Involvement of multiple protein kinase C isoforms in the ACTH secretory pathway of AtT-20 cells

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1 The mouse AtT-20/D16-16 anterior pituitary tumour cell line was used as a model system for the study of protein kinase C (PKC)-mediated enhancement of calcium- and guanine nucleotide-evoked adrenocorticotrophin (ACTH) secretion.

2 A profile of the PKC isozymes present in AtT-20 cells was obtained by Western blotting analysis and it was found that AtT-20 cells express the α, β, ε and ζ isoforms of PKC.

3 PKC isozymes were activated by the use of substances reported to activate particular isoforms of the enzyme. The effects of these substances were investigated in both intact and electrically-permeabilized cell. Phorbol 12, 13-diacylglycerol (PMA; EC50 = 1 ± 0.05 nM), which activates all isoforms of PKC except the ζ isoform, and GTP-γ-S (10 ± 0.5 nM), which activates the α, β and γ isoforms and 12-deoxyphorbol 13-phenylacetate 20-acetate (dPPA; EC50 = 3 ± 0.5 nM), a β-selective isozyme activator) all stimulated ACTH secretion from intact cells in a concentration-dependent manner. Maximal TMX stimulated ACTH secretion was of a similar degree to that obtained in response to PMA but maximal dPPA-stimulated ACTH secretion was only 60–70% of that obtained in response to PMA or TMX.

4 Calcium stimulated ACTH secretion from electrically-permeabilized cells over the concentration-range of 100 nM to 10 μM. PMA (100 nM), TMX (100 μM) but not dPPA (100 nM) enhanced the amount of ACTH secreted at every concentration of calcium investigated. PMA (100 nM) and TMX (100 nM) significantly enhanced ACTH secretion in the effective absence of calcium (i.e. where the free calcium concentration is 1 nM).

5 GTP-γ-S stimulated ACTH secretion from permeabilized cells in a concentration-dependent manner with a threshold of 1 μM. PMA (100 nM), TMX (100 μM) but not dPPA (100 nM) increased the amount of ACTH secretion evoked by every concentration of GTP-γ-S investigated.

6 The PKC inhibitor, chelerythrine chloride (10 μM), blocked the PMA (100 nM)-evoked enhancement of calcium- and GTP-γ-S-stimulated ACTH secretion but did not significantly alter calcium- or GTP-γ-S evoked secretion itself.

7 The present paper indicates that AtT-20 cells express multiple isoforms of PKC and that these act at different sites in the secretory pathway for ACTH secretion. The α and ε isoforms of PKC can act distal to calcium entry to modulate the ability of increased cytosolic calcium concentrations to stimulate ACTH secretion. This site of action is either at the level of, or at some stage distal to, the GTP-binding protein which mediates the effects of calcium upon ACTH secretion. The β isozyme of PKC may act at a stage early in the secretory pathway to regulate the cytosolic calcium concentration.

Keywords: Phorbol esters; protein kinase C; calcium; G-protein; anterior pituitary; ACTH

Introduction

Adrenocorticotrophin (ACTH) secretion from anterior pituitary corticotrophs is stimulated by hypothalamic neuropeptides including corticotrophin-releasing factor (CRF) and arginine vasopressin (AVP) (Axelrod & Reisine, 1984; Antoni, 1986). The stimulus-secretion coupling mechanisms mediating the stimulatory effects of these agents on ACTH secretion remain to be completely clarified. CRF acts via the adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA) pathway in both anterior pituitary corticotrophs (Aguilera et al., 1983) and in a tumour cell line of the anterior pituitary, AtT-20, which consists of a homogeneous population of ACTH-secreting cells (Luini et al., 1985; Guild & Reisine, 1987). Cyclic AMP has a dual action both enhancing calcium influx into corticotrophs and potentiating the effect of such an increment in cytosolic calcium upon the secretory apparatus (Luini et al., 1985; Guild et al., 1986; Guild & Reisine, 1987; Guild, 1991). The nature of AVP's signal-transduction pathway is less clear than that of CRF but AVP does activate protein kinase C (PKC) in pituitary corticotrophs (Abou-Samra et al., 1986). Although AtT-20 cells do not express functional vasopressin receptors, stimulants of PKC have been shown to alter cytosolic calcium concentrations and stimulate ACTH release from these cells (Reisine & Guild, 1987). Activation of these two protein kinases influences the calcium messenger system and both have a 'pre-' and 'post-' calcium site of action to regulate calcium influx and the effect of particular cytosolic calcium concentrations upon ACTH secretion (Reisine & Guild, 1987; Guild, 1991; McFerran & Guild, 1994).

The mechanisms linking changes in cytosolic calcium concentration to changes in hormone secretion remain largely unknown but it has been suggested that a late stage in stimulus-secretion coupling may involve a direct regulation of exocytosis by guanosine 5'-triphosphate (GTP)-binding proteins, dubbed Gβγ by Gomperts and his co-workers (Gomperts, 1990). Such a GTP-binding protein has been reported to mediate the effects of calcium upon the secretory apparatus in AtT-20 cells and may be a target for regulation by PKA (Guild, 1991) and PKC (McFerran & Guild, 1994).
Interestingly, PKA and PKC act through distinct mechanisms to influence cytosolic calcium concentrations in AtT-20 cells (Reisine & Guild, 1987; Reisine, 1989) and both have distinct 'post-calcium' sites of action at a late stage in the secretory process, potentiating the effects of calcium and guanine nucleotides upon the secretory apparatus (Guild, 1991; McFerran & Guild, 1994).

Several isoforms of PKC have been identified and characterized (for review, see Hug & Sarre, 1993) and have been specifically implicated as having distinct roles in signal transduction, stimulus-secretion coupling and growth (Housey et al., 1988; Naor et al., 1989; Akita et al., 1990; Pai et al., 1991; Shtulman et al., 1992; Leli et al., 1992). In view of the evidence supporting the presence and active role in secretion of pharmacologically distinct forms of PKC in rat anterior pituitary cells (Thomson et al., 1993), the present study profiled the PKC isoforms present in AtT-20 cells and used activators of isoforms of PKC (Ryves et al., 1991) to investigate the contribution of these PKC isoforms to the regulation of ACTH secretion. In particular, the possibility that different PKC isoforms are responsible for the 'pre-' and 'post-' calcium sites of regulation of ACTH secretion was targeted. The distinction between such a 'pre-' and 'post-' calcium site of action required a direct manipulation of the intracellular environment. This was provided by the use of electrically permeabilized cells, a technique which has been used previously to gain access to the cytosol in AtT-20 cells (Guild, 1991). The present paper indicates that AtT-20 cells express different isoforms of PKC and that these act at different sites in the secretory pathway for ACTH.

**Methods**

**Culture of AtT-20 cells**

Cells of the mouse AtT-20/D16-16 pituitary tumour were grown and subcultured in Dulbecco's Modified Eagle's Medium (DMEM) (glucose 4.5 g l⁻¹) supplemented with 10% (w:v) foetal calf serum as previously described (Reisine, 1984). Cells to be used in ACTH release experiments from intact cells were plated onto 24 well (16 mm diameter) multwell plates (Costar, U.S.A.) at an initial cell density of 10⁵ cells/well and were used 5–7 days after subculturing (80–90% confluency). Cells to be used in experiments involving electrically permeabilized cells and the Western blot studies were plated in 75 cm² flasks (Nunc, Gibco, U.K.) at an initial density of 2 x 10⁶ cells/flask and were used 7–9 days after subculturing (80–90% confluency).

**Preparation of AtT-20 cells for ACTH release experiments**

**Intact cell preparations** The culture medium was removed, cells adhering to the substrate in each well were washed 3 times with 1 ml of DMEM supplemented with 0.1% (w:v) bovine serum albumin (DMEM/BSA) and then incubated for 1 h in 1 ml of fresh DMEM/BSA at 37°C in a humidified atmosphere of 10% CO₂ in air. The DMEM/BSA was then decanted and replaced with 1 ml of fresh DMEM/BSA.

**Permeabilized cell preparations** The culture medium was removed and the cells liberated from the substrate by trypsin (0.025%, v:v) (Gibco BRL) and then washed twice by centrifugation (200 g, 5 min)/resuspension in a balanced salt solution of the following composition (mM): NaCl 145, KCl 5.6, CaCl₂ 2, MgCl₂ 0.5, glucose 5.6, HEPES 5, sodium ascorbate 0.5, BSA 0.1% (w:v); pH 7.4. After washing, the cells were suspended at a density of 10⁶ cells ml⁻¹ in this buffer and incubated for a further 30 min at 37°C. The cell suspension was then centrifuged (200 g, 5 min) and the pericellular pellet washed twice by resuspension/centrifugation (200 g, 5 min) in the standard permeabilization buffer of the follow-

ing composition (mM): potassium glutamate 129, PIPES (potassium salt) 20, glucose 5, ATP 5, EGTA 5, MgCl₂ 1, BSA 0.1% (w:v), pH 6.6. The cells were then resuspended in this buffer at a density of 10⁷ ml⁻¹ and electrically permeabilized by subjecting to intense electric fields of brief duration (Knight & Baker, 1982). The optimum permeabilisation parameters of 10 discharges of 2500 V cm⁻¹, previously determined (Guild, 1991), were adopted in these experiments.

**Activation of PKC isoforms by drugs**

PKC isoforms were activated by the use of substances previously reported to activate particular isoforms of the enzyme (Ryves et al., 1991). The activators of PKC used in these experiments were: phorbol 12-myristate 13-acetate (PMA) (which activates all isoforms of PKC, except the δ isoform and is used here as non-selective, general activator of the enzyme); thymeleatoxin (TMX) (which reportedly activates the α, β and γ isoforms) and 12-deoxyxphorbol 13-phynyleatoct 20-acetate (dPAA) (a reportedly δ-selective isoform activator).

**Measurement of stimulated ACTH secretion from intact cells**

The ability of activators of protein kinase C to stimulate ACTH secretion from intact AtT-20 cells remaining attached to the culture dishes was measured as previously described (Reisine & Guild, 1987; McFerran & Guild, 1994). Briefly, drugs were added to the 1 ml of DMEM/BSA bathing the cells in the wells of the culture dishes. Zero time samples were taken at this point and the remaining cells incubated for 1 h at 37°C in a humidified atmosphere of 10% CO₂ in air. In each experiment, sextuplicate samples were run for each condition. Incubations were terminated by removing the DMEM/BSA bathing the cells, centrifugation (10,000 g, 20 s) of this sample and the removal of the supernatant. The ACTH content of the supernatant was measured by radioimmunoassay.

**Measurement of calcium- and guanine nucleotide-stimulated ACTH from permeabilized AtT-20 cells**

The standard protocol for the determination of ACTH secretion from permeabilized AtT-20 cells was as follows: permeabilized cells were suspended at a cell density of 10⁵ cells ml⁻¹ in either a series of calcium- and EGTA buffers chosen to give a free calcium concentration in the range 1 nM–10 μM for experiments investigating the effects of calcium or in a permeabilization buffer with a free calcium concentration of 1 nM for experiments investigating the effects of guanine nucleotides and prepared as previously described (Guild, 1991). At this point, the zero time samples were centrifuged (200 g, 5 min) and an aliquot of the supernatant was stored for subsequent measurement of ACTH content. The cell suspensions were incubated at 37°C for 30 min at which point incubations were terminated by centrifugation (200 g, 5 min) and removal of the supernatant. The ACTH content was measured by radioimmunoassay. In each experiment, sextuplicate samples were run for each condition. The modifications to this standard protocol which were made to permit the measurement of the effect of the protein kinase activators upon calcium- and guanine nucleotide-stimulated ACTH secretion from permeabilized AtT-20 cells are described in the legends to the figures.

**Detection of PKC isoforms in AtT-20 cells and rat tissues**

Adult male Sprague-Dawley rats were killed by excess CO₂ inhalation and dissections performed. The tissues were
Dounce homogenized in 2 vol ice-cold homogenisation buffer of the following composition: Tris·HCl 20 mM (pH 7.4), ethyleneglycol-bis(β-aminoethyl ether) N,N,N′,N′′-tetraacetic acid (EGTA) 1 mM, dithiothreitol 1 mM, Na F 100 mM, supplemented with a protease inhibitor cocktail of aprotinin, trypsin inhibitor, benzamidine, leupeptin and 4-amidinophenyl-methanesulphonylfluoride at 100, 100, 250, 100 and 100 μg ml⁻¹ respectively (Boehringer Mannheim Corp., Indianapolis, IN, U.S.A.). The homogenates were then boiled for 10 min in sample buffer (5% wt:vol sodium dodecylsulphate, Tris 125 mM, glycine 10% wt:vol, 2-mercaptoethanol 2% vol:vol, bromophenyl blue 2 μl ml⁻¹ (final concentrations)). AtT-20 cell monolayers were washed once and scraped into ice-cold phosphate buffered saline and treated with sample buffer as described above.

Sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western analysis was performed essentially as previously described (Strulovicci et al., 1989; 1991) with the following modifications: blocking of non-specific protein binding sites was performed by 5% (wt:vol) non-fat dried milk (in phosphate buffered saline without calcium or magnesium salts supplemented with 0.5% (vol:vol) Tween-20); specific antibody binding was visualised using protein-A horseradish peroxidase followed by enhanced chemiluminescence and autoradiographic exposure. Antisera were used at the following dilutions: 1:50 anti-PKC-α, anti-PKC-β and anti-PKC-γ; 1:100 anti-PKC-ε, 1:1000 anti-PKC-η and 2 μg ml⁻¹ anti-PKC-δ and anti-PKC-ζ.

The effect of the PKC inhibitor chelerythrine chloride upon the PMA enhancement of calcium- and guanine nucleotide-stimulated ACTH secretion

The PKC inhibitor, chelerythrine chloride (Herbert et al., 1990) was used to investigate whether the observed PMA enhancement of calcium and GTP-γ-S-stimulated ACTH secretion was via a stimulation of PKC. This was tested by measuring calcium- and GTP-γ-S-stimulated ACTH secretion from permeabilized cells in the presence and absence of PMA (100 nM) and the effect of co-incubation with chelerythrine chloride (10 μM).

Radioimmunossay

The radioimmunoassay for ACTH was performed as previously described (Reisine, 1984). [125I]-ACTH for radioimmunoassay use was produced using the iodogen reagent (1,3,4,6-tetrachloro-3,6-6-diphenylglycoluril) which was first described as a reagent for iodination by Fraker & Speeck (1978). The amount of ACTH released was expressed as the amount present at the end of the specified incubation period less the amount present at zero time.

Statistics

In each experiment sextuplicate determinations for each experimental condition were made and each experiment was repeated three times, on different days. ACTH secretion is expressed as the means ± s.e.mean of the mean values from these 3 separate experiments. Statistical significance was tested by the use of ANOVA tests with Scheffe's F-test post hoc analysis. A P value <0.05 was considered significant and is the definition of the term used here. Two-way ANOVA was used to test for a statistically significant interaction between two groups (i.e. the concentration-response curves to calcium ions or GTP-γ-S in the presence and absence of PKC activators) and the significance of these interactions is specified.

Materials

The following substances (with their sources) were used: DMEM, foetal calf serum and trypsin/EDTA were purchased from Gibco BRL, U.K.; human ACTH antiserum and human ACTH for standards was the gift of the National Hormone and Pituitary programme, Baltimore, MD, USA. Polyclonal antiserum to PKC-α, -β, γ, ε were prepared as previously described (Strulovicci et al., 1989; 1991). Other antibodies were purchased from Gibco BRL (Gaithersburg, MD, U.S.A.; PKC-δ and ζ) or was a kind gift from Dr Harold Mishak (NIH, Bethesda, MD, U.S.A.; PKC-η). Immunobilon-P polyvinylidene difluoride microporous transfer membranes were obtained from Millipore, Bedford, MA, U.S.A. Protein A conjugated to peroxidase was bought from Calbiochem, San Diego, CA, U.S.A. Enhanced chemiluminescence materials were purchased from Amersharm International, Aylesbury, U.K.; phorbol 12-myristate 13-acetate was purchased from Sigma UK, thymeleatoxin, 12-deoxyphorbol 13-phenylacetate 20-acetate and chelerythrine chloride were purchased from Calbiochem-Novabiochem UK. Guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) was obtained from Boehringer Mannheim, UK. All other chemicals used were of Analar grade and readily commercially available.

Results

The isoforms of PKC present in AtT-20 cells identified by Western blotting studies

PKC isoform immunoreactivity of whole AtT-20 cells was detected by Western analysis using isoform-specific antiserum (Figure 1). Detectable amounts of PKCs α, β, ε and ζ were observed whereas γ, δ and η could not be detected under the
The effect of PKC activators upon ACTH secretion from intact AtT-20 cells

PMA (1 pM–100 nM) stimulated a concentration-dependent (EC50 = 1 ± 0.1 nM, n = 3) increase in ACTH secretion (significant (P < 0.05) at concentrations of PMA of 100 pm and above) (Figure 2). These results are consistent with previous studies showing an activation of PKC by PMA to stimulate ACTH secretion from AtT-20 cells (Reisine & Guild, 1987; Reisine, 1989; McFerran & Guild, 1994). TMX (10 pm–1 μM) and dPPA (10 pm–1 μM) both stimulated ACTH secretion in a concentration-dependent manner (EC50 = 10 ± 0.5 nM (n = 3) and 3 ± 0.5 nM (n = 3) respectively and significant (P < 0.05) at concentrations of TMX and dPPA of 10 pm and above) (Figure 2). Maximal TMX-stimulated ACTH secretion was of a similar degree to that obtained in response to PMA but maximal dPPA-stimulated ACTH secretion was only 60–70% of that obtained in response to PMA or TMX (Figure 2).

The effect of PKC activators upon calcium-evoked ACTH secretion from permeabilized AtT-20 cells

Calcium-evoked ACTH secretion from permeabilized AtT-20 cells was dependent upon the concentration of free calcium in the permeabilization medium (Figure 3). Calcium stimulated ACTH secretion in a concentration-dependent manner between 100 nM and 10 μM (significant (P < 0.05) stimulation at concentrations of free calcium ions of 1 μM and above). Co-incubation with PMA (100 nM) resulted in an enhanced ACTH secretion at all concentrations of calcium investigated (Figure 3a). PMA (100 nM) enhanced ACTH secretion in the effective absence of calcium (i.e. where the free calcium concentration is 1 nM). A comparison of the calcium ion concentration-response curves in the presence and absence of PMA (100 nM) by two way ANOVA test revealed that calcium ion-stimulated ACTH secretion was significantly (P < 0.0001) enhanced by co-incubation with PMA (100 nM) and furthermore there was a significant (P < 0.0017) interaction between the calcium ion concentration-response curves in the presence and absence of TMX (100 nM). The results are expressed as the mean ± s.e. mean from 3 separate experiments. Absence of error bars indicate that they lie within the symbol used. Calcium stimulated ACTH secretion in a concentration-dependent manner between 100 nM and 10 μM (significant (P < 0.05) stimulation at concentrations of free calcium ions of 1 μM and above). A comparison of the calcium ion concentration-response curves in the presence and absence of TMX (100 nM) by two way ANOVA test revealed that calcium ion-stimulated ACTH secretion was significantly (P < 0.0001) enhanced by co-incubation with PMA (100 nM) and furthermore there was a significant (P < 0.0017) interaction between PMA and calcium ions upon ACTH secretion. (b) Co-incubation with dPPA (100 nM) did not significantly alter calcium-evoked ACTH secretion at any concentration of calcium investigated. (c) A comparison of the calcium ion concentration-response curves in the presence and absence of TMX (100 nM) by two way ANOVA test revealed that calcium ion-stimulated ACTH secretion was significantly (P < 0.0001) enhanced by co-incubation with TMX (100 nM) and furthermore there was a significant (P < 0.0377) interaction between TMX and calcium ions upon ACTH secretion.
tion between PMA and calcium ions upon ACTH secretion. In contrast to PMA, co-incubation with dPPA (100 nM) did not significantly alter calcium-evoked ACTH secretion at any concentration of calcium investigated (Figure 3b). Co-incubation with TMX (100 nM) resulted in an enhanced ACTH secretion at all concentrations of calcium investigated (Figure 3c). A comparison of the calcium ion concentration-response curves in the presence and absence of TMX (100 nM) by two way ANOVA test revealed that calcium ion-stimulated ACTH secretion was significantly \( P < 0.0001 \) enhanced by co-incubation with TMX (100 nM) and furthermore there was a significant \( P < 0.0377 \) interaction between TMX and calcium ions upon ACTH secretion.

The effect of PKC activators upon guanine nucleotide-evoked ACTH secretion from permeabilized AtT-20 cells

GTP-\( \gamma \)-S stimulated ACTH secretion, in the absence of calcium, in a concentration-dependent manner (significant \( P < 0.05 \) stimulation at concentrations of GTP-\( \gamma \)-S of 10 \( \mu \)M and above) (Figure 4). PMA (100 nM) stimulated ACTH secretion in the absence of GTP-\( \gamma \)-S and enhanced the stimulated ACTH secretion at every concentration of the nucleotide investigated (Figure 4a). A comparison of the GTP-\( \gamma \)-S concentration-response curves in the presence and absence of PMA (100 nM) by two way ANOVA test revealed that GTP-\( \gamma \)-S-stimulated ACTH secretion was significantly \( P < 0.0001 \) enhanced by co-incubation with PMA (100 nM) but that there was no significant interaction between GTP-\( \gamma \)-S and PMA. In contrast, co-incubation with dPPA (100 nM) did not alter GTP-\( \gamma \)-S-evoked ACTH secretion at any concentration of the nucleotide investigated (Figure 4b). Co-incubation with TMX (100 nM) significantly enhanced ACTH secretion at all concentrations of GTP-\( \gamma \)-S investigated (Figure 4c) in a manner similar to, but to a lesser extent than, that observed with PMA. A comparison of the GTP-\( \gamma \)-S concentration-response curves in the presence and absence of TMX (100 nM) by two way ANOVA test revealed that GTP-\( \gamma \)-S-stimulated ACTH secretion was significantly \( P < 0.0001 \) enhanced by co-incubation with TMX (100 nM) but that there was no significant interaction between GTP-\( \gamma \)-S and TMX. Thus PMA and TMX but not dPPA enhanced both calcium- and guanine nucleotide-evoked ACTH secretion.

The effect of higher concentrations of dPPA upon calcium- and guanine nucleotide-evoked ACTH secretion from permeabilised AtT-20 cells

The effect of concentrations of dPPA greater than 100 nM (shown to be supra-maximal upon ACTH secretion from intact cells, Figure 1) upon calcium ion (10 \( \mu \)M)- and GTP-\( \gamma \)-S (100 \( \mu \)M)-evoked ACTH secretion was investigated (Table 1). dPPA did not significantly enhance control, calcium ion- or GTP-\( \gamma \)-S-evoked secretion at a concentration of 100 nM (consistent with the results presented in Figures 3 and 4). However, dPPA did significantly \( P < 0.05 \) enhance calcium ion-evoked secretion and GTP-\( \gamma \)-S-evoked secretion significantly \( P < 0.05 \) at a concentration of 10 \( \mu \)M (Table 1). These results indicate that high concentrations of dPPA, much greater than those reported for its selective action upon the \( \beta \) isofrom of PKC, can enhance both calcium ion- and GTP-\( \gamma \)-S-evoked ACTH secretion from permeabilized AtT-20 cells.

The effect of cherythryline chloride upon calcium- and guanine nucleotide-evoked ACTH secretion from permeabilised AtT-20 cells

Calcium stimulated ACTH secretion in a concentration-dependent manner between 1 nM and 10 \( \mu \)M (significant \( P < 0.05 \) stimulation at concentrations of free calcium ions of 1 \( \mu \)M and above) (Figure 5a). Co-incubation with PMA (100 nM) resulted in an enhanced ACTH secretion at all concentrations of calcium investigated (Figure 5a). The PKC inhibitor, cherythryline chloride (10 \( \mu \)M) did not significantly

![Figure 4](image-url)  Effect of PMA, TMX and dPPA on GTP-\( \gamma \)-S-stimulated ACTH secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated, as described in the Methods, in standard permeabilization medium supplemented with the indicated concentration of GTP-\( \gamma \)-S either in the presence (A) or absence (B) of PMA (100 nM). a), dPPA (100 nM, b) or TMX (100 nM, c). The results are expressed as the mean ± s.e. mean from 3 separate experiments. Absence of error bars indicate that they lie within the symbol used. GTP-\( \gamma \)-S stimulated ACTH secretion, in the absence of calcium, in a concentration-dependent manner (significant \( P < 0.05 \) stimulation at concentrations of GTP-\( \gamma \)-S of 10 \( \mu \)M and above). (a) A comparison of the GTP-\( \gamma \)-S concentration-response curves in the presence and absence of PMA (100 nM) by two way ANOVA test revealed that GTP-\( \gamma \)-S-stimulated ACTH secretion was significantly \( P < 0.0001 \) enhanced by co-incubation with PMA (100 nM) but that there was no significant interaction between GTP-\( \gamma \)-S and PMA (b) Co-incubation with dPPA (100 nM) did not alter GTP-\( \gamma \)-S-evoked ACTH secretion at any concentration of the nucleotide investigated. (c) A comparison of the GTP-\( \gamma \)-S concentration-response curves in the presence and absence of TMX (100 nM) by two way ANOVA test revealed that GTP-\( \gamma \)-S-stimulated ACTH secretion was significantly \( P < 0.0001 \) enhanced by co-incubation with TMX (100 nM) but that there was no significant interaction between GTP-\( \gamma \)-S and TMX.
alter the ACTH secretion obtained at any concentration of calcium (Figure 5a) but inhibited the PMA-evoked enhancement of ACTH secretion at all of the calcium concentrations (Figure 5a). A comparison of the calcium ion concentration-response curves in the presence and absence of PMA (100 nM), chelerythrine chloride (10 μM) or the combination of PMA and chelerythrine chloride by two way ANOVA test revealed that calcium ion-stimulated ACTH secretion was significantly (P < 0.0001) enhanced by co-incubation with PMA (100 nM) but not by chelerythrine chloride or the PMA and chelerythrine chloride combination.

GTP-γ-S stimulated ACTH secretion in the absence of calcium in a concentration-dependent manner with a threshold of 1 μM (Figure 5b). PMA (100 nM) stimulated ACTH secretion in the absence of GTP-γ-S and significantly enhanced GTP-γ-S-stimulated ACTH secretion (Figure 5b). Chelerythrine chloride (10 μM) did not significantly alter GTP-γ-S-stimulated ACTH secretion but inhibited the PMA (100 nM)-evoked enhancement of GTP-γ-S-evoked ACTH secretion. A comparison of the GTP-γ-S concentration-response curves in the presence and absence of PMA (100 nM), chelerythrine chloride (10 μM) or the combination of PMA and chelerythrine chloride by two way ANOVA test revealed that GTP-γ-S-stimulated ACTH secretion was significantly (P < 0.0001) enhanced by co-incubation with PMA (100 nM) but not by chelerythrine chloride or the PMA and chelerythrine chloride combination. These results indicate that PMA acts via an activation of PKC to evoke the observed enhancement of calcium- and guanine nucleotide-evoked ACTH secretion in this system.

Discussion

The present study adds to the considerable body of evidence supporting an involvement of PKC in the stimulation of ACTH release from both the normal corticotroph (Abou-Samra et al., 1986) and the model for this secretory cell, the AT-20 cell line (Reisine & Guild, 1987; Reisine, 1989; McFerran & Guild, 1994). PKC interacts with the ACTH secretory pathway at multiple points as shown by its 'pre- and post-'calcium site of action to regulate calcium influx and the effect of particular cytosolic calcium concentrations upon ACTH secretion (Reisine & Guild, 1987; McFerran & Guild, 1994). Since PKC is now known to consist of several different isoenzymes (for review, see Hug & Sarre, 1993), the question arose as to whether different isozymes of PKC could act at different points in the stimulus-secretion coupling pathway in AT-20 cells. The family of PKC isozymes can be divided into two main groups, the calcium-dependent or conventional PKC's (cPKC's) and the calcium-independent or novel PKC's (nPKC's) (Ohno et al., 1991). Several isoforms of PKC have been specifically implicated as having

![Figure 5](attachment:image.png)

Figure 5 Effect of chelerythrine chloride upon the ability of PMA to potentiate calcium- and GTP-γ-S-evoked ACTH secretion from permeabilized AT-20 cells. (a) Permeabilized cells were incubated in standard permeabilization medium containing calcium-EGTA buffers designed to give the indicated free calcium concentrations either in the presence (●) or absence (□) of PMA (100 nM). The effects of chelerythrine chloride (10 μM) upon ACTH secretion evoked by these calcium concentrations (●) and upon the combination of calcium plus PMA (100 nM) (▲) were measured. The results are expressed as the mean ± s.e.mean from 3 separate experiments. Absence of error bars indicate that they lie within the symbol used. Calcium stimulated ACTH secretion in a concentration-dependent manner between 1 μM and 10 μM (significant (P < 0.05) stimulation at concentrations of free calcium ions of 1 μM and above). A comparison of the calcium ion concentration-response curves in the presence and absence of PMA (100 nM), chelerythrine chloride (10 μM) or the combination of PMA and chelerythrine chloride by two way ANOVA test revealed that calcium ion-stimulated ACTH secretion was significantly (P < 0.0001) enhanced by co-incubation with PMA (100 nM) but not by chelerythrine chloride or the PMA and chelerythrine chloride combination. (b) Permeabilized cells were incubated in standard permeabilization medium containing calcium-EGTA buffers designed to give 1 nM free calcium supplemented with the indicated concentrations of GTP-γ-S concentrations either in the presence (●) or absence (□) of PMA (100 nM). The effects of chelerythrine chloride (10 μM) upon ACTH secretion evoked by these GTP-γ-S concentrations (●) and upon the combination of GTP-γ-S plus PMA (100 nM) (▲) were measured. The results are expressed as the mean ± s.e.mean from 3 separate experiments. Absence of error bars indicate that they lie within the symbol used. GTP-γ-S stimulated ACTH secretion in the absence of calcium in a concentration-dependent manner with a threshold of 1 μM. A comparison of the GTP-γ-S concentration-response curves in the presence and absence of PMA (100 nM), chelerythrine chloride (10 μM) or the combination of PMA and chelerythrine chloride by two way ANOVA test revealed that GTP-γ-S-stimulated ACTH secretion was significantly (P < 0.0001) enhanced by co-incubation with PMA (100 nM) but not by chelerythrine chloride or the PMA and chelerythrine chloride combination.

Table 1 Permeabilized cells were incubated in standard permeabilization medium or medium supplemented with GTP-γ-S (100 μM) or calcium ions (10 μM) as indicated: the effect of these treatments in the presence of the indicated concentrations of dPPA upon ACTH secretion was measured as described in the Methods.

<table>
<thead>
<tr>
<th>[dPPA]</th>
<th>ACTH secretion (pg/10^12 cells)</th>
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<tr>
<td></td>
<td>Control GTP (100 μM) Calcium (10 μM)</td>
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<tr>
<td>0</td>
<td>373 ± 37 840 ± 30 737 ± 68</td>
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<tr>
<td>100 nM</td>
<td>390 ± 40 963 ± 103 860 ± 95</td>
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<td>1 μM</td>
<td>460 ± 46 1040 ± 81 1120 ± 113</td>
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<td>10 μM</td>
<td>490 ± 16 1250 ± 180 1240 ± 100</td>
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ACTH release is expressed as the mean ± s.e.mean from 3 separate experiments. In the absence of dPPA, GTP-γ-S and calcium ions significantly (P < 0.05) stimulated ACTH secretion above the control value. dPPA did not significantly enhance control, calcium ion- or GTP-γ-S-evoked secretion at a concentration of 100 μM but did significantly (P < 0.05) enhance calcium ion- and GTP-γ-S-evoked secretion at 10 μM dPPA.
distinct roles in signal-transduction, stimulus-secretion coupling and growth (Housey et al., 1988; Naor et al., 1989; Akita et al., 1990; Pai et al., 1991; Sharma et al., 1991; Kiley et al., 1992; Leli et al., 1992). In view of all of these findings, it was decided to profile the PKC isozymes present in AtT-20 cells by Western blotting techniques as part of an attempt to establish physiological roles for these isozymes in stimulus-secretion coupling for ACTH secretion. The cPKCs probed for in this study were the α, β and γ isozymes and the nPKCs probed for were the δ, ε, η and ζ isozymes.

The present study indicates that AtT-20 cells contain detectable amounts of PKC α, β, ε and ζ whereas γ, δ and η could not be detected under the conditions used in this study. Thus AtT-20 contains both calcium-dependent isozymes and the ε and ζ calcium-independent nPKCs. This profile of PKC isozymes is identical to another strain of AtT-20 cells, the AtT-20/D16-V line (unpublished observations). PKC isozymes were activated by the use of substances previously reported to activate selectively particular isozymes of the enzyme (Ryves et al., 1991). PMA activates all isozymes of PKC and was used here as a general activator of both cPKCs and nPKCs (although calcium is required for maximal stimulation of cPKCs by PMA, (Ryves et al., 1991) and the ζ isozyme is reported to be insensitive to PMA (see, Hug & Sarre, 1993)). TMX was used in an attempt to activate selectively the α and β isozymes and dPPA was used in an attempt to activate selectively the β, ε isozyme. This study used pharmacological agents to investigate if and where the desired PKC isozyme contributes to the regulation of ACTH secretion from AtT-20 cells. This approach was used recently to support the presence and active role in secretion of pharmacologically distinct forms of PKC in rat anterior pituitary cells (Thomson et al., 1993). However, attributing physiologically relevant roles to PKC isozymes identified by molecular biological and biochemical techniques remains uncertain and may change in the future.

PMA and TMX stimulated ACTH secretion from intact AtT-20 cells indicating that activation of all PKC isozymes or only the α and β isozymes can elicit the full PKC-activated ACTH secretion from these cells. The ability of dPPA (and presumably the β isozyme) to evoke a secretory response which was only 60–70% of that obtained to the other two agents may indicate that this isozyme can only evoke a partial stimulation of the secretory pathway. However, intact cells do not permit the differentiation between a 'pre-' and 'post-' calcium site of action by these isozymes and therefore the data from intact cells has to be viewed in concert with that from a cell preparation where the complicating influence of the cell membrane upon calcium metabolism can be circumvented. Such a situation is obtained by the use of electrically permeabilized cells, a technique which has been used previously to gain access to the cytosol in AtT-20 cells (Guild, 1991). The ability of calcium over the physiological range of 100 nM to 10 μM to stimulate ACTH secretion from permeabilized AtT-20 cells is entirely consistent with previous studies using digitonin-(Luini & De Matteis, 1988) and electrically-(Guild, 1991) permeabilized AtT-20 cells. In these studies a free calcium concentration of 10 μM produced maximal stimulation of ACTH.

PMA and TMX but not dPPA enhanced calcium-dependent ACTH secretion from permeabilized AtT-20 cells. These data confirm the previously suggested 'post'- calcium point of control of the secretory pathway for PKC in AtT-20 cells (Reisine & Guild, 1987; Reisine, 1989; McFerran & Guild, 1994) but indicate that the β isozyme of PKC may not contribute to this site of regulation. The role of the β, isozyme of PKC in the stimulus-secretion coupling pathway for ACTH may be at an early stage to stimulate calcium entry into the cell across the plasma membrane (Reisine & Guild, 1987; Reisine, 1989), as reported previously in anterior pituitary tissues (MacEwan & Mitchell, 1991; MacEwan et al., 1991). An action of PMA upon both potassium and calcium channels in the plasma membranes has been shown in AtT-20 cells (Reisine & Guild, 1987; Reisine, 1989) and either of these actions may be due to the action of the β, isozyme. It may be that the α and ε isozymes (or any, as yet, uncharacterized isozymes) of PKC are responsible for the 'post'- calcium site of enhancement of ACTH secretion from AtT-20 cells (Reisine & Guild, 1987; McFerran & Guild, 1994). This involvement of both calcium-dependent and calcium-independent isozymes of PKC in regulating exocytosis has been reported in both rat basophilic RBL-2H3 cells (Ozawa et al., 1992) and GH3C1 rat anterior pituitary tumour cells (Akita et al., 1990).

The ability of TMX to stimulate ACTH secretion from permeabilized AtT-20 cells in the absence of calcium was surprising in the light of the evidence that TMX was selective for the calcium-dependent isozymes of PKC (Ryves et al., 1991). This may indicate that TMX is not as selective an activator of these isozymes of PKC as had been believed (it may even be a partial agonist on the ε isozyme) or that calcium-dependent isozymes of PKC can be partially activated in the absence of calcium. Interestingly, TMX was recently shown to bind to the ε isofrom of PKC but was 10–20 fold less potent in its binding to this isozyme than its binding to the α and β, isozymes (Kazanietz et al., 1993). Although binding to the ε isofrom does not indicate whether or not TMX can activate this isozyme, it does reinforce the caution required in claiming selectivity in the spectrum of activation of PKC isoforms made for these agents. This is also true of the claim of selectivity of activation of dPPA made originally upon data obtained in in vitro studies (Ryves et al., 1991). dPPA stimulated PKC-β, kinase activity at concentrations of 20 nM but did not activate other isoforms of PKC at concentrations up to 2 μM in vitro (Ryves et al., 1991). A recent report by some of the same investigators has claimed that dPPA is not a PKC-β, selective activator when used in intact cells (Kiley et al., 1994). The ability of dPPA to activate other PKC isoforms (and perhaps even uncharacterized PKC isoforms from a digitonin-soluble fraction) in that study, however, required concentrations of dPPA in excess of 100 nM (Kiley et al., 1994). This non-selective activation of PKC isoforms in intact cells, therefore, was observed at concentrations of dPPA above that used for selective activation of PKC-β, in the present study. Consistent with this, in the present study, is that higher concentrations of dPPA (i.e. 10 μM) did produce an enhancement of calcium ion- and GTP-γ-S-evoked ACTH secretion indicating that this agent will activate other isoforms of PKC when present in sufficient quantities. Nonetheless, the present study demonstrates a difference between the actions of PMA and TMX and those of dPPA which would not be the case if these agents were non-selective in their activation of PKC isoforms.

The ability of PMA and TMX but not dPPA to potentiate GTP-γ-S-evoked ACTH secretion suggests that, in AtT-20 cells, the 'post'-calcium site of interaction between calcium and PKC is either at the level of or at some stage distal to Gε. The results indicate that the β isozyme of PKC may not contribute at this site of regulation. The ability of some activators of PKC to stimulate ACTH secretion from permeabilized cells in the absence of calcium and added GTP-γ-S raises the possibility that PKC may mediate the effects of the calcium-Gε system upon secretion. A previous report from this laboratory (McFerran & Guild, 1994) did not support the hypothesis that PKC mediates the effect of calcium-Gε system upon secretion. It would appear that PKC plays a modulatory role in regulating secretion and is not necessary for secretion per se.

This and previous studies (Guild, 1991; McFerran & Guild, 1994) suggest that not only does a G-protein directly regulate exocytosis but its action is regulated by second messenger systems perhaps by increasing the readily releasable pool of stored, endogenous hormone (Dannies, 1982). It is clear that calcium alone cannot stimulate the maximal possible hormone secretion and that co-operation
with PKC and PKA, which may increase the 'efficacy' of the calcium-$G_\text{G}$ system, increased the amount of hormone secreted in response to a particular concentration of calcium (Guild, 1991; McFerran & Guild, 1994). This study also indicates that different isoforms of PKC may act at different points in the stimulus-secretion pathway to regulate ACTH secretion. It is tempting to speculate that different isoforms of PKC contribute to the secretory response depending upon the free cytosolic calcium concentration. At lower calcium concentrations (around the resting calcium concentration of 100 nm) the $\alpha$-isofrom of PKC may have an important role in stimulating ACTH secretion under circumstances where there is no rise in the cytosolic calcium concentration. As the cytosolic calcium concentration increases above resting values the calcium-dependent cPKCs (here the $\alpha$ and $\beta$ isofroms detected to be present in AtT-20 cells) may start to exert a greater influence over ACTH secretion. This differential interaction between PKC isoforms and the cytosolic free calcium may go some way to explaining the need for the multiplicity of PKC isoforms present in AtT-20 cells. In addition, the ability of PKC to potentiate calcium-activated secretion may explain the ability of vasopressin to potentiate corticotrophin-releasing hormone (CRF)-stimulated ACTH secretion from dispersed anterior pituitary cells (Gillies et al., 1982). This suggestion is based upon the observation that vasopressin stimulates PKC activity in pituitary corticotrophs (Abou-Samra et al., 1986) and that CRF stimulates cyclic AMP production and an increase in cytosolic calcium ion concentration in AtT-20 cells (Guild & Reisine, 1987). Hence the potential for an interaction between PKC- and PKA-stimulated mechanisms is provided by these two neuropeptides.

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References


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