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Effects of adenosine 3′:5′-cyclic monophosphate and guanine nucleotides on calcium-evoked ACTH release from electrically permeabilized 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 investigation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-mediated enhancement of calcium-evoked adrenocorticotrophin (ACTH) secretion.

2 AtT-20 cells were permeabilized by subjecting the cells to intense electric fields. Exposure of permeabilized cells to calcium (1 mM) in the external medium significantly stimulated ACTH secretion over the first 20 min of exposure. This calcium-stimulated ACTH secretion was dependent upon the presence of MgATP (5 mM).

3 The amount of ACTH secreted, in a 20 min incubation at 37°C, from permeabilized cells depended upon the free calcium concentration in the permeabilization medium. Calcium stimulated ACTH secretion from permeabilized cells in the concentration range of 10^{-7}–10^{-5} M (half maximal = 7×10^{-7} M). Cyclic AMP(10^{-4} M) increased the amount of ACTH secreted at each effective concentration of calcium. However, cyclic AMP did not alter the potency of calcium as a stimulant of ACTH secretion.

4 Guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S, 10^{-4} M) stimulated ACTH secretion from permeabilized cells in the absence of calcium and was additive with calcium-evoked ACTH secretion up to a maximum which could be stimulated by calcium acting singly. Guanosine 5′-O-(2-thiodiphosphate) (GDP-β-S, 10^{-4} M) inhibited calcium-evoked ACTH secretion from permeabilized cells.

5 GTP-γ-S stimulated ACTH secretion from permeabilized cells in a concentration-dependent manner. The nucleotide significantly stimulated ACTH secretion at concentrations of 10^{-5} M and above. Cyclic AMP (10^{-4} M) increased the amount of ACTH secretion evoked by effective concentrations of GTP-γ-S.

6 The results of the present study support the hypothesis that, in AtT-20 cells, cyclic AMP is acting at some site, distal to calcium entry, which modulates the ability of an increase in cytosolic calcium concentration to stimulate ACTH secretion. One such site may be a GTP-binding protein which the present study suggests may mediate the effects of calcium upon the secretory apparatus. These GTP-binding proteins may be a target for regulation by cyclic AMP.

Keywords: Cyclic AMP; calcium; G-proteins; anterior pituitary tumour cell line; ACTH

Introduction

Corticotrophin-releasing factor (CRF), the most potent and effective natural stimulant of adrenocorticotrophin (ACTH) release, activates both adenylate cyclase and adenosine 3′:5′-cyclic monophosphate (cyclic AMP)-dependent protein kinase (PKA) in homogenates of rat anterior pituitary (Aguilera *et al.*, 1983) and a tumour cell line of the mouse anterior pituitary (AtT-20/D16-16) (Miyazaki *et al.*, 1984; Litvin *et al.*, 1984). Other agents that increase the cellular content of cyclic AMP, such as forskolin, and 8-bromo-cyclic AMP, also stimulate ACTH secretion from corticotrophs and AtT-20 cells (Aguilera *et al.*, 1983; Axelrod & Reisine, 1984; Heisler & Reisine, 1984; Litvin *et al.*, 1984; Miyazaki *et al.*, 1984). These findings suggest that elevation of cellular cyclic AMP content and activation of PKA may be an important step in the stimulation of ACTH secretion by CRF.

Extracellular calcium is necessary for the stimulation of ACTH secretion but not the stimulation of cyclic AMP production (Axelrod & Reisine, 1984; Miyazaki *et al.*, 1984). Furthermore, CRF, forskolin and 8 bromo-cyclic AMP also elevate cytosolic calcium concentrations in AtT-20 cells (Luini *et al.*, 1985; Reisine & Guild, 1985; Guild *et al.*, 1986; Guild & Reisine, 1987). Since an increase in the cellular levels of calcium has been proposed as a necessary and sufficient stimulus to trigger hormone secretion (Douglas, 1968), it is conceivable that cyclic AMP influences hormone secretion via an interaction with the calcium messenger system in AtT-20 cells.

The interaction between cyclic AMP and calcium in AtT-20 cells in on at least at two levels. Firstly, cyclic AMP potentiates voltage-dependent calcium entry into AtT-20 cells and raises cytosolic free calcium concentrations (Luini *et al.*, 1985; Guild & Reisine, 1987). Secondly, cyclic AMP potentiates the ability of a particular increase in cytosolic calcium concentration to stimulate ACTH secretion (Guild *et al.*, 1986). These data suggest that an additional important action of this cyclic nucleotide is distal to the entry of calcium into the cytosol to modulate the action of changes in cytosolic calcium, including those evoked by cyclic AMP itself, upon the secretory apparatus. In the present study, the electrical permeabilization technique (Knight & Baker, 1982) was used to test this hypothesis further. This technique has been used previously in this laboratory to investigate the interaction between cyclic AMP and calcium upon hormone secretion from dispersed cells of the intermediate lobe of the rat pituitary (Yammamoto *et al.*, 1987) and the 7315c rat anterior pituitary tumour cell line (Guild *et al.*, 1988). These previous findings strongly suggested a post-calcium site of action in the stimulus-secretion coupling pathway for cyclic AMP-mediated stimulation of hormone secretion.

The mechanisms linking changes in cytosolic calcium concentration to changes in hormone secretion remain largely unknown and therefore possible targets for cyclic AMP-dependent phosphorylation are not immediately obvious. There is increasing evidence that GTP-binding proteins are also involved in mediating the effects of second messengers upon cellular function. GTP analogues have been reported to stimulate exocytosis from secretory cells by a mechanism independent of their actions upon the signal-transduction processes (Burgoyne, 1987). It has been suggested that a late stage in the stimulus-secretion coupling may involve a direct regulation of exocytosis by GTP-binding proteins, dubbed G_E by Gomperts and his co-workers (Barrowman *et al.*, 1986). Pre-

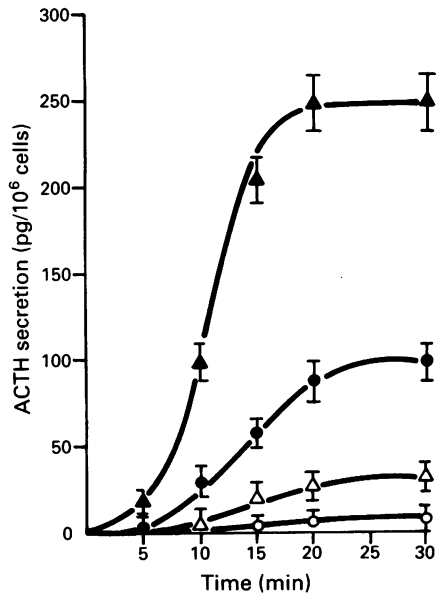

Figure 1 Time course of adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Cells, permeabilized as described in the methods (5 discharges of 2.5 kV cm^{-1}), were incubated for the indicated time periods in standard permeabilization medium modified to contain (1) no calcium and no MgATP (○), (2) no calcium but MgATP (5 mM) (●), (3) calcium (1 mM) but no MgATP (△), or (4) calcium (1 mM) and MgATP (5 mM) (▲). The amount of ACTH secreted into the medium during each time period was determined by radioimmunoassay, as described in the methods. The results are expressed as the means from 3 separate experiments; s.e.mean shown by the vertical bars.

vious findings have suggested that a GTP-binding protein may mediate the effects of changes in cytosolic free calcium concentration on hormone secretion (Luini & De Matteis, 1988; 1990).

The principal aim of this study was to investigate the post-calcium site of action of cyclic AMP in AtT-20 cells (Guild *et al.*, 1986) and to test whether this is at the level of a GTP-binding protein. The results of the present study support the hypothesis that, in AtT-20 cells, cyclic AMP is acting at some site distal to changes in the free cytosolic calcium concentration to potentiate calcium-evoked ACTH secretion. Furthermore, GTP-binding proteins mediate the effect of calcium upon the secretory apparatus. A site of regulation by cyclic AMP may lie at or beyond the level of these GTP-binding proteins controlling secretion.

Methods

Culture of AtT-20 cells

Cells of the mouse AtT-20/D16-16 pituitary tumour were grown and subcultured in Dulbecco's Modified Eagle's Medium (DMEM) containing glucose 4.5 g l^{-1} and supplemented with 10% (w/v) foetal calf serum, as previously described (Reisine, 1984). Cells were plated in 75 cm^2 flasks (Nunc, Gibco, UK) at an initial density of 2 × 10^6 cells/flask and were used 7–9 days after subculturing (80–90% confluency). Routinely between 10–20 × 10^6 cells were harvested from each culture flask.

Preparation of AtT-20 cells for experiments

The culture medium was removed, cells adhering to the substrate were washed 3 times with 10 ml of DMEM supplemented with 0.1% (w/v) bovine serum albumin (DMEM/BSA) and then incubated for 1 h in 10 ml of fresh DMEM/BSA at 37°C in a humidified atmosphere of 5% CO_2 in air. The DMEM/BSA was then decanted and the cells liberated from the substrate by Trypsin/EDTA. The cells were washed twice by centrifugation (200 *g*, 5 min) and 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 and BSA 0.1% (w/v); pH 7.4. After washing, the cells were suspended at a density of 10^6 cells ml^{-1} in this buffer and incubated for a further 30 min at 37°C. The cell suspension was then centrifuged (200 *g*, 5 min) and the cell pellet washed twice by resuspension/centrifugation (200 *g*, 5 min) in the standard permeabilization buffer of the following composition (mM): potassium glutamate 129, PIPES (potassium salt) 20, glucose 5, ATP 5, EGTA 5, BSA 0.1% (w/v); pH 6.6. The cells were finally resuspended in this buffer at a density of 10^7 ml^{-1} and electrically permeabilized by subjection to intense electric fields of brief duration (Knight & Baker, 1982). The nuclear stain, ethidium bromide, is normally impermeant and fluorescent only when in contact with nucleic acid. It can, therefore, be used as an indicator of cell permeabilization. To determine the parameters that lead to 100% permeabilization, the cells were subjected to varying numbers of discharges of varying voltages and subsequently stained with ethidium bromide (50 μM) as previously described (Guild *et al.*, 1988). Optimum permeabilization parameters were also determined for the ability of calcium, added to the external medium, to stimulate ACTH secretion from permeabilized cells as previously described (Guild *et al.*, 1988). For both indicators of permeabilization, optimum parameters were determined to be 5 discharges of 2500 V cm^{-1} (data not shown) and were subsequently adopted in these experiments. The time period for which the cells remained permeabilized was also determined by methods previously described (Guild *et al.*, 1988) and it was found that AtT-20 cells remained permeabilized for 60 min (data not shown).

Measurement of calcium-stimulated ACTH from permeabilized AtT-20 cells

The standard protocol for the determination of ACTH secretion from permeabilized AtT-20 cells was as follows: permeabilized cells were suspended at a cell density of 10^6 cells ml^{-1} in a series of calcium-EGTA buffers chosen to give a free calcium concentration in the range 10^{-9}–10^{-3} M. At this point, the zero time samples were centrifuged (10,000 *g*, 30 s) and an aliquot of the supernatant was stored for subsequent measurement of ACTH content. The cell suspensions were incubated at 37°C for 20 min at which point incubations were terminated by centrifugation (10,000 *g*, 30 s) and removal of the supernatant. The ACTH content of the supernatant was measured by radioimmunoassay. In each experiment, sextuplicate samples were run for each condition. The modifications to this standard protocol which were made to permit the measurement of the effect of cyclic AMP and guanine nucleotides, upon calcium-stimulated ACTH secretion from permeabilized AtT-20 cells, are described in the legends to the figures.

Preparation of calcium-EGTA buffers

In experiments involving the exposure of permeabilized cells to a range of free calcium concentrations in the external medium, calcium-EGTA buffers were employed to obtain the desired free calcium concentration (Portzehl *et al.*, 1964). Various quantities of volumetric 1 M $CaCl_2$ and $MgCl_2$ were added to the standard permeabilization buffer to give the required free calcium concentration and a free magnesium concentration of 1 mM when in equilibrium with 5 mM EGTA and 5 mM MgATP (present in the standard buffer) at pH 6.6. The exact quantity of $CaCl_2$ and $MgCl_2$ to be added was calculated by use of a computer programme written on the basis of previously published programmes for hand held calculators (Perrin & Sayce, 1967; Fabiato & Fabiato, 1979). The free calcium concentration of these Ca-EGTA buffers was checked

by a calcium-sensitive electrode (Affolter & Siegel, 1979) and calibrated by use of calcium standards (Tsien & Rink, 1980).

Radioimmunoassays

The radioimmunossay for ACTH was performed as previously described (Reisine, 1984). The amount of ACTH released by 10^6 cells was expressed as the amount present at the end of the specified incubation period less the amount present at zero time.

Statistics

Each experiment was repeated three times, on different days. A two-sided, 0.05 level, paired *t* test was used to determine if the effect of a treatment was significant.

Materials

The following substances (with their sources) were used: cyclic AMP, ATP, BSA (fraction V) from Sigma, UK; guanosine 5'-O-(3-thiotriphosphate) (GTP-γ-S) and guanosine 5'-O-(2-thiodiphosphate) (GDP-β-S) from Boehringer Mannheim, UK; DMEM, foetal calf serum and trypsin/EDTA were purchased from GIBCO, UK; human ACTH antiserum and human ACTH for standards was the gift of the National Hormone and Pituitary programme, Baltimore, MD, U.S.A.; [^{125}I]-ACTH tracer was the gift of Dr F. Antoni, University of Edinburgh. All other chemicals used were of Analar grade and readily commercially available.

Results

Characterization of calcium-stimulated ACTH secretion

Permeabilized AtT-20 cells secreted ACTH in response to 1 mM external calcium (Figure 1). The ability of calcium to stimulate ACTH secretion was dependent upon the presence of MgATP (5 mM) in the incubation medium. Calcium (1 mM) stimulated an ACTH secretion of 251 ± 10 pg/10^6 cells in a 20 min period in the presence of MgATP but only 24 ± 3 pg/10^6 cells in the absence of MgATP. Interestingly, MgATP alone was able to stimulate significantly ACTH secretion from permeabilized AtT-20 cells in an apparently calcium-independent manner but to a much lesser extent than in the presence of calcium. The time course studies revealed that significant calcium-stimulated ACTH secretion was detectable after 5 min of exposure and that it reached a plateau after 20 min. Consequently, 20 min was chosen as the incubation period for subsequent experiments.

Calcium-evoked ACTH secretion from permeabilized AtT-20 cells was dependent upon the concentration of free calcium in the permeabilization medium (Figure 2). Calcium stimulated ACTH secretion in a concentration-dependent manner between 10^{-7} and 10^{-5} M ($EC_{50} = 7 \times 10^{-7}$ M). Cyclic AMP (10^{-4} M) enhanced calcium-dependent ACTH secretion from permeabilized AtT-20 cells. The amount of ACTH secreted at each effective concentration of calcium was increased (the maximum effect was a 2 fold increase). Cyclic AMP did not significantly change either the EC_{50} of the calcium concentration-effect curve ($EC_{50} = 7 \pm 1 \times 10^{-7}$ M in the absence and $5 \pm 1 \times 10^{-7}$ M in the presence of cyclic AMP) or the concentration of calcium which caused a maximal effect.

The effect of guanine nucleotides upon ACTH secretion from permeabilized AtT-20 cells

The non-hydrolysable GTP analogue, GTP-γ-S (10^{-4} M), which persistently activates GTP-binding proteins, signifi-

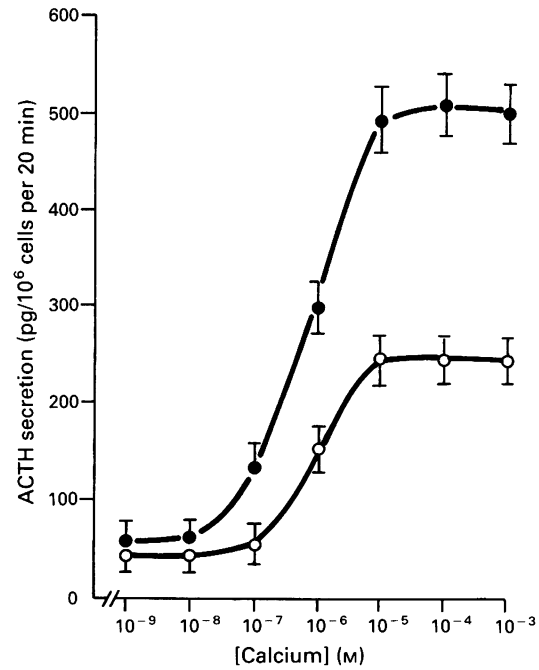

Figure 2 Effect of adenosine 3':5'-cyclic monophosphate (cyclic AMP) on calcium-dependent adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium containing various proportions of calcium and EGTA, such that the indicated concentrations of free calcium resulted, either in the presence (●) or absence (○) of cyclic AMP (10^{-4} M). The amount of ACTH secreted was determined. The results are expressed as the means from 3 separate experiments; s.e.mean shown by vertical bars.

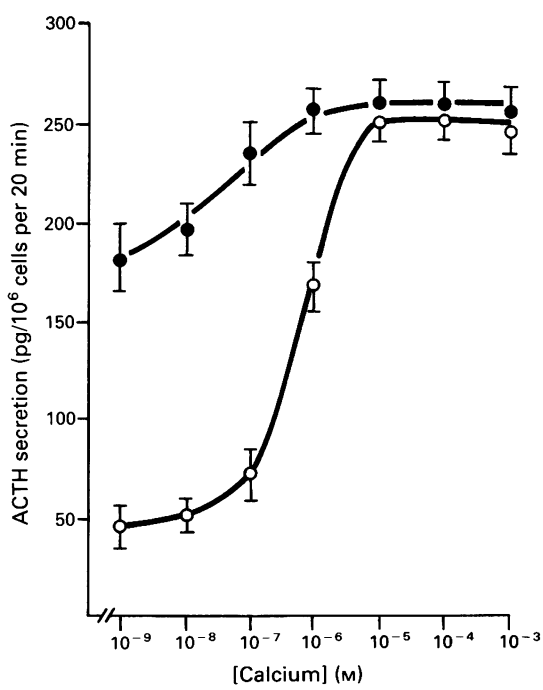

Figure 3 Effect of guanosine 5'-0-(3-thiotriphosphate (GTP-γ-S) on calcium-dependent adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium containing various proportions of calcium and EGTA, such that the indicated concentrations of free calcium resulted, either in the presence (●)or absence (○)of GTP-γ-S (10^{-4} M). The amount of ACTH secreted was determined. The results are expressed as the means from 3 separate experiments; s.e. mean shown by vertical bars.

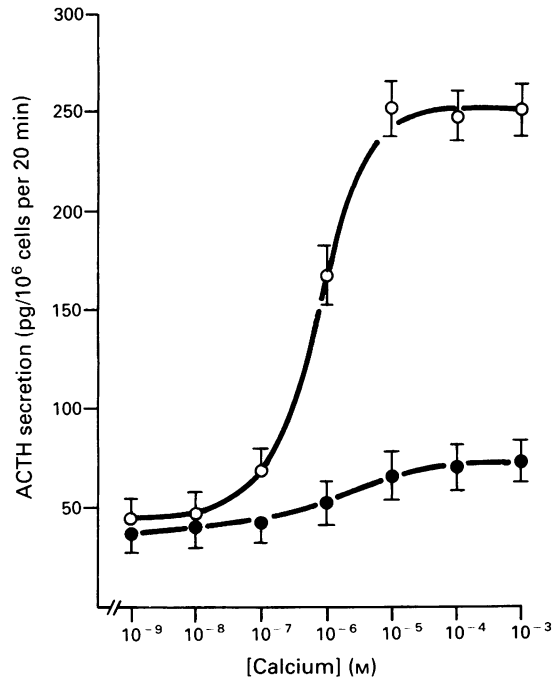

Figure 4 Effect of guanosine 5'-0-(2-thiodiphosphate) (GDP-β-S) on calcium-dependent adrenocorticotrophin (ACTH) secretion from permeabilized AtT-20 cells. Permeabilized cells were incubated in standard permeabilization medium containing various proportions of calcium and EGTA, such that the indicated concentrations of free calcium resulted, either in the presence (●) or absence (○) of GDP-β-S (10^{-4} M). The amount of ACTH secreted was determined. The results are expressed as the means from 3 separate experiments; s.e.mean shown by vertical bars.

cantly stimulated ACTH secretion in the absence of calcium (free calcium concentration of 10^{-9} M, Figure 3). This effect of GTP-γ-S (10^{-4} M) was additive with the stimulation of ACTH secretion evoked by increasing concentrations of free calcium

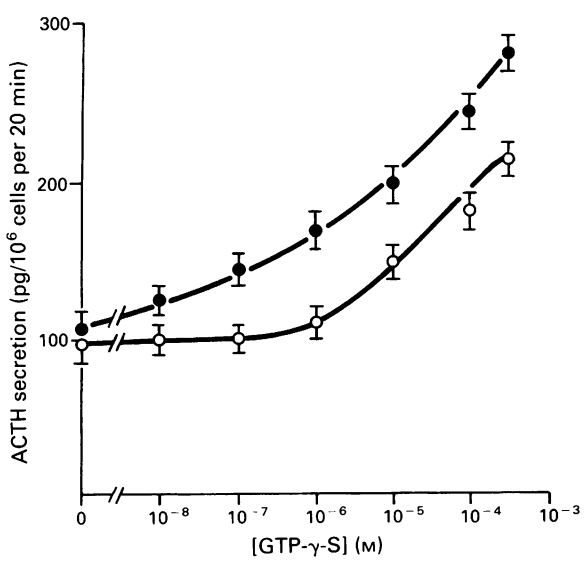

Figure 5 Effect of adenosine 3':5'-cyclic monophosphate (cyclic AMP) on guanosine 5'-0-(3-thiotriphosphate) (GTP-γ-S)-stimulated adrenocorticotrophin (ACTH) secretion from permeabilised AtT-20 cells. Permeabilised cells were incubated in the standard permeabilization medium supplemented with the indicated concentrations of GTP-γ-S either in the presence (●) or absence (○) of cyclic AMP (10^{-4} M). The amount of ACTH secreted was determined. The results are expressed as the means for 3 separate experiments; s.e.mean shown by the vertical bars.

but only up to a maximal value which could be achieved by calcium acting alone. The stable GDP analogue, GDP-β-S (10^{-4} M), used as a method of competing with GTP and thus inhibiting the activation of GTP-binding proteins, inhibited calcium-evoked ACTH secretion (Figure 4). The stimulation of ACTH secretion evoked by 10^{-5} M free calcium was inhibited by 80% in the presence of GDP-β-S (10^{-4} M). The stimulation of ACTH secretion by GTP-γ-S in the absence of calcium was concentration-dependent (Figure 5). The threshold concentration for GTP-γ-S stimulation of ACTH secretion was 10^{-6} M and would appear to be approaching a maximal effect at 3×10^{-4} M. Cyclic AMP (10^{-4} M) enhanced the stimulation of ACTH secretion stimulated by GTP-β-S at all concentrations of the nucleotide investigated. Thus cyclic AMP potentiated both calcium- and guanine nucleotide-stimulated ACTH secretion.

Discussion

Permeabilizing AtT-20 cells by use of the high voltage technique did not impair their ability to undergo exocytosis. Permeabilized tumour cells maintained a secretory response which was dependent upon the free cytosolic calcium concentration and the presence of MgATP. This latter finding is in agreement with previous reports from both this (Yamamoto *et al.*, 1987; Guild *et al.*, 1988) and other laboratories (Knight & Baker, 1982; Bittner *et al.*, 1986; Vallar *et al.*, 1987) in which MgATP has been found to be necessary for secretion in several cell types including digitonin-permeabilized AtT-20 cells (Luini & De Matteis, 1988). Tumour cells permeabilized in the absence of calcium and MgATP did not release ACTH to any significant extent, suggesting that release due to cellular damage was not significant.

Permeabilized AtT-20 cells exhibited increased ACTH secretion when the intracellular calcium concentration was increased between 10^{-7} and 10^{-5} M ($EC_{50} = 7 \times 10^{-7}$ M). These data are entirely consistent with those obtained from several cell types by various permeabilization techniques (for review see Knight & Scrutton, 1986). In addition, the calcium concentration range of 10^{-7}–10^{-5} M is similar to that found in digitonin-permeabilized AtT-20 cells (Luini & De Matteis, 1988) and our own results in dispersed intermediate lobe pituitary cells (Yamamoto *et al.*, 1987) and electrically-permeabilized 7315c cells (Guild *et al.*, 1988). These comparisons indicate that electrically-permeabilized AtT-20 cells may be a useful system for the further investigation of the effects of cyclic AMP upon calcium-evoked ACTH secretion.

Cyclic AMP enhanced calcium-dependent secretion from permeabilized AtT-20 cells. This implies that, in permeabilized AtT-20 cells, cyclic AMP does not enhance hormone secretion by increasing the cytosolic calcium concentration, since any such increases would be buffered by the calcium-EGTA buffers (which are designed to maintain the desired free calcium concentrations). By implication, increased calcium entry into the cytosol from either intra- or extracellular sources (Luini *et al.*, 1985; Guild *et al.*, 1986; Guild & Reisine, 1987; Reisine & Guild, 1987) may not be the only mechanism by which cyclic AMP enhances secretion and an ability to enhance ACTH secretion evoked by a particular increment in cytosolic calcium may be an important additional mechanism. This is consistent with our previous work with intact AtT-20 cells (Guild *et al.*, 1986). If cyclic AMP were to increase the cytosolic calcium concentration in a compartment critical for secretion, then calcium would be more potent in stimulating secretion in its presence. Since this was not observed, the principal site of action of cyclic AMP in enhancing ACTH secretion from permeabilized AtT-20 cells must be distal to the entry of calcium into the cytosol. The fact that cyclic AMP increases the capacity of the AtT-20 cell to release ACTH in

response to a particular increment in cytosolic calcium leads to the speculation that cyclic AMP alters a rate-limiting step in the calcium-dependent release pathway or alters the fusion of secretory vesicles with the cell membrane. It was this speculation which led to the studies of the effects of guanine nucleotides in permeabilized AtT-20 cells.

A new role for GTP-binding proteins has emerged from the use of GTP analogues in permeabilized secretory cells (Burgoyne, 1987) in which they have been reported to stimulate exocytosis by a mechanism independent of their actions upon signal-transduction processes (Bittner *et al.*, 1986; Howell *et al.*, 1987; Vallar *et al.*, 1987). Both membrane fusion and microtubule assembly are sensitive to guanine nucleotides and it is therefore possible that one of these could be the site at which the control of exocytosis is exerted (Barrowman *et al.*, 1986). A postulated GTP-binding protein involved in the control of exocytotic secretion has been dubbed G_E by Gomperts and his co-workers (Barrowman *et al.*, 1986) implying that it is directly involved in membrane fusion in exocytosis. Recent studies in digitonin-permeabilized AtT-20 cells have indicated a role for GTP-binding proteins, perhaps G_E, in the stimulation of ACTH secretion in this cell line (Luini & De Matteis, 1988; 1990). We have shown here that GTP-γ-S is able to stimulate ACTH secretion, in a concentration-dependent manner, in the absence of calcium. In addition, GDP-β-S inhibited calcium-evoked ACTH secretion. These findings strongly support the contention that a GTP-binding protein(s) mediates the stimulation of ACTH secretion evoked by increases in cytosolic calcium concentration.

The nature of the GTP-binding protein directly regulating exocytosis remains unknown. The *ras* (*Ha-*, *Ki*, and *N-ras*) and *ras*-like genes (*rho*, *ral*, *R-ras*, Yptl, *rab2* and *Sec4*) encode an evolutionarily conserved family of proteins, with molecular weights of 20–25,000, which exhibit both GTP/GDP binding and GTPase activities (Barbacid, 1987). It has been suggested that the product of a *ras* gene is a GTP-binding protein involved in the regulation of exocytosis based on the evidence that microinjection of the protein product of a human *ras* gene resulted in degranulation of mast cells (Bar-Sagi & Feramisco, 1986). Interestingly small molecular weight GTP-binding proteins have been implicated as molecular switches in the processing, packaging and exocytosis in constitutive exocytosis in yeast (Salminen & Novick, 1987; Segev *et al.*, 1988; Bourne *et al.*, 1990). Furthermore, the Sec4 gene product Sec4p, rapidly associates with the cytoplasmic surface of secretory vesicles as well as the inner surface of the plasma membrane of the yeast cells (Goud *et al.*, 1988) and has been proposed as important in the promotion of fusion of the secretory vesicle and plasma membranes (Bourne *et al.*, 1990). However, any comparison with the secretory system investigated here is severely limited by the lack of a stimulated exocytotic pathway in yeast and the fact that constitutive membrane traffic in yeast is inhibited by GTP-γ-S (Bourne, 1988).

The ability of cyclic AMP to potentiate GTP-γ-S-evoked ACTH secretion suggests that the site of interaction between calcium and cyclic AMP is either at or at some point distal to the GTP-binding protein(s) mediating the effects of calcium upon the secretory apparatus. Interestingly, small molecular weight GTP-binding proteins have been identified as PKA phosphorylation targets (Lazarowski *et al.*, 1989; Nagata *et al.*, 1989). Furthermore, PKA phosphorylates proteins in intact AtT-20 cells of molecular weight in the range 20–25,000 Da (Bishop *et al.*, 1987; Rougon *et al.*, 1989). It is therefore an attractive idea that cyclic AMP could be acting at the level of a small molecular weight GTP-binding protein or the proteins associated with the regulation of their activity (for review see Bourne *et al.*, 1990) to produce the observed ability of this cyclic nucleotide to potentiate calcium-evoked hormone secretion.

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