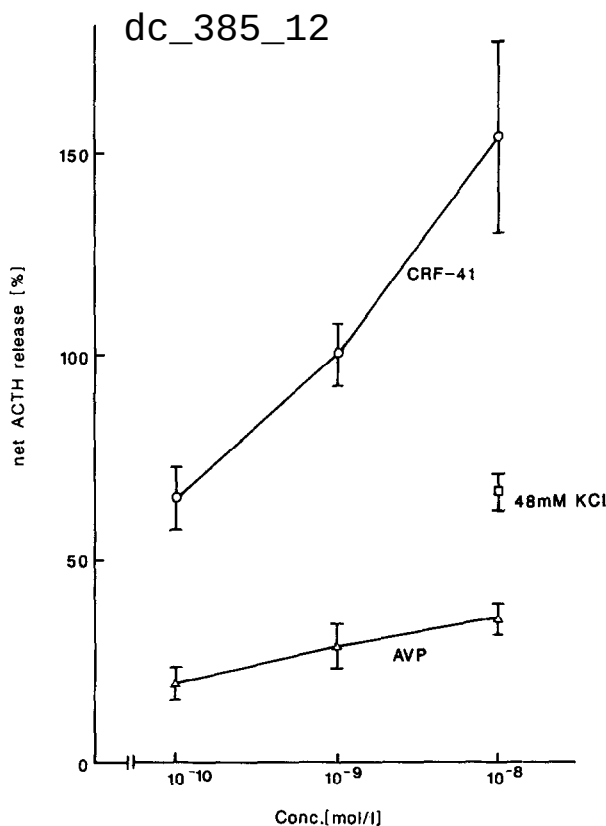


FIGURE 1. The effect of CRF-41, vasopressin and KCl on ACTH release by perfused rat anterior pituitary cell columns. The secretagogues were applied for 5 min. Net ACTH release is expressed as the percentile of the response to 10^{-9} mol/l CRF-41 (net ACTH release per 15 min = 370 ± 28 fmol, mean $\pm$ S.E.M.) which was run in all columns. $n=4$ /group. Basal release is usually about 10% of the 10^{-9} mol/l CRF-41 stimulated response.

trations of CRF-41 and vasopressin stimulated ACTH release in a dose-dependent manner, CRF-41 being the more potent secretagogue (FIG 1); 48 mmol/l KCl was also effective (FIG 1). Administration of 8-BrGMP 10 min before and during the 5 min application of CRF-41 (10^{-10} mol/l) reduced the ACTH response to this stimulus in a dose-dependent manner (FIG 2). Vasopressin-induced ACTH release was diminished by 8-BrGMP as well as di-BucGMP (FIG 2 & 3). The 8-BrGMP analog (10^{-4} mol/l) also suppressed ACTH release induced by the combination of CRF-41 (5×10^{-11} mol/l) and vasopressin (5×10^{-10} mol/l), which is the physiological cocktail of these stimulators found in pituitary portal blood, to $25 \pm 11\%$ (mean $\pm$ S.E.M. $n=6$, $P<0.05$) of the control stimulus. The ACTH response to 48 mmol/l KCl was reduced to $55 \pm 6\%$ (mean $\pm$ S.E.M., $n=8$, $P<0.01$) of the control stimulus by 8-BrGMP at 10^{-5} mol/l. No consistent effect of cGMP analogs on basal ACTH release could be discerned.

Atriopeptin, administered in the same temporal pattern as the cGMP analogs, markedly inhibited the ACTH response to vasopressin (FIG 3 and Table I).

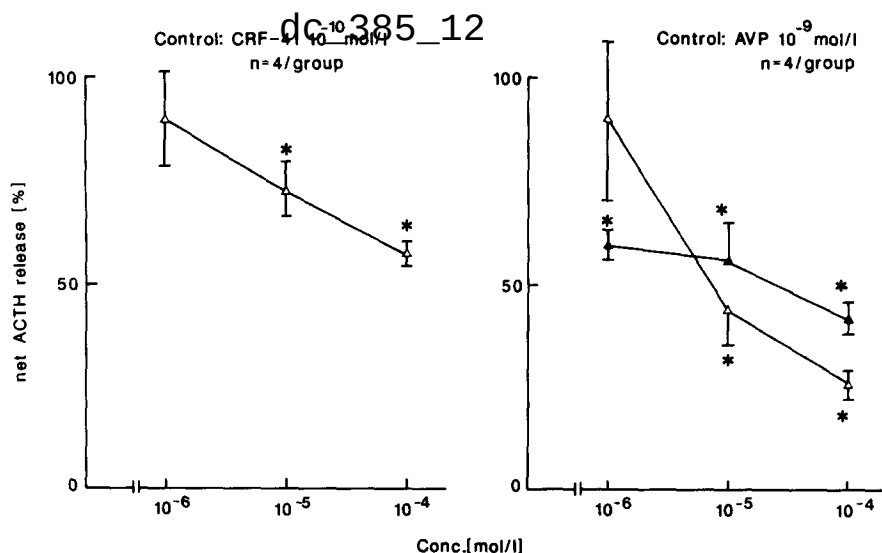


FIGURE 2. The effects of membrane permeant analogs of cGMP on ACTH release induced by 10^{-10} mol/l CRF-41 (left panel, net ACTH to control stimulus = 275 ± 33 fmol/15 min, mean $\pm$ S.E.M.) and 10^{-9} mol/l arginine-vasopressin (right panel, net ACTH to control stimulus = 212 ± 14 fmol/15 min {di-BucGMP experiment} and 120 ± 13 fmol/15 min {8-Br-cGMP experiment}). Data are means, the bars represent the S.E.M., n=4 in all groups. Symbols: (Δ) 8-Br-cGMP, (▲) di-BucGMP, * $P < 0.05$, when compared with the control stimulus by Quade's test.

The nitroso compounds glyceroltrinitrate and sodium nitroprusside were also effective inhibitors of vasopressin-stimulated ACTH release (Table I).

DISCUSSION

The data presented here suggest that a rise of intracellular cGMP levels strongly suppresses ACTH release induced by three secretagogues that utilize distinct transmembrane signaling mechanisms [1,16].

The concentrations of CRF-41 and vasopressin used are in the range of the concentrations of these peptides in the hypophyseal portal blood of anaesthetized rats [cf.2,17] or conscious rams exposed to stressful stimuli [18]. It seems therefore reasonable to suggest that elevation of intracellular cGMP levels may inhibit ACTH release under physiological conditions.

The mechanism of cGMP action was not addressed in the present study. However, it is known that a common requirement for the ACTH stimulating action of CRF-41, vasopressin and KCl is an increase of cytoplasmic free Ca^{2+} [cf.1], and it is now well established that cGMP reduces the stimulus induced rise in intracellular free Ca^{2+} levels [19-21]. This mechanism could also explain its inhibitory effects on secretagogue-stimulated ACTH release.

Current evidence indicates that most tissues contain two distinct types of guanylate cyclase, a particulate enzyme in the plasma membrane and a soluble cytosolic species [11]. Atriopeptin is one of the few agents known to stimulate particulate guanylate cyclase [cf.11], and also increases cGMP

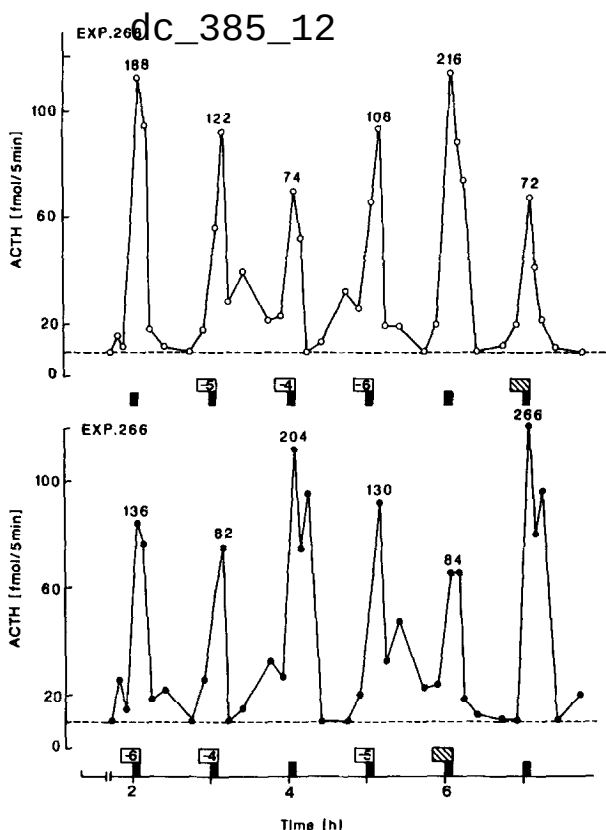


FIGURE 3. The effects of di-BucGMP and atriopeptin on ACTH release stimulated by 10^{-9} mol/l vasopressin. Data from two individual experiments are shown, which are included in the summaries presented in FIG 2. Black boxes indicate the application of vasopressin (5 min), boxes with numbers show the application of di-BucGMP the number is the power of 10 to give the concentration in mol/l, striped boxes indicate 10^{-7} mol/l atriopeptin (Sigma). The numbers on the top of secretory peaks show the net amount of ACTH released over 15 min in the respective peaks, the dashed line indicates the limit of detection of the radioimmunoassay.

levels in anterior pituitary cell cultures [7,10]. However, despite the increase in cGMP levels no prominent effect on CRF-41 stimulated ACTH release was detected, as measured in static incubations lasting 3 h [6,7,9,10]. In the present study we have used acutely dispersed cells in a perfusion system, and applied stimuli of short duration to demonstrate a strong inhibitory action of atriopeptin on secretagogue induced ACTH release. These differences in methodology may well explain the failure of previous workers to reveal the inhibitory effect of the peptide.

Atriopeptin is found in hypothalamic neurons that project to the external layer of the median eminence [cf. 22 and references therein]. Thus, the present data raise the possibility that atriopeptin may function as a hypothalamic corticotropin-release inhibiting factor. Alternatively, since atriopeptin is a circulating hormone [23] it may antagonize fluid and salt retention caused by the excess production of adrenocortical mineralocorticoids brought

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Table I. Effects of activators of guanylate cyclase on ACTH release induced by a 5 minute exposure to vasopressin (10^{-9} mol/l). The columns were treated with the guanylate cyclase activators 10 min before and 5 min during the application of vasopressin. Data are means $\pm$ S.E.M., expressed as percentage of the control stimulus (vasopressin alone) in the respective column. Experiments were performed in a balanced complete block design, n is the number observations per group, * means $P < 0.05$ using Wilcoxon's matched pairs, signed ranks test or Quade's 2-way analysis of variance as applicable

Compound	net ACTH release [%]	n
Glyceroltrinitrate 10^{-5} mol/l	47 $\pm$ 6 *	6
Sodiumnitroprusside 10^{-6} mol/l	42 $\pm$ 5 *	4
10^{-5} mol/l	59 $\pm$ 10 *	4
Atriopeptin (Bachem) 10^{-8} mol/l	37 $\pm$ 6 *	4
10^{-7} mol/l	19 $\pm$ 5 *	4

about by increased release of ACTH. However, further studies with more physiological concentrations of the peptide are required to establish the validity of these hypotheses.

Glyceroltrinitrate and sodium nitroprusside, activators of soluble guanylate cyclase in several types of tissue [cf.11] also decreased ACTH release induced by vasopressin. It appears therefore, that activation of both types of guanylate cyclase suppresses ACTH release, and in subsequent studies it should be possible to examine the regulation of the soluble enzyme in anterior pituitary corticotroph cells.

In summary, the present data show that a rise in intracellular levels of cGMP strongly inhibits the secretagogue-induced release of ACTH. Further work is warranted to identify bioactive compounds that regulate cGMP metabolism in anterior pituitary corticotrophs.

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Atriopeptins are potent inhibitors of ACTH secretion by rat anterior pituitary cells *in vitro*: involvement of the atrial natriuretic factor receptor domain of membrane-bound guanylyl cyclase

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ABSTRACT

The current concept of the regulation of adeno-hypophysial corticotrophin secretion assigns a stimulatory role to neuropeptides of hypothalamic origin and an inhibitory function to adrenocortical glucocorticoid hormones. It has been shown that in isolated perfused rat anterior pituitary cells atriopeptins (1–28) and (5–28), but not (5–25), potently suppress corticotrophin secretion elicited by 41-residue corticotrophin-releasing factor and arginine vasopressin. The action of

atriopeptins is associated with a marked increase in the cellular content of cyclic GMP. Moreover, 8-Br-cyclic GMP, a cell membrane permeant analogue of cyclic GMP, also inhibited secretagogue-stimulated corticotrophin release. It is suggested that atriopeptins of hypothalamic and perhaps of cardiac origin may be functionally significant corticotrophin-release inhibiting factors.

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INTRODUCTION

Forty-one residue corticotrophin-releasing factor (CRF-41) and arginine vasopressin (AVP) are the principal hypothalamic stimuli of anterior pituitary adrenocorticotrophin (ACTH) secretion (Antoni, 1986; Rivier & Plotsky, 1986). These neurohormones are co-secreted into hypophysial portal blood by hypophysiotrophic neurones (Antoni, 1986; Whitnall, 1988) and synergistically stimulate ACTH release (Gillies, Linton & Lowry, 1982).

Previously, we have shown *in vitro* that cyclic GMP (cGMP) derivatives that permeate the cell membrane inhibit the release of ACTH (Antoni & Dayanithi, 1989a) elicited by the combined stimulus of 0.05 nmol CRF-41/l and 0.5 nmol AVP/l (CRF-41/AVP), which is the physiological ACTH-releasing cocktail in the hypophysial portal blood of rats (Plotsky, 1987; Fink, Robinson & Tannahill, 1988) and sheep (Caraty, Grino, Locatelli & Oliver, 1988; Engler, Pham, Fullerton *et al.* 1989) subjected to stressful stimuli.

In the search for physiologically relevant substances that may regulate the production of cGMP in adeno-hypophysial corticotroph cells we examined whether atriopeptins, which are well-known activators of particulate guanylyl cyclase in various tissues (Waldman & Murad, 1987; Goetz, 1988), can influence the secretion of ACTH.

MATERIALS AND METHODS

Isolated anterior pituitary cells were prepared from the adeno-hypophyses of female Wistar rats (250–350 g body weight) by digestion with trypsin (Dayanithi & Antoni, 1989). The cells were mixed with preswollen Sephadex G-10 (Pharmacia, Milton Keynes, Bucks, U.K.) and loaded into columns which were perfused at a rate of 0.3 ml/min with Dulbecco's Minimal Essential Medium (GIBCO, Paisley, Strathclyde, U.K.) containing 2.5 g bovine serum albumin/l, 10^5 U penicillin/l, 1 g streptomycin/l and 2.5 mg amphoteri-

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cin B/l (Gillies *et al.* 1982; Dayanithi & Antoni, 1989). Fractions of the column effluent were collected every 2.5 min and ACTH content was determined by radioimmunoassay (Dayanithi & Antoni, 1989).

Unless stated otherwise, the columns were primed before the experimental treatments as follows. At 1 h after the start of the perfusion CRF-41 (1 nmol/l; Bachem, Saffron Walden, Essex, U.K.) was applied to the cell columns for 5 min, followed by 0.5 nmol CRF-41/l and 0.5 nmol AVP/l (Ferring, Malmö, Sweden) for 5 min at 1.5 h and 2.5 min at 2 h after the start of perfusion. This priming procedure (treatment A) was necessary to ensure that the response of ACTH to CRF-41/AVP was uniform during the course of the experiment. Another priming protocol, treatment B, involved applying CRF-41/AVP for 5 min at 0.5, 1 and 1.5 h and for 2.5 min at 2 h after the start of the perfusion. This protocol also produced virtually uniform net responses of ACTH to a repeated challenge with CRF-41/AVP.

Following priming, experimental treatments were applied in a randomized block design. The cells were exposed to rat atriopeptins (all from CRB, Cambridge, Cambs, U.K.) for 10 min before and 2.5 min during stimulation with CRF-41/AVP. In the experiments shown in Fig. 2 atriopeptin or 8-Br-cGMP were given as a continuous infusion starting 10 min before the second stimulation with CRF-41/AVP and terminating after the fourth stimulation period.

Net ACTH release was calculated (Dayanithi & Antoni, 1989) and, because of the variation in the net ACTH release between experiments, data were standardized within each column by expressing net ACTH release as a percentage of the net hormonal response to the control CRF-41/AVP stimulus. Statistical analysis was by Quade's two-way non-parametric analysis of variance for repeated measures (Conover, 1980).

For the measurement of cGMP the cells (without Sephadex G-10) were preincubated for 2 h in the medium used for perfusion experiments, then collected by centrifugation and resuspended in medium containing mol iso-butyl-methyl xanthine (5×10^{-4} mol/l) at 10^6 cells/ml. Aliquots (0.5 ml) of the cell suspension were incubated with various concentrations of atriopeptins at 37 °C for 20 min after which the tubes were placed on ice, and the cells pelleted at 100 *g*. The supernatant was aspirated and 0.25 ml 6% (w/v) trichloroacetic acid added to the pellet which was sonicated. After centrifugation, the supernatant was collected and extracted with 0.5 ml of a mixture of tri-*N*-octyl-amine and 1,1,2-tri-chloro-tri-fluoro-ethane (2:2:7:8, v/v; Aldrich Chemical Co., Gillingham, Dorset, U.K.) to remove the tri-chloroacetic acid (Martin, Morgan, Smith & White, 1986). The cGMP content of the aqueous upper phase was determined

by radioimmunoassay after acetylation (Harper & Brooker, 1975), the cross-reaction with acetylated cAMP was less than 0.1%.

RESULTS

Figure 1 characterizes the CRF-41/AVP stimulus in relation to baseline secretion.

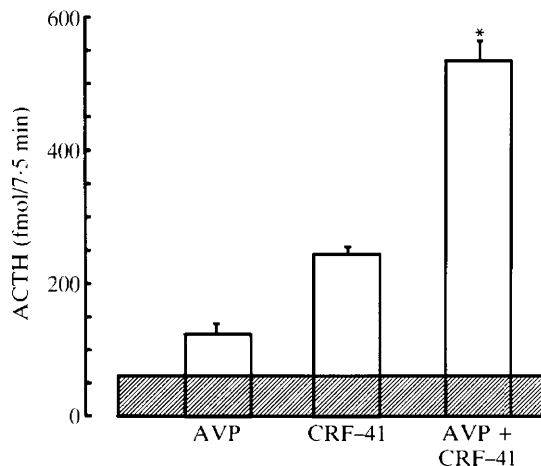


FIGURE 1. Effects of arginine vasopressin (AVP; 0.5 nmol/l) and 41-residue corticotrophin-releasing factor (CRF-41; 0.05 nmol/l) or their combination on the release of ACTH by perfused isolated rat anterior pituitary cells. Values are means $\pm$ S.E.M. ($n=4$ /group), the shaded bar is the basal hormone release inclusive of S.E.M. * $P < 0.01$ compared with CRF-41 or AVP alone (Quade's test).

Atriopeptin(1–28) markedly suppressed ACTH secretion evoked by CRF-41/AVP (Fig. 2). A cell membrane permeant analogue of cGMP, 8-Br-cGMP, also inhibited the hormonal response to CRF-41/AVP (Fig. 2). The effect of atriopeptin(1–28) on secretagogue-evoked ACTH release was concentration dependent (Fig. 3a), the maximal effect being an approximately 70% reduction of hormone release at 100 nmol inhibitor/l. Atriopeptin(1–28) increased the cGMP content of anterior pituitary cells (Fig. 3b).

Atriopeptin(5–28), the predominant molecular species found in the hypothalamus (Nakao, Morii, Itoh *et al.* 1986), also inhibited ACTH release and increased the accumulation of cGMP (Fig. 3).

Importantly, atriopeptin(5–25), an analogue which had only a minor effect on cellular cGMP content, was a weak inhibitor of secretagogue-stimulated ACTH secretion (Fig. 3). Overall, atriopeptins had no consistent effects on the basal secretion of ACTH, which was generally in the range of the limit of detection of the ACTH assay (0.5 fmol/tube). The relatively

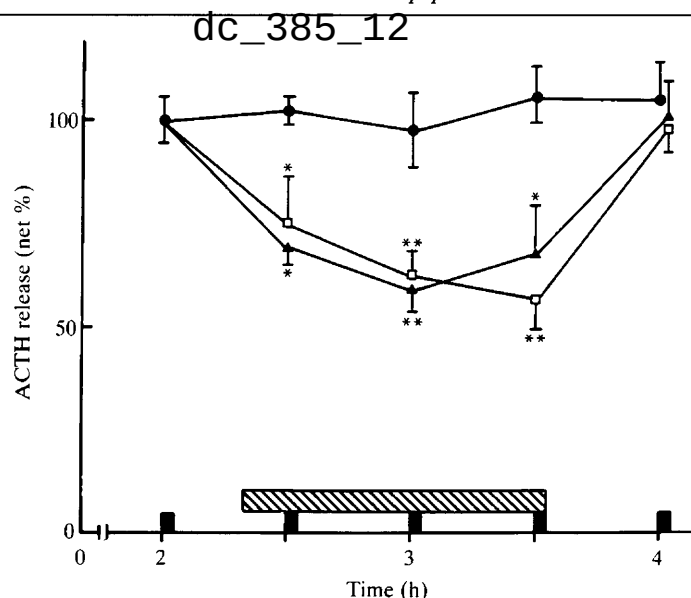


FIGURE 2. Effect of atriopeptin(1-28; □) (0.5 nmol/l) and 8-Br-guanosine 3',5' monophosphate (8-Br-cGMP; ▲) (10 μ mol/l) on the release of ACTH evoked by the combined stimulus of 41-residue corticotrophin-releasing factor (0.05 nmol/l) and arginine vasopressin (0.05 nmol/l) (solid boxes) in perfused isolated rat anterior pituitary cells. The hatched bar indicates the application of the inhibitor compounds. Values are means $\pm$ S.E.M. ($n=4$ /group). * $P<0.05$, ** $P<0.01$ compared with control (●) group (Quade's test).

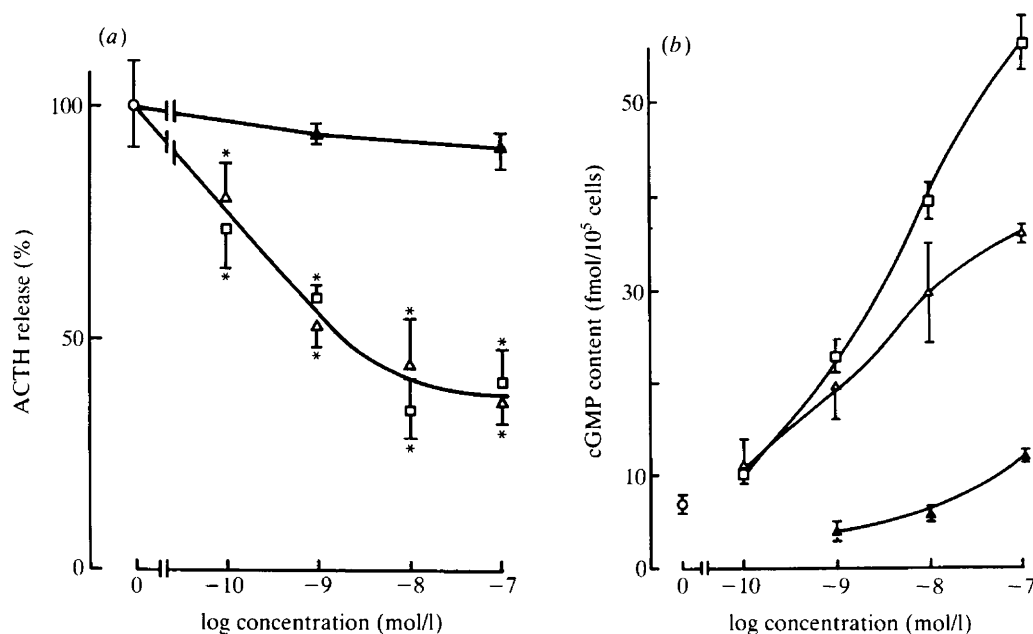


FIGURE 3. Effects of atriopeptins(1-28; Δ), (5-28; □) and (5-25; ▲) on (a) ACTH release evoked by the combined stimulus of 41-residue corticotrophin-releasing factor (0.05 nmol/l) and arginine vasopressin (0.05 nmol/l) in perfused isolated rat anterior pituitary cells and (b) cyclic GMP (cGMP) content of the cells in static incubation for 20 min. Values are means $\pm$ S.E.M. (a) $n=4$ /group (b) $n=3$ /group. * $P<0.05$ compared with control value (Quade's test).

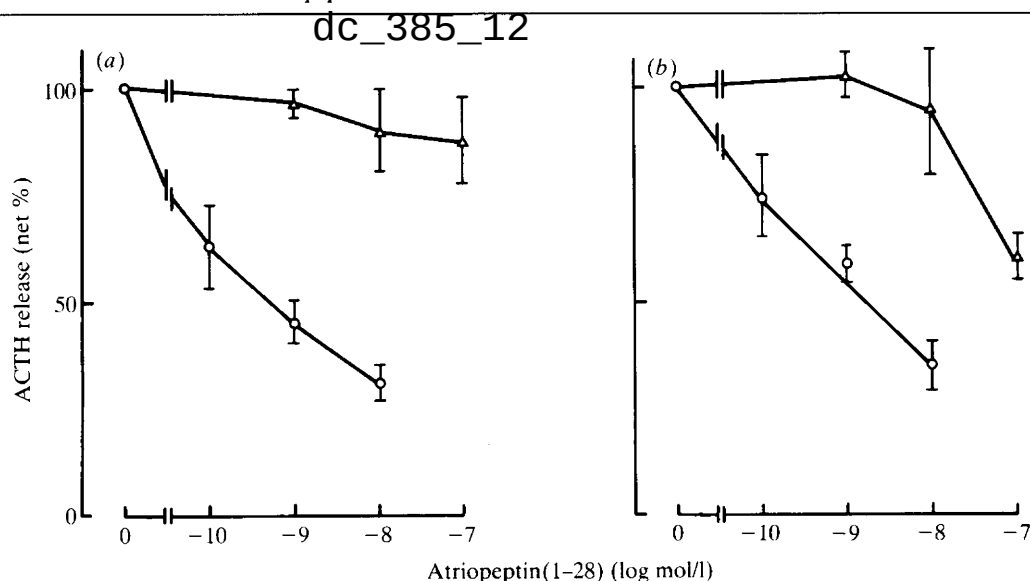


FIGURE 4. Effect of priming protocol on isolated perfused rat anterior pituitary cells. Cells became refractory to inhibition by (a) atriopeptin(1–28) or (b) atriopeptin(5–28); the control stimulus is the combination of 41-residue corticotrophin-releasing factor (0.05 nmol/l) and arginine vasopressin (0.05 nmol/l) (CRF-41/AVP). Values are means $\pm$ S.E.M. ($n=4$ /group). Treatment A (○) involved applying 0.5 nmol CRF-41/l for 5 min at 1 h after the start of perfusion followed by CRF-41/AVP for 5 min at 1.5 h and 2.5 min at 2 h. For treatment B (△) CRF-41/AVP was given for 5 min every 30 min up to 1.5 h and for 2.5 min at 2 h before experimental treatments were started. For further details on priming consult Materials and Methods.

poor precision of these measurements may, therefore, have masked an inhibitory action of atriopeptins on basal ACTH release.

Finally, it was found that atriopeptin(1–28) as well as atriopeptin(5–28) were relatively ineffective in cells primed according to treatment B, as compared with their efficacy in cells primed according to treatment A (Fig. 4).

DISCUSSION

The results presented here indicate that atriopeptins inhibit ACTH secretion evoked by CRF-41/AVP through the atriopeptin receptor domain of the membrane-bound guanylyl cyclase protein (Leitman, Andersen, Kuno *et al.* 1986; Scarborough, Schenk, McEnroe *et al.* 1986; Chinkers, Garbers, Chang *et al.* 1989).

Several groups have previously examined the effects of atriopeptins on pituitary hormone secretion and the increase of cGMP production has been reported (Heisler, Simard, Assayag *et al.* 1986; Shibasaki, Naruse, Yamauchio *et al.* 1986; Abou-Samra, Catt & Aguilera, 1987; Hashimoto, Hattori, Suemaru *et al.* 1987; King & Baertschi, 1989). However, the release of ACTH was either unaltered by atriopeptins (Heisler *et al.* 1986; Abou-Samra *et al.* 1987;

Hashimoto *et al.* 1987) or a minor inhibitory effect occurred which could not be correlated with the activation of guanylyl cyclase (Shibasaki *et al.* 1986; King & Baertschi, 1989), indicating that the action of atriopeptin is not through atriopeptin receptors coupled with guanylyl cyclase. Finally, two other previous studies have reported that atriopeptin actually stimulates ACTH release (Horváth, Ertl & Schally, 1986; Koch & Lutz-Bucher, 1989).

In contrast, in the present study atriopeptins were found to be potent inhibitors of ACTH release, their action is related to the increase of cellular cGMP content, and is also mimicked by a membrane permeant analogue of cGMP (Antoni & Dayanithi, 1989a) thus bearing the hallmarks of an atriopeptin-A (previously B-atriopeptin) receptor effect (Leitman *et al.* 1986; Scarborough *et al.* 1986; Chang, Lowe, Lewis *et al.* 1989).

The probable explanation of this discrepancy lies in the nature of the in-vitro models used. All of the previous studies were carried out on primary cultures of pituitary cells in static incubation, whereas we have used acutely isolated perfused cells. Precedents for marked alterations in the function properties of corticotroph cells in primary culture when compared with freshly dispersed cells are known (Antoni, 1986; Boyle, Yamamoto, Chen *et al.* 1988), the reason for the lack of responsiveness to atriopeptin, however, is not clear

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at present. It appears that cell surface receptors for the peptide are present in cultured pituitary cells, since the increase of cGMP production upon exposure to atriopeptin has been consistently demonstrated (Heisler *et al.* 1986; Abou-Samra *et al.* 1987), although the possibility of a selective loss of atriopeptin receptors in corticotrophs cannot be excluded at present. It seems more plausible, however, that the target(s) of cGMP action, e.g. protein kinase G, or an ion channel, may be modified during culturing.

With respect to these possibilities, it has been shown that at least two types of potassium ion channels are required for the ACTH-release inhibiting action of atriopeptin (Antoni & Dayanithi, 1989b). The plasticity of ion channels in various types of cell are well documented, and conditions of cell culture may produce an ion channel profile which is quite different from that found *in vivo* (Lewis & Cahalan, 1988; Gilly & Brisman, 1989). A decrease in the cellular concentration of cGMP-dependent protein kinase has been demonstrated in cultured vascular smooth muscle cells and appears to be the cause of the poor responsiveness of these cells to atriopeptins (Cornwell & Lincoln, 1989). Finally, evidence has been presented that the type of atriopeptin receptor expressed by vascular smooth muscle cells is altered during culturing (Uchida, Mizuno, Shimonaka *et al.* 1989). Further work is clearly required to elucidate the precise nature of the changes in the properties of corticotrophs during cell culture which lead to the poor response to atriopeptin. It is, however, tempting to speculate that, in our experiments, treatment of the cells with secretagogue pulses soon after trypsinization may have accelerated the development of these changes.

From the point of view of physiological function, the results presented here indicate that atriopeptins of cardiac (cf. Goetz, 1988) as well as hypothalamic (Nakao *et al.* 1986) origin may effectively inhibit the pituitary secretion of ACTH.

The concentration of atriopeptins in the systemic circulation is normally below 0.1 nmol/l (cf. Goetz, 1988). It appears, therefore, that atriopeptins released from the heart may only influence ACTH secretion when the release of atriopeptin is enhanced, as in the case of increased intravascular fluid volume (cf. Goetz, 1988). Given the ability of ACTH to stimulate the production of mineralocorticoid hormones such a mechanism may contribute to the suppressive effect of atriopeptins on adrenocortical function, and thus promote the reduction of extracellular fluid volume.

Functional evidence for a hypothalamic inhibitor of ACTH secretion that acts in a tonic manner has been presented (Engler, Pham, Fullerton *et al.* 1988). Atriopeptin-containing terminal axons that originate in the hypothalamic para- and periventricular nuclei have been observed in the immediate vicinity of pri-

mary portal capillaries in the external lamina of the median eminence (Kawata, Nakao, Morii *et al.* 1985; Skofitsch, Jacobowitz, Eskay & Zamir, 1986; Standaert, Needleman & Saper, 1986; Palkovits, Eskay & Antoni, 1987), indicating that atriopeptin is released into the hypophysial portal circulation. We have shown here that atriopeptin(5–28), the major form of atriopeptin in the hypothalamus, is a potent inhibitor of CRF-41/AVP-stimulated ACTH secretion. We suggest, therefore, that atriopeptin is a hypothalamic corticotrophin-release inhibiting hormone.

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Atriopeptin Inhibits Stimulated Secretion of Adrenocorticotropin in Rats: Evidence for a Pituitary Site of Action*

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ABSTRACT. The aim of this study was to resolve previous controversies regarding the effect of atriopeptin on the secretion of ACTH *in vivo*. Male Wistar rats were used throughout. The animals were subjected to lesioning of the hypothalamic paraventricular nucleus (PVN) or sham operation and implanted with indwelling jugular cannulae 5 days later for blood sampling and drug infusion. Two days after the insertion of the cannulae the animals were treated with saline or 103-126 amino acid residue atriopeptin iv: a bolus injection was given (200 or 40 pmol/rat) followed by an infusion (40 or 8 pmol/min) which was maintained for the entire duration of the experiment (70 min). Ten minutes after the bolus of atriopeptin the animals received iv a combination of 1 pmol 41-residue CRF and 10 pmol arginine vasopressin (CRF/AVP) to stimulate ACTH secretion. Serial blood samples (0.1 ml) were obtained at -10 min and immediately before the injection of CRF/AVP and at 5, 10, 20, 30, and 60 min afterwards. Plasma ACTH concentration was measured by RIA.

In sham-operated rats CRF/AVP caused a 4-fold increase in plasma ACTH which peaked at 5 min and returned to baseline by 60 min. In sham-operated rats the higher dose of atriopeptin (200 pmol bolus, 40 pmol/min infusion) did not alter the effect of the stimulus between 5 and 30 min, and augmented plasma

ACTH at 60 min. The smaller dose of atriopeptin reduced plasma ACTH at 10 and 20 min by 54% and 48%, respectively, and also decreased by 48% the net amount of ACTH released over 30 min in response to CRF/AVP. When given alone, the higher dose of atriopeptin caused a persistent (60 min) 10-13% reduction of mean arterial blood pressure, while the lower dose decreased blood pressure by about 9% for less than 10 min. In parallel, the higher dose of atriopeptin increased plasma ACTH concentration while the lower dose produced no change.

In PVN-lesioned rats the CRF/AVP induced ACTH response was similar to that seen in sham-operated controls. Only the higher dose of atriopeptin was tested, and this markedly reduced CRF/AVP stimulated ACTH secretion at 5-60 min after CRF/AVP. Given alone, atriopeptin had no marked effect on plasma ACTH in PVN-lesioned rats, while its hypotensive action was similar to that in sham-operated animals.

It is concluded that: 1) Atriopeptin inhibits stimulated ACTH secretion in rats; 2) High systemic doses of atriopeptin that cause persistent hypotension release endogenous ACTH-releasing factors of paraventricular nucleus origin, and this may mask an inhibitory action of the peptide at the pituitary level; 3) Atriopeptin may function as a hypothalamic corticotropin release-inhibiting factor. (*Endocrinology* 127: 3003-3008, 1990)

THE secretion of ACTH by the anterior pituitary gland is stimulated by the hypothalamic neurohormones CRF and arginine-vasopressin (AVP), and inhibited by adrenocortical glucocorticoid hormones (1-4). There is also a considerable body of evidence for the existence of an as yet unidentified hypothalamic corticotropin-release inhibiting factor (CIF) (5-7).

Recent studies have revealed a novel, cGMP-mediated mechanism of inhibiting ACTH release by isolated rat anterior pituitary cells *in vitro* (8, 9). Atriopeptin, an activator of particulate guanylyl cyclase is a potent stimulus of cGMP production in anterior pituitary cells (10-

12) and inhibits ACTH release evoked by CRF and AVP at subnanomolar concentrations (12-15). Since atriopeptin is found in the hypothalamo-hypophysial tract (16-19) and is released into hypophysial portal blood (20) it is a candidate for the role of the purported corticotropin release-inhibiting substance. However, *in vivo* studies of the action of atriopeptin on pituitary-adrenocortical function generally do not support this notion (21-25), although some workers have reported a reduction of stimulated ACTH (26) or corticosterone (27) secretion in rats.

In the present study we have sought to resolve this issue by examining the effect of atriopeptin on plasma ACTH levels in rats injected with CRF and AVP. A possible source of the discrepancies in previous studies appeared to be the hypotensive action of atriopeptin which may stimulate the secretion of endogenous ACTH

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releasing peptides (28, 29). Therefore, some of the experiments were carried out in animals in which the main source of hypophysiotropic CRF, the hypothalamic paraventricular nucleus had been destroyed.

Materials and Methods

Animals

Male Wistar rats (190–220 g BW, supplied by Harlan-Olac (Bicester, Oxon, UK) or LATI (Gödöllő, Hungary) were housed under standard lighting (0700–1900 h) in a temperature-controlled (23 C) room. Pelleted rat chow and tap water were available *ad libitum*. The animals were anesthetized with pentobarbital (40 mg/kg ip) and subjected to surgical lesioning or sham lesioning of the hypothalamic paraventricular nucleus (PVN), which is the main site of production of hypophysiotropic CRF (30). The procedures for the lesioning of the PVN and the histological verification of the lesions have been previously described in detail (31). Briefly, animals were anesthetized with pentobarbital and placed in a stereotaxic frame. A specially designed microknife was lowered to the base of the skull through a burrhole behind the Bregma suture, rotated 360° and withdrawn. For sham lesions the knife was lowered 5 mm below the surface of the brain and withdrawn. The site of the lesion was verified in each animal by microscopic examination of serial 50 μ m frontal sections of the frozen hypothalamus that were prepared on a sliding microtome and stained with cresyl violet. Rostrally, the lesion usually damaged or destroyed the anterior commissural nucleus while posteriorly it extended into the dorsomedial nucleus. Laterally it did not reach beyond the fornix. Results from animals with a complete lesion of the PVN are reported.

Experimental treatments for monitoring plasma ACTH

Five days after hypothalamic surgery, the rats were again anesthetized with pentobarbital and two cannulae were introduced through a cut in the right jugular vein: a Silastic cannula (od: 0.047 in; id: 0.025 in; Dow Corning, Midland, MI) for blood sampling and a PP10 polypropylene cannula (Portex, Hythe, Kent, UK) for parallel drug infusion. The cannulae were secured in the vein, externalized sc at the back of the skull, and fixed to the bone with an anchoring screw and dental cement. Subsequently the animals were caged singly, and two days later extension tubings were attached between 0900 and 1030 h to the cannulae for blood sampling and continuous drug infusion. The animals were left undisturbed for 4 h, then 0.1 ml blood was withdrawn for measuring initial plasma ACTH concentration, and iv treatment with atriopeptin (residues 103–126 supplied by Cambridge Research Biochemicals, Cambridge, UK) was started. The treatment consisted of a bolus injection (200 or 40 pmol/rat in 0.1 ml saline) through the blood sampling cannula while a constant infusion of 40 or 8 pmol/min in a vol of 0.005 ml/min saline was started through the infusion cannula. Ten minutes later blood was sampled again and a mixture of 1 pmol CRF/10 pmol AVP (CRF/AVP, both peptides from Bachem, Saffron Walden, UK) in 0.2 ml saline was injected. Further blood samples (0.1 ml) were withdrawn 5, 10, 20, 30, and 60 min after the injection of CRF/AVP. Blood was collected

into chilled tubes containing Na₂EDTA and centrifuged. The plasma was collected and stored at –20 C. The concentration of ACTH in plasma was measured by direct RIA as described previously (32). In order to exclude the possibility that the changes in plasma ACTH are due to changes in the volume of distribution and/or the metabolism of ACTH we have determined the half-life of radioiodinated ACTH in conscious cannulated rats. The animals were implanted with jugular cannulae and treated with CRF/AVP and saline or the larger dosage regimen of ANF as above. Radioiodinated ACTH (cca 0.5 μ Ci) (32) was coinjected with CRF/AVP, blood samples (0.1 ml) were taken 1, 3, 5, 10, 20, 30, and 60 min after the injection of the radiolabel. The radioactivity in plasma was counted in a LKB Clinib-counter at 70% efficiency. The rate of disappearance of the radiolabel from plasma was identical in saline and atriopeptin treated rats, the apparent half-life was 2.6 min.

Measurement of mean arterial blood pressure (MABP)

In separate experiments MABP was monitored through an arterial catheter (PP30), implanted into the left carotid artery under pentobarbital anesthesia 1 day before the experiment, using a Statham pressure transducer and a Hellige recorder. The rats also received jugular cannulae as above. On the day of the experiment at least 2 h were allowed for the stabilization of MABP, then blood sampling and treatment with atriopeptin was started as described above except that atriopeptin or saline treatment was initiated at time 0 instead of –10 min. An additional set of rats treated with the high ANF dosage regimen and a bolus of CRF/AVP was also monitored for MABP.

Data analysis

Data were evaluated by the Kruskal-Wallis test followed by multiple comparisons as described by Conover (33) or by Mann-Whitney U test, as appropriate.

Results

Paradox effects of atriopeptin in sham-operated rats

In sham-operated rats iv injection of (CRF/AVP) produced a steep rise of plasma ACTH concentration which peaked at 5 min after injection and declined to baseline by 60 min. Treatment with atriopeptin that consisted of an iv bolus of 200 pmol 10 min before CRF/AVP and an infusion of 40 pmol/min for the entire duration of the experiment, had no effect on the peak ACTH response and prolonged the duration of the increase above basal secretion (Fig. 1). Lowering the dose of atriopeptin to 40 pmol bolus and 8 pmol/min infusion caused a significant reduction of plasma ACTH concentration at 10 and 20 min after CRF/AVP. Moreover, the average net ACTH response (area under the curve) over 30 min was about 50% less than that in controls (Fig. 1, *inset*).

When atriopeptin was given alone the higher dose of the peptide significantly increased plasma ACTH levels, whereas the lower dose had no effect (Table 1). Plasma ACTH levels tended to rise in all groups with time,

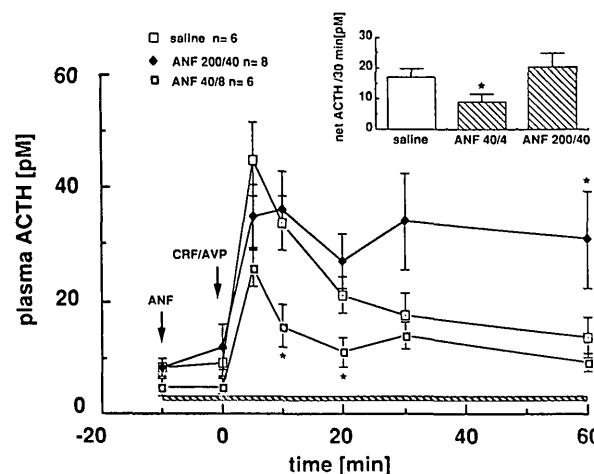


FIG. 1. The effect of atriopeptin on plasma ACTH in conscious sham-lesioned male rats injected at time zero with 1 pmol CRF and 10 pmol AVP (CRF/AVP) iv. Atriopeptin was given iv as a bolus injection (arrow) followed by a continuous infusion at a rate of 0.005 ml/min (striped bar). Control animals received saline. Data are means $\pm$ SEM. *, $P < 0.05$ when compared with saline treated controls at the same time point by Kruskal-Wallis test. Inset, Plasma ACTH expressed as the average increase over baseline during a period of 30 min after the injection of CRF/AVP. Data are means $\pm$ SEM. *, $P < 0.05$ by Kruskal-Wallis test.

possibly because of the mild hypovolemia caused by repeated blood sampling. MABP was persistently suppressed by the higher dosage regimen of atriopeptin, while the lower dose produced only a transient reduction (Fig. 2a). Injection of CRF/AVP 10 min after the start of the infusion of the higher dose of ANF caused a marked increase in MABP (control: 115.2 ± 4.5 ; 10 min after atriopeptin: 97 ± 4.1 ; peak at 0.83 ± 0.04 min after CRF/AVP: 117.2 ± 4.6 ; data in mmHg, mean $\pm$ SEM $n = 5$) which returned to the low, atriopeptin treated values by 3.8 ± 0.3 min after the injection of CRF/AVP and remained suppressed during the infusion of atriopeptin.

Marked inhibition of ACTH secretion by atriopeptin in PVN-lesioned rats

The data presented above raised the possibility that atriopeptin may facilitate the release of endogenous CRF

and AVP possibly through its hypotensive action and thus an inhibitory effect at the pituitary level would be obscured. Therefore, a series of experiments were performed in PVN-lesioned animals in which over 90% of the CRF is depleted from the median eminence (34–36) as well as from the pituitary portal circulation (37).

In PVN-lesioned rats the plasma ACTH response to CRF/AVP was similar to that of intact rats in all respects (Fig. 3). Only the higher dose of atriopeptin (200 pmol bolus, 40 pmol/min infusion) was applied which decreased the ACTH response between 5 and 60 min, but had no effect on the initial hormone levels (Fig. 3). On its own, the same dosage regimen of atriopeptin had no substantial effect on plasma ACTH (Table 1), but markedly reduced mean arterial pressure during the entire period of observation (Fig. 2b).

Discussion

The experiments presented here show that atriopeptin is an inhibitor of stimulated ACTH release *in vivo*.

Clinical studies have reported either no effect of atriopeptin on plasma cortisol (24) or found a marked inhibition (23, 25), without a change in ACTH (23). These studies did not examine the effect of atriopeptin on stimulated ACTH secretion, furthermore, a direct action of the peptide on adrenocortical cells could not be excluded.

A previous study in the rat reported no effect of atriopeptin on ACTH release induced by CRF or AVP (22), however, atriopeptin was only given as a bolus in this study and was probably eliminated from the circulation by the time the secretagogue stimuli were applied (38–40). The same workers have reported that atriopeptin administered in the same manner inhibits ACTH secretion evoked by hemorrhage (26). Another group of investigators (27) found that atriopeptin given intracerebroventricularly suppressed the increase of plasma corticosterone concentration elicited by intrathecal angiotensin II. In the sheep atriopeptin enhanced hemorrhage induced ACTH and cortisol secretion (21). The dose of

TABLE 1. Effect of atriopeptin on plasma ACTH in conscious, cannulated sham-operated, or PVN-lesioned rats

Treatment	Time (min)							n
	-10	0	5	10	20	30	60	
Sham-op								
Saline	4.5 ± 1.2	6.9 ± 2.5	7.5 ± 2.1	11.8 ± 3.0	6.7 ± 1.6	17 ± 4.6	20.6 ± 3.4	5
ANF40/8	5.3 ± 1.9	8.6 ± 1.8	10.0 ± 2.5	9.4 ± 3.5	10.9 ± 3.1	14.5 ± 3.4	32.2 ± 6.1	6
ANF200/40	3.0 ± 0.5	9 ± 3.8	12.4 ± 3.3	12.6 ± 3.6	12.6 ± 5.4	14.1 ± 4.6	69.9 ± 17.2^a	4
PVN-L								
Saline	4.5 ± 1.7	6.8 ± 1.3	7.3 ± 1.1	6.3 ± 1.4	9 ± 1.9	9.66 ± 2.9	9.24 ± 1	5
ANF200/40	2.8 ± 1.1	4.1 ± 2	4.7 ± 1.4	4.5 ± 1.4	6.6 ± 1.8	6.7 ± 2.0	4.3 ± 1.1^a	5

Atriopeptin or saline was given at -10 min iv as a 0.1 ml bolus injection (200 or 40 pmol/rat) followed by a continuous infusion at a rate of 0.005 ml/min (40 or 8 pmol/min · rat). Saline (0.2 ml) was also injected iv at time zero. Data are means (pm) $\pm$ SEM.

^a $P < 0.05$ vs. saline-treated controls using Kruskal-Wallis test or *U* test.

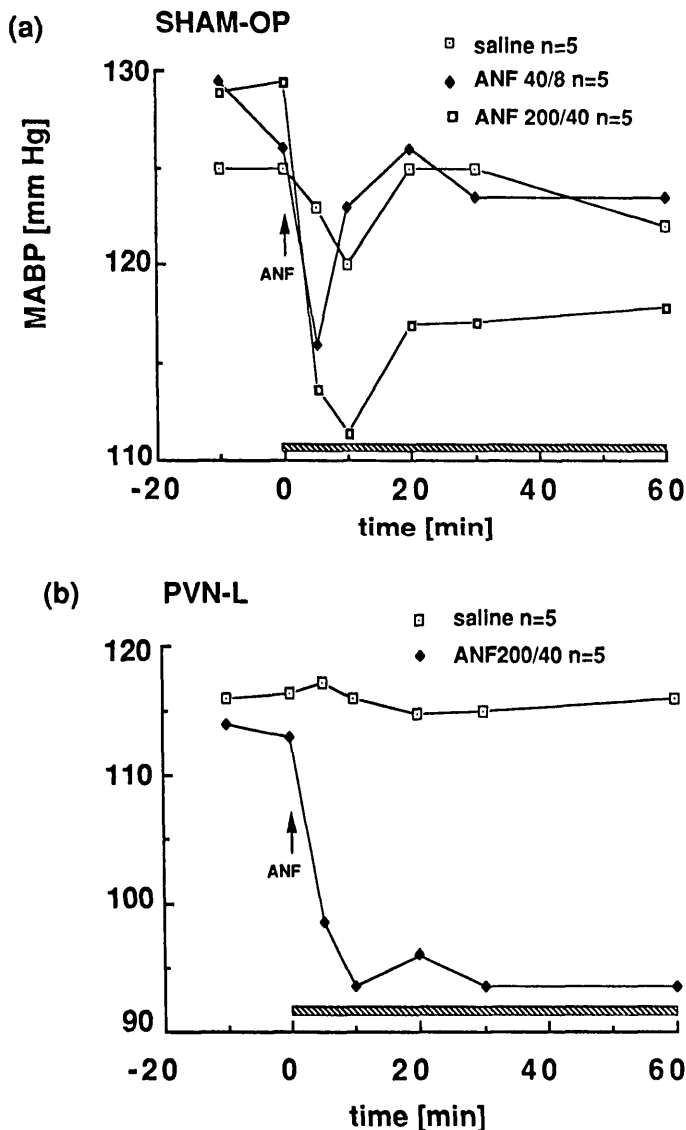


FIG. 2. MABP in (a) sham-operated rats and (b) paraventricular nucleus-lesioned rats. Atriopeptin was given as iv bolus (0.1 ml) at time zero followed by an infusion (striped bar) in a volume of 0.005 ml/min, controls received saline. Means, the standard errors were all less than 4% of their respective means.

atriopeptin used caused persistent hypotension (15% decrease in MABP) and stimulated ACTH as well as cortisol release (21). The site of action of atriopeptin was not clarified in any of these studies.

Our present data in rats confirm that if the administration of atriopeptin causes persistent hypotension, no inhibition of CRF/AVP evoked ACTH release occurs, in fact, an enhancement of the response is found at a later time point. However, a lower dose of atriopeptin, which caused only a transient drop of blood pressure markedly blunted the ACTH response to CRF/AVP. In addition, ACTH secretion was enhanced by the higher, strongly hypotensive dose of atriopeptin given alone, but not by the lower dose of the peptide which was only mildly

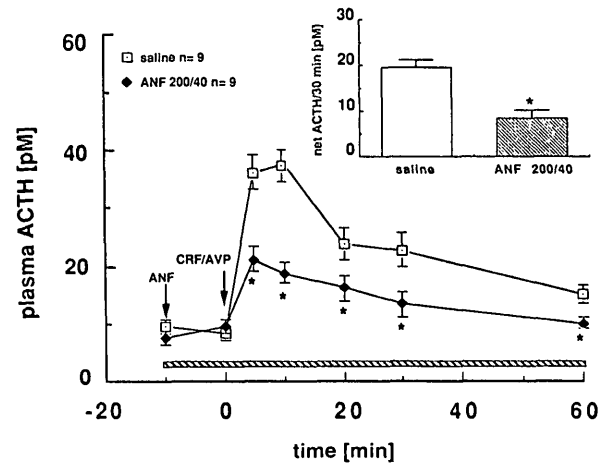


FIG. 3. The effect of atriopeptin on plasma ACTH in conscious paraventricular nucleus-lesioned male rats injected at time zero with 1 pmol CRF and 10 pmol AVP (CRF/AVP) iv. Atriopeptin was given iv as a bolus injection (arrow) followed by a continuous infusion at a rate of 0.005 ml/min (striped bar). Control animals received saline. Data are means $\pm$ SEM. *, $P < 0.05$ when compared with saline-treated controls at the same time point by U test. Inset, Plasma ACTH expressed as the average increase over baseline during a period of 30 min after the injection of CRF/AVP. Data are means $\pm$ SEM. *, $P < 0.05$ by U test.

hypotensive and inhibited CRF/AVP induced ACTH release. The enhancement of ACTH secretion by atriopeptin was manifested only about 1 h after the treatment with the peptide was initiated. This is possibly due to the complex interaction between factors favoring an increase (hypotension, mild hypovolemia, mild stress due to cannulation, approach of the circadian peak in hypothalamo-pituitary-adrenocortical activity), or inhibition (exogenous atriopeptin, endogenous glucocorticoids) of ACTH secretion. Note that while the intensity of the stimuli favoring an increase is likely to increase as the experiment progresses with time, the inhibitory factors are constant.

A plausible explanation of these findings is that at high concentrations the cardiovascular effects of atriopeptin facilitate the release of hypothalamic ACTH-releasing hormones into the hypophyseal portal system thereby enhancing the secretagogue drive to adenohypophyseal corticotrophs. This may fully mask the inhibitory effect of the peptide on ACTH release at the pituitary level.

With respect to this hypothesis, it is well established that hypotension due to vasodilatation evokes a reflex release of hypophysiotropic CRF and AVP from the paraventricular hypothalamo-hypophyseal pathway (29), as well as that of AVP by magnocellular neurones (28). Both of these mechanisms are thought to contribute to the increase of ACTH release in response to hypotension caused by vasodilatation (28, 29). Other factors, such as hypovolemia and changes in blood pH caused by pro-

longed exposure to high peripheral levels of atriopeptin may also contribute to enhance CRF and AVP release (28, 29).

The experiments with PVN-lesioned rats support the hypothesis that atriopeptin elicits ACTH secretion by mobilizing hypothalamic ACTH secretagogues.

Lesioning of the paraventricular nucleus eliminates over 90% of the hypophysiotropic CRF and AVP producing parvicellular neurons (2). The size of the plasma ACTH response to CRF/AVP in PVN-lesioned rats was comparable to that seen in sham-lesioned rats. In contrast, atriopeptin at a dose which actually enhanced CRF/AVP-stimulated ACTH release in sham-operated rats, was strongly inhibitory in PVN-lesioned animals. Moreover, the infusion of atriopeptin on its own did not increase plasma ACTH in PVN-lesioned rats despite a marked decrease of MABP which was similar to that seen in sham-operated rats.

These data conform with the hypothesis that the ACTH stimulating action of atriopeptin requires neurohormones derived from the PVN. Thus, the results presented here indicate that atriopeptin inhibits secretagogue stimulated ACTH secretion at the pituitary level, but this effect may be masked if the dose of the peptide is sufficient to provoke CRF and AVP secretion.

The concentration of atriopeptin in plasma was not measured in our studies, but based on the roughly 30 sec half-life of the peptide in the circulation of the rat (38–40), we estimate that the levels attained at the time of the CRF/AVP injection are between 0.1 and 1 nmol/liter. Such concentrations have been measured in the hypophysial portal blood of anesthetized, surgically stressed rats (20), and also suppress CRF/AVP stimulated ACTH secretion by rat anterior pituitary cells *in vitro* (12–15).

In summary, our findings show that atriopeptin is a potent inhibitor of stimulus evoked ACTH secretion *in vivo* indicating that it may function as a hypothalamic CIF.

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Atriopeptin: An Endogenous Corticotropin-Release Inhibiting Hormone.

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Activation of the hypothalamic-pituitary-adrenocortical axis is a major component of the body's response to stress. Current theories on the pathophysiology of disorders associated with hyperfunction of the axis, such as depression and Cushing's disease, are based on the concept that anterior pituitary adrenocorticotropin (ACTH) secretion is stimulated by hypothalamic corticotropin-releasing hormones and inhibited by adrenal corticosteroids. Hypothalamic inhibitory control of pituitary ACTH secretion has been also postulated, but has not gained general acceptance because of the lack of definitive evidence for a corticotropin-release inhibiting hormone. It is shown here that in conscious rats stress-induced secretion of ACTH and corticosterone is markedly enhanced by the immunoneutralisation of atriopeptin. Therefore, we propose that atriopeptin is a physiologically relevant corticotropin-release inhibiting hormone.

Evidence suggesting an inhibitory component of the hypothalamic control of ACTH secretion dates back to the early sixties *cf.* Ref. 1. A considerable body of data now indicates that the mediator of this inhibition may be atriopeptin (atrial natriuretic peptide, ANP) produced in the hypothalamo-hypophyseal pathway to the external zone of the median eminence *cf.* Ref. 2. Although earlier studies have suggested that ANP has no significant action on pituitary hormone release [3,4] more recent systematic analysis has shown that at physiologically relevant subnanomolar concentrations ANP inhibits stimulated ACTH secretion *in vitro* [5-9] as well as *in vivo* [10] by a direct action on the anterior pituitary gland. In anterior pituitary cells ANP suppresses ACTH release by activating particulate guanylyl cyclase, a mechanism distinct from the nuclear receptor-mediated effects of glucocorticoid hormones, the classical inhibitors of ACTH release *cf.* Refs. 6 & 11. Finally, ANP is released into the hypophyseal portal circulation by hypothalamic neurons [12].

The outstanding pivotal question is whether or not endogenous ANP is involved in the control of ACTH secretion? Since no effective antagonist of ANP action is available at present we have addressed this problem by injecting immunoneutralising antisera against ANP into conscious chronically cannulated rats subjected to stress.

Materials and Methods

Animals All procedures with animals were in accordance with the Cruelty to Animals Act 1986 (U.K.). Male Wistar-derived rats (Animal House, Western General Hospital, Edinburgh, 200-250 g BW) were anesthetized with halothane and equipped with an intraarterial catheter in the right external carotid artery. The cannula was exteriorized by suturing to the skin of the neck and flushed daily with 0.3 ml of 5000 IU/ml heparin. Animals were housed in individual cages in a controlled environment (lights on 05.00-19.00 h). At least 48h after the implantation of the cannula an extension tubing was attached for the collection of blood samples. Two h afterwards blood samples (0.25 ml; volume replaced with saline) were obtained in the following order: 10 min and immediately before the stress stimulus. Anti-ANP antisera or pre-immune rabbit serum (0.2 ml/rat) were injected through the carotid cannula at 2min before stress. Stress consisted of brief immobilisation and the intraperitoneal injection of 0.2 ml of 0.9% NaCl. Further blood samples were obtained 5, 10, 20, 30, and 60 min afterwards. All experiments were performed between 09.00 and 13.00 h. ACTH in

plasma was determined by direct radioimmunoassay using antiserum 8514 as described previously, with minor modifications to ensure that the anti-ANP rabbit serum in the samples did not interfere with the measurement of ACTH [10]. Plasma corticosterone was also measured by radioimmunoassay [13].

Sera Antisera against ANP were raised in New Zealand white rabbits using various synthetic rat ANP analogues (Peninsula Labs, St. Helen's, Merseyside, U.K.) conjugated to mammalian purified protein derivative or bovine thyroglobulin with glutaraldehyde. The titer and specificity of the antisera were characterized in radioimmunoassay using ¹²⁵I-ANP(99-126) as tracer. The sera were also screened for immunoneutralising activity *in vitro* at 1/100 and 1/300 dilutions by examining the effects of antiserum on the ANP-induced accumulation of cyclic guanosine 3':5' monophosphate (cGMP) in suspensions of rat anterior pituitary cells as previously described [6]. Four different antisera (Pink, Orange, RD and RU) all directed against the C-terminus of ANP, blocked the effects of ANP on pituitary cGMP accumulation *in vitro* and were selected for *in vivo* studies. Specificity studies with these sera at 1/400-fold dilution showed negligible cross reaction (< 1%) with human brain natriuretic peptide, somatostatin and vasopressin. None of the antisera recognized ANP (99-109) and all reacted avidly with ANP (111-126). Each antiserum was injected into three rats and the data are reported as a single anti-ANP serum-treated group.

Results

A submaximal stress stimulus (intraperitoneal injection of saline into previously handled rats) was used in order to reveal any facilitation of the ACTH response by anti-ANP serum. Intraperitoneal injection of saline increased plasma ACTH at least 2-fold above basal in 5 out of 9 control rats receiving pre-immune rabbit serum and in 8 out of 12 rats treated with antiserum against ANP, ($p > 0.1$ by 2x2 contingency table and chi-square test) indicating that the threshold of the stress response was not changed by the injection of anti-ANP serum. In the rats that showed an increase of plasma ACTH upon stress the size of the hormonal response at 5 and 10 min after stress was significantly enhanced by the administration of anti-ANP serum (Fig 1a). Overall, the amount of ACTH secreted after stress was three times greater in the group treated with anti-ANP serum (Fig 1a, *inset*). By 60 min plasma ACTH returned to baseline levels in both groups. The plasma corticosterone response in these animals was also significantly enhanced at 5, 10 and 20 min when compared with controls (Fig 1b).

Administration of ANP antiserum to unstressed rats

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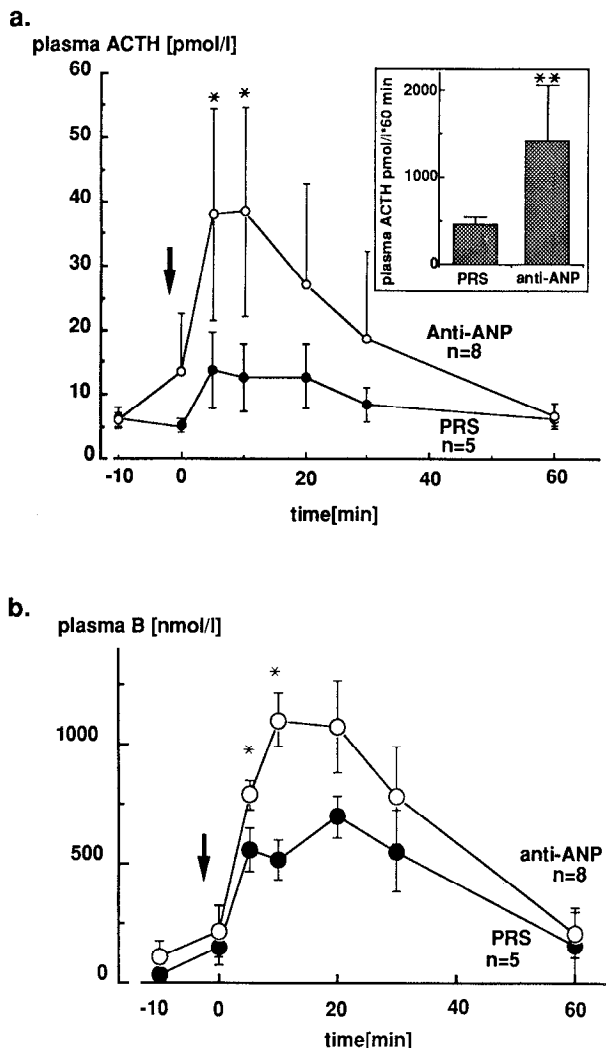


Figure 1. a.) Effect of pre-immune rabbit serum (PRS) and anti-ANP sera (anti-ANP) on stress-induced ACTH secretion in conscious cannulated male rats. Sera (0.2 ml) were given intraarterially at -2 min (arrow). Data are geometric means bars show SEM; * $p < 0.05$, Student's t-test performed after log-transformation of the data which was necessary to achieve homogeneity of variances. Inset: Data from the same experimental series shown as area under the curve between 0 and 60 min. Data are geometrical means bars show SEM; ** $p < 0.01$, compared with pre-immune serum group, Student's t-test after log transformation of data. b.) Plasma corticosterone in the same groups of animals as in a. Data are geometric means bars show SEM; * $p < 0.05$, Student's t-test performed after log-transformation of the data. Data are geometrical means bars show SEM.

caused no marked changes in plasma ACTH or corticosterone showing that a stress stimulus is required to reveal the effect of endogenous ANP (Data not shown).

Discussion

These data demonstrate that endogenous ANP plays an important role in determining the size of the pituitary-adrenocortical response to stress. With respect to the origin of ANP involved in the regulation of ACTH secretion two sources deserve consideration: the heart *cf.* Refs. 14 & 15 and the hypothalamo-hypophyseal system [16-18]. Atrialpeptin in the peripheral circulation

originates largely from the heart, but ANP levels in peripheral blood are normally very low (0.001-0.01 nM) and generally do not change in response to acute stressful stimuli *cf.* Ref. 19. Atrialpeptin is released from the hypothalamo-hypophyseal pathway into hypophyseal portal blood attaining concentrations (0.1-1 nM) that are relevant for ANP mediated inhibition of pituitary ACTH secretion *in vitro* [5,6] as well as *in vivo* [10]. Moreover, the release of ANP by hypothalamic cultures is enhanced by glucocorticoids suggesting that adrenocortical feedback inhibition of the hypothalamic-pituitary-adrenocortical axis may involve an action of steroids on the hypothalamic ANP system [20].

In summary, the present data show that endogenous ANP is a physiologically relevant inhibitory factor in the control of pituitary ACTH secretion during stress. The site of action of endogenous ANP, whether at the pituitary gland or in the hypothalamus, is not resolved by the present study. However, on the basis of previous evidence it seems reasonable to suggest that this ANP originates from the hypothalamo-hypophyseal tract to the external zone of the median eminence [12,16-18], acts directly at the pituitary level to suppress ACTH secretion [6,8,10,21] and thus constitutes a hypothalamic corticotropin-release inhibiting pathway.

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