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Effects of protein kinase C activators upon the late stages of 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 phorbol 12-myristate 13-acetate (PMA)-mediated enhancement of calcium-evoked adrenocorticotrophin (ACTH) secretion.

2 PMA stimulated ACTH secretion from intact cells in a concentration-dependent manner. Other phorbol esters; phorbol 12,13-dibutyrate (PDBu) and phorbol 12,13-didecanoate (PDD) and diacylglycerol analogues; 1-oleoyl-2-acetyl-sn-glycerol (OAG) and 1,2-dioctanoyl-sn-glycerol (DOG) also stimulated ACTH release from intact AtT-20 cells. This would suggest that activation of protein kinase C (PKC) stimulates ACTH secretion from AtT-20 cells.

3 Calcium stimulated ACTH secretion from electrically-permeabilized cells over the concentration-range of 10^{-7} M to 10^{-5} M. PMA (10^{-7} M) enhanced the amount of ACTH secreted at every concentration of calcium investigated. The PKC inhibitor, chelerythrine (10^{-5} M) blocked the PMA (10^{-7} M)-evoked enhancement of calcium (10^{-5} M)-stimulated ACTH secretion but did not alter significantly the calcium (10^{-5} M)-evoked secretion itself. This suggests that PKC modulates the secretory response to increases in intracellular calcium but does not mediate the effects of calcium.

4 Guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S, 10^{-5} M) stimulated ACTH secretion from permeabilized cells in the absence of calcium and was additive with calcium-evoked ACTH secretion up to a maximum value which could be achieved by calcium acting alone. This suggests that a GTP-binding protein mediates the secretory response to increases in the intracellular calcium. PMA (10^{-7} M) enhanced ACTH secretion stimulated by the combination of calcium and GTP-γ-S (10^{-5} M).

5 GTP-γ-S stimulated ACTH secretion from permeabilized cells in a concentration-dependent manner with a threshold of 10^{-6} M. PMA (10^{-7} M) increased the amount of ACTH secretion evoked by every concentration of GTP-γ-S investigated. Chelerythrine (10^{-5} M) blocked the PMA (10^{-7} M)-evoked enhancement of GTP-γ-S (10^{-4} M)-stimulated ACTH secretion but did not significantly alter GTP-γ-S (10^{-4} M)-evoked secretion itself. This suggests that PKC modulates the secretory response to GTP-γ-S but does not mediate the effects of GTP-γ-S.

6 GTP-γ-S (10^{-8}–10^{-4} M) stimulated ACTH secretion from permeabilized cells either in the presence or absence of ATP (5 mM) indicating that its effects on secretion are ATP-independent.

7 The results of the present study support the hypothesis that, in AtT-20 cells, PMA is acting at some site distal to calcium entry which modulates the ability of an increase in cytosolic calcium concentration to stimulate ACTH secretion. This site of action is either at the level of or at some stage distal to a GTP-binding protein which mediates the effects of calcium upon secretion.

8 PMA, unlike adenosine 3':5'-cyclic monophosphate (cyclic AMP) (Guild, 1991), can stimulate ACTH secretion from permeabilized cells in the absence of added calcium and guanine nucleotides which suggests that PMA and cyclic AMP are acting through distinct mechanisms at this post calcium site of action.

Keywords: PMA; protein kinase C; calcium; G-protein; anterior pituitary cell line; adrenocorticotrophin (ACTH)

Introduction

Calcium has long been established as a trigger to hormone secretion (Douglas, 1968) and supporting evidence for this comes from the use of permeabilized cells in which the cytosolic free calcium concentration can be controlled. Calcium has been shown to stimulate secretion from a variety of permeabilized cell types (for review see Knight & Scrutton, 1986) including adrenocorticotrophin (ACTH) secretion from the mouse AtT-20/D16-16 anterior pituitary cell line, a model system for the normal corticotroph (Luini & De Matteis, 1988; Guild, 1991). The ability of a particular concentration of calcium to stimulate hormone secretion can however be influenced by various factors including other second messengers.

The adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase A (PKA), which mediates the effects of changes in cellular cyclic AMP content (for review see Taylor *et al.*, 1990), stimulates ACTH secretion from AtT-20 cells and from rat anterior pituitary corticotrophs (Aguilera *et al.*, 1983; Axelrod & Reisine, 1984; Heisler & Reisine, 1984; Miyazaki *et al.*, 1984). The action of this enzyme upon the secretory pathway in AtT-20 cells is thought to be due, at least partly, to an interaction with the calcium messenger system on at least two levels. Cyclic AMP enhances calcium entry via voltage-dependent calcium channels, thus raising intracellular calcium levels (Luini *et al.*, 1985; Guild & Reisine, 1987). In addition cyclic AMP potentiates the ability of a particular increase in cytosolic calcium levels to stimulate ACTH secretion (Guild *et al.*, 1986; Guild, 1991). These results suggest two points of regulation for PKA including a post calcium site of action (Guild, 1991).

Calcium stimulates hormone secretion in AtT-20 cells via stimulation of a guanosine 5'-triphosphate (GTP)-binding protein and therefore intracellular concentrations of GTP

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analogues such as guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) can stimulate ACTH secretion from AtT-20 cells in the absence of calcium (Luini & De Matteis, 1988; 1990; Guild, 1991). This GTP-binding protein involved in the late stages of the secretory pathway, termed G_E by Gomperts and co-workers, is present in a variety of secretory cells (for review see Gomperts, 1990). Cyclic AMP enhances GTP-γ-S stimulated ACTH secretion from AtT-20 cells suggesting this post calcium site of action is either at the level of G_E or at some stage distal to this protein (Guild, 1991).

Protein kinase C (PKC) is a kinase which is also regulated by second messenger action (for review, see Hug & Sarre, 1993). This enzyme also plays an important role in ACTH secretion from AtT-20 cells (Reisine & Guild, 1987) and in the normal corticotroph (Abou-Samra *et al.*, 1986). The ability of PKC to stimulate ACTH secretion from AtT-20 cells is again due, at least partly, to an interaction with the calcium messenger system. Phorbol esters such as phorbol 12-myristate 13-acetate (PMA), activators of PKC (for review, see Hug & Sarre, 1993), stimulate a rapid increase in intracellular free calcium levels in this cell line (Reisine & Guild, 1987; Reisine, 1989) and thus stimulate hormone secretion. This increase in cytosolic calcium levels induced by PMA in AtT-20 cells is however transient but the induced ACTH secretion is sustained (Reisine & Guild, 1987) suggesting that the rise in cytosolic calcium cannot account completely for the triggered hormone secretion. This study, therefore, investigated the possibility that PKC has a similar post calcium site of action to that of PKA.

The results of the present study suggest that, in AtT-20 cells, PMA is acting at some stage distal to changes in free calcium concentrations to modulate calcium-evoked ACTH secretion. In addition, such an action may be at the level of, or at some stage distal to G_E, suggesting that PKC does have a similar post calcium site of action to that of PKA. However PMA stimulated ACTH secretion in the absence of calcium and guanine nucleotides which was not the case for cyclic AMP (Guild, 1991) suggesting that PKA and PKC may be acting through distinct mechanisms. PKC acts to modulate the regulation of ACTH secretion by the calcium/G_E system but is not essential to the stimulation of secretion by this system. Preliminary accounts of this study have been communicated to the British Pharmacological Society (McFerran *et al.*, 1994).

Methods

Culture of AtT-20 cells

Cells of the mouse AtT-20/D16-16 pituitary tumour cell line were grown and subcultured in Dulbecco's modified Eagle's medium (DMEM) (4500 mg glucose 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 in 24-well (16 mm diameter) multiwell plates (Costar, U.S.A.) at an initial density of 10^5 cells/well and were used 5–7 days after subculturing (80–90% confluency). Cells to be used in experiments involving electrically permeabilized cells were plated in 75 cm^2 flasks (Nunc, Gibco, UK) at an initial density of 2×10^6 cells/flask and were used 7–9 days after subculturing (80–90% confluency).

Preparation of AtT-20 cells

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% CO_2 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, cells adhering to the substrate were liberated by trypsin (0.05% w/v)/EDTA (1 mM). The cells were washed twice by centrifugation (200 *g*, 5 min)/resuspension in a balanced salt solution of the following composition (mM): NaCl 145, KCl 5.6, $CaCl_2$ 2, $MgCl_2$ 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 10^6 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 cell pellet washed twice by resuspension/centrifugation (200 *g*, 5 min) in the standard permeabilization buffer of the following composition (mM): potassium glutamate 129, PIPES (potassium salt) 20; glucose 5; ATP 5; EGTA 5; BSA 0.1% (w/v); pH 6.6. The cells were finally resuspended in this buffer at a density of 4×10^7 cells ml^{-1} and electrically permeabilized by subjection to intense electric fields of brief duration (Knight & Baker, 1982). Optimum permeabilization parameters were determined as previously described (Guild, 1991) and were found to be 10 discharges each of 2500 V cm^{-1}. These parameters were adopted in these experiments.

Measurement of stimulated ACTH secretion from intact cells

The ability of phorbol esters and diacylglycerol analogues to stimulate ACTH secretion from intact AtT-20 cells remaining attached to the culture dishes was measured as previously described (Hook *et al.*, 1982). 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% CO_2 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 stimulated ACTH secretion from permeabilized AtT-20 cells

Calcium-stimulated ACTH secretion Permeabilized cells were suspended at a density of 10^5 cells ml^{-1} in a series of calcium-EGTA buffers chosen to give free calcium concentrations over the range of 10^{-9}–10^{-5} M as previously described (Guild, 1991). At this point the zero time samples were centrifuged (200 *g*, 5 min) and samples of the supernatant 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, 4°C) and samples of the supernatant stored for subsequent measurement of ACTH content. In experiments investigating the effects of phorbol esters, diacylglycerol analogues and GTP-γ-S upon calcium-evoked ACTH secretion, the calcium-EGTA buffers were supplemented by PMA, PDD, PDBu, DOG, OAG or GTP-γ-S as indicated in the legends to the figures. In each experiment sextuplicate samples of the supernatant were removed from each condition and the ACTH content measured by radioimmunoassay.

Guanine nucleotide-stimulated ACTH secretion Permeabilized cells were suspended at a density of 10^5 cells ml^{-1} in a calcium-EGTA buffer (10^{-9} M) supplemented with GTP-γ-S (10^{-8}–10^{-4} M) as previously described (Guild, 1991). The effect of PMA upon GTP-γ-S (10^{-8}–10^{-4} M)-evoked ACTH secretion were investigated by co-incubation of PMA with GTP-γ-S (10^{-8}–10^{-4} M). At this point the zero time samples were centrifuged (200 *g*, 5 min) and samples of the supernatant 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, 4°C) and samples of the supernatant stored for subsequent measurement of ACTH content. In each experiment sextuplicate samples of the supernatant were removed from each condition and the ACTH content measured by radioimmunoassay.

The effect of inhibiting PKC upon calcium- and guanine nucleotide-stimulated ACTH secretion

The PKC inhibitor, chelerythrine (Herbert *et al.*, 1990), was used to investigate whether calcium and GTP-γ-S stimulated PKC to evoke ACTH secretion. This possibility of an indirect regulation of ACTH secretion, mediated by PKC, by these agents was tested by measuring calcium- and guanine nucleotide-stimulated ACTH secretion (as described above) in the presence and absence of chelerythrine (10^{-5} M). In addition, the effect of omitting ATP from the permeabilization media, and hence depriving protein kinases of ATP for their actions, upon guanine nucleotide-stimulated ACTH secretion was investigated.

Radioimmunoassay

The radioimmunoassay for ACTH was performed as previously described (Reisine, 1984). [^{125}I]-ACTH for radioimmunoassay use was produced using the lodogen reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) which was first described as a reagent for iodination by Fraker & Speck (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

A two-sided, 0.05 level, paired *t* test was used to determine if the effect of a treatment was significant.

Materials

The following substances (with their sources) were used: ATP, bovine serum albumin (BSA) (fraction V), 4α phorbol, phorbol 12,13-dibutyrate (PDBu), phorbol 12,13-didecanoate (PDD), phorbol 12-myristate 13-acetate (PMA) from Sigma, UK; 1-oleoyl-2-acetyl-sn-glycerol (OAG), 1,2-dioctanoyl-sn-glycerol (DOG) and chelerythrine chloride from Calbiochem-NovaBiochem, UK; guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) from Boehringer Mannheim, UK; DMEM, foetal calf serum and trypsin EDTA from GIBCO, UK; human ACTH antiserum and human ACTH standards were a gift of the National Hormone and Pituitary programme, Baltimore, MD, U.S.A.; Iodogen iodination reagent from Pierce & Warriner. All other chemicals were of Analar grade and readily commercially available.

Results

The effect of PKC activators upon ACTH secretion from intact AtT-20 cells

PMA (10^{-12}–10^{-7} M) stimulated a concentration-dependent increase in ACTH secretion (Figure 1) as previously shown (Reisine & Guild, 1987). ACTH secretion in response to PMA was significantly greater than control at concentrations of 10^{-10} M and above with a 4 fold increase over basal obtained at maximum. A supramaximal concentration of 10^{-7} M was chosen for subsequent use of PMA in the following experiments. The phorbol esters phorbol 12,13 didecanoate (PDD) and phorbol 12,13 dibutyrate (PDBu) (Table 1) and the diacylglycerol analogues 1-oleoyl-2-acetyl-sn-glycerol (OAG) and 1,2-dioctanoyl-sn-glycerol (DOG) (Table 2), at concentrations of 10^{-7} M and 10^{-4} M respectively, produced a similar significant increase in ACTH secretion (4–5 fold over basal). However, the inactive 4 α-phorbol did not stimulate secretion to the same extent (Table 1).

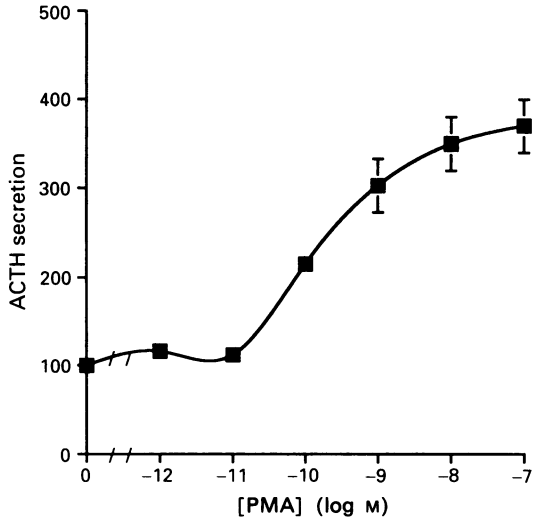

Figure 1 Effect of phorbol 12-myristate 13-acetate (PMA) on adrenocorticotrophin (ACTH) secretion from intact AtT-20 cells. Intact cells were incubated for 1 h in Dulbecco's Modified Eagles Medium with 0.1% bovine serum albumin (DMEM/BSA) supplemented with the indicated concentration of PMA. The hormone release results were standardized and expressed as % of the control responses, the control responses being that obtained in the absence of PMA. 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.

Table 1 Effects of phorbol 12,13-dibutyrate (PDBu), phorbol 12,13-dideconoate (PDD) and 4 α phorbol on adrenocorticotrophin (ACTH) secretion from intact AtT-20 cells

Phorbol ester (10^{-7} M)	*ACTH secretion* (% of control)
PDBu	413 ± 26
PDD	437 ± 64
4 α phorbol	148 ± 28

Intact cells were incubated for 1 h in Dulbecco's Modified Eagles Medium with 0.1% bovine serum albumin (DMEM/BSA) in the absence of phorbol ester or in the presence of 10^{-7} M phorbol ester. The hormone release results were standardized and expressed as % of the control responses, the control responses being that obtained in the absence of phorbol ester. The results are expressed as the mean ± s.e.mean from 3 separate experiments.

Table 2 Effects of 1-oleoyl-2-acetyl-sn-glycerol (OAG) and 1,2-dioctanoyl-sn-glycerol (DOG) on adrenocorticotrophin (ACTH) secretion from intact AtT-20 cells

Diacylglycerol analogue (10^{-4} M)	*ACTH secretion* (% of control)
OAG	398 ± 31
DOG	456 ± 75

Intact cells were incubated for 1 h in Dulbecco's Modified Eagles Medium with 0.1% bovine serum albumin (DMEM/BSA) in the absence of diacylglycerol analogue or in the presence of 10^{-4} M diacylglycerol analogue. The hormone release results were standardized and expressed as % of the control responses, the control responses being those obtained in the absence of diacylglycerol analogue. The results are expressed as the mean ± s.e.mean from 3 separate experiments.

The effect of PMA and chelerythrine 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 2). Calcium stimulated ACTH secretion in a concentration-dependent manner between 10^{-7} and 10^{-5} M as previously shown (Guild, 1991). ACTH secretion in response to free calcium concentrations of 10^{-6} M and above were significantly greater than the 10^{-9} M calcium control. PMA (10^{-7} M) significantly enhanced calcium evoked ACTH secretion from permeabilized AtT-20 cells at all concentrations of calcium investigated (Figure 2). Importantly, PMA significantly enhanced ACTH secretion in the effective absence of calcium (free calcium concentration of 10^{-9} M). The protein kinase c inhibitor, chelerythrine (10^{-5} M), did not significantly alter the ACTH secretion obtained at either 10^{-9} M (cont.) or 10^{-5} M (Ca^{2+}) calcium (Figure 3a) but inhibited the PMA (10^{-7} M)-evoked enhancement of ACTH secretion at both of these calcium concentrations (Figure 3a). This suggests that PKC modulates the secretory response to increases in intracellular calcium but does not mediate the effects of calcium.

The effect of guanine nucleotides and PMA upon calcium-evoked ACTH secretion from permeabilized AtT-20 cells

GTP-γ-S (10^{-5} M), a non-hydrolysable GTP analogue which persistently activates GTP-binding proteins, significantly stimulated ACTH secretion in the absence of calcium (free calcium concentration of 10^{-9} M, Figure 4). When co-incubated with a range of free calcium concentrations this effect of GTP-γ-S (10^{-5} M) was additive with that of calcium up to a maximum value which could be produced by calcium acting alone, obtained at 10^{-5} M calcium, at which point there was no significant difference between ACTH secretion obtained in the presence or absence of GTP-γ-S (10^{-5} M) (Figure 4). This is consistent with the concept that a GTP-binding protein mediates the effect of calcium upon ACTH secretion in AtT-20 cells. Co-incubation of PMA (10^{-7} M) with either a range of free calcium concentrations alone or with a range of free calcium concentrations supplemented with GTP-γ-S (10^{-5} M) resulted in a significant enhancement of both calcium (10^{-9}–10^{-5} M)-evoked ACTH secretion and ACTH secretion in response to the combination of calcium (10^{-9}–10^{-5} M) and GTP-γ-S (10^{-5} M) (Figure 4). The potentiation of calcium (10^{-9}–10^{-5} M)-evoked ACTH secretion by PMA (10^{-7} M) remained significant at all concentrations of calcium investigated. The enhancement by PMA (10^{-7} M) of the calcium (10^{-9}–10^{-5} M)/GTP-γ-S (10^{-5} M) combination was significant at all calcium concentrations except 10^{-8} M calcium. There was no significant difference between ACTH secretion obtained in response to the combination of calcium

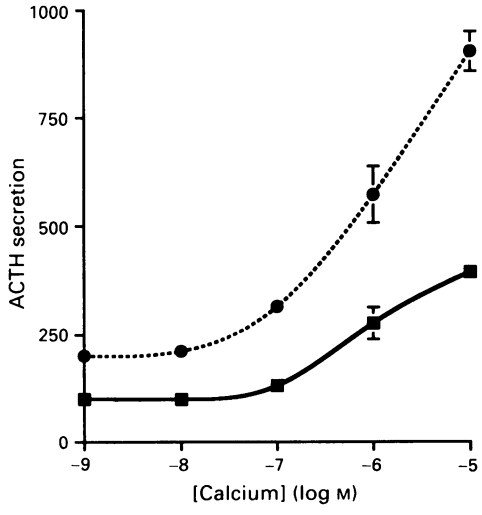

Figure 2 Effect of phorbol 12-myristate 13-acetate (PMA) on calcium-dependent adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium containing various proportions of calcium and EGTA, such that the indicated concentrations of free calcium resulted, either in the presence (●) or absence (■) of PMA (10^{-7} M). The hormone release results were standardized and expressed as % of the control responses, the control responses being those obtained in the absence of calcium (10^{-9} M). 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.

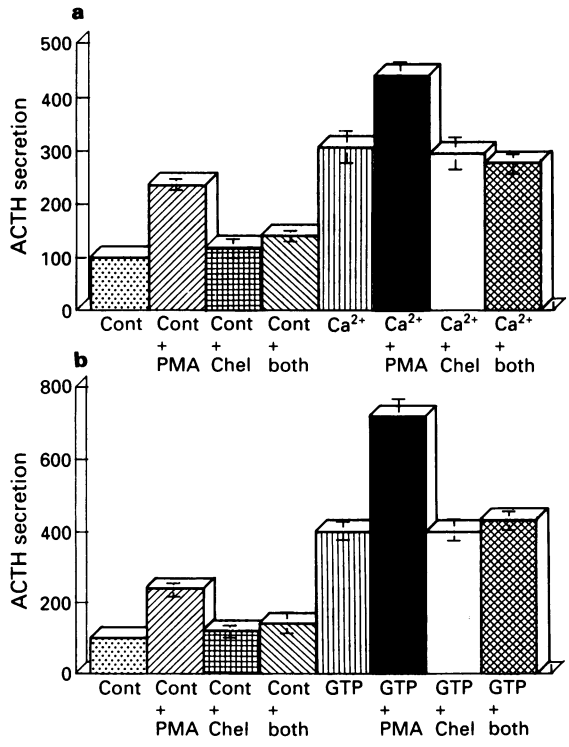

Figure 3 Effect of chelerythrine upon calcium- and guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S)-dependent adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells and the ability of phorbol 12-myristate 13-acetate (PMA) to potentiate their effects. (a) Permeabilized cells were incubated in standard permeabilization medium containing calcium-EGTA buffers designed to give 10^{-9} M free calcium (Cont) or 10^{-5} M free calcium (Ca^{2+}) either in the presence (Cont + PMA, Ca^{2+} + PMA) or absence (Cont, Ca^{2+}) of PMA (10^{-7} M). The effects of chelerythrine (10^{-5} M) at 10^{-9} M free calcium (Cont + Chel) and at 10^{-5} M free calcium (Ca^{2+} + Chel) and upon combination of 10^{-9} M free calcium plus PMA (10^{-7} M) (Cont + both) and 10^{-5} M free calcium plus PMA (10^{-7} M) (Ca^{2+} + both) were measured. The hormone release results were standardized and expressed as % of the control responses, the control responses being those obtained in the absence of calcium (10^{-9} M). The results are expressed as the mean ± s.e.mean from 3 separate experiments. (b) Permeabilized cells were incubated in standard permeabilization medium containing calcium-EGTA buffers designed to give 10^{-9} M free calcium (Cont) alone or supplemented with GTP-γ-S (10^{-4} M) (GTP) either in the presence (Cont + PMA, GTP + PMA) or absence (Cont, GTP) of PMA (10^{-7} M). The effects of chelerythrine (10^{-5} M) at 10^{-9} M free calcium (Cont + Chel) and GTP-γ-S (10^{-4} M) (GTP + Chel) and upon combination of 10^{-9} M free calcium plus PMA (10^{-7} M) (Cont + both) and GTP-γ-S (10^{-4} M) plus PMA (10^{-7} M) (GTP + both) were measured. The hormone release results were standardized and expressed as % of the control responses, the control responses being those obtained in the absence of calcium (10^{-9} M). The results are expressed as the mean ± s.e.mean from 3 separate experiments.

(10^{-5} M) and PMA (10^{-7} M) with that of the combination of calcium (10^{-5} M, PMA 10^{-7} M) and GTP-γ-S (10^{-5} M). A maximum ACTH secretion could be achieved by calcium and PMA together at which point there was no significant difference between ACTH secretion obtained in response to calcium and PMA (10^{-7} M) in the presence or absence of GTP-γ-S (10^{-5} M) (Figure 4).

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

GTP-γ-S stimulated ACTH secretion, in the absence of calcium, in a concentration-dependent manner with a threshold of 10^{-6} M and which became significant at concentrations of GTP-γ-S above 10^{-5} M (Figure 5). PMA (10^{-7} M) significantly stimulated ACTH secretion in the absence of GTP-γ-S and significantly enhanced the stimulated ACTH secretion at every concentration of the nucleotide investigated. Thus PMA enhanced both calcium and guanine nucleotide-evoked ACTH secretion. The protein kinase c inhibitor, chelerythrine (10^{-5} M), did not significantly alter the ACTH secretion obtained either in the absence (cont) or presence of 10^{-4} M GTP-γ-S (GTP) (Figure 3b) but inhibited the PMA (10^{-7} M)-evoked enhancement of ACTH secretion at both of these conditions (Figure 3b). This suggests that PKC modulates the secretory response to GTP-γ-S but does not mediate the effects of the nucleotide nor presumably the GTP-binding protein(s) activated by this agent.

Guanine nucleotide-evoked ACTH secretion in the presence and absence of ATP

GTP-γ-S (10^{-8}–10^{-4} M) stimulated ACTH secretion from permeabilized AtT-20 cells both in the presence and absence of ATP (5 mM) (Figure 6). The omission of ATP from the permeabilization medium significantly reduced the ACTH secretion obtained in the absence of GTP-γ-S and had the effect of reducing the base line from which GTP-γ-S stimulated ACTH secretion from permeabilized cells. Importantly, GTP-γ-S stimulated ACTH secretion to a similar degree at 10^{-4} M either in the presence or absence of ATP (5 mM). Thus GTP-γ-S-stimulated ACTH secretion in AtT-20 cells, unlike calcium-stimulated ACTH secretion (Guild,

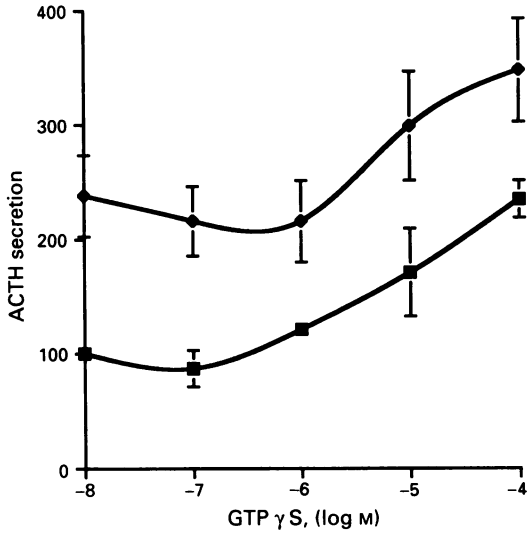

Figure 5 Effect of phorbol 12-myristate 13-acetate (PMA) on guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S)-stimulated adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of GTP-γ-S either in the presence (◆) or absence (■) of PMA (10^{-7} M). The hormone release results were standardized and expressed as % of the control responses, the control responses being those obtained in the presence of GTP-γ-S (10^{-8} M). 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.

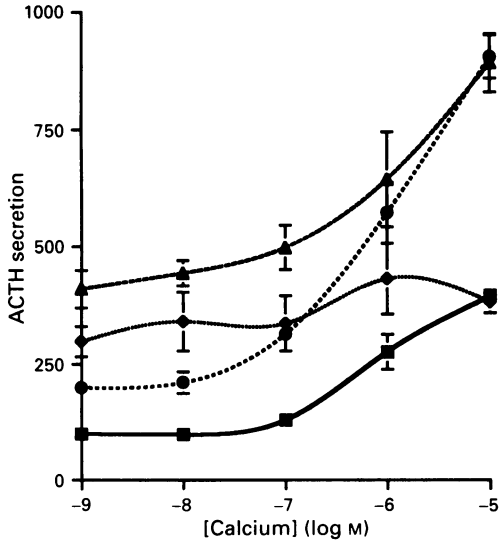

Figure 4 Effects of phorbol 12-myristate 13-acetate (PMA) and guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) on calcium-dependent adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium containing various amounts of calcium and EGTA, such that the indicated concentrations of free calcium resulted, in the presence of 10^{-7} M PMA (●), 10^{-5} M GTP-γ-S (◆), both 10^{-7} M PMA and 10^{-5} M GTP-γ-S (▲) or in the absence of both PMA and GTP-γ-S (■). The hormone release results were standardized and expressed as % of the control responses, the control responses being that obtained in the absence of calcium (10^{-9} M). 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.

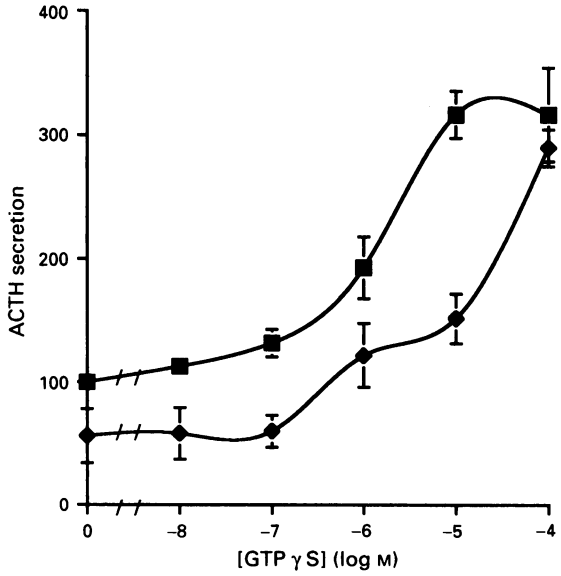

Figure 6 Guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S)-dependent ACTH secretion from permeabilized AtT-20 cells in the presence and absence of ATP. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of GTP-γ-S either in the presence (■) or absence (◆) of 5 mM ATP. The hormone release results were standardized and expressed as % of the control responses, the control responses being those obtained in the absence of GTP-γ-S in the presence of 5 mM ATP. 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.

1991), is not dependent upon the presence of ATP in the incubation medium. ATP could provide a source of GTP, via the enzyme nucleoside diphosphate kinase (Gomperts, 1990), and so result in a 'basal' stimulation of secretion via the G_E system upon which added GTP-γ-S exerted its effect.

Discussion

PMA, PDBu and PDD all stimulated ACTH secretion from intact AtT-20 cells. The ability of analogues of diacylglycerol; the physiological activator of PKC (Hug & Sarre, 1993), OAG and DOG, to stimulate a similar secretory response coupled with the inability of 4 α-phorbol, an inactive phorbol ester, to produce such a response suggest that the effects of PMA upon ACTH secretion from AtT-20 cells are mediated by PKC and are not due to a simple biophysical effect. The ability of PMA to stimulate ACTH secretion from AtT-20 cells is consistent with studies using the normal corticotroph (Abou-Samra *et al.*, 1986). PMA was chosen as a representative phorbol ester for further experiments to complement these studies in the normal corticotroph and previous studies in AtT-20 cells (Reisine & Guild, 1987; Reisine, 1989).

In order to determine if PMA was acting at a late stage in the secretory pathway, a more direct manipulation of the intracellular environment was required. 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 ability of calcium over the physiological range of 10^{-7} to 10^{-5} M 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 and in a variety of other permeabilized cell types (for review, see Knight & Scrutton, 1986). In these studies a free calcium concentration of 10^{-5} M produced maximal stimulation.

PMA enhanced calcium-dependent ACTH secretion from permeabilized AtT-20 cells. This suggests that, in permeabilized AtT-20 cells, PMA does not enhance ACTH secretion by increasing the cytosolic calcium concentration, since any change in calcium concentration would be buffered by the calcium-EGTA buffers which are designed for this purpose. Therefore PMA, in addition to stimulating calcium entry into the cell across the plasma membrane (Reisine & Guild, 1987; Reisine, 1989), may enhance ACTH secretion from AtT-20 cells by an action distal to changes in the cytosolic calcium concentration. Previous studies show PMA induced a transient rise in the cytosolic calcium concentration in AtT-20 cells however the resultant ACTH secretion is sustained (Reisine & Guild, 1987). This additional post calcium site of action, therefore, may be responsible for the more sustained nature of the ACTH secretion induced by PMA.

It is difficult to interpret apparent additivity of secretory responses in studies of this sort and caution should be exercised when making claims about physiological mechanisms underlying these observations. Notwithstanding these limitations, in this study there was an apparent interaction between calcium and PMA which was additive at lower calcium concentrations and which become synergistic at higher calcium concentrations. Whilst acknowledging the dangers, a possible explanation for this can be put forward. PKC is now known to consist of a group of at least nine different PKC isoenzymes (for review, see Hug & Sarre, 1993). This family of isozymes can be divided into two main groups, the calcium-dependent or conventional PKCs (cPKCs) and the calcium-independent or novel PKCs (nPKCs) (Ohno *et al.*, 1991). PMA is able to stimulate both cPKCs and nPKCs although calcium is required for maximal stimulation of cPKCs by PMA (Ryves *et al.*, 1991). Therefore the additive enhancement by PMA of calcium-stimulated secretion seen at lower calcium concentrations may be due to stimulation of nPKCs which are active in the absence of calcium and partial stimulation of cPKCs. The synergistic interaction between calcium and PMA seen at higher calcium concentrations could therefore be due to stimulation of both nPKCs and cPKCs which become fully stimulated by PMA at higher calcium concentrations (Ryves *et al.*, 1991).

The ability of GTP-γ-S to enhance calcium-stimulated secretion confirms a previous report from this lab suggesting a GTP-binding mediates the effects of calcium upon ACTH secretion from AtT-20 cells (Guild, 1991). The ability of PMA to potentiate the calcium/GTP-binding protein system suggests that, in AtT-20 cells, the post-calcium site of interaction between calcium and PMA is either at the level of, or at some stage distal to, G_E. Cyclic AMP has been shown to have a similar post-calcium site of action (Guild, 1991), but cyclic AMP requires the stimulation of ACTH secretion by either calcium or guanine nucleotides in order for this post-calcium potentiation of ACTH secretion from AtT-20 cells to be manifest (Guild, 1991). In contrast, this study shows that PMA is able to stimulate ACTH secretion from permeabilized AtT-20 cells in the absence of both calcium and guanine nucleotides. Therefore both PMA and cyclic AMP enhance the calcium/GTP-binding protein system; however, PMA has the additional action of enhancing secretion in the absence of calcium or GTP-γ-S. This important difference may suggest that although cyclic AMP and PMA both have similar post-calcium sites of action they are acting through distinct mechanisms to enhance calcium-stimulated ACTH secretion. This has already been shown to be the case at an earlier stage in the stimulus-secretion coupling pathway. PMA and cyclic AMP both stimulate ACTH secretion from AtT-20 cells by enhancing calcium entry into the cell across the plasma membrane but do so through distinct mechanisms (Reisine & Guild, 1987, Reisine, 1989).

The ability of PMA 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-G_E system upon secretion. This argument was used to support the idea that a GTP binding protein mediated the effects of calcium upon the secretory apparatus of AtT-20 cells (this study and Guild, 1991) as added guanine nucleotides stimulated a step distal to that regulated by calcium. The data in this study, however, do not support the hypothesis that PKC mediates the effect of calcium-G_E system upon secretion. Firstly, PMA potentiates calcium-evoked ACTH secretion and the combination of the two evoked much greater ACTH secretion than could be obtained by calcium alone. If PKC mediated calcium-evoked ACTH secretion then addition of PMA and activation of PKC would show the same pattern of interaction upon hormone secretion as seen with GTP-γ-S and calcium. Secondly, the PKC inhibitor, chelerythrine, did not inhibit calcium- or GTP-γ-S-evoked ACTH secretion from AtT-20 cells. This agent did, however, inhibit the PMA-evoked potentiation of both calcium- and GTP-γ-S-evoked ACTH secretion from AtT-20 cells indicating that it did indeed inhibit PKC in AtT-20 cells but that this did not result in any significant attenuation of the secretory response to activation of the calcium-G_E system. Another possibility for the actions of PMA in the absence of calcium and added GTP-γ-S is that PKC, in addition to an action at a late stage (i.e. post G_E) in the secretory pathway, may also stimulate a parallel pathway to promote ACTH secretion. From the data presented in this study, it would appear that PKC plays a modulatory role in regulating secretion and is not necessary for secretion. In this sense AtT-20 cells are similar to adrenal chromaffin, PC12 cells, neuroendocrine and mast cells (for review, see Burgoyne & Morgan, 1993).

GTP-γ-S is able to stimulate ACTH secretion from permeabilized AtT-20 cells, under the present conditions, in the absence of ATP to a similar degree to that obtained in the presence of ATP. This argues against the involvement of a protein kinase in mediating the effect of G_E upon secretion

and leads us to believe that ATP in this system serves mainly as a modulator as suggested for rat mast cells (Gomperts, 1990). We have previously demonstrated that calcium-evoked secretion from permeabilized AtT-20 cells is dependent upon the presence of ATP in the permeabilization media (Guild, 1991). We believe that ATP in this case provides a source of GTP via the enzyme nucleoside diphosphate kinase (Gomperts, 1990) and so permits the stimulation of secretion via the calcium/G_E system when activated by calcium. These results are similar to those obtained in permeabilized rat mast cells (Lillie & Gomperts, 1992; Koffer & Churcher, 1993) and in adrenal chromaffin cells (reviewed in Burgoyne & Morgan, 1993).

The nature of the GTP-binding protein (G_E) controlling the late stage of ACTH secretion in AtT-20 cells is as yet unclear. Synaptic vesicles and secretory granules possess both monomeric ras-like (Burgoyne & Morgan, 1989; Darchen *et al.*, 1990; Fischer von Mollard *et al.*, 1991) and trimeric (Toutant *et al.*, 1987) GTP-binding proteins. Rab 3 proteins (monomeric ras-like GTP-binding proteins) have been shown in AtT-20 cells to be important for localisation, sequestration and storage of secretory vesicles (Ngsee *et al.*, 1993). An inhibitory form of G_E (G_{Ei}), thought to be a trimeric protein, is responsible for inhibition of secretion in AtT-20 cells (Luini & DeMatteis, 1988) and in chromaffin cells (Vitale *et al.*, 1993). Whether the stimulatory form of G_E (G_{Es}) in AtT-20 cells is similarly a trimeric GTP-binding protein has yet to be determined.

The results of this and a previous study (Guild, 1991) 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 hormone (Dannies, 1982). It is clear that calcium alone cannot stimulate the maximal possible hormone secretion and that co-operation with PKC and PKA increases the amount of hormone secreted in response to a particular concentration of calcium. Interestingly, in mast cells GTP-γ-S-stimulated secretion requires a phosphorylated 'primed' state but no MgATP needs to be utilized during the process of exocytosis (Koffer & Churcher, 1993). It is tempting to speculate that in AtT-20 pituitary cells the protein kinases modulate the secretory response by maintaining a 'primed' state and the size of the readily-releasable pool of hormone but the exocytotic machinery is directly controlled by the calcium/G_E system (Gomperts, 1990).

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