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A direct inhibitory action of prostaglandins upon ACTH secretion at the late stages of the 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 the effects of prostaglandins upon the late stages of the adrenocorticotrophin (ACTH) secretory pathway.

2 Calcium (1 nM–100 μM), guanosine-5′-O-(3-thiotriphosphate) (GTP-γ-S) (1–100 μM) and mastoparan (1 and 10 μM) all stimulated ACTH secretion from permeabilized AtT-20 cells in a concentration-dependent manner. GTP-γ-S and mastoparan stimulated ACTH secretion from permeabilized cells in the absence of calcium. Co-incubation with prostaglandins E_1 and E_2 (PGE_1, PGE_2) (10 μM) but not prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) (10 μM) significantly inhibited calcium-, GTP-γ-S and mastoparan-evoked secretion by 30–50%.

3 The effects of PGE_1 and PGE_2 upon GTP-γ-S (100 μM)-, calcium (10 μM)- and mastoparan (10 μM)-evoked secretion were concentration-dependent. PGE_1 significantly inhibited GTP-γ-S- and calcium-evoked secretion at concentrations of PGE_1 above 1 μM but mastoparan-evoked secretion only at the highest concentration of PGE_1 investigated (10 μM). PGE_2 was much more potent than PGE_1 and significantly inhibited GTP-γ-S- and calcium-evoked secretion at 10 nM and above and mastoparan-evoked secretion above 1 μM.

4 The inhibitory effects of PGE_1 and PGE_2 upon calcium-, GTP-γ-S- and mastoparan-stimulated ACTH secretion from permeabilized cells were pertussis toxin (PTX) sensitive.

5 In intact cells PGE_1, PGE_2 and $PGF_{2\alpha}$ (1 nM–10 μM) acting singly had little or no effect upon ACTH secretion. However, only PGE_2 (1 nM–10 μM) significantly inhibited corticotrophin-releasing factor-41 (CRF-41) (100 nM)-evoked secretion in a concentration dependent manner.

6 The present study finds that prostaglandins of the E series exert an inhibitory action, *via* a pertussis toxin-sensitive GTP-binding (G)-protein, in the late stages of the ACTH secretory pathway distal to the G-exocytosis (Ge)/calcium point of control.
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Keywords: ACTH; anterior pituitary; calcium; prostaglandins; G-proteins; exocytosis

Abbreviations: ACTH, adrenocorticotrophic hormone; CRF-41, corticotrophin-releasing factor-41; DMEM, Dulbecco's modified Eagle's medium; Ge, G-exocytosis; G-protein, GTP-binding protein; GTP-γ-S, guanosine-5′-O-(3-thiotriphosphate; PIPES, piperazine-N,N′-bis[2-ethanesulphonic acid]; prostaglandins E_1, E_2 and $F_{2\alpha}$, PGE_1, PGE_2, $PGF_{2\alpha}$; PTX, pertussis toxin; s.e.mean, standard error of the mean

Introduction

Adrenocorticotrophin (ACTH) secretion from anterior pituitary corticotrophs is under the control of a variety of hypothalamic neuropeptides, neurotransmitters and circulating hormones (for review see Axelrod & Reisine, 1984). It is an advantage in studies of stimulus-secretion coupling mechanisms to have homogenous populations of cells and such a population of corticotrophs can be provided by a pituitary tumour cell lines such as the AtT-20 cell line (Sabol, 1980). In this cell line signal-transduction studies have demonstrated a role for G-proteins, termed Ge (Gomperts, 1990), in mediating the ability of calcium ions to stimulate secretion (Guild, 1991; Erlich *et al*., 1998; Luini & Dematteis, 1988; 1990; McFerran & Guild, 1995). One of the agents used to activate Ge in AtT-20 cells, mastoparan (McFerran & Guild, 1995; Erlich *et al*., 1998), has also been reported to directly activate phospholipase A_2 (Argiolas & Pisano, 1983). The significance of this lies in the evidence that activation of phospholipase A_2, the liberation of arachidonic acid and further metabolism of this product may have an important role in stimulus-secretion coupling in AtT-20 cells (Abou-Samra *et al*., 1986; Hamill *et al*., 1981; Luini & Axelrod, 1985; Won & Orth, 1994).

A recent study from this laboratory addressed the question as to whether the actions of mastoparan could be partly due to an activation of phospholipase A_2 and the liberation of arachidonic acid as well as to an activation of Ge (Guild, 2001). No late stage role for phospholipase A_2 or its products in mediating the stimulation of ACTH secretion was found but an inhibitory late-stage action distal to the calcium/Ge point of control was, however, indicated (Guild, 2001). Thus the role of phospholipase A_2 in stimulus-secretion coupling is a complex one. There are actions of its products at earlier stages of the secretory pathway to stimulate or facilitate calcium-evoked secretion (Abou-Samra *et al*., 1986; Hamill *et al*., 1981; Luini & Axelrod, 1985;

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Won & Orth, 1994) and actions at the late stages of the secretory pathway to inhibit secretion (Guild, 2001). The ability of the cyclo-oxygenase inhibitor ibuprofen to prevent the late stage inhibition of calcium/Ge-evoked- secretion by phospholipase A_2 and its products suggests that it is a prostaglandin that is responsible for this inhibitory late stage regulation (Guild, 2001). This is consistent with the reports of an inhibitory autocrine action of prostaglandins in the ACTH secretory pathway (Vlaskovska & Knepel, 1984; Vlaskovska *et al*., 1984) and suggests that this action is at least partly exerted at the late stages of the secretory pathway.

The present study investigated the possibility of a late-stage inhibition of ACTH secretion and finds that prostaglandins of the E series exert an inhibitory action distal to the Ge/calcium point of control. Furthermore, this inhibitory action is *via* a PTX-sensitive G-protein and supports the contention that there is a role for both stimulatory and inhibitory heterotrimeric G-proteins in the direct control of exocytosis (Erlich *et al*., 1998).

Methods

Culture of AtT-20 cells

Cells of the mouse AtT-20 anterior pituitary tumour cell line were grown and sub-cultured in Dulbecco's modified Eagle's medium (DMEM) (4500 mg glucose l^{-1}) supplemented with 10% (v v^{-1}) foetal calf serum as previously described (Reisine, 1984). Cells to be used in experiments involving electropermeabilization were plated in 75 cm^2 flasks (Nunc, Gibco, U.K.) at an initial density of 2×10^6 cells per flask and were used 7–9 days after sub-culturing (80–90% confluency). Routinely between $8-14 \times 10^6$ cells were harvested from each culture flask. Cells to be used in ACTH release experiments from intact cells were plated in 24-well (16 mm diameter) multiwell plates (Corning, U.S.A.) at an initial density of 10^5 cells per well and were used 7–9 days after sub-culturing (80–90% confluency).

Preparation of cells

For intact cells the culture medium was removed from the multiwell plates and cells adhering to the well surface were washed twice with 1 ml of DMEM supplemented with 0.1% (w v^{-1}) BSA (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.

AtT-20 cells were prepared for permeabilization as previously described (Guild, 1991). In brief cells were liberated from the substrate, washed twice by centrifugation ($200 \times g$, 5 min)/resuspension in a balanced salt solution of the following composition (mM): NaCl 145, KCl 5.6, $CaCl_2$ 0.5, glucose 5.6, HEPES 5, BSA 0.1% (w v^{-1}): pH 7.4. and suspended at a density of 10^6 cells ml^{-1} and incubated for a further 30 min at 37°C. The cell suspension was then centrifuged ($200 \times g$, 5 min) and washed twice by centrifugation ($200 \times g$, 5 min)/resuspension in a standard permeabilization buffer of the following composition (mM): potassium glutamate 129, piperazine-N,N′-bis[2-ethanesulphonic acid] (PIPES) (potassium salt), 20, glucose 5, ATP 5, MgCl 1, EGTA 5. BSA 0.1 % (w v^{-1}); pH 6.6. The cells were finally resuspended at a density of 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). The standard permeabilization medium was essentially calcium free with a free calcium concentration of 1 nM.

The effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ upon basal and CRF-stimulated ACTH secretion from intact cells

In intact cell experiments prostaglandins E_1, E_2 and $F_{2\alpha}$ were added to the 1 ml of DMEM/BSA bathing the cells in the wells of the culture dishes to give the concentrations indicated in the figure legends. Zero time samples were taken at this point and the remaining cells incubated for 2 h at 37°C in a humidified atmosphere of 10% CO_2 in air. Additionally, CRF (100 nM) was co-incubated with prostaglandins E_1, E_2 and $F_{2\alpha}$ at the concentrations indicated in the figure legends as above. Incubations were terminated by removing the DMEM/BSA from the wells, centrifugation ($10{,}000 \times g$, 30 s) of this sample and the removal of the supernatant. In each experiment, six replicate samples of each condition were analysed. The ACTH content of the supernatant was measured by radioimmunoassay.

The effects of prostaglandins E_1, E_2 and $F_{2\alpha}$ upon calcium-, GTP-γ-S- and mastoparan-stimulated ACTH secretion from permeabilized cells

Permeabilized cells were suspended at a density of 10^7 cells ml^{-1} in the standard permeabilization medium either in the presence or absence of prostaglandins E_1, E_2 and $F_{2\alpha}$ (10 μM) and pre-incubated at 37°C for 15 min. These pre-treated, permeabilized cells were subsequently challenged by further suspension at a density of 10^5 cells ml^{-1} in either (1) standard permeabilization medium to investigate the effect of the Ge activators, GTP-γ-S- and mastoparan or (2) a series of calcium/EGTA buffers designed to give a free calcium concentration in the range of 1 nM–100 μM as previously described (McFerran & Guild, 1996). Zero time samples were centrifuged ($200 \times g$, 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 either in the continued presence or absence of prostaglandins E_1, E_2 and $F_{2\alpha}$ (10 μM) at which point incubations were terminated by centrifugation ($200 \times g$, 5 min) and removal of the supernatant. The ACTH content of which was measured by radioimmunoassay.

The concentration-dependency of the effects of the prostaglandins E_1, E_2 and $F_{2\alpha}$ upon calcium (10 μM)-, GTP-γ-S (100 μM)- and mastoparan (10 μM)-stimulated ACTH secretion from permeabilized cells

Permeabilized cells were suspended at a density of 10^7 cells ml^{-1} in the standard permeabilization medium either in the presence or absence of various concentrations of prostaglandins E_1, E_2 and $F_{2\alpha}$ (1 nM–10 μM) and pre-incubated at 37°C for 15 min. These pre-treated, permea-

bilized cells were subsequently challenged by further suspension at a density of 10^5 cells ml^{-1} in the appropriate permeabilization medium in the presence and absence of calcium (10 μM), GTP-γ-S (100 μM) or mastoparan (10 μM). These experimental incubations were also performed either in the continued presence or absence of prostaglandins E_1, E_2 and $F_{2\alpha}$ (1 nM – 10 μM) as appropriate. At this point, the zero time samples were centrifuged ($200 \times g$, 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 ($200 \times g$, 5 min) and removal of the supernatant. The ACTH content of which was measured by radioimmunoassay.

The PTX-sensitivity of the actions of prostaglandins

The effect of PTX pre-treatment upon the secretory responsiveness of AtT-20 cells was investigated by using cells which had been grown in medium supplemented with 1 μg ml^{-1} PTX for 16 h as previously described (Erlich *et al*., 1998).

Radioimmunoassays

The radioimmunoassay for ACTH performed as previously described (McFerran & Guild, 1995). [^{125}I]-ACTH was produced using the Iodogen reagent first described as an agent for iodination by Fraker & Speck (1978). The amount of ACTH secreted was expressed as the amount present at the end of the incubation period less the amount present at zero time.

Statistics

In each experiment sextuplicate determinations at each experimental condition were made and each experiment was repeated three times on different days. ACTH secretion is expressed as the mean ± standard error of the mean (s.e mean). from these three experiments. Statistical significance was determined by means of by use of analysis of variance (ANOVA) tests with Scheffe's *F*-test *post hoc* analysis. The statistical significance of a particular treatment was determined using a two-way ANOVA test. In both cases a *P* value less than or equal to 0.05 was considered significant and is used in the text to signify such.

Materials

The following substances (with their sources) were; PTX, mastoparan, adenosine 5′-triphosphate (ATP), BSA (fraction V), from Sigma, U.K.; GTP-γ-S from Boehringer Mannheim, U.K.; CRF-41 from Peninsula Labs, U.K.; DMEM, foetal calf serum and trypsin/EDTA from GIBCO, U.K.; human ACTH antiserum and human ACTH standards were a gift of the National Hormone and Pituitary programme, Baltimore, MD, U.S.A.; anti-rabbit IgG was a gift of the Diagnositcs Scotland, Carluke, Lanarkshire, U.K.; Iodogen iodination reagent from Pierce and Warriner. All other chemicals were of Analar grade and readily commercially available.

Results

The effect of prostaglandins E_1, E_2 and $F_{2\alpha}$ upon calcium ion-, GTP-γ-S - and mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells

The amount of ACTH secreted from permeabilized AtT-20 cells was dependent upon the concentration of free calcium ions in the permeabilization medium (Figure 1). ACTH secretion was stimulated significantly above that in calcium-free conditions (1 nM) at concentrations of 1 μM calcium ions and above (threshold of 100 nM). Maximal ACTH secretion was observed at concentrations of 10 μM and above. Co-incubation with prostaglandins E_1 and E_2 (10 μM) significantly inhibited calcium-evoked secretion (Figure 1). This inhibition was in the region of 30 – 50%. The prostaglandin $PGF_{2\alpha}$ was without any significant effect upon calcium-evoked ACTH secretion (data not shown).

GTP-γ-S stimulated ACTH secretion in the absence of calcium in a concentration-dependent manner (Figure 2A). This stimulation was significantly greater than control at concentrations of 10 μM and above. Mastoparan (1 and 10 μM) also stimulated ACTH secretion from permeabilized cells in the absence of calcium (Figure 2B). This stimulation was significantly greater than control at both concentrations of the peptide investigated. Co-incubation with prostaglandins E_1 and E_2 (10 μM) significantly inhibited both GTP-γ-S- and mastoparan-evoked secretion (Figure 2A,B). The prostaglandin $PGF_{2\alpha}$ was without any significant effect upon GTP-γ-S- and mastoparan -evoked ACTH secretion (data not shown).

Concentration-dependency of the effects of prostaglandins E_1, E_2

These effects of prostaglandins E_1 and E_2 upon GTP-γ-S (100 μM)- and mastoparan-evoked secretion were concen-

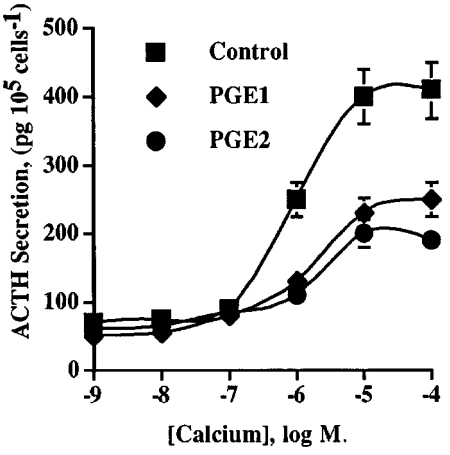

Figure 1 The effects of calcium ions in the presence of the prostaglandins E_1 and E_2 (10 μM) upon secretion from permeabilized cells. Permeabilized cells were pre-incubated and incubated in a series of calcium-EGTA buffers with the indicated free calcium ion concentrations alone or in the presence of PGE_1 (10 μM) or PGE_2 (10 μM) as described in Methods. The results are expressed as the mean ± s.e.mean from three separate experiments. Absence of error bars indicates that they lie within the symbol used.

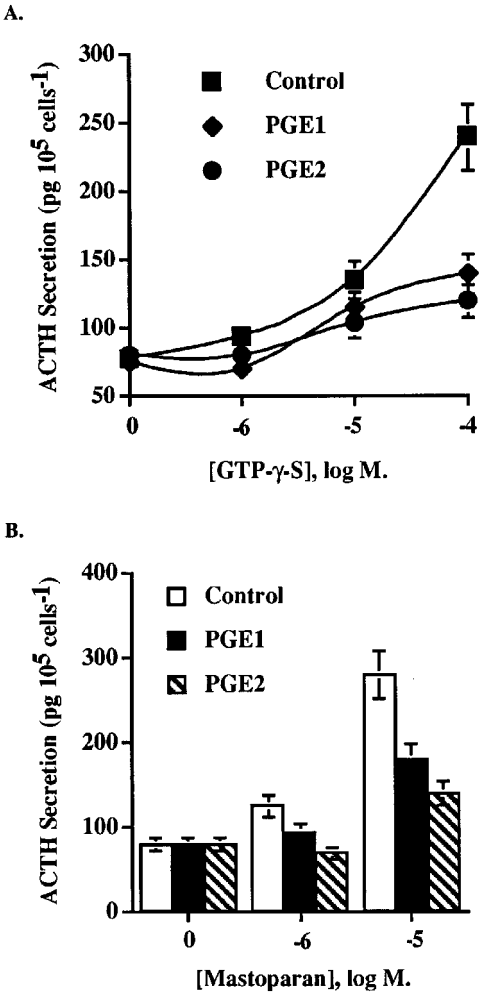

Figure 2 The effect of prostaglandins E_1 and E_2 (10 μM) upon GTP-γ-S- and mastoparan-evoked ACTH secretion from permeabilized cells. Permeabilized cells were pre-incubated and incubated in a calcium-EGTA buffer containing a free calcium ion concentration of 1 nM supplemented with the indicated concentrations of GTP-γ-S (A) or mastoparan (B) either in the absence or presence of PGE_1 (10 μM) or PGE_2 (10 μM) as described in Methods. The results are expressed as the mean$\pm$s.e.mean from three separate experiments. Absence of error bars indicates that they lie within the symbol used.

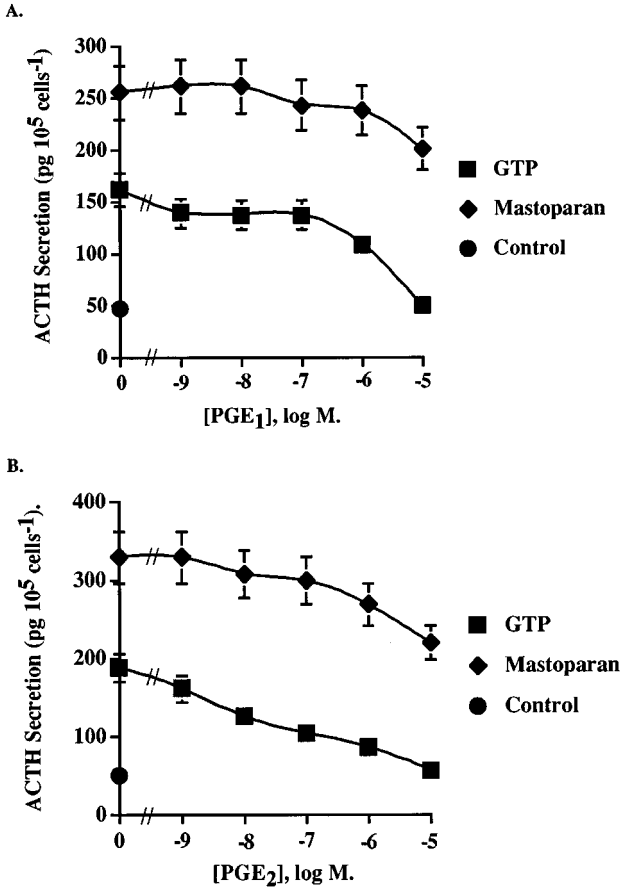

Figure 3 The concentration-dependency of the effects of prostaglandins E_1 and E_2 upon GTP-γ-S- and mastoparan-evoked ACTH secretion from permeabilized cells. Permeabilized cells were pre-incubated and incubated in a calcium-EGTA buffer containing a free calcium ion concentration of 1 nM alone or supplemented with GTP-γ-S (100 μM) or mastoparan (10 μM) co-incubated with the indicated concentrations of PGE_1 (A), or PGE_2 (B) as described in Methods. The results are expressed as the mean$\pm$s.e.mean from three separate experiments. Absence of error bars indicates that they lie within the symbol used.

tration-dependent (Figure 3A,B). Differences, however, were observed in the potencies of prostaglandins E_1 and E_2-mediated inhibitions of GTP-γ-S (100 μM)- and mastoparan (10 μM)-evoked secretion. PGE_1 significantly inhibited GTP-γ-S (10 μM)-evoked secretion at concentrations of 1 μM and above and mastoparan (10 μM)-evoked secretion only at the highest concentration of PGE_1 investigated (i.e. 10 μM). PGE_2, however, significantly inhibited GTP-γ-S (10 μM)-evoked secretion at concentrations of 10 nM and above and mastoparan (10 μM)-evoked secretion at the concentration of PGE_2 above 1 μM. Thus PGE_2 was in the order of 10–100 times more potent in inhibiting Ge-evoked secretion than was PGE_1. A similar concentration-dependency for the effects of prostaglandins E_1 and E_2 upon calcium (10 μM)-evoked secretion was observed (Table 1). The concentration of PGE_1 and PGE_2 used in the experiments summarized in Figures 1 and 2 (i.e. 10 μM) was thus supramaximal for PGE_2 but sub-maximal for PGE_1.

The effects of PTX pretreatment upon prostaglandins E_1 and E_2-mediated inhibition of calcium ion-, GTP-γ-S - and mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells

The inhibitory effects of PGE_1 and PGE_2 upon calcium-, GTP-γ-S- and mastoparan-stimulated ACTH secretion from permeabilized cells were PTX-sensitive. Calcium (10 μM), GTP-γ-S (100 μM) and mastoparan (10 μM) all stimulated ACTH secretion (Figure 4A,B). Co-incubation with either PGE_1 (10 μM) (Figure 4A) or PGE_2 (10 μM) (Figure 4B) inhibited this stimulation of secretion by all three agents in the previously observed range of 30–50%. Pre-treatment of the cells with 1 μg ml^{-1} PTX for 16 h, however, prevented the inhibition of calcium-, GTP-γ-S- and mastoparan-evoked secretion by both PGE_1 and PGE_2 (Figure 4A,B).

Table 1 The concentration-dependency of the effects of prostaglandins E_1 and E_2 upon calcium-evoked ACTH secretion from permeabilized cells

Treatment	*ACTH secretion* (pg 10^5 cells^{-1})
Control	48±3
Calcium (10 μM)	182±15
Calcium (10 μM) + PGE_1 (1 nM)	180±14
Calcium (10 μM) + PGE_1 (10 nM)	168±14
Calcium (10 μM) + PGE_1 (100 nM)	158±13
Calcium (10 μM) + PGE_1 (1 μM)	120±10
Calcium (10 μM) + PGE_1 (10 μM)	90±4
Calcium (10 μM)	179±19
Calcium (10 μM) + PGE_2 (1 nM)	162±15
Calcium (10 μM) + PGE_2 (10 nM)	126±12
Calcium (10 μM) + PGE_2 (100 nM)	105±10
Calcium (10 μM) + PGE_2 (1 μM)	87±9
Calcium (10 μM) + PGE_2 (10 μM)	77±4

Permeabilized cells were pre-incubated and incubated in a calcium-EGTA buffer containing a free calcium ion concentration of 10 μM alone or co-incubated with the indicated concentrations of PGE_1 or PGE_2 as described in Methods. The results are expressed as the mean±s.e.mean from three separate experiments.

The effect of prostaglandins E_1, E_2 and $F_{2\alpha}$ upon basal and CRF-evoked ACTH secretion from intact AtT-20 cells

The effects of PGE_1, PGE_2 and $PGF_{2\alpha}$ (1 nM–10 μM) acting alone were investigated and small (20–30%) but significant increases in ACTH secretion were observed at a concentration of 10 μM of PGE_2 and $PGF_{2\alpha}$ only (Table 2). Despite both PGE_1 and PGE_2 inhibiting calcium-, GTP-γ-S- and mastoparan-stimulated ACTH secretion from permeabilized cells only PGE_2 significantly inhibited CRF, the most potent and effective natural stimulant of ACTH secretion *in vivo* (Vale *et al*., 1981)-evoked secretion from intact cells (Table 3). This effect of PGE_2 was seen at concentrations of 100 nM and above.

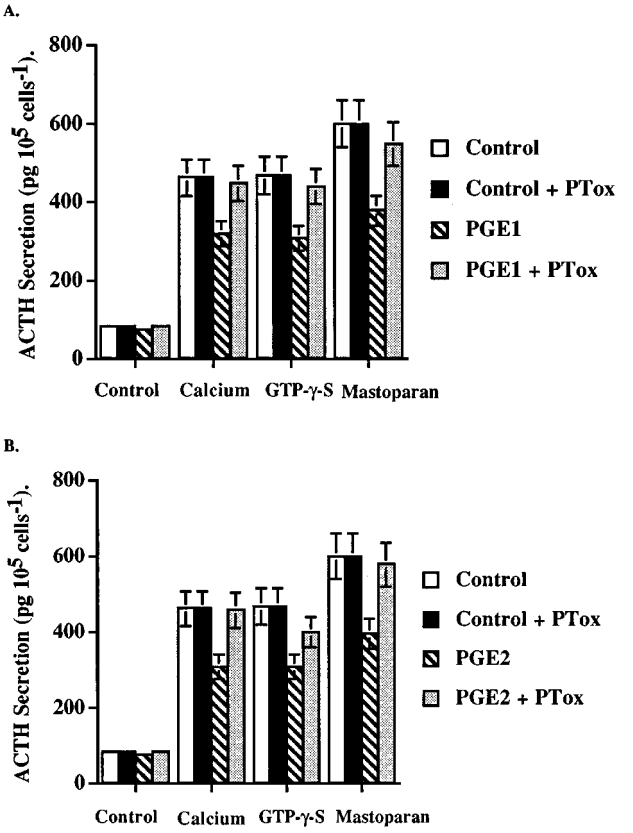

Figure 4 The effect of PTX pre-treatment upon PGE_1 (10 μM)- and PGE_2 (10 μM)-evoked inhibition of calcium (10 μM)-, GTP-γ-S (100 μM)- and mastoparan (10 μM)-stimulated ACTH secretion. Cells were treated with pertussis toxin (1 μg ml^{-1}, 16 h) or vehicle alone as indicated in A and B. Permeabilized cells were then pre-incubated as described in Methods and then suspended in either a calcium-EGTA buffer containing a free calcium ion concentration of 10 μM alone (Calcium) or one containing 1 nM alone (Control) or supplemented with GTP-γ-S (100 μM) (GTP-γ-S) or mastoparan (10 μM) (Mastoparan) as indicated either in the absence or presence of PGE_1 (10 μM) (A) or PGE_2 (10 μM) (B) as described in Methods. The results are expressed as the mean±s.e.mean from three separate experiments. Absence of error bars indicates that they lie within the symbol used.

Discussion

Activation of phospholipase A_2 and the liberation of arachidonic acid and its products has been shown to have an important role in stimulus-secretion coupling for regulated ACTH secretion (Abou-Samra *et al*., 1986; Hamill *et al*., 1981; Luini & Axelrod, 1985; Won & Orth, 1994). Calcium-dependent activation of a membrane bound phospholipase A_2 and the liberation of arachidonic acid has been reported in AtT-20 cells (Hamill *et al*., 1981). Furthermore, the metabolites of arachidonic acid formed *via* the cytochrome P-450 epoxygenase and/or lipoxygenase pathway are reportedly involved in the stimulation of ACTH secretion from AtT-20 cells (Luini & Axelrod, 1985). However, not all of the actions of the products of arachidonic acid are stimulatory because prostaglandins can be inhibitory in the ACTH secretory pathway (Vlaskovska & Knepel, 1984; Vlaskovska *et al*., 1984). Nonetheless, this evidence suggests that activation of phospholipase A_2, the liberation of arachidonic acid and further metabolism of this product may have an important role in stimulus-secretion coupling in AtT-20 cells. The details of these roles are still being elucidated.

A limitation of the use of intact cells in studies of the roles of phospholipase A_2 and arachidonic acid in stimulus-secretion coupling mechanisms for ACTH secretion is that all aspects of these mechanisms will be manifest in the overall secretory response. A combination of the use of intact and permeabilized cells permits a greater focus upon early and/or late stages of the secretory pathway. This approach has revealed an inhibitory late-stage action of phospholipase A_2 and its products upon the ACTH secretory pathway (Guild, 2001). Furthermore, in the same study the ability of the cyclo-oxygenase inhibitor ibuprofen to prevent this inhibition of calcium/Ge-evoked secretion suggests that it is a prostaglandin that is responsible for this inhibitory late stage regulation (Guild, 2001).

The present study confirms this hypothesis and illustrates a late stage inhibitory action by prostaglandins of the E series upon ACTH secretion from AtT-20 cells. Prostaglandin involvement in the regulation of ACTH secretion has been

Table 2 The effect of prostaglandins E_1, E_2 and $F_{2\alpha}$ upon basal ACTH secretion from intact AtT-20 cells

Treatment	*ACTH secretion* (pg well^{-1})
Control	501+31
CRF 100 nM	912+65
PGE_1 1 nM	510±25
PGE_1 10 nM	480±31
PGE_1 100 nM	516±28
PGE_1 1 μM	528±22
PGE_1 10 μM	473±21
PGE_2 1 nM	493±18
PGE_2 10 nM	493±24
PGE_2 100 nM	421±44
PGE_2 1 μM	461±35
PGE_2 10 μM	612±33
$PGF_{2\alpha}$ 1 nM	490±26
$PGF_{2\alpha}$ 10 nM	474±28
$PGF_{2\alpha}$ 100 nM	462±32
$PGF_{2\alpha}$ 1 μM	542±34
$PGF_{2\alpha}$ 10 μM	603±31

Intact cells were incubated with the indicated concentrations of drugs as described in Methods. The results are expressed as the mean±s.e.mean from three separate experiments.

Table 3 The effect of prostaglandin E_2 upon CRF41-evoked ACTH secretion from intact AtT-20 cells

Treatment	*ACTH secretion* (pg well^{-1})
Control	649±40
CRF(100 nM)	1582±81
CRF (100 nM) + PGE_2 1 nM	1462±75
CRF (100 nM) + PGE_2 10 nM	1413±73
CRF (100 nM) + PGE_2 100 nM	1362±63
CRF (100 nM) + PGE_2 1 μM	1350±61
CRF (100 nM) + PGE_2 10 μM	1312±63

Intact cells were incubated with the indicated concentrations of drugs as described in Methods. The results are expressed as the mean±s.e.mean from three separate experiments.

investigated for many years (Hedge, 1977; Labrie *et al*., 1982). Prostaglandins stimulate ACTH secretion but this effect is now mainly considered to be *via* a central action to stimulate CRF secretion from the hypothalamus (De Wied *et al*., 1969; Hedge & Hanson, 1972; Peng *et al*., 1970; Thompson & Hedge, 1978). Nonetheless, the presence of prostaglandins and prostaglandin receptors within the anterior pituitary have been demonstrated (Labrie *et al*., 1982; Malet *et al*., 1982; Ojeda *et al*., 1978; Pilote *et al*., 1982). However, conflicting reports on the actions of prostaglandins upon ACTH secretion at the level of the pituitary itself have been made. In fact, stimulatory actions (Buckingham & Hodges, 1977; Labrie *et al*., 1982), inhibitory actions (Hedge, 1976; Vale *et al*., 1978; Vlaskovska & Knepel, 1984; Vlaskovska *et al*., 1984) and no affect at all (De Wied *et al*., 1969) have all been found.

Intravenous injections of prostaglandins E_1, E_2 and $F_{2\alpha}$ into a rat were reported to be equipotent in stimulating ACTH secretion (Nasushita *et al*., 1997). Although not anticipating a stimulatory action of these prostaglandins, this study chose to compare all three of these prostaglandins upon ACTH secretion from intact AtT-20 cells. The effects of PGE_1, PGE_2 and $PGF_{2\alpha}$ acting alone were investigated and a stimulatory action for PGE_2 and $PGF_{2\alpha}$ were observed at only the highest concentration investigated. On the other hand PGE_2 significantly inhibited CRF-evoked secretion from intact cells confirming the inhibitory role for this prostaglandin already reported (Vlaskovska & Knepel, 1984; Vlaskovska *et al*., 1984). There may indeed be both stimulatory and inhibitory actions of prostaglandins upon ACTH secretion and the net response may depend upon whether or not ACTH secretion is being stimulated by a secretagogue or not. The stimulatory actions of prostaglandins may be exerted at an early, pre-calcium/Ge stage in the secretory pathway and so are bypassed in permeabilized cells. The inhibitory actions may be partly exerted at the post-calcium/Ge stage in the secretory pathway so are observed in permeabilized cells. This may explain the contradictory findings in the literature and be an inherent problem with the use of intact cells where both components are competing for dominance. This study was able to avoid this problem in part with the use of permeabilized cells that for all intents and purposes revealed only the inhibitory actions of prostaglandins upon a stimulated secretory system. The inhibitory action of PGE_2 is very clearly shown when ACTH secretion is stimulated in intact cells by CRF-41 and in permeabilized cells by calcium/Ge activators. In the basal secretory state in intact cells there may be a stimulatory and inhibitory component to its actions that cancel each other out under the conditions used in these experiments.

In our hands $PGF_{2\alpha}$ was ineffective in producing any inhibition of CRF-41-evoked secretion from intact cells or calcium/Ge-evoked secretion from permeabilized cells despite $PGF_{2\alpha}$ having been reported to inhibit CRF-evoked secretion from dispersed intact rat anterior pituitary cells (Cowell *et al*., 1991). This difference may be a species difference as AtT-20 cells are from a mouse or may be result of the transformation of mouse corticotrophs into a tumour cell line.

PGE_1 and PGE_2 both inhibited calcium-, GTP-γ-S- and mastoparan-evoked ACTH secretion from permeabilized cells. This indicates a post calcium and post-Ge (Erlich *et al*., 1998) site of inhibition by these arachidonic acid products. This separates the action of prostaglandins from the pre-calcium actions of other arachidonic acid products which stimulate secretion perhaps by enhancing the increases in intracellular calcium that stimulate ACTH secretion in these cells (Abou-Samra *et al*., 1986; Hamill *et al*., 1981; Luini & Axelrod, 1985; Won & Orth, 1994). This study exploited the great advantage of the use of permeabilized AtT-20 cells where ACTH secretion can be stimulated by G-protein activating agents in the complete absence of calcium ions. PGE_1 and PGE_2 were not equipotent in inhibiting Ge-controlled secretion. PGE_2 was much more potent than PGE_1 and this study supports the involvement of PGE_2 rather than PGE_1 or $PGF_{2\alpha}$. It is possible that the actions of PGE_1 are really *via* an action on PGE_2 receptors and that this is only manifest in permeabilized cells where the inhibitory action of prostaglandins is revealed unopposed by any stimulatory actions. In intact cells only PGE_2 significantly inhibits CRF-evoked secretion as expected.

This study also found that GTP-γ-S-evoked secretion is more susceptible to inhibition by PGE_2 than is mastoparan-evoked secretion. In our hands, PGE_2 inhibited mastoparan-evoked ACTH secretion to a lesser degree and at higher

concentrations than it did GTP-γ-S-evoked secretion. The reason for this may lie in the fact that mastoparan is reported to directly activate phospholipase A2 (Argiolas & Pisano, 1983) as well as Ge (McFerran & Guild, 1995). There may be two antagonistic actions of mastoparan going on simultaneously namely stimulation of secretion *via* Ge and inhibition of secretion *via* PGE_2 production (Guild, 2001). There could be a tonic inhibition of mastoparan-evoked secretion *via* endogenous PGE_2 production which is not the case with GTP-γ-S and so exogenously-added PGE_2 is less effective in inhibiting mastoparan-evoked secretion. Consistent with this hypothesis is the observation that the cyclo-oxygenase inhibitor ibuprofen enhances mastoparan-evoked ACTH secretion (Guild, 2001).

The data obtained in this study is consistent with the reports of an inhibitory autocrine action of prostaglandins in the ACTH secretory pathway (Vlaskovska & Knepel, 1984; Vlaskovska *et al*., 1984) and suggests that this action is at least partly exerted at the late stages of the secretory pathway. This study agrees with the findings of Vlaskovska and co-workers that it is PGE_2 that has this action. This action would be *via* prostaglandin receptors acting *via* heterotrimeric G-proteins. This is consistent with evidence in AtT-20 cells for heterotrimeric G-protein involvement in an inhibition of exocytosis (Gei) where we see a direct receptor-mediated, PTX-sensitive, inhibition *via* G-proteins of the late stages of the secretory pathway (Luini & De Matteis, 1988; 1990). This parallels the evidence for such a direct inhibitory control of exocytosis in chromaffin cells (Gasman *et al*., 1997; Vitale *et al*., 1993; 1994; 1995; 1996; 1997) and insulin secreting cells (Lang *et al*., 1995). Our evidence suggests that a Ge involved in the stimulation of secretion in AtT-20 cells could also be a heterotrimeric G-protein (Erlich *et al*., 1998; Guild, 1991; McFerran & Guild, 1995) indicating that there may be a dual regulation of ACTH secretion by G-proteins at the late stages of the exocytotic pathway with both a stimulatory Ges and an inhibitory Gei, (both possibly heterotrimeric) contributing to the control of the fusion machinery. The current study finds no late stage role for phospholipase A_2 or its products in mediating the stimulation of ACTH secretion by the calcium/Ge system but does indicate a separate late stage inhibitory action of prostaglandins distal to the calcium/Ge point of control.

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