British Journal of Pharmacology (1995) 115, 696–702

Effects of mastoparan 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 the effects of mastoparan upon the late stages of the adrenocorticotrophin (ACTH) secretory pathway.

2 Mastoparan ($10^{-8}-10^{-5}$ M), an activator of heterotrimeric guanosine 5'-triphosphate binding proteins (G-proteins), stimulated ACTH secretion from electrically-permeabilized AtT-20 cells in a concentration-dependent manner in the effective absence of calcium ions with a threshold of 10^{-6} M. Guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) ($10^{-8}-10^{-4}$ M) also stimulated ACTH secretion from electrically-permeabilized AtT-20 cells in a concentration-dependent manner in the effective absence of calcium ions with a threshold of 10^{-6} M. This GTP-γ-S-evoked secretion is consistent with previous studies which demonstrated that a G-protein, termed G_E, mediates calcium evoked ACTH secretion from AtT-20 cells. GTP-γ-S-evoked secretion however was not as great as that obtained in response to mastoparan.

3 Both mastoparan (10^{-5} M) and GTP-γ-S (10^{-4}M) stimulated ACTH secretion from electrically-permeabilized AtT20 cells in a time-dependent manner. A time of 30 min was adopted as the standard incubation period for the study of both mastoparan and GTP-γ-S-stimulated ACTH secretion from permeabilized AtT-20 cells.

4 Mastoparan ($10^{-8}-10^{-5}$ M) stimulated ACTH secretion from permeabilized AtT-20 cells to the same extent in the presence and absence of the protein kinase C (PKC) inhibitor, chelerythrine chloride (10^{-5} M).

5 Mastoparan ($10^{-8}-10^{-5}$ M)-stimulated ACTH secretion from permeabilized AtT-20 cells was significantly reduced in the presence of guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S, 10^{-4} M).

6 The mastoparan analogue, Mas-7 ($10^{-8}-10^{-5}$ M) stimulated ACTH secretion from permeabilized AtT-20 cells to a greater extent than mastoparan ($10^{-8}-10^{-5}$ M) however, the mastoparan analogue Mas-17 ($10^{-8}-10^{-5}$ M) had no effect upon ACTH secretion from permeabilized AtT-20 cells.

7 Mastoparan ($10^{-8}-10^{-5}$ M) stimulated ACTH secretion from permeabilized AtT-20 cells in the presence and absence of ATP, normally present in the standard permeabilization medium at a concentration of 5 mM. Mastoparan ($10^{-8}-10^{-5}$ M)-stimulated ACTH secretion as well as control secretion was reduced when ATP was omitted.

8 The results of the present study demonstrate that mastoparan stimulated ACTH secretion from permeabilized AtT-20 cells and displayed characteristics consistent with calcium ion- and GTP-γ-S-stimulated ACTH secretion from permeabilized AtT-20 cells. This suggests that in permeabilized AtT-20 cells, mastoparan directly activates G_E and that this G-protein may be a heterotrimeric G-protein. This study also suggests mastoparan may be a useful alternative to GTP-γ-S as a means of directly activating G_E.

Keywords: Heterotrimeric G-protein; mastoparan; anterior pituitary cell line; ACTH

Introduction

Increasing the concentration of free calcium ions in the cytosol of secretory cells has long been established as a trigger to exocytosis (Douglas, 1968) with much supporting evidence emerging from the use of a variety of permeabilized cell types in which the cytosolic free calcium ion concentration can be controlled (for review see Knight & Scrutton, 1986). One such cell type used in permeabilization studies is the adrenocorticotrophin (ACTH)-secreting mouse AtT-20/D16-16 anterior pituitary tumour cell line, a model system for the study of the normal corticotroph (Luini & DeMatteis, 1988; 1990; Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). Permeabilized AtT-20 cells release ACTH in response to increasing cytosolic free calcium ion levels (Luini & DeMatteis, 1988; 1990; Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994); however, the mechanism by which calcium ions stimulate secretion from AtT-20 cells is not fully understood. Calcium ions have been shown to mediate their effects upon hormone secretion via stimulation of a guanosine 5'-triphosphate binding protein (G-protein) in a variety of permeabilized cell systems (for review see Gomperts, 1990). This has been demonstrated in permeabilized AtT-20 cells by the ability of the non-hydrolysable GTP analogue guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) to stimulate ACTH secretion in the effective absence of calcium ions (Luini & DeMatteis, 1988; 1990; Guild, 1991; McFerran & Guild, 1994). This G-protein mediating calcium ion-stimulated hormone secretion has been termed G_E by Gomperts (1990).

Two families of G-protein have been proposed as candidates for G_E. These are heterotrimeric G-proteins composed of three distinct subunits termed α, β and γ (for reviews see Gilman, 1987; Taylor, 1990) and small molecular weight monomeric Ras-like G-proteins (for review see Hall, 1990). Both heterotrimeric G-proteins (Toutant *et al.*, 1987) and monomeric G-proteins (Burgoyne & Morgan, 1989; Darchen *et al.*, 1990; Fischer von Mollard, 1991) have been located on intracellular membranes and implicated in vesicular traffic. In AtT-20 cells monomeric Ras-like proteins, in particular rab 3, have been shown to play a role in localization, sequestration

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and storage of secretory vesicles (Ngsee *et al.*, 1993). In addition an inhibitory form of G_E (G_{Ei}), which mediates somatostatin inhibition of ACTH secretion, has been identified in AtT-20 cells and is thought to be heterotrimeric (Luini & DeMatteis, 1988; 1990). The object of this study therefore was to characterize further the stimulatory form of G_E (G_{Es}) which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells.

The G-protein which mediates calcium ion-stimulated ACTH secretion from AtT-20 cells was further investigated by use of mastoparan and related peptides. Mastoparan is an amphiphilic tetradecapeptide originally isolated from wasp venom with a wide variety of actions some of which have been attributed to the activation of heterotrimeric G-proteins by a mechanism similar to that of agonist-bound receptors (Higashijima *et al.*, 1988; 1990; Weingarten *et al.*, 1990). This study demonstrates that mastoparan is able to stimulate secretion from permeabilized AtT-20 cells independently of changes in the cytosolic calcium ion level and protein kinase C (PKC) and that this action is mediated, at least partly, by a G-protein. Mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells displayed characteristics consistent with previous studies investigating calcium ion- and GTP-γ-S-stimulated ACTH secretion from permeabilized AtT-20 cells which originally established the existence of G_E in AtT-20 cells (Guild, 1991; McFerran & Guild, 1994). The results of this study suggest that, in AtT-20 cells, mastopaparan is acting by a direct action upon G_E and are consistent with G_E present in this cell line being a heterotrimeric G-protein. This study also suggests that mastoparan may be a useful alternative to GTP-γ-S as a means of directly activating G_E.

Methods

Culture of AtT-20 cells

Cells of the mouse AtT-20/D16-16 anterior pituitary tumour cell line were grown and subcultured 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 were plated in 75 cm^2 flasks (Nunc, Gibco, U.K.) at an initial density of 2×10^6 cells/flask and were used upon reaching 80–90% confluency.

Preparation of AtT-20 cells

The culture medium was removed, cells adhering to the substrate were liberated with 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, bovine serum albumin (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, $MgCl_2$ 1, 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 3000 V cm^{-1}. These parameters were adopted in this study.

Measurement of stimulated ACTH secretion from permeabilized AtT-20 cells

Permeabilized cells were suspended at a density of 10^5 cells ml^{-1} in calcium/EGTA buffers designed to establish and maintain the desired cytosolic free calcium concentration as previously described (Guild, 1991). The standard permeabilization medium was designed to maintain a cytosolic free calcium level of 10^{-9} M (effectively zero calcium). All experiments involving permeabilized cells were carried out in this standard permeabilization medium. These incubations were supplemented, as indicated in the figure legends, with the G-protein activators guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S), mastoparan and mastoparan analogues. The stable guanosine 5'-diphosphate (GDP) analogue guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S), used as a means of inhibiting G-proteins by its ability to compete with GTP, was also investigated. At this point zero time incubations 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 (with the exception of time course experiments) 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 six samples were run for each condition and the ACTH content measured by radioimmunoassay.

The effect of protein kinase C inhibition upon mastoparan-evoked ACTH secretion from permeabilized AtT-20 cells

GTP-γ-S is able to stimulate ACTH secretion from permeabilized AtT-20 cells independently of protein kinase C (PKC). The PKC inhibitor, chelerythrine chloride (Herbert *et al.*, 1990), was used to investigate whether mastoparan-evoked ACTH secretion is similarly independent of PKC. Mastoparan-evoked ACTH secretion was measured (as described above) in the presence and absence of chelerythrine chloride (10^{-5} M). At this concentration, chelerythrine chloride selectively inhibits PKC (Herbert *et al.*, 1990) and has been shown to be effective in inhibiting PKC-stimulated ACTH secretion from permeabilized AtT-20 cells (McFerran & Guild, 1994). In addition, the effect of omitting ATP from the permeabilization media upon mastoparan-stimulated ACTH secretion was also investigated.

Radioimmunoassay

ACTH secretion was measured by radioimmunoassay (RIA) based upon the previously described method of Antoni *et al.* (1983). Dilutions of sample and antiserum were made in RIA buffer of the following composition: sodium phosphate 0.05M, polyethylene glycol 8000 6% (w/v), BSA, 0.1% (w/v), Triton-X 100 0.1% (v/v), EDTA 2.5 mM, pH 7.4. The incubation mixture contained a total volume of 300 μl RIA buffer, consisting of 100 μl of human ACTH 1-39 standard or unknown sample, 100 μl antiserum (rabbit antihuman-ACTH) at a final dilution of 1:32000 in RIA buffer, and approximately 10000 c.p.m. [^{125}I]-ACTH in 100 μl RIA buffer. [^{125}I]-ACTH was produced using the iodogen reagent (1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril) which was first described as a reagent for iodination by Fraker & Speck (1978). Samples were then incubated for 16–24 h at 4°C. Donkey anti-rabbit IgG (100 μl), at a dilution of 1:10 in RIA buffer containing 0.5% normal rabbit serum, was added to each tube and samples were further incubated at room temperature for 3 h; 1 ml 3% (w/v) polyethylene glycol was added to each tube and samples were centrifuged at 4°C, 3200 *g* for 30 min. The supernatants were then decanted and the precipitates counted by means of a gamma counter. The amount of ACTH released during experimental procedures was expressed as the amount present at the end of the specified incubation period less the amount present at zero time (with the exception of time course experiments).

Statistics

In each experiment six determinations for each experimental condition were made and each experiment was repeated three

times on different days. ACTH secretion is expressed as the mean ± s.e. mean from these three experiments. Statistical significance was determined by use of ANOVA tests with Scheffe's *F*-test *post hoc* analysis. The statistical significance of a particular treatment was determined by a two-way ANOVA test. In both cases a *P* value less than or equal to 0.05 was considered significant.

Materials

The following substances (with their sources) were used: ATP, bovine serum albumin (fraction V), mastoparan (Ile-Asn-Leu-Lys-Ala-Leu-Ala-Ala-Leu-Ala-Lys-Lys-Ile-Leu-NH_2) from Sigma, U.K.; guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) and guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) from Boehringer Mannheim, U.K.; Mas-7 and Mas-17 from Peninsula Laboratories; chelerythrine chloride from Calbiochem-Novabiochem, U.K.; 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.; anti-rabbit IgG was a gift of the Scottish antibody production unit, Carluke, Lanarkshire, U.K.; iodogen iodination reagent from Pierce & Warriner. All other chemicals were of Analar grade and readily commercially available.

Results

The effects of mastoparan and GTP-γ-S upon ACTH secretion from permeabilized AtT-20 cells

GTP-γ-S (10^{-6}–10^{-4} M) significantly stimulated ACTH secretion from electrically-permeabilized AtT-20 cells, in the effective absence of calcium (free calcium concentration of 10^{-9} M), in a concentration-dependent manner with a threshold of 10^{-6}M (Figure 1) but lower concentrations (10^{-8}–10^{-7} M) were ineffective in this regard as previously reported (Guild, 1991; McFerran & Guild, 1994). Mastoparan (10^{-6}–10^{-5} M) also stimulated ACTH secretion, in the effective absence of calcium (free calcium concentration of 10^{-9} M), in a concentration-dependent manner with a threshold of 10^{-6} M but lower concentrations (10^{-8}–10^{-7} M) were ineffective in this regard (Figure 1). ACTH secretion evoked by mastoparan was significantly ($P<0.001$) greater than that evoked by GTP-γ-S.

Mastoparan (10^{-5} M) (Figure 2a) significantly ($P<0.001$) stimulated ACTH secretion from permeabilized AtT-20 cells in

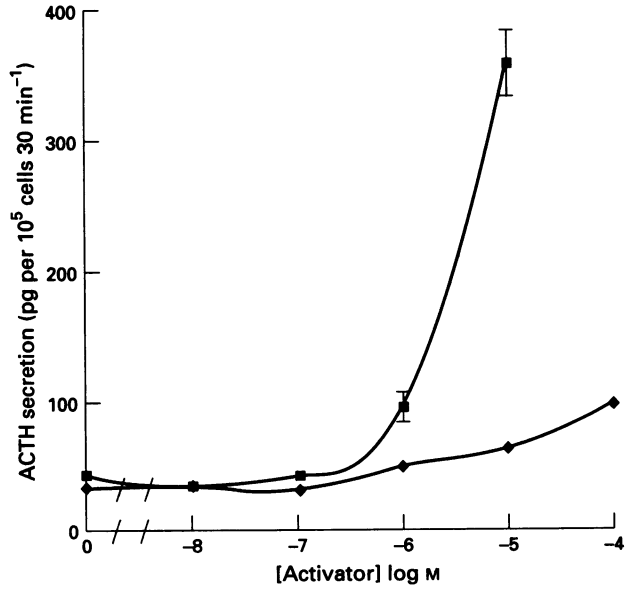

Figure 1 Effect of mastoparan (■) and GTP-γ-S (◆) on adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of mastoparan and GTP-γ-S and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

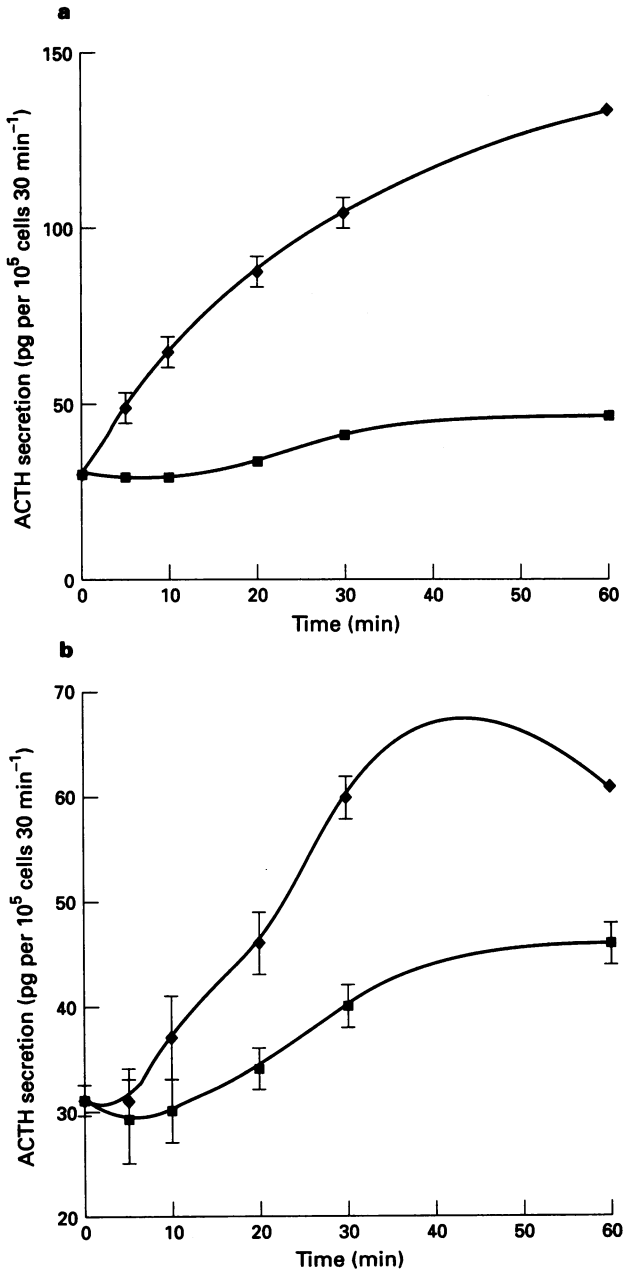

Figure 2 Time course of adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. (a) Permeabilized cells were incubated for the indicated time periods in standard permeabilization medium in the presence (◆) or absence (■) of 10^{-5} M mastoparan and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicate that they lie within the symbol. (b) Permeabilized cells were incubated in standard permeabilization medium for the indicated time periods in the presence (◆) or absence (■) of 10^{-4}M GTP-γ-S and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

the effective absence of calcium in a time-dependent manner. Secretion in response to mastoparan (10^{-5} M) after 30 min was 104 ± 6 pg per 10^5cells compared to a control secretion of 40 ± 3 pg per 10^5 cells. GTP-γ-S (10^{-4} M) (Figure 2b) also significantly ($P<0.01$) stimulated ACTH secretion from permeabilized AtT-20 cells in the effective absence of calcium in a time-dependant manner. Secretion to GTP-γ-S (10^{-4} M) after 30 min was 60 ± 2 pg per 10^5 cells compared to a control secretion of 40 ± 2 pg per 10^5 cells. In both cases 30 min was chosen as the standard incubation period for subsequent experiments.

Characterization of mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells

Mastoparan (10^{-5} M) significantly stimulated ACTH secretion from permeabilized AtT-20 cells in both the presence and absence of the stable GDP analogue GDP-β-S (10^{-4} M). However, GDP-β-S significantly ($P>0.001$) reduced mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells. Secretion evoked by 10^{-5} M mastoparan was reduced from 340 ± 9 pg per 10^5 cells to 211 ± 23 pg per 10^5 cells (Figure 3). GDP-β-S at a higher concentration of 10^{-3} M did not reduce ACTH secretion evoked by mastoparan 10^{-5} M to a greater extent than 10^{-4} M GDP-β-S (data not shown) indicating that this was the maximal inhibition obtained by the use of this guanine nucleotide analogue.

Chelerythrine chloride (10^{-5} M), a potent protein kinase C inhibitor, had no significant effect upon control secretion or mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells (data not shown). ACTH secretion in response to 10^{-5} M mastoparan was 321 ± 11 pg per 10^5 cells in the absence of chelerythrine chloride and 307 ± 22 pg per 10^5 cells in the presence of chelerythrine chloride.

Mastoparan significantly stimulated ACTH secretion from permeabilized AtT-20 cells in both the presence and absence of ATP normally present in the standard permeabilization medium at a concentration of 5 mM (Figure 4). Omitting ATP from the standard permeabilization medium however significantly ($P<0.01$) reduced secretion in response to mastoparan. Secretion obtained in the absence of mastoparan was reduced from 31 ± 3 pg per 10^5 cells to 13 pg per 10^5 cells. ACTH secretion in response to 10^{-5} M mastoparan was reduced from 274 ± 13 pg per 10^5 cells in the presence of ATP to 128 ± 11 pg per 10^5 cells in the absence of ATP (Figure 4).

The effects of mastoparan analogues, Mas-7 and Mas-17, upon ACTH secretion from permeabilized AtT-20 cells

The effects of two analogues of mastoparan upon ACTH secretion from permeabilized AtT-20 cells were investigated. The mastoparan analogue Mas-7 has been reported to be a highly active G-protein activator whereas the mastoparan analogue Mas-17 is unable to activate G-proteins (Higashijima *et al.*, 1990). Mas-7 (10^{-7} M – 10^{-5} M) stimulated ACTH secretion, in the effective absence of calcium (free calcium concentration of 10^{-9} M), in a concentration-dependent manner with a threshold of 10^{-7} M (Figure 5) but lower concentrations (10^{-8} M) were ineffective in this regard. ACTH secretion in response to Mas-7 (10^{-5} M) was 430 ± 13 pg per 10^5 cells compared to 291 ± 11 pg per 10^5 cells obtained in response to mastoparan (10^{-5} M). In contrast Mas-17 (10^{-8} M – 10^{-5} M) was unable to stimulate ACTH secretion from permeabilized AtT-20 cells (Figure 5). No significant difference was observed between control secretion and secretion in response to any concentration of Mas-17.

Discussion

The aim of this study was to determine whether mastoparan is able to evoke ACTH secretion from AtT-20 cells by an action at a late stage in the secretory pathway distal to changes in cytosolic calcium ion levels. Electrical permeabilization provided a means of directly manipulating the intracellular environment and studying the effects of mastoparan at this late stage in the secretory pathway. Electrical permeabilization has

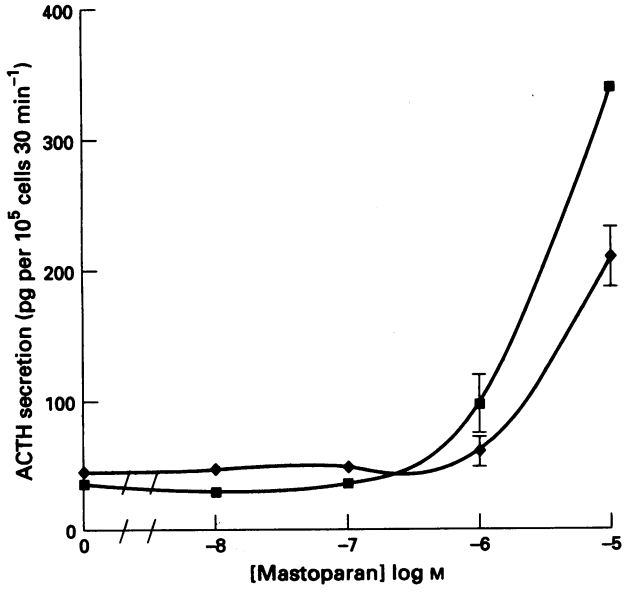

Figure 3 Effect of GDP-β-S upon mastoparan-evoked adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of mastoparan in the absence (■) or presence (◆) of 10^{-4}M GDP-β-S and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

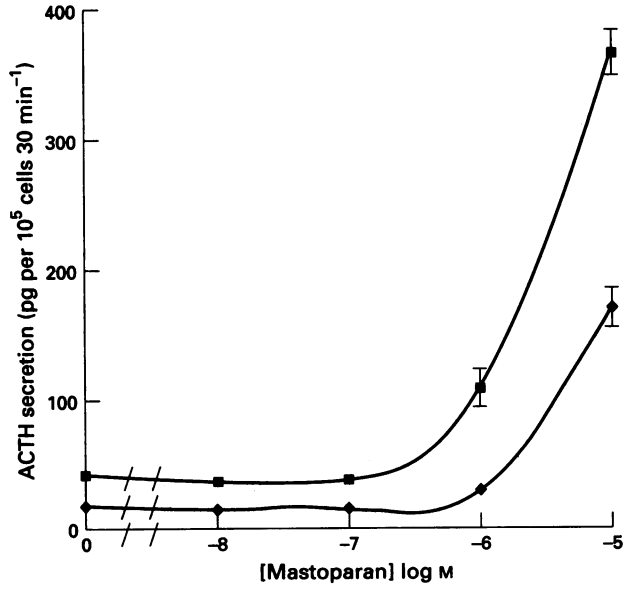

Figure 4 Mastoparan-evoked adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells in the presence and absence of ATP. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of mastoparan in the absence (◆) or presence (■) of 5 mM ATP and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

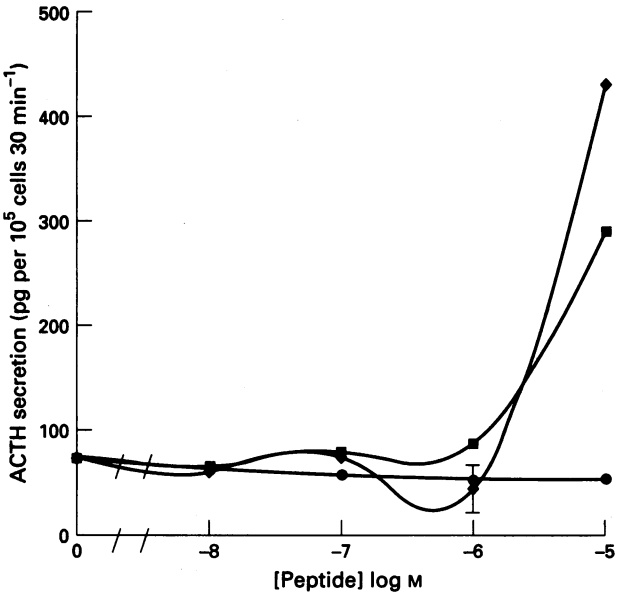

Figure 5 Effect of mastoparan (■), Mas-7 (◆) and Mas-17 (●) on adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium supplemented with the indicated concentration of peptide and ACTH secretion measured as described in the methods. ACTH release (pg per 10^5 cells) is expressed as the mean ± s.e. mean from at least 3 separate experiments; absence of error bars indicates that they lie within the symbol.

previously been used to gain access to the cytosol of AtT-20 cells without impairing the ability of these cells to undergo exocytosis (Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). Mastoparan stimulated ACTH secretion from permeabilized AtT-20 cells in a concentration-dependent manner in the effective absence of free calcium ions which are themselves able to stimulate ACTH secretion from electrically-permeabilized AtT-20 cells (Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). Any changes in free calcium ion concentrations induced by mastoparan would have been buffered by the calcium/EGTA buffers designed to establish and maintain the required cytosolic free calcium ion concentration which in the case of this study was 10^{-9} M (effectively zero); therefore mastoparan evoked ACTH secretion from permeabilized AtT-20 cells is calcium-independent.

Adenosine 3':5'-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA) is also known to stimulate ACTH secretion from permeabilized AtT-20 cells; however, this evoked secretion does not occur in the absence of either calcium ions or guanine nucleotides (Guild, 1991). Since mastoparan stimulated secretion in the absence of guanine nucleotides and the effective absence of calcium ions it is probable that activation of PKA by means of cyclic AMP generation does not contribute to these actions of mastoparan.

In contrast to cyclic AMP, phorbol 12-myristate 13-acetate (PMA), a protein kinase C (PKC) activator, has been shown to stimulate ACTH secretion from permeabilized AtT-20 cells independently of calcium ions and guanine nucleotides (McFerran & Guild, 1994). The possibility that mastoparan was acting via PKC activation was therefore investigated. Mastoparan was able to stimulate ACTH secretion from permeabilized AtT-20 cells to the same extent in the presence or absence of chelerythrine chloride, a potent inhibitor of PKC (Herbert *et al.*, 1990). Chelerythrine chloride, at the concentration used in this study, has been shown to be an effective PKC inhibitor in AtT-20 cells by completely inhibiting PMA-evoked ACTH secretion from permeabilized AtT-20 cells (McFerran & Guild, 1994). Mastoparan is therefore able to stimulate ACTH secretion from AtT-20 cells independently of PKC.

Mastoparan stimulates secretion from a number of cell types including histamine from mast cells (Argiolas & Pisano, 1984; Mousli *et al.*, 1989; Bueb *et al.*, 1990) (after which the peptide is named), catecholamines from chromaffin cells (Kuroda *et al.*, 1980), insulin from pancreatic islets (Komatsu *et al.*, 1992; Yokokawa *et al.*, 1989) and the RINm5F β-cell line (Komatsu *et al.*, 1993), prolactin from anterior pituitary lactotrophs (Kurihara *et al.*, 1986), 5-hydroxytryptamine from platelets (Ozaki *et al.*, 1990) and surfactant from pulmonary alveolar cells (Joyce-Brady *et al.*, 1991). Some of these actions of mastoparan have recently been attributed to the activation of heterotrimeric G-proteins by the ability of this protein to form a highly structured α-helix in the phospholipid membrane which resembles the intracellular loops of G-protein-coupled receptors and as a result is able to activate heterotrimeric G-proteins in a similar fashion to that of agonist-bound receptors (Higashijima *et al.*, 1988; 1990; Weingarten *et al.*, 1990).

However, mastoparan has been reported to have a number of actions which may not be mediated by G-proteins. These actions include; non-specific cell lysis in chromaffin cells (Wilson, 1989); binding to calmodulin (Malenick & Anderson, 1983); direct activation of phospholipases A_2 (Argiolas & Pisano, 1983); and C (Wallace & Carter, 1989); stimulation of nucleoside diphosphate kinase (Kikkawa *et al.*, 1992); inhibition of PKC, Ca/CaM kinase II, Na-K ATPase and the Na pump (Raynor *et al.*, 1991). Some of these actions will not be relevant in the permeabilized cell system used here where changes in the cytosolic free calcium concentration, calmodulin activity, membrane-bound ion channels and pumps are circumvented. It is apparent from this diversity of action that great caution must be exerted when interpreting the sites and mechanism of action of mastoparan in any secretory system.

It was important, therefore, to establish the degree to which the effects of mastoparan in AtT-20 cells were due to G-protein activation as opposed to a non G-protein-mediated event. The stable GDP analogue GDP-β-S, which inhibits GTP activation of G-proteins by means of competitive antagonism, partially inhibited (50%) mastoparan-evoked ACTH secretion from permeabilized AtT-20 cells. Mastoparan stimulated ACTH secretion from AtT-20 cells is therefore mediated, at least partly, by a G-protein. GDP-β-S was used to assess G-protein contribution to the actions of mastoparan in pancreatic β-cells where the results (showing a similar degree of attenuation of mastoparan's actions by GDP-β-S to those seen here) indicate that mastoparan stimulates insulin secretion by a mechanism that is independent of changes in cytosolic calcium ions or PKC activation and is dependent, at least partly, upon activation of a G-protein at a late stage in the secretory pathway (Jones *et al.*, 1993). The results of the present study are consistent with previous studies showing that calcium ion-evoked ACTH secretion from permeabilized AtT-20 cells is also inhibited by GDP-β-S (Guild, 1991). In addition Mas-17, an analogue of mastoparan unable to activate G-proteins (Higashijima *et al.*, 1990), was unable to stimulate ACTH secretion from permeabilized AtT-20 cells. This is in contrast to the mastoparan analogue Mas-7, a highly active G-proteins activator (Higashijima *et al.*, 1990), which stimulated ACTH secretion to an even greater extent than mastoparan. This again suggests that the ability of mastoparan to stimulate ACTH secretion from permeabilized AtT-20 cells independently of changes in calcium and PKC is, at least partly, due to a direct activation of a G-protein. It is not possible at this stage to identify the mechanism(s) underlying the apparent G-protein-independent actions of mastoparan in these cells.

This study is consistent with previous studies using the same system in which guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) (a non hydrolysable GTP analogue) similarly stimulated ACTH secretion independently of calcium and PKC by activation of G_E (Luini & DeMatteis, 1988; 1990; Guild, 1991; McFerran & Guild, 1994). It can therefore be concluded that mastoparan-stimulated ACTH secretion from permeabilized AtT-20 cells is also partly mediated by G_E. Mastoparan has been postulated to activate G_E directly in a number of other

secretory cells including pancreatic β-cells (Jones *et al.*, 1993), mast cells (Aridor *et al.*, 1990) and platelets (Wheeler-Jones *et al.*, 1992). In addition the ability of mastoparan to stimulate ACTH secretion from permeabilized AtT-20 cells may suggest that this G-protein may belong to the heterotrimeric family of G-proteins.

Both small molecular G-proteins and heterotrimeric G-proteins are present on intracellular organelles and are thought to play an important role in intracellular membrane trafficking (for review see Pfeffer, 1992). Small molecular weight G-proteins participating in membrane traffic are postulated to cycle between a GTP and a GDP bound state (for review see Pfeffer, 1992) therefore the non-hydrolysable GTP analogue, GTP-γ-S, would arrest this cycle in the GTP bound conformation and result in an inhibition of this process. In contrast heterotrimeric G-proteins upon binding GTP-γ-S become persistently activated (for reviews see Gilman, 1987; Taylor, 1990). This and other reports from this laboratory (Guild, 1991; McFerran & Guild, 1994) demonstrate that GTP-γ-S is able to stimulate ACTH secretion from AtT-20 cells and is thought to do so by a direct action upon G_E. This evidence is therefore consistent with G_E belonging to the heterotrimeric and not the small molecular weight family of G-proteins.

Mastoparan-evoked ACTH secretion from permeabilized AtT-20 cells was significantly, but not completely, reduced when ATP was omitted from the permeabilization medium. This is again consistent with calcium ion-evoked ACTH secretion from permeabilized AtT-20 cells which displayed a similar ATP-dependency (Guild, 1991) suggesting that calcium and mastoparan are acting through similar mechanisms. One possible explanation for this ATP-dependency is that ATP, via conversion by the ubiquitous enzyme nucleoside diphosphate kinase, provides a source of GTP which is otherwise absent from the permeabilization medium (Gomperts, 1990) and as a result mastoparan or calcium can activate G_E. However, it should be noted that there remained a significant mastoparan-stimulated ACTH secretion in the absence of ATP. This may be due to mastoparan-induced activation of nucleoside diphosphate kinase (Kikkawa *et al.*, 1992) and subsequent generation of GTP from residual cellular ATP to permit G_E-evoked ACTH secretion or may be due to an action completely independent of G_E.

Mastoparan evoked ACTH secretion from permeabilized AtT-20 cells with characteristics consistent with previous studies investigating calcium ion- and GTP-γ-S-evoked ACTH secretion from the same system (Guild, 1991; Gilkes *et al.*, 1992; McFerran & Guild, 1994). The results of this study therefore confirm G_E is present in AtT-20 cells and are also consistent with G_E belonging to the heterotrimeric family of G-proteins. Therefore mastoparan may provide a useful alternative to GTP-γ-S as a means of directly activating G_E, with the advantages that it appears to stimulate secretion to a greater extent and may be more selective for G_E than GTP-γ-S.

The authors would like to acknowledge gratefully the financial support of the Wellcome Trust, the Royal Society and the Nuffield Foundation. B.W.M. is supported by a Maitland-Ramsay scholarship.

References

ANTONI, F.A., HOLMES, M.C. & JONES, M.T. (1983). Oxytocin as well as vasopressin potentiates ovine CRF *in vitro*. *Peptides*, **4**, 411–415.

ARGIOLAS, A. & PISANO, J.J. (1983). Facilitation of phospholipase A_2 activity by mastoparan, a new class of mast cell degranulating peptides from wasp venom. *J. Biol. Chem.*, **258**, 13697–13702.

ARIDOR, M., TRAUB, L.M. & SAGI-EISENBERG, R. (1990). Exocytosis in mast cells by basic secretagogues; evidence for direct activation of GTP-binding proteins. *J. Cell. Biol.*, **111**, 909–917.

BUEB, J.L., MOUSLI, M., ROUOT, B. & LANDRY, Y. (1990). Activation of G_i-like proteins, a receptor-independent effect of kinins in mast cells. *Mol. Pharmacol.*, **38**, 816–822.

BURGOYNE, R.D. & MORGAN, A. (1989). Low molecular mass GTP-binding proteins of adrenal chromaffin cells are present on the secretory granule. *FEBS Lett.*, **245**, 122–126.

DARCHEN, F., ZAHRAOUI, A., HAMMEL, F., MONTEILS, M.P., TAVITAIN, A. & SCHERMAN, D. (1990). Association of the GTP-binding protein rab 3A with bovine adrenal chromaffin granules. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 5692–5696.

DOUGLAS, W. (1968). Stimulus-secretion coupling: The concept and clues from chromaffin and other cells. *Br. J. Pharmacol.*, **34**, 451–474.

FISCHER VON MOLLARD, G., SUDHOF, T.C. & JAHN, R. (1991). A small GTP-binding protein dissociates from synaptic vesicles during exocytosis. *Nature*, **349**, 79–81.

FRAKER, P. & SPECK, J. (1978). Protein and cell membrane iodination with a sparingly soluble chloramide, 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril. *Biochem. Biophys. Res. Commun.*, **80**, 849–857.

GILKES, A.F., MACKAY, K.B., CRAMB, G. & GUILD, S.B. (1992). Atrial natriuretic peptide effects in AtT-20 pituitary tumour cells. *Mol. Cell. Endocrinol.*, **89**, 39–45.

GILMAN, A.G. (1987). G proteins: Transducers of receptor-generated signals. *Annu. Rev. Biochem.*, **56**, 615–649.

GOMPERTS, B.D. (1990). G_E: GTP-binding protein mediating exocytosis. *Annu. Rev. Physiol.*, **52**, 591–606.

GUILD, S. (1991). Effects of adenosine 3':5'-cyclic monophosphate and guanine nucleotides on calcium-evoked ACTH release from electrically-permeabilised AtT-20 cells. *Br. J. Pharmacol.*, **109**, 117–122.

HALL, A. (1990). The cellular functions of small GTP-binding proteins. *Science*, **249**, 635–640.

HERBERT, J.M., AUGEREAU, J.M., GLEYE, J. & MAFFRAND, J.P. (1990). Chelerythrine is a potent and specific inhibitor of protein kinase-C. *Biochem. Biophys. Res. Commun.*, **172**, 993–999.

HIGASHIJIMA, T., BURNIER, J. & ROSS, E.M. (1990). Regulation of Gi and Go by mastoparan, related amphiphilic peptides, and hydrophobic amines; Mechanisms and structural determinants of activity. *J. Biol. Chem.*, **265**, 14176–14186.

HIGASHIJIMA, T., UZU, S., NAKAJIMA, T. & ROSS, E.M. (1988). Mastoparan, a peptide toxin from wasp venom, mimics receptors by activating GTP-binding regulatory proteins (G proteins). *J. Biol. Chem.*, **263**, 6491–6494.

JONES, P.M., MANN, F.M., PERSAUD, S.J. & WHEELER-JONES, C.P.D. (1993). Mastoparan stimulates insulin secretion from pancreatic β-cells by effects at a late stage in the secretory pathway. *Mol. Cell. Endocrinol.*, **94**, 97–103.

JOYCE-BRADY, M., RUBINS, J.B., PANCHENKO, M.P., BERNARDO, J., STEELE, M.P., KOLM, L., SIMONS, E.R. & DICKEY, B.F. (1991). Mechanisms of mastoparan-stimulated surfactant secretion from isolated pulmonary alveolar type 2 cells. *J. Biol. Chem.*, **266**, 6859–6865.

KIKKAWA, S., TAKAHASHI, K., SHIMADA, N., MICHIO, U., KIMURA, N. & KATADA, T. (1992). Activation of nucleoside diphosphate kinase by mastoparan, a peptide isolated from wasp venom. *FEBS Lett.*, **305**, 237–240.

KNIGHT, D.E. & BAKER, P.F. (1982). Calcium-dependence of catecholamine release from adrenal medulary cells after exposure to intense electric fields. *J. Memb. Biol.*, **68**, 107–140.

KNIGHT, D.E. & SCRUTTON, M.C. (1986). Gaining access to the cytosol: The technique and some applications of electropermeabilization. *Biochem. J.*, **234**, 497–450.

KOMATSU, M., AIZAWA, T., YOKOKAWA, N., SATO, Y., OKADA, N., TAKASU, N. & YAMADA, T. (1992). Mastoparan-induced hormone release from rat pancreatic islets. *Endocrinology*, **130**, 221–228.

KOMATSU, M., MCDERMOTT, A.M., GILLISON, S.L. & SHARP, G.W.G. (1993). Mastoparan stimulates exocytosis at a Ca^{2+}-independent late site in stimulus secretion coupling; Studies with the RINm5F β-cell line. *J. Biol. Chem.*, **268**, 23297–23306.

KURIHARA, H., KITAJIMA, K., SENDA, T., FUITA, H. & NAKAJIMA, T. (1986). Multigranular exocytosis induced phospholipase A_2-activators, melittin and mastoparan, in rat anterior pituitary cells. *Cell Tissue Res.*, **243**, 311–316.

KURODA, Y., YOSHIOKA, M., KUMAKURA, K., KOBAYASHI, K. & NAKAJIMA, T. (1980). Effects of peptides on the release of catecholamines and adenine nucleotides from cultured adrenal chromaffin cells. *Proc. Jpn. Acad. Ser. B Physiol. Biol. Sci.*, **56**, 660–664.

LUINI, A. & DE MATTEIS, M.A. (1988). Dual regulation of ACTH secretion by guanine nucleotides in permeabilised AtT-20 cells. *Cell. Mol. Biol.*, **8**, 129–138.

LUINI, A. & DE MATTEIS, M.A. (1990). Evidence that a receptor-linked G-protein inhibits exocytosis by post-second messenger mechanism in AtT-20 cells. *J. Neurochem.*, **54**, 30–38.

MALENICK, D.A. & ANDERSON, S.R. (1983). High affinity binding of mastoparan by calmodulin. *Biochem. Biophys. Res. Commun.*, **114**, 50–54.

MCFERRAN, B.W. & GUILD, S.B. (1994). Effects of protein kinase C activators upon the late stages of the ACTH secretory pathway of AtT-20 cells. *Br. J. Pharmacol.*, **113**, 171–178.

MOUSLI, M., BRONNER, C., BUEB, J.L., TSCHIRHART, E., GIES, J.P. & LANDRY, Y. (1989). Activation of rat peritoneal mast cells by substance P and mastoparan. *J. Pharmacol. Exp. Ther.*, **250**, 329–335.

NGSEE, J.K., FLEMING, A.M. & SCHELLER, R.H. (1993). A rab protein regulates the localisation of secretory granules in AtT-20 cells. *Mol. Biol. Cell.*, **4**, 747–756.

OZAKI, Y., MATSUMOTO, Y., YATOMI, Y., HIGASHIHARA, M., KARIYA, T. & KUME, S. (1990). Mastoparan, a wasp venom, activates platelets via pertussis toxin sensitive GTP-binding proteins. *Biochem. Biophys. Res. Commun.*, **170**, 779–785.

PFEFFER, S.R. (1992). GTP-binding proteins in intracellular transport. *Trends Biochem. Sci.*, **2**, 41–46.

RAYNOR, R.L., ZHENG, B. & KUO, J.F. (1991). Membrane interactions of amphiphilic polypeptides mastoparan, melittin, polymixin B and cardiotoxin. Differential inhibition of protein kinase C, Ca^{2+}/calmodulin-dependent protein kinase II and synaptosomal membrane Na,K-ATPase, and Na^{+} pump and differentiation of HL60 cells. *J. Biol. Chem.*, **266**, 2753–2758.

REISINE, T. (1984). Somatostatin desensitisation: Loss of the ability of somatostatin to inhibit cAMP accumulation and ACTH release. *J. Pharmacol. Exp. Ther.*, **229**, 14–20.

TAYLOR, C.W. (1990). The role of G proteins in transmembrane signalling. *Biochem. J.*, **272**, 1–13.

TOUTANT, M., AUNIS, D., BOCKAERT, J., HOMBURGER, V. & ROUOT, B. (1987). Presence of three pertussis toxin substrates and $G_o\alpha$ immunoreactivity in both plasma and granule membranes of chromaffin cells. *FEBS Lett.*, **215**, 339–344.

WALLACE, M.A. & CARTER, H.R. (1989). Effects of the wasp venom peptide, mastoparan, on a phosphoinositide-specific phospholipase-c purified from rabbit brain membranes. *Biochem. Biophys. Acta*, **1006**, 311–316.

WEINGARTEN, R., RANSNAS, L., MUELLER, H., SKLAR, L.A. & BOKOCH, G.M. (1990). Mastoparan interacts with the carboxy terminus of the α subunit of G_i. *J. Biol. Chem.*, **265**, 11044–11049.

WHEELER-JONES, C.P.D., SAERMARK, T., KAKKAR, V.V. & AUTHI, K.S. (1992). Mastoparan promotes exocytosis and increases intracellular cyclic AMP in human platelets; Evidence for a G_E-like mechanism of secretion. *Biochem. J.*, **281**, 465–472.

WILSON, S. (1989). Effects of mastoparan on catecholamine release from chromaffin cells. *FEBS Lett.*, **247**, 239–241.

YOKOKAWA, N., KOMATSU, M., TAKEDA, T., AIZAWA, T. & YAMADA, T. (1989). Mastoparan, a wasp venom, stimulates insulin release by pancreatic islets through pertussis toxin sensitive GTP-binding protein. *Biochem. Biophys. Res. Commun.*, **158**, 712–716.

(Received November 28, 1994
Revised February 22, 1995
Accepted March 7, 1995)