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Effects of phospholipase A_2 activating peptides upon GTP-binding protein-evoked adrenocorticotrophin secretion

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Abstract

A GTP-binding protein (G-protein), termed G-exocytosis (Ge), mediates the effects of calcium ions in the late stages of the adrenocorticotrophin (ACTH) secretory pathway. An activator of Ge, mastoparan, also stimulates phospholipase A_2 and so a comparison of other phospholipase A_2-activating peptides, melittin and phospholipase A_2-activating peptide was made with mastoparan to assess whether phospholipase A_2 activation was an important component of Ge-evoked secretion. All three peptides stimulated ACTH secretion in the effective absence of calcium ions from permeabilised cells, actions potentiated by a phospholipase A_2 inhibitor. Ca^{2+}-evoked secretion from permeabilised cells was similarly potentiated by a phospholipase A_2 inhibitor. Furthermore, arachidonic acid inhibited Ca^{2+}- and Ge-evoked ACTH secretion, an action blocked by the cyclo-oxygenase inhibitor ibuprofen. This study suggests that the products of phospholipase A_2-generated arachidonic metabolism may exert an inhibitory action on the late post-Ca^{2+} stages of the ACTH secretory pathway and that prostaglandins may be the active agents in this capacity.

Keywords: G-protein; Phospholipase A_2; Exocytosis; ACTH (adrenocorticotrophin); Anterior pituitary

1. Introduction

Adrenocorticotrophin (ACTH) secretion from anterior pituitary corticotrophs is an important part of the body's response to stress and is under the control of a variety of hypothalamic neuropeptides, neurotransmitters and circulating hormones (for review, see Axelrod and Reisine, 1984). Any investigation of the intracellular stimulus-secretion coupling mediating the effects of these regulators of ACTH secretion is difficult in view of the heterogeneous nature of the cell populations of the anterior pituitary. Homogenous populations of cells can be provided by pituitary tumour cell lines, one of which, the AtT-20 cell line, can act as a model for the pituitary corticotroph (Sabol, 1980). Ca^{2+} has long been established as a trigger to hormone secretion (Douglas, 1968) with supporting evidence emerging from the use of permeabilised cells in which the cytosolic free calcium ion concentration can be controlled (for review, see Knight and Scrutton, 1986). Ca^{2+} stimulates ACTH secretion from permeabilised AtT-20 cells (Guild, 1991; Luini and Dematteis, 1988; McFerran and Guild, 1994, 1995a; McFerran et al., 1995).

Permeabilising cells also permits the introduction of normally impermeant substances into the cytosol and such an approach may yield details about the post-Ca^{2+} stimulus-secretion coupling mechanisms. Studies using the non-hydrolysable GTP analogue GTP-γ-S have demonstrated a role for GTP-binding (G)-proteins, termed G-exocytosis (Ge), in mediating the ability of calcium ions to stimulate secretion in a variety of secretory cell types (for review, see Gomperts, 1990) including AtT-20 cells (Guild, 1991; Luini and Dematteis, 1988, 1990; McFerran and Guild, 1995a). Further results suggesting that Ge belongs to the heterotrimeric family of G-proteins is partly based on the use of mastoparan and related peptides (Erlich et al., 1998; McFerran and Guild, 1995a,b; McFerran et al., 1995). Mastoparan is an amphiphilic tetradecapeptide with actions attributed to the activation of heterotrimeric G-proteins by a mechanism similar to agonist-bound receptors (Higashijima et al., 1988, 1990; Weingarten et al., 1990).

Mastoparan has also been reported to directly activate phospholipase A_2 (Argiolas and Pisano, 1983). The significance of this lies in the fact that activation of phospholipase A_2 and the liberation of arachidonic acid and its products have been shown to have an important role in mediating Ca^{2+}'s stimulatory effects upon regulated ACTH (Abou-Samra et al., 1986; Hamill et al., 1981;

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Luini and Axelrod, 1985; Won and Orth, 1994). The current study investigated whether mastoparan's actions in stimulating ACTH secretion from permeabilised cells could be due to a stimulation of phospholipase A_2 as well as Ge. To this end other phospholipase A_2-activating peptides such as melittin and phospholipase A_2-activating peptide were investigated and compared to mastoparan itself (Argiolas and Pisano, 1983; Peitsch et al., 1993). The effect of phospholipase A_2 inhibitors ONO-RS-082 (Konrad et al., 1992) and the arachidonic acid analogue arachidonyl trifluoromethyl ketone (AACOCF3) (Street et al., 1993) upon the ability of these phospholipase A_2-activating peptides and the Ca^{2+}/Ge system to stimulate secretion was also studied. The current study finds no late stage role for phospholipase A_2 or its products in mediating the stimulation of ACTH secretion by mastoparan and the Ca^{2+}/Ge system but does indicate a separate late stage inhibitory action of cyclo-oxygenase products distal to the Ca^{2+}/Ge point of control.

2. Material and methods

2.1. 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) foetal calf serum as previously described (Reisine, 1984). Cells to be used in experiments involving electropermeabilisation were plated in 75 cm^2 flasks (Nunc, Gibco, UK) 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 and 14×10^6 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, USA) at an initial density of 10^5 cells per well and were used 7–9 days after sub-culturing (80–90% confluency).

2.2. Preparation of cells

For intact cell preparations, cells grown in 24 well multiwell plates were used. 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) 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.

AtT-20 cells were prepared for permeabilisation 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, bovine serum albumin 0.1% (w/v): 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 permeabilisation buffer of the following composition (mM): potassium glutamate 129, piperazine-*N*,*N*′-bis[2-ethanesulfonic acid] (PIPES) (potassium salt) 20, glucose 5, ATP 5, MgCl 1, EGTA 5, bovine serum albumin 0.1% (w/v); pH 6.6. The cells were finally resuspended at a density of 10^7 cells ml^{-1} and electrically permeabilised by subjection to intense electric fields of brief duration (Knight and Baker, 1982). Optimum permeabilisation parameters were determined as previously described (Guild, 1991). The standard permeabilisation medium was essentially Ca^{2+} free with a free Ca^{2+} concentration of 10^{-9} M.

2.3. The effects of melittin, mastoparan phospholipase A_2-activating peptide on ACTH secretion from intact and permeabilised cells

In intact cell experiments mellitin, mastoparan or phospholipase A_2-activating peptide (10^{-7}–10^{-5} M) were added to the 1 ml of DMEM/BSA bathing the cells in the wells of the culture dishes to give the concentration indicated in the figure legends. Zero time samples were taken at this point and the remaining cells incubated for 3 h at 37 °C in a humidified atmosphere of 10% CO_2 in air. 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

Permeabilised cells were suspended at a density of 10^5 cells ml^{-1} in the standard permeabilisation medium in the presence and absence of mellitin, mastoparan or phospholipase A_2-activating peptide (10^{-7}–10^{-5} M) as previously described (McFerran and 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; other samples were incubated at 37 °C for 30 min and their ACTH content was measured by radioimmunoassay. The effects of the phospholipase A_2 inhibitor ONO-RS-082 upon melittin, mastoparan and phospholipase A_2-activating peptide-stimulated secretion was investigated by pre-incubation of the permeabilised cells in standard permeabilisation medium in the presence and absence of ONO-RS-082 (10^{-4} M) for 15 min. Subsequently cells were incubated in the presence and absence of melittin, mastoparan or phospholipase A_2-activating peptide (10^{-7}–10^{-5} M) with or without ONO-RS-082 (10^{-4} M) as described above.

2.4. The effects of calcium ions upon ACTH secretion

Permeabilised cells were suspended at a density of 10^5 cells ml^{-1} in a series of Ca^{2+}-EGTA buffers with free calcium ion concentration in the range of 10^{-9}–10^{-3} M. 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 remaining test samples were incubated at 37 °C for 30 min and their ACTH content was measured by radioimmunoassay. The effect of arachidonic acid or the arachidonic acid analogue AACOCF3 upon Ca^{2+}-stimulated ACTH secretion was investigated by a modification of the above procedures. Permeabilised cells were suspended at a density of 10^5 cells ml^{-1} in the standard permeabilisation medium in the presence or absence of arachidonic acid or AACOCF3 (10^{-4} M) and pre-incubated at 37 °C for 30 min. These pre-treated, permeabilised cells were subsequently challenged by further suspension in a series of EGTA buffers supplemented with vehicle alone, arachidonic acid or AACOCF3 (10^{-4} M) as appropriate and incubated as described above. The ability of calcium ions to stimulate ACTH secretion from permeabilised cells in the presence of the PLA_2-stimulating peptides mellitin, mastoparan or phospholipase A_2-activating peptide (all at 10^{-5} M) was investigated by incubating cells in the Ca^{2+}-EGTA buffers described above supplemented with one of the three PLA_2-stimulating peptides.

2.5. The effect of arachidonic acid upon Ge-evoked ACTH secretion

Permeabilised cells were suspended at a density of 10^5 cells ml^{-1} in the standard permeabilisation medium either in the presence or absence of arachidonic acid (10^{-4} M) and pre-incubated at 37 °C for 30 min. These pre-treated, permeabilised cells were subsequently challenged by further suspension in the standard permeabilisation medium in the presence and absence of GTP-γ-S (10^{-6}–10^{-4} M) or mastoparan (10^{-6}–10^{-5} M). These experimental incubations were also performed either in the continued presence and absence of arachidonic acid (10^{-4} M) 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 effects of the cyclo-oxygenase inhibitor ibuprofen upon GTP-γ-S (10^{-6}–10^{-4} M) and mastoparan (10^{-6}–10^{-5} M)-evoked secretion in the presence and absence of arachidonic acid was investigated by repeating the above procedures for GTP-γ-S or mastoparan + arachidonic acid (10^{-4} M) in the presence of ibuprofen (10^{-4} M). The above experiments investigating the effects of arachidonic acid and ibuprofen upon Ge-evoked secretion were repeated for the actions of melittin (10^{-5} M).

2.6. Radioimmunoassays

The radioimmunoassay for ACTH performed as previously described (McFerran and Guild, 1995a). [^{125}I]ACTH was produced using the Iodogen reagent first described as an agent for iodination by Fraker and 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.

2.7. 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 ± S.E.M. 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.

2.8. Materials

The following substances (with their sources) were used: arachidonic acid, mastoparan, ibuprofen, adenosine 5′-triphosphate (ATP), bovine serum albumin (BSA) (fraction V), from Sigma, UK; guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) from Boehringer Mannheim, UK; melittin, phospholipase A_2-activating peptide, ONO-RS-082, arachidonyl trifluoromethyl ketone (AACOCF3) from Alexis (UK); DMEM, foetal calf serum and trypsin/EDTA from Gibco; human ACTH antiserum and human ACTH standards were a gift of the National Hormone and Pituitary programme, Baltimore, MD, USA; anti-rabbit IgG was a gift of the Scottish antibody production unit, Carluke, Lanarkshire, UK; Iodogen iodination reagent from Pierce and Warriner. All other chemicals were of Analar grade and readily commercially available.

3. Results

3.1. The effects of the phospholipase A_2 activating peptides melittin, mastoparan and phospholipase A_2-activating peptide upon ACTH secretion from intact and permeabilised AtT-20 cells

Melittin and mastoparan stimulated ACTH secretion from intact cells in a concentration-dependent manner with a significant stimulation above control at concentrations of 10^{-6} M and above (Fig. 1, Panel A). Phospholipase

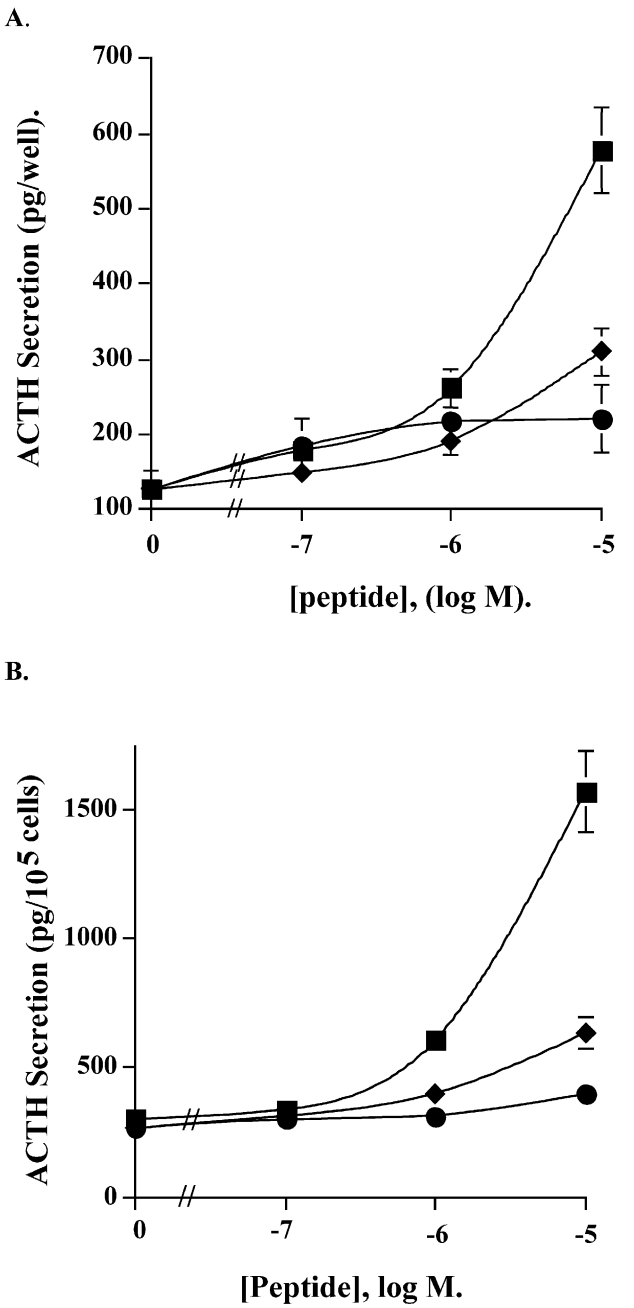

Fig. 1. Effect of mastoparan, melittin phospholipase A_2-activating peptide upon ACTH secretion from intact and permeabilised AtT-20 cells. Panel A: Intact cells were incubated with the indicated concentrations of melittin (■), mastoparan (♦) and phospholipase A_2-activating peptide (●) as described in Section 2. Panel B: Permeabilised cells were incubated in the standard permeabilisation medium, as described in Section 2, supplemented with the indicated concentrations of melittin (■), mastoparan (♦) and phospholipase A_2-activating peptide (●). ACTH release in both panels is expressed as the mean ± S.E.M. Absence of error bars indicates that they lie within the point.

A_2-activating peptide also stimulated ACTH secretion from intact cells but this stimulation above basal was only significantly above control at a concentration of 10^{-5} M (Fig. 1, Panel A). Melittin-evoked secretion was significantly greater than mastoparan at all effective concentrations of the peptides. Phospholipase A_2-activating peptide evoked the smallest increase in ACTH secretion of the three peptides investigated (a doubling of ACTH secretion compared to a threefold increase in secretion in response to mastoparan and a fivefold increase in response to mellitin; Fig. 1, Panel A).

Melittin and mastoparan stimulated ACTH secretion from permeabilised cells, in the effective absence of calcium ions, in a concentration dependent manner with a significant stimulation above basal at concentrations of 10^{-6} M and above (Fig. 1, Panel B). Melittin-evoked secretion was significantly greater than mastoparan at all effective concentrations of the peptides. Phospholipase A_2-activating peptide also stimulated ACTH secretion from permeabilised cells in the effective absence of calcium ions but this stimulation above basal was only significantly above control at a concentration of 10^{-5} M (Fig. 1, Panel B and Table 1). Phospholipase A_2-activating peptide evoked the smallest increase in ACTH secretion of the three peptides investigated (a 50% increase over basal compared to a 15-fold increase over basal in response to mellitin). Melittin, mastoparan and phospholipase A_2-activating peptide (10^{-5} M)-evoked secretion were all potentiated by co-incubation with the phospholipase A_2 inhibitor ONO-RS-082 (10^{-4} M) (Table 1). The degree of enhancement of secretion by co-incubation with ONO-RS-082 with these peptides was variable ranging from a 20% enhancement of basal secretion to a 50% enhancement of mellitin-evoked secretion (Table 1). Thus an inhibition of phospholipase A_2 results in an enhancement of secretion which means that the stimulation of ACTH secretion by these three peptides is therefore not likely to be due to an activation of phospholipase A_2.

3.2. The effects of calcium ions, arachidonic acid and AACOCF3 upon ACTH secretion from permeabilised cells

The amount of ACTH secreted from permeabilised AtT-20 cells was dependent upon the concentration of free

Table 1
The effect of melittin, mastoparan phospholipase A_2-activating peptide upon ACTH secretion in the presence and absence of ONO-RS-082

Phospholipase A_2-activating peptide	Control secretion (pg/10^5 cells)	+ ONO-RS-082 (10^{-4} M) (pg/10^5 cells)
Control	105 ± 9	120 ± 8
Melittin (10^{-5} M)	1496 ± 75	2216 ± 110
Mastoparan (10^{-5} M)	613 ± 26	875 ± 60
phospholipase A_2-activating peptide (10^{-5} M)	155 ± 12	201 ± 15

Permeabilised cells were incubated in the standard permeabilisation medium, as described in Section 2, supplemented with the indicated concentrations of melittin, mastoparan and phospholipase A_2-activating peptide either in the presence or absence of ONO-RS-082. ACTH release is expressed as the mean ± S.E.M. from three separate experiments.

calcium ions in the permeabilisation medium (Fig. 2). ACTH secretion was stimulated significantly above that in Ca^{2+}-free conditions (10^{-9} M) at concentrations of 10^{-6} M calcium ions and above (threshold of 10^{-7} M). Maximal ACTH secretion was observed at concentrations of 10^{-5} M and above. Co-incubation with arachidonic acid (10^{-4} M) significantly inhibited Ca^{2+}-evoked secretion (Fig. 2). This effect of arachidonic acid was concentration-dependent (data not shown) with 10^{-4} M chosen as a supramaximal concentration which gave a 30–40% reduction in Ca^{2+}-evoked secretion. The arachidonic acid analogue AACOCF3 (10^{-4} M), a phospholipase A_2 inhibitor, significantly enhanced ACTH secretion at every concentration of Ca^{2+} investigated including effectively Ca^{2+}-free conditions (Fig. 2). Thus, activation of phospholipase A_2 and generation of arachidonic acid acts at a post-Ca^{2+} stage to inhibit Ca^{2+}-evoked ACTH secretion. Any inhibition of phospholipase A_2 and the prevention of generation of products made from arachidonic acid removes a tonic inhibition of secretion.

3.3. The effects of calcium ions in the presence of the PLA_2 activating peptides melittin, mastoparan and phospholipase A_2-activating peptide upon secretion from permeabilised cells

Mastoparan (10^{-5} M) stimulated ACTH secretion from permeabilised cells in the absence of Ca^{2+} (10^{-9} M free Ca^{2+}) to a level that was not significantly different from that achieved by the maximally effective concentration of calcium ions acting alone (10^{-5} M and above) (Fig. 3). Mastoparan and Ca^{2+} were not additive upon ACTH secretion and either stimulant was able to achieve the same maximal ACTH secretion (Fig. 3). Melittin (10^{-5} M) stimulated ACTH secretion from permeabilised cells in the absence of Ca^{2+} but this was to a level significantly greater than that which could be achieved by Ca^{2+} alone (Fig. 3). Melittin and Ca^{2+} were not additive upon ACTH secretion. In contrast to the other two peptides, co-incubation with phospholipase A_2-activating peptide (10^{-5} M) significantly inhibited Ca^{2+}-evoked secretion (Fig. 3).

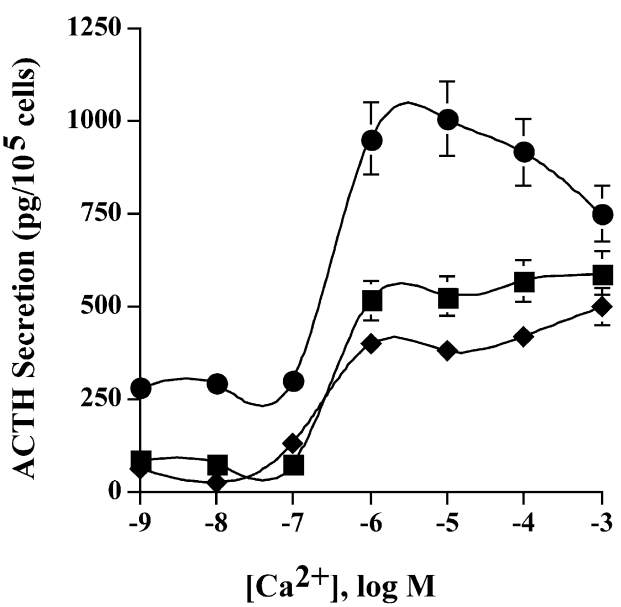

Fig. 2. The effects of calcium ions, arachidonic acid and AACOCF3 upon ACTH secretion from permeabilised cells. Permeabilised cells were incubated in a series of EGTA buffers with the indicated free calcium ion concentrations in the either in the presence (♦) or absence (■) of arachidonic acid (10^{-4} M) or presence of AACOCF3 (●) as described in Section 2. The results are expressed as the mean ± S.E.M. from three separate experiments. Absence of error bars indicates that they lie within the symbol used.

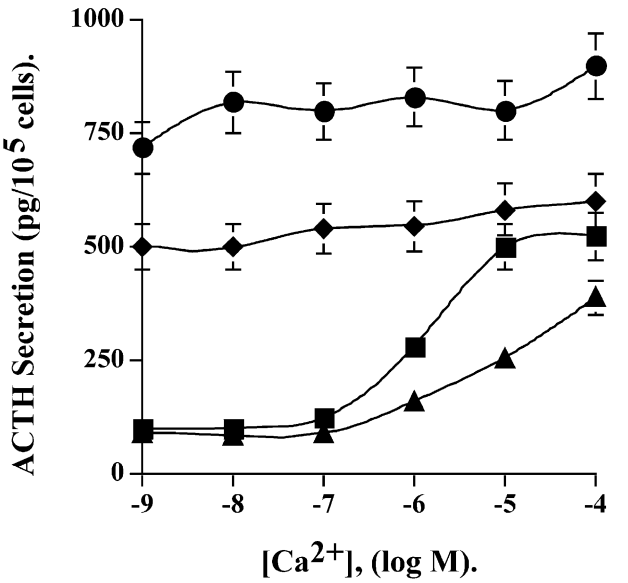

Fig. 3. The effects of calcium ions in the presence of the PLA_2 activating peptides melittin, mastoparan and phospholipase A_2-activating peptide upon secretion from permeabilised cells. Permeabilised cells were incubated in a series of Ca^{2+}-EGTA buffers with the indicated free calcium ion concentrations alone (■) or in the presence of mastoparan (10^{-5} M) (♦) melittin (10^{-5} M) (●) or phospholipase A_2-activating peptide (▲) as described in Section 2. The results are expressed as the mean ± S.E.M. from three separate experiments. Absence of error bars indicates that they lie within the symbol used.

3.4. The effect of arachidonic acid upon GTP-γ-S-, mastoparan-, and melittin-evoked ACTH secretion from permeabilised cells

GTP-γ-S stimulated ACTH secretion in the absence of Ca^{2+} in a concentration-dependent manner (Fig. 4, Panel A). This stimulation was significantly greater than control at concentrations of 10^{-5} M and above. Mastoparan (10^{-6} and 10^{-5} M) also stimulated ACTH secretion from permeabilised cells in the absence of Ca^{2+} (Fig. 4, Panel B). This stimulation was significantly greater than control at both concentrations of the peptide investigated. The absolute values for the maximal secretory responses to mastoparan (10^{-5} M) and GTP-γ-S (10^{-4} M) are given in

Table 2. This confirms the ability of G-proteins to stimulate ACTH secretion in the absence of calcium ions. Arachidonic acid (10^{-4} M) significantly inhibited both GTP-γ-S- and mastoparan-evoked secretion (Fig. 4, Panels A and B) paralleling its actions upon Ca^{2+}-evoked secretion. Arachidonic acid inhibited Ge-evoked secretion by 30–40%. These inhibitory actions of arachidonic acid were not observed in the presence of the cyclo-oxygenase inhibitor ibuprofen (10^{-4} M) (Table 2).

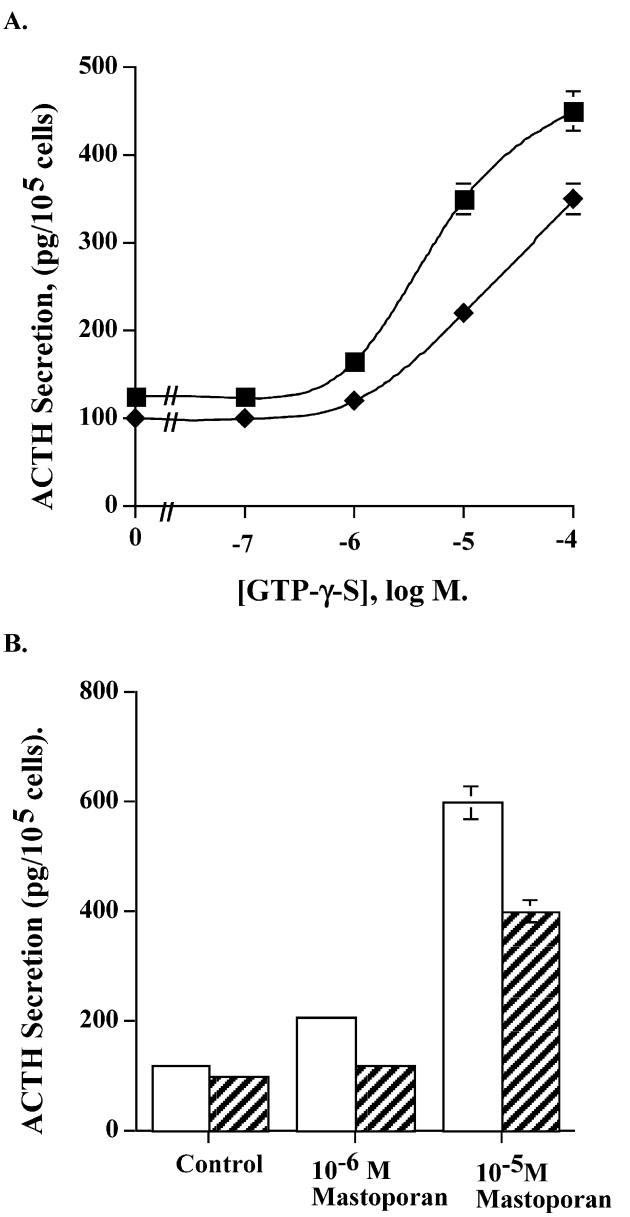

Fig. 4. The effect of arachidonic acid upon GTP-γ-S- and mastoparan-evoked ACTH secretion from permeabilised cells. Permeabilised cells were suspended in a Ca^{2+}-EGTA buffer containing a free calcium ion concentration of 10^{-9} M supplemented with the indicated concentrations of GTP-γ-S (Panel A) or mastoparan (Panel B) either in the absence (■ Panel A, open bars Panel B) or presence (◆ Panel A, hatched bars Panel B) of arachidonic acid (10^{-4} M) as described in the methods. The results are expressed as the mean ± S.E.M. from three separate experiments. Absence of error bars indicates that they lie within the symbol used.

Table 2
The effect of arachidonic acid upon GTP-γ-S-, mastoparan- and melittin-evoked ACTH secretion from permeabilised cells in the presence and absence of ibuprofen

	Control secretion (pg/10^5 cells)	+ ibuprofen (10^{-4} M) (pg/10^5 cells)
Control	86 ± 6	119 ± 7
Control + arachidonic acid (10^{-4} M)	78 ± 5	120 ± 6
GTP-γ-S (10^{-4} M)	464 ± 23	526 ± 28
GTP-γ-S (10^{-4} M) + arachidonic acid (10^{-4} M)	311 ± 17	530 ± 26
Mastoparan (10^{-5} M)	606 ± 35	670 ± 40
Mastoparan (10^{-5} M) + arachidonic acid (10^{-4} M)	398 ± 20	680 ± 33
Melittin (10^{-5} M)	1246 ± 41	1398 ± 63
Melittin (10^{-5} M) + arachidonic acid (10^{-4} M)	872 ± 35	1350 ± 73

Permeabilised cells were incubated in the standard permeabilisation medium, as described in Section 2, supplemented with the indicated concentrations of GTP-γ-S, mastoparan and melittin either in the presence or absence of arachidonic acid and/or ibuprofen. ACTH release is expressed as the mean ± S.E.M. of the secretion obtained above that obtained in the absence of any compound.

Melittin (10^{-5} M)-evoked secretion from permeabilised cells was also significantly inhibited by arachidonic acid (10^{-4} M) (Table 2). Again these inhibitory actions of arachidonic acid were not observed in the presence of the cyclo-oxygenase inhibitor ibuprofen (10^{-4} M) (Table 2).

The products of phospholipase A_2-generated arachidonic metabolism may thus exert an inhibitory action on the late post-Ca^{2+} stages of the ACTH secretory pathway and prostaglandins may be the active agents in this capacity.

4. Discussion

A role for G-proteins as important regulators of exocytosis emerged from the use of non-hydrolysable GTP analogues in permeabilised secretory cells (Gomperts, 1990). They stimulated exocytosis by a mechanism independent of their actions upon the early events in signal-transduction processes and were dubbed G-exocytosis (Ge) to reflect this. An involvement of such Ge proteins in the control of ACTH secretion has been shown in AtT-20 cells (Erlich et al., 1998; Guild, 1991; Luini and Dematteis, 1988, 1990; McFerran and Guild, 1994, 1995a,b). Mastoparan is able to stimulate ACTH secretion from permeabilised AtT-20 cells by a direct activation of Ge (Erlich et al., 1998; McFerran and Guild, 1995a) which lends weight

to the hypothesis that a heterotrimeric G-protein is involved at some stage in the stimulus-secretion coupling mechanisms the effects of Ca^{2+} upon ACTH secretion.

The mechanisms that link Ge to the exocytotic machinery remain unclear. Interestingly, mastoparan has also been reported to directly activate phospholipase A_2 (Argiolas and Pisano, 1983) and, furthermore, 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 and Axelrod, 1985; Won and Orth, 1994). Ca^{2+}-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 and Axelrod, 1985). All of 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.

This study 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. To this end, other phospholipase A_2-activating peptides such as melittin and phospholipase A_2-activating peptide were investigated and compared to mastoparan itself (Argiolas and Pisano, 1983; Peitsch et al., 1993). All three peptides stimulated ACTH secretion from intact cells. Melittin stimulated the largest increase in secretion and phospholipase A_2-activating peptide evoking the smallest increase in secretion. However, the limitation of the use of intact cells is that any actions of these peptides upon all parts of the stimulus-secretion coupling mechanisms for ACTH secretion will be manifest in the overall secretory response. This study, therefore, focused upon the late stages of the secretory pathway where a role for Ge is indicated and therefore investigated the effects of these peptides in permeabilised cells. In this study, all three peptides had such a late stage post-Ca^{2+} action upon ACTH secretion. Melittin and phospholipase A_2-activating peptide both stimulated ACTH secretion from permeabilised cells in the absence of calcium ions albeit to very different extents compared to mastoparan. Melittin has been previously shown to stimulate ACTH secretion from intact AtT-20 cells (Heisler et al., 1982) and from anterior pituitary cultures (Abou-Samra et al., 1986) but this is perhaps the first demonstration that melittin can stimulate ACTH secretion in the absence of calcium ions. Phospholipase A_2-activating peptide also stimulated ACTH secretion in the absence of calcium ions but to a very limited extent and much less than either melittin or mastoparan. Phospholipase A_2-activating peptide was identified as a phospholipase A_2-activating in studies looking at the effects of melittin itself (Peitsch et al., 1993). As these two peptides were shown to have sequence homology in their phospholipase A_2-activating regions, it would be expected that they would have more similarity in their effectiveness in activating phospholipase A_2. Furthermore, mastoparan is reported to be as potent a phospholipase A_2-activator as melittin (Argiolas and Pisano, 1983) yet it produced a much lesser effect on ACTH secretion. There is however an alternative explanation for the actions of melittin and phospholipase A_2-activating peptide. Melittin can also stimulate the activity of heterotrimeric G-proteins (Higashijima et al., 1988) indicating that the Ca^{2+}-independent secretion of ACTH by this peptide may be due to its activation of Ge.

A variety of phospholipase A_2 inhibitors (ONO-RS-082, OBAA, U73122 and the arachidonic acid analogue AACOCF3) are available with differing spectrums of activity against different isozymes of phospholipase A_2 (Banga et al., 1986; Bleasdale et al., 1990; Kohler et al., 1991; Street et al., 1993). This study chose to investigate the effects of two of these compounds. The effect of ONO-RS-082 (Konrad et al., 1992) upon the ability of these phospholipase A_2-activating peptides to stimulate secretion was studied. Rather than inhibit the actions of these three peptides, as would be expected if they were acting primarily through a stimulation of phospholipase A_2, this compound actually potentiated the stimulation of ACTH secretion obtained by all three agents. This suggests that there may be two antagonistic actions of these peptides going on simultaneously. One is a stimulation of secretion via an activation of Ge not phospholipase A_2 and the other is an inhibitory action on secretion via the metabolic products of arachidonic acid released by the stimulation of phospholipase A_2. The net secretory response is the result of these two opposing actions. The effect of inhibiting phospholipase A_2 is revealing a greater stimulation of secretion by Ge.

This inhibitory late-stage action of phospholipase A_2 and its products was further investigated by looking at the effects of phospholipase A_2-activating peptides and arachidonic acid upon Ca^{2+}- and Ge-evoked secretion. Mastoparan was able to substitute for Ca^{2+} in stimulating ACTH secretion from permeabilised cells. The mastoparan-sensitive pool of ACTH was the same as that sensitive to increases in free calcium ion. These results parallel the interaction between GTP-γ-S and Ca^{2+} that first suggested that a G-protein mediates the effects of Ca^{2+} upon the secretory apparatus in AtT-20 cells (Guild, 1991). Melittin, however, stimulated ACTH secretion to a level greater than that achieved by Ca^{2+} alone suggesting that this peptide has actions additional to stimulating Ge. Thus, melittin is not an agent that can be used to mimic the secretory response obtained to increases in intracellular Ca^{2+} as is the case for GTP-γ-S and mastoparan.

In contrast, phospholipase A_2-activating peptide inhibited Ca^{2+}-evoked ACTH secretion from permeabilised cells

indicating that it poorly activates, if at all, Ge, and that it acts, presumably via a stimulation of phospholipase A_2, to generate arachidonic acid products that inhibit ACTH secretion. These inhibitory products must act at the late post-Ca^{2+} stages of the secretory pathway and that if there are any stimulatory arachidonic acid products these must act pre-Ca^{2+} in the ACTH secretory pathway.

Arachidonic acid itself inhibited both Ca^{2+}/Ge-evoked secretion and melittin-evoked secretion suggesting that a product of its further metabolism does exert a late stage inhibitory action on secretion. This action of arachidonic acid is in contrast to the stimulation of ACTH secretion seen in response to arachidonic acid in intact pituitary cultures (Abou-Samra et al., 1986). Furthermore, it has been reported that there was no effect of arachidonic acid upon basal and CRF-evoked secretion from intact AtT-20 cells (Pompeo et al., 1997). This may not be inconsistent with the results of this study as the secretory studies of Pompeo et al. (1997) was in intact cells where arachidonic acid could be metabolised to both stimulatory and inhibitory products. These authors did not look at the effect of exogenously added arachidonic acid upon Ca^{2+} or GTP-γ-S-evoked ACTH secretion from permeabilised cells. Inhibitory products acting at the late post-Ca^{2+} stages of the secretory may cancel out the actions of any stimulatory arachidonic acid products acting pre-Ca^{2+} in the ACTH secretory pathway. Thus in intact cells variable results may be obtained depending upon experimental conditions. This study reveals a late-stage inhibitory action of arachidonic acid metabolites as an early-stage action can be removed in permeabilised cells.

Consistent with this late stage inhibitory action of arachidonic acid is the observation that the arachidonic acid analogue, arachidonyl trifluoromethyl ketone (AACOCF3), which is in fact an inhibitor of the 85 kDa cytosolic isoform of phospholipase A_2 (Street et al., 1993), potentiated Ca^{2+}-evoked ACTH secretion from permeabilised cells. Thus, in this experimental system where it is possible to focus upon the post-Ca^{2+} and Ge steps in the stimulus-secretion coupling pathway, there is a point of inhibitory control by the phospholipase A_2 and its products. Thus the role of phospholipase A_2 in stimulus-secretion coupling is a complex one with actions of its products at earlier stages of the secretory pathway to stimulate or facilitate Ca^{2+}-evoked secretion (Abou-Samra et al., 1986; Hamill et al., 1981; Luini and Axelrod, 1985; Won and Orth, 1994) and actions of its products at the late stages of the secretory pathway to inhibit secretion. The ability of the cyclo-oxygenase inhibitor ibuprofen to prevent arachidonic acid's inhibition of Ca^{2+}/Ge-evoked and melittin-evoked secretion suggests that it is a prostaglandin that is responsible for this inhibitory late stage regulation.

The data obtained in this study is consistent with the reports of an inhibitory autocrine action of prostaglandins in the ACTH secretory pathway (Vlaskovska and 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 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, pertussis-toxin-sensitive, inhibition via G-proteins of the late stages of the secretory pathway (Luini and Dematteis, 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). As our evidence also 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 and Guild, 1995a), 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 mastoparan and the Ca^{2+}/Ge system but does indicate a separate late stage inhibitory action of cyclo-oxygenase products distal to the Ca^{2+}/Ge point of control.

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