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Effects of wortmannin upon the late stages of the secretory pathway of AtT-20 cells

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Abstract

Heterotrimeric GTP-binding (G) proteins, termed Ge, have a role in the late stages of the adrenocorticotrophin (ACTH) secretory pathway in the mouse AtT-20/D16-16 anterior pituitary tumour cell line. The wortmannin sensitivity of Ge-controlled mechanisms in AtT-20 cells was investigated to provide information on the possible mechanisms linking Ge with secretion. Permeabilised cells exposed to calcium ions (10^{-9} to 10^{-3} M), guanosine 5′-*O*-(3-thiotriphosphate) (GTP-γ-S) (10^{-8} to 10^{-4} M) and mastoparan (10^{-8} to 10^{-5} M) demonstrated a significant and concentration-dependent stimulation of ACTH secretion from non-stimulated levels for all three agents. Coincubation with wortmannin (10^{-5} M) significantly inhibited both calcium-independent and -stimulated secretion. The effect of wortmannin was concentration-dependent being maximal at 10^{-6} M. The study shows that wortmannin inhibits both calcium-independent and -stimulated secretion from permeabilised AtT-20 cells indicating a role for phosphatidylinositol-3 kinase in determining the size of the readily releasable pool of ACTH and/or in mediating calcium/Ge-evoked secretion from this pool.

Keywords: G-protein; Wortmannin; Phosphatidylinositol 3-kinase; Exocytosis; ACTH (adrenocorticotrophin); Pituitary, anterior

1. Introduction

The stimulus-secretion coupling mechanisms for adrenocorticotrophin (ACTH) secretion from anterior pituitary corticotrophs have been investigated in the mouse AtT-20/D16-16 anterior pituitary tumour cell line (Sabol, 1980). This is a clonal homogenous population of endocrine cells that has proved useful in the study of corticotrophin-releasing hormone-41 (CRH-41)-regulated ACTH secretion (Luini et al., 1985; Guild and Reisine, 1987; Luini and DeMatteis, 1988; Guild, 1991; McFerran and Guild, 1994, 1995a,b; McFerran et al., 1995). CRH-41 acts via the cAMP-dependent protein kinase pathway in both anterior pituitary corticotrophs (Aguilera et al., 1983) and in AtT-20 cells (Heisler and Reisine, 1984; Miyazaki et al., 1984; Luini et al., 1985; Guild and Reisine, 1987). The connection between the initial changes in cellular cAMP levels and the stimulation of ACTH secretion lies in an interaction between cAMP and the cytosolic free calcium ion concentration. cAMP has a dual action to both enhance calcium ion influx into corticotrophs, which stimulates secretion and to potentiate the effect of such an increment in cytosolic calcium ions upon the secretory apparatus (Luini et al., 1985; Guild et al., 1986; Guild and Reisine, 1987; Guild, 1991). Stimulants of protein kinase C have also been shown to alter cytosolic calcium ion concentrations and stimulate ACTH release from these cells (Reisine and Guild, 1987; McFerran and Guild, 1996).

Calcium 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). Calcium stimulates ACTH secretion from permeabilised AtT-20 cells (Guild, 1991; Luini and DeMatteis, 1988; McFerran and Guild, 1994, 1995a,b; McFerran et al., 1995). Permeabilising cells also permits the introduction of normally impermeant substances into the cytosol and such an

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approach may yield details about the post-calcium stimulus-secretion coupling mechanisms. Studies using the non-hydrolysable GTP analogue guanosine 5′-*O*-(3-thiotriphosphate (GTP-γ-S) have demonstrated a role for GTP-binding (G) proteins, termed Ge, in mediating the ability of calcium ions to stimulate secretion in a variety of secretory cell types including AtT-20 cells (Gomperts, 1990; Guild, 1991; Luini and DeMatteis, 1988, 1990; McFerran and Guild, 1994). Previous results are consistent with Ge in this cell line being a heterotrimeric G-protein (Guild, 1991; McFerran and Guild, 1995a; Erlich et al., 1998). The mechanisms that link Ge to secretion remain unknown.

The capacity of the calcium/Ge system to evoke secretion can be regulated by both cAMP-dependent protein kinase (Guild, 1991) and protein kinase C (McFerran and Guild, 1994) clearly supporting the suggested 'post-calcium' roles for these kinases in the secretory pathway of these cells. Interestingly, a study of the phosphorylation states of proteins in AtT-20 cells in response to stimulants of secretion revealed an increased phosphorylation of some proteins but also a decreased phosphorylation of others (Bishop et al., 1987). This suggests that phosphorylation and dephosphorylation may play an important role in the regulation of secretion. The involvement of kinases and phosphatases in mediating and regulating the actions of Ge upon ACTH secretion has been shown in AtT-20 cells (McFerran and Guild, 1995b, 1996).

Additional protein kinase candidates for involvement in the late stages of the stimulus-secretion coupling mechanisms for ACTH secretion have been sought in the present study. Wortmannin is reported to inhibit myosin light chain kinase and phsophotidylinositol-3-kinase (Nakanishi et al., 1992; Yano et al., 1993). These two protein kinases are involved in stimulus-secretion coupling in anterior pituitary cells and an action by wortmannin in such a system has been reported (Roa et al., 1997). That study, however, was conducted in intact cells where the roles and positions of phosphatidylinositol 3-kinase and myosin light chain kinase in stimulus-secretion coupling are more difficult to determine. This study, however, used a permeabilised cell population to permit an investigation of the late stages of the secretory pathway. Such an experimental system permits an investigation of the post-calcium, Ge-regulated steps of the secretory pathway. The wortmannin sensitivity of Ge-controlled mechanisms in AtT-20 cells was, therefore, investigated to provide further information upon the possible late stage mechanisms linking Ge with secretion. This study shows that wortmannin inhibits both calcium-independent, constitutive secretion and calcium/Ge-stimulated secretion from permeabilised AtT-20 cells. The wortmannin-sensitive kinase responsible for this action is likely to be phosphatidylinositol 3-kinase, which may have a role in determining the size of the readily releasable pool of ACTH and/or in mediating calcium- and G-protein-evoked secretion from this pool.

2. Material and methods

2.1. Culture of AtT-20 cells

Cells of the mouse AtT-20/D16-16 anterior pituitary tumour cell line were grown 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 per flask and were used upon reaching 80–90% confluency.

2.2. Measurement of stimulated ACTH from permeabilised AtT-20 cells

AtT-20 cells were prepared for permeabilisation as previously described (Guild, 1991). Cells were liberated form 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 calcium free with a free calcium concentration of 10^{-9} M.

Permeabilised cells were suspended in a calcium-EGTA buffer containing a free calcium ion concentration of 10^{-9} M either in the presence or absence of wortmannin (10^{-8} to 10^{-5} M) and pre-incubated at 37°C for 30 min. These pre-treated, permeabilised cells were subsequently challenged by stimulants by: (1) further suspension in a series of calcium-EGTA buffers with free calcium ion concentration in the range of 10^{-9} to 10^{-4} M or (2) in a calcium-EGTA buffer containing a free calcium ion concentration of 10^{-9} M in the presence and absence of GTP-γ-S (10^{-6} to 10^{-4} M) or (3) mastoparan (10^{-6} to 10^{-5} M)). These experimental incubations were also performed either in the presence and absence of wortmannin (10^{-8} to 10^{-5} M). 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. In each experiment, sextuplicate samples were run for each condition.

2.3. Radioimmunoassays

The radioimmunoassay for ACTH performed as previously described (McFerran and Guild, 1995a,b). [^{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.

2.4. 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.

2.5. Materials

The following substances (with their sources) were used: forskolin, mastoparan, adenosine 5′-triphosphate (ATP), Bovine serum albumin (fraction V), from Sigma (UK); guanosine 5′-O-(3-thiotriphosphate) (GTP-γ-S) from Boehringer Mannheim, UK; human ACTH antiserum and human ACTH standards were a gift of the National Hormone and Pituitary programme (Baltimore, MD, USA); anti-rabbit immunoglobulin G (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 concentration-dependency of the effects of wortmannin upon stimulated and basal secretion

The effects of wortmannin were concentration-dependent (Fig. 1). Calcium-dependent (10^{-5} M) and calcium-independent (10^{-9} M calcium) ACTH secretion were significantly inhibited by wortmannin at concentrations of wortmannin of 10^{-8} M and above (Fig. 1). The effects of wortmannin were maximal by 10^{-6} M. Similar results for the effects of wortmannin upon GTP-γ-S (10^{-4} M)- and mastoparan (10^{-5} M)-evoked secretion were obtained (data not shown). Consequently, a concentration of 10^{-5} M wortmannin was chosen as a supramaximal concentration for use in the experiments summarised in Figs. 2–4.

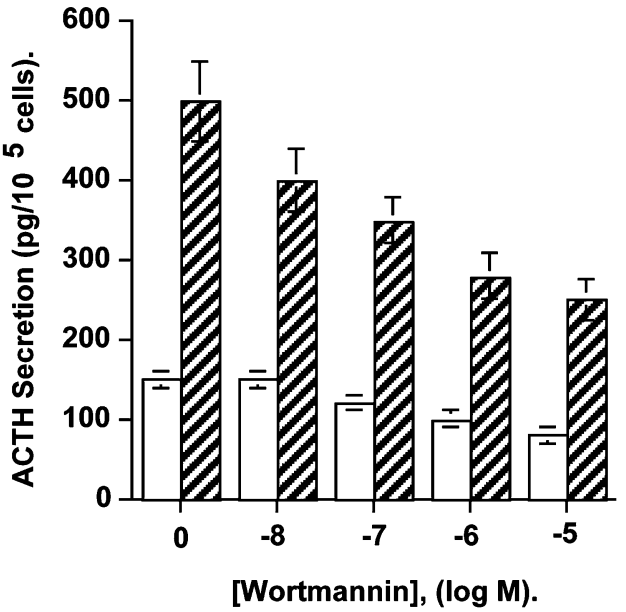

Fig. 1. The concentration dependency of the effect of wortmannin upon calcium (10^{-5} M)-stimulated ACTH secretion. Permeabilised cells were suspended in a 10^{-9} M calcium-EGTA buffer in the presence of the indicated concentrations of wortmannin and pre-incubated at 37°C for 30 min. These pre-treated cells were then suspended either in a 10^{-9} M (basal, open bars) or 10^{-5} M (calcium-stimulated, hatched bars) calcium-EGTA buffer and further incubated in the presence of the indicated concentrations of wortmannin as described in Material and methods. The results are expressed as the mean ± S.E.M. from three separate experiments; S.E.M. is shown by the vertical bars. Absence of error bars indicates that they lie within the symbol used.

3.2. The effect of wortmannin upon calcium-evoked secretion

Permeabilised cells exposed to calcium ions (10^{-9} to 10^{-3} M) demonstrated a significant, concentration-dependent stimulation of ACTH secretion over calcium-independent basal, which was observed over the concentration range of 10^{-7} to 10^{-5} M (Fig. 2). In the presence of wortmannin (10^{-5} M), ACTH secretion was significantly reduced at all concentrations of calcium tested (Fig. 2). Calcium (10^{-5} M)-evoked secretion was significantly reduced from 410 ± 35 to 200 ± 2 pg/10^5 cells/30 min in the presence of wortmannin (Fig. 2), an inhibition in the range of 50%. No change in the concentration-dependency of calcium-evoked secretion was observed.

3.3. The effect of wortmannin upon GTP-γ-S-evoked secretion

Permeabilised cells exposed to GTP-γ-S (10^{-6} to 10^{-4} M) demonstrated a significant, concentration-dependent stimulation of ACTH secretion over basal, which was observed over the concentration range of 10^{-5} to 10^{-4} M (Fig. 3). In the presence of wortmannin (10^{-5} M), GTP-γ-S-evoked ACTH secretion was significantly reduced at all

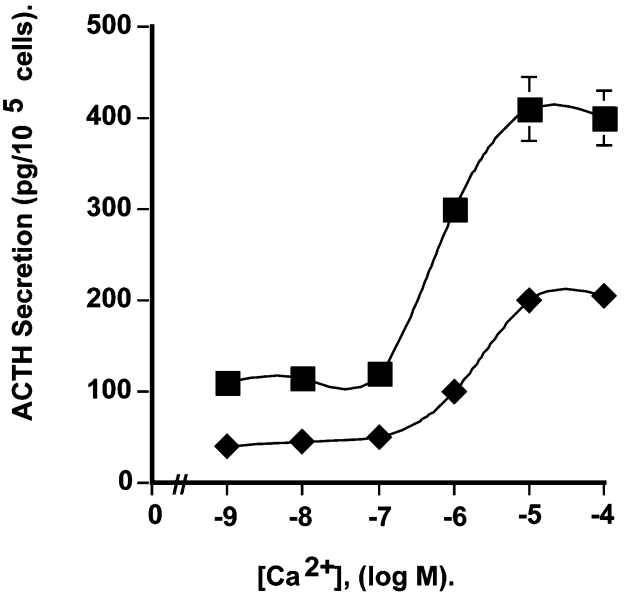

Fig. 2. Effect of wortmannin upon calcium-stimulated ACTH secretion. Permeabilised cells were incubated in a series of calcium-EGTA buffers with the indicated free calcium ion concentrations in either the presence (♦) or absence (■) of wortmannin (10^{-5} M) as described in Material and methods. The results are expressed as the mean ± S.E.M. from three separate experiments; S.E.M. is shown by the vertical bars. Absence of error bars indicates that they lie within the symbol used.

concentrations of the nucleotide tested (Fig. 3). GTP-γ-S (10^{-4} M)-evoked ACTH was significantly reduced from

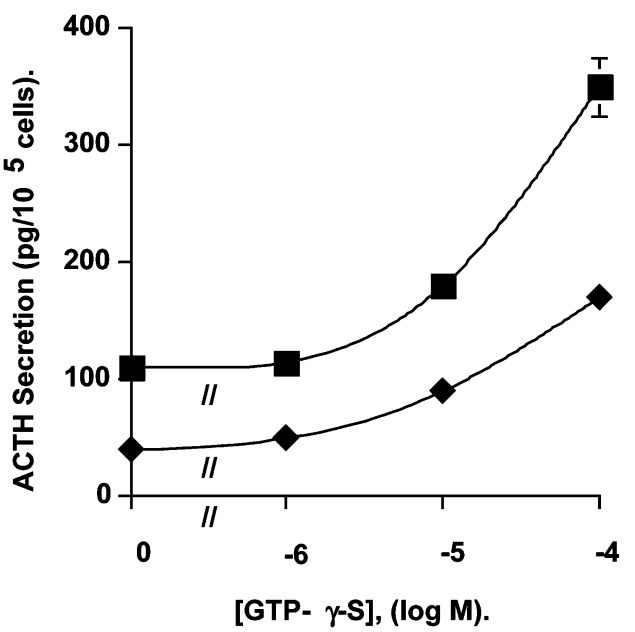

Fig. 3. Effect of wortmannin upon GTP-γ-S-stimulated ACTH secretion. Permeabilised cells were suspended in a calcium-EGTA buffer containing a free calcium ion concentration of 10^{-9} M supplemented with the indicated concentrations of GTP-γ-S, either in the presence (♦) or absence (■) of wortmannin (10^{-5} M) as described in Material and methods. The results are expressed as the mean ± S.E.M. from three separate experiments; S.E.M. is shown by the vertical bars. Absence of error bars indicates that they lie within the symbol used.

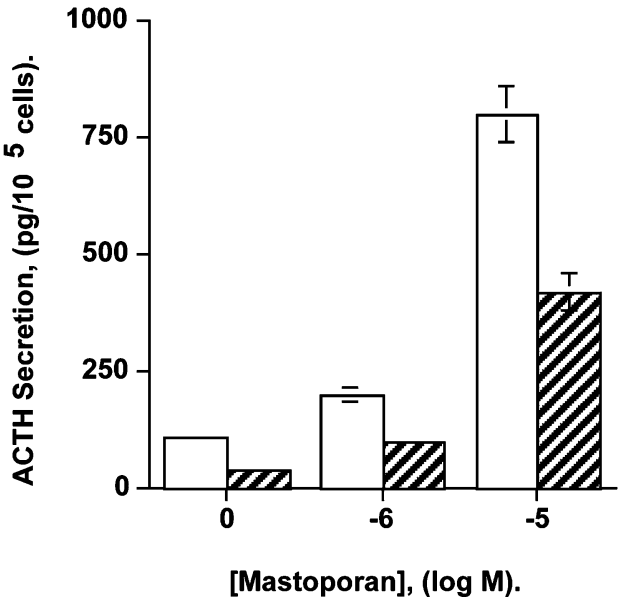

Fig. 4. Effect of wortmannin upon calcium-stimulated ACTH secretion. Permeabilised cells were suspended in a calcium-EGTA buffer containing a free calcium ion concentration of 10^{-9} M supplemented with the indicated concentrations of mastoparan, either in the presence (hatched bars) or absence (open bars) of wortmannin (10^{-5} M) as described in Material and methods. The results are expressed as the mean ± S.E.M. from three separate experiments; S.E.M. is shown by the vertical bars. Absence of error bars indicates that they lie within the symbol used.

350 ± 25 pg/10^5 cells/30 at 10^{-4} M to 170 ± 15 pg/10^5 cells/30 min in the presence of wortmannin (10^{-5} M), an inhibition in the range of 50%.

3.4. The effect of wortmannin upon mastoparan-evoked secretion

Permeabilised cells exposed to mastoparan (10^{-6} to 10^{-5} M) demonstrated a significant, concentration-dependent stimulation of ACTH secretion over basal, which was observed using the concentrations of mastoparan tested, 10^{-6} and 10^{-5} M (Fig. 4). In the presence of wortmannin (10^{-5} M), mastoparan-evoked ACTH secretion was significantly reduced at the two concentrations of the peptide tested (Fig. 4). Mastoparan(10^{-5} M)-evoked secretion was significantly reduced from 800 ± 60 to 420 ± 40 pg/10^5 cells/30 min in the presence of wortmannin (10^{-5} M) (Fig. 4), an inhibition in the range of 50%. Mastoparan evoked the largest increase in ACTH secretion over basal of the three stimulants tested (Figs. 2–4).

4. Discussion

A central observation in stimulus-secretion coupling is that raising the concentration of free calcium ions in the cytosol of secretory cells is a trigger to exocytosis (for

review see Clapham, 1995). The elucidation of the machinery linking this change in cytosolic calcium to secretion was aided by the emergence of the soluble *N*-ethylamide sensitive factor-attachment protein receptor (SNARE) hypothesis, which has provided a model for the molecular machinery surrounding the docking, priming and fusion events of exocytosis itself (Sollner et al., 1993; Sollner, 1995). Although the elements of this 'fusion machine' appear common to all secretory systems, diversity emerges in the mechanisms employed to control it in regulated secretion. These mechanisms not only ensure secretion only under appropriate stimulatory conditions but also reflect the nature of the exocytotic response required from such different secretory systems as nerves, neuroendocrine and endocrine cells (Edwardson and Marciniak, 1995; Lang, 1999; Martin, 1997).

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. Two families of G-proteins—heterotrimeric (Ahnert-Hilger et al., 1994; Aridor et al., 1993; Donaldson et al., 1991; Lyte et al., 1992; Pinxteren et al., 1998; Stow et al., 1991; Toutant et al., 1987) and monomeric (Ahnert-Hilger et al., 1994; Brown et al., 1998; Burgoyne and Morgan, 1989; Darchen et al., 1990; Fischer Von Mollard., 1994; O'Sullivan et al., 1996)—have been put forward as potential candidates for a Ge. An involvement of such Ge proteins in the control of ACTH secretion has been shown in AtT-20 cells (Guild, 1991; Luini and DeMatteis, 1988, 1990; McFerran and Guild, 1994, 1995a,b; Erlich et al., 1998). There is strong evidence in AtT-20 cells for heterotrimeric G-protein involvement in an inhibition of exocytosis where a direct receptor-mediated, pertussis toxin-sensitive, inhibition via G-proteins of the late stages of the secretory pathway is observed (Luini and DeMatteis, 1988, 1990). Evidence also suggests that a Ge involved in the stimulation of secretion in AtT-20 cells could also be a heterotrimeric G-protein (Guild, 1991; McFerran and Guild, 1995a; Erlich et al., 1998). Thus, there may be a dual regulation of ACTH secretion by G-proteins at the late stages of the exocytotic pathway with both a stimulatory Ge and an inhibitory Ge (both possibly heterotrimeric) contributing to the control of the fusion machinery. The present study confirms the ability of calcium ions and Ge-activating compounds to stimulate ACTH secretion from permeabilised cells (Guild, 1991; McFerran and Guild, 1995a,b). Furthermore, it confirms a calcium/Ge-independent, constitutive element of secretion from permeabilised cells observed in the complete absence of cytosolic free calcium ions.

The mechanisms distal to Ge and which connect it to the fusion machinery are still unclear and may involve protein kinase cascades. We have shown that the protein kinases cAMP-dependent protein kinase and protein kinase C are involved in the post-calcium regulation of ACTH secretion and partly regulate the size of the readily releasable pool of ACTH acted upon by the calcium/Ge system (McFerran and Guild, 1996). However, activation of either or both of these kinases cannot substitute for the activation of the calcium/Ge system and so cannot be the direct mediators of calcium's action on the fusion machinery (Guild, 1991; McFerran and Guild, 1994, 1996). Additional protein kinase candidates for involvement in the late stages of the stimulus-secretion coupling mechanisms for ACTH secretion have, therefore, been sought in the present study.

Wortmannin is reported to inhibit myosin light chain kinase and phosophotidylinositol-3-kinase (Nakanishi et al., 1992; Yano et al., 1993). The significance of this fact is that these two protein kinases are involved in stimulus-secretion coupling in secretory cells (Hirasawa et al., 1997; Marquandt et al., 1996; Nakanishi and Stojilkovich, 1997; Roa et al., 1997). Furthermore, an action by wortmannin in anterior pituitary cells has been reported (Nakanishi and Stojilkovich, 1997; Roa et al., 1997). What is unknown and what this study was designed to address is which of these kinases or indeed whether both of these are involved in the late stages of secretion where Ge has a role. Previous reports have used intact cells where it is difficult to answer that question. This study used a permeabilised cell population that permits an investigation of the late stages of the secretory pathway. The ability to investigate the effects of wortmannin in a calcium-free environment in such an experimental system has allowed one previously reported mechanism of action for the drug to be eliminated. The results of the present study indicate that wortmannin inhibits both calcium-independent and calcium/Ge-evoked ACTH secretion from permeabilised AtT-20 cells. The short-term incubations with wortmannin in these experiments would argue against an action of wortmannin upon ACTH synthesis and overall ACTH stores. The results indicate that one or both of phosphatidylinositol 3-kinase and myosin light chain kinase play an important role in the late stages of stimulus-secretion coupling in AtT-20 cells.

A previous study of the wortmannin sensitivity of anterior pituitary regulated secretion concluded that the actions of wortmannin were due to an action of the inhibitor upon myosin light chain kinase (Roa et al., 1997). Myosin light chain kinase is a calcium-sensitive enzyme thus providing one link between cytosolic free calcium ions and secretion (Roa et al., 1997). Myosin light chain kinase is activated by the calcium/calmodulin complex and so a decrease in or absence of cytosolic calcium ions leads to dissociation of the kinase/calmodulin complex as well as isomerisation of myosin light chain kinase to its inactive form (Sweeney et al., 1993). It would seem very unlikely that there is any myosin light chain kinase activity at 1 nM calcium ion concentration. However, in the present study GTP-γ-S and

mastoparan were able to stimulate ACTH secretion in the effective absence of calcium by an action on Ge which is distal to changes in cytosolic free calcium concentration. Thus, an action upon secretion via myosin light chain kinase is unlikely when these two calcium-independent stimulants are used. Thus, the ability of wortmannin to inhibit both GTP-γ-S and mastoparan-evoked secretion is not likely to be due to its ability to inhibit myosin light chain kinase. Furthermore, wortmannin inhibits Ge-regulated secretion in this study at concentrations well below those reported to inhibit myosin light chain kinase (Cross et al., 1995; Nakanishi et al., 1992; Roa et al., 1997) and which are consistent with an action on phosphatidylinositol 3-kinase (Yano et al., 1993). As Ge is a heterotrimeric G-protein in AtT-20 cells (McFerran and Guild, 1995a,b) and one class of phosphatidylinositol 3-kinases is regulated by heterotrimeric G-protein β/γ subunits (Vanhaesbroeck et al., 1997) there could be a role for phosphatidylinositol 3-kinase as a link between Ge and exocytosis.

Phosphatidylinositol 3-kinase is a heterodimer complex composed of a 110-kDa catalytic subunit and an 85-kDa regulatory subunit (Downes and Carter, 1991; Kapeller and Cantley, 1994). The catalytic subunit has a lipid kinase activity that phosphorylates the 3 position of the inositol ring. Phosphatidylinositol 3-kinase utilises phosphatidylinositol 4,5-bis phosphate (PIP_2) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Stephens et al., 1991; Carter et al., 1994). PIP_3 belongs to a class of phosphoinositides that is not a target for PLC and that is essential components for vesicular trafficking (Shepherd et al., 1996). A role for inositol phospholipids in calcium-regulated exocytosis was demonstrated in permeabilised chromaffin cells (Eberhard et al., 1990) where the involvement of phosphatidyl inositol 4-phosphate (PIP) and $PIP_2$was indicated. In PC12 cells, calcium-regulated exocytosis is blocked by anti-PIP_2 antibodies (Hay et al., 1995). Furthermore, proteins involved in phosphoinositide metabolism are necessary for stimulated exocytosis (Hay et al., 1995; Wiedemann et al., 1996). Evidence specifically for the involvement of phosphatidylinositol 3-kinase in the secretory pathway was indicated when a yeast protein involved in vacuolar sorting in yeast turned out to be phosphatidylinositol 3-kinase (Schu et al., 1993). This was followed by the finding that wortmannin inhibited receptor-mediated endocytosis and endosome fusion (Jones and Clague, 1995). A role for phosphatidylinositol 3-kinase in regulated exocytosis comes from the observation that wortmannin inhibits calcium-dependent catecholamine release in adrenal chromaffin cells (Ohara-Imaizumi et al., 1992). An examination of the subcellular location and distribution of phosphatidylinositol 3-kinase during regulated exocytosis in chromaffin cells suggests that phosphatidylinositol 3-kinase is an important component of stimulus-secretion coupling for catecholamine secretion (Chasserot-Golaz et al., 1998).

The present study indicates a strong possibility of an involvement of phosphatidylinositol 3-kinase in the late stages of the ACTH secretory pathway and thus in an endocrine cell. A connection between phosphatidylinositol 3-kinase and the upstream calcium/Ge system has already been postulated but the connection between phosphatidylinositol 3-kinase and the downstream fusion machine is still not clear. The ratio of PIP_2 to PIP_3 may be important in determining the fusogenic nature of the lipid bilayers and so the fusion of secretory granules with the cell membrane (Chasserot-Golaz et al., 1998). Furthermore, the calcium-sensor synaptotagmin, which regulates the SNARE complex (Sudhof and Rizo, 1996), interacts with PIP_2 and PIP_3 in a calcium-dependent manner and may switch between them during exocytosis (Schiavo et al., 1996). These observations may provide a link between phosphatidylinositol 3-kinase and the late stages of regulated exocytosis.

Interestingly, a recent report has implicated phosphatidylinositol 3-kinase in stimulus-secretion coupling in parafollicular cells where it activates an isoform of protein kinase C, protein kinase C ζ, which partly mediates calcium-evoked secretion in these cells (Liu et al., 2000). Protein kinase C contributes to the ACTH secretory response of AtT-20 cells by an action at a late stage in the stimulus-secretion coupling pathway (McFerran and Guild, 1996). Although protein kinase C is capable of stimulating ACTH secretion from AtT-20 cells in the effective absence of calcium ions the secretory response evoked by this route is greatly enhanced by the presence of calcium ions. It therefore appears that calcium ions can still be regarded as the central intracellular regulator of ACTH release from AtT-20 cells however cooperation with protein kinase C is required to uncover what might be a maximal secretory response. Of relevance here is that a major isoform of protein kinase C expressed in AtT-20 cells is protein kinase C ζ (McFerran et al., 1995) which provides yet another potential link between phosphatidylinositol 3-kinase and secretion in these cells.

In conclusion, this study shows that wortmannin inhibits both calcium/Ge-independent and calcium/Ge-stimulated secretion from permeabilised AtT-20 cells indicating a role for wortmannin-sensitive kinases in the late stages of the secretory pathway in endocrine cells. The evidence favours phosphatidylinositol 3-kinase rather than myosin light chain kinase as the kinase of importance here for this action of wortmannin upon the late stages of the secretory pathway. In view of the central role of phosphoinositides and phosphatidylinositol 3-kinase in the late stages of exocytosis, inhibiting phosphatidylinositol 3-kinase by wortmannin would be expected to inhibit exocytosis overall and manifest itself in a reduction in both the basal, constitutive-like and the stimulated secretion of hormone. This role of phosphatidylinositol 3-kinase may be in (1) determining the size of the readily releasable pool of ACTH and/or (2) mediating calcium- and G-protein-

evoked secretion from this pool and/or (3) being part of the 'fusion machine' itself.

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