Exocytosis in chromaffin cells: evidence for a MgATP-independent step that requires a pertussis toxin-sensitive GTP-binding protein

Nicolas VITALE, Danièle THIERSE, Dominique AUNIS and Marie-France BADER

Institut National de la Santé et de la Recherche Médicale, U-338 Biologie de la Communication Cellulaire, 5 rue Blaise Pascal, 67084 Strasbourg Cedex, France

We have previously described that mastoparan, an amphiphilic tetradecapeptide that activates heterotrimeric G-proteins, inhibits Ca^{2+} -induced MgATP-dependent secretion from streptolysin-O-permeabilized chromaffin cells [Vitale, Mukai, Rouot, Thiersé, Aunis and Bader (1993) *J. Biol. Chem.* **268**, 14715–14723]. Our observations suggest the involvement of an inhibitory G_o -protein, possibly located on the membrane of secretory granules, in the final stages of the exocytotic pathway in chromaffin cells. Here, we demonstrate that mastoparan is also able to stimulate the Ca^{2+} -dependent secretion of catecholamines in the absence of MgATP in the medium. This MgATP-independent secretion is totally blocked by tetanus toxin, a potent inhibitor of exocytosis in all neurosecretory cells so far investigated, suggesting that the mastoparan target is a component of the exocytotic machinery. Mas17, a mastoparan analogue inactive on G-proteins, had no effect on catecholamine secretion whereas both Mas7, a highly active analogue of mastoparan, and AIF_4^- , which selectively activates trimeric G-proteins, triggered MgATP-independent secretion. Non-hydrolysable GTP analogues (GTP[S] and p[NH]ppG) mimicked the dual effects of mastoparan on secretion: they inhibited

exocytosis in the presence of MgATP and stimulated MgATP-independent secretion. The different potencies displayed by these two analogues suggest the involvement of two distinct G-proteins. Accordingly, the mastoparan-induced MgATP-independent secretion is highly sensitive to pertussis toxin (PTX) whereas the inhibition by mastoparan of secretion in the presence of MgATP is resistant to PTX treatment. When permeabilized cells were incubated with mastoparan, the release of arachidonic acid increased in a PTX-sensitive manner. 7,7-Dimethyl-5,8-eicosadienoic acid, a potent inhibitor of intracellular phospholipase A_2 , inhibited both the arachidonate release and the MgATP-independent catecholamine secretion evoked by mastoparan. In contrast, neomycin, an inhibitor of phospholipase C, had no significant effect on either the release of arachidonic acid or the secretion of catecholamines provoked by mastoparan. We conclude that two distinct heterotrimeric G-proteins act in series in the exocytotic pathway in chromaffin cells: one controls an ATP-dependent priming step through an effector pathway that remains to be determined, and the second is involved in a late Ca^{2+} -dependent step which does not require MgATP but possibly involves the generation of arachidonic acid.

INTRODUCTION

Among the factors regulating vesicular traffic, the small Ras-like GTPases have received particular attention. These proteins seem to be involved in various steps of the secretory pathway including transport of vesicles from the endoplasmic reticulum to the Golgi, transport within the Golgi, formation of secretory vesicles and transport to the plasma membrane (Goda and Pfeffer, 1989; Balch, 1990, 1992; Gruenberg and Clague, 1992; Rothman and Orci, 1992). Several recent reports indicate that another class of GTP-binding (G) protein, the heterotrimeric G-proteins, may participate in the regulation of vesicular traffic. These proteins have previously been studied in detail as key components of the machinery mediating signal transduction at the plasma membrane (Gilman, 1987; Taylor, 1990), but they may also play some part in intracellular regulation(s) and/or function(s) (Barr et al., 1992). For instance, heterotrimeric G-proteins are found not only at the plasma membrane but also on intracellular membranes. Pertussis toxin (PTX)-sensitive G-proteins have been detected on the endoplasmic reticulum (Audigier et al., 1988), on endosomes (Ali, N. et al., 1989) and on the Golgi complex (Ercolani, 1990), and we have shown their presence on membranes of chromaffin secretory granules (Toutant et al., 1987). These observations suggest that trimeric G-proteins are involved in the regulation of some function(s) of these organelles. Indeed, studies using transfected cells (Stow et al., 1991) and cell-free systems (Barr et al., 1991; Donaldson et al., 1991) show that

a PTX-sensitive trimeric G-protein is involved in the formation of secretory vesicles from the Golgi complex. Furthermore fusion of endosomes, a process known to involve the Ras-like GTPase rab5 (Gorvel et al., 1991), appears to be regulated by a trimeric G-protein (Colombo et al., 1992). These results raise the intriguing possibility that members of the heterotrimeric G-protein family may be implicated in intracellular transport and membrane trafficking.

A regulatory role for a trimeric G_o -protein in the direct control of exocytosis in adrenal chromaffin cells was first suggested by Ohara-Imaizumi et al. (1992). We recently investigated the function(s) of trimeric G-proteins in regulated exocytosis from permeabilized chromaffin cells using mastoparan and AIF_4^- (Vitale et al., 1993). Mastoparan is an amphiphilic tetradecapeptide from wasp venom with a wide variety of cellular actions including mast-cell degranulation (Argiolas and Pisano, 1983; Aridor et al., 1990; Bueb et al., 1990), induction of insulin and 5-hydroxytryptamine (serotonin) secretion (Yokokawa et al., 1989; Ozaki et al., 1990) and stimulation of phospholipase C and phospholipase A_2 (Argiolas and Pisano, 1983; Okano et al., 1985; Norgauer et al., 1992). These effects of mastoparan have been attributed to the activation of heterotrimeric G-proteins as the peptide stimulates GTPase activity of G_i - and G_o -proteins by a mechanism that is virtually identical with that of agonist-bound receptors (Higashijima et al., 1988, 1990; Weingarten et al., 1990). AIF_4^- does not bind to GTPases of the Ras superfamily (Kahn, 1991) but activates selectively trimeric G-proteins by

mimicking the γ -phosphoryl group of GTP (Higashijima et al., 1991). Both mastoparan and AlF_4^- stimulate GTPase activity in chromaffin granule membranes and inhibit Ca^{2+} -evoked catecholamine secretion in permeabilized chromaffin cells (Vitale et al., 1993). Moreover specific antibodies raised against the C-terminus of $\text{G}_{\alpha s}$ reverse both the inhibitory effects of mastoparan on secretion and the stimulatory effects of mastoparan on chromaffin granule-associated G-proteins (Vitale et al., 1993). These findings strengthen the idea of a role for a G_o -protein, possibly located on the membrane of secretory granules, in the final stages of exocytosis in chromaffin cells.

Regulated secretion requires both Ca^{2+} and MgATP. Kinetic studies in permeabilized chromaffin cells (Bittner and Holz, 1992a,b) and patch-clamp analysis of exocytosis in chromaffin cells (Neher and Zucker, 1993) and pituitary cells (Thomas et al., 1993) have led to the realization that exocytosis involves several distinct kinetic steps, each having different Ca^{2+} - and MgATP-dependences. Studies in diverse secretory systems (Vilmart-Seuwen et al., 1986; Holz et al., 1989; Howell et al., 1989; Lumpert et al., 1990; Hay and Martin, 1992) confirm that ATP may act before Ca^{2+} in the sequence of events leading to exocytosis, by maintaining a 'primed' state of the secretory apparatus. Low concentrations of Ca^{2+} seem to be optimal for modulating this ATP-dependent priming reaction, whereas high Ca^{2+} concentrations, in the 100 μM range, are required to trigger the final fusion step in regulated secretion (Augustine and Neher, 1992; Bittner and Holz, 1992b; Neher and Zucker 1993; Thomas et al., 1993). Given the possibility that secretion can be dissected into distinct ATP-dependent and ATP-independent phases in permeabilized cell models (Holz et al., 1989; Bittner and Holz, 1992b), we explored the possibility that trimeric G-proteins may be selectively involved in one or another phase of the secretory machinery. The results presented here show that activation of a PTX-sensitive G-protein with mastoparan or AlF_4^- promotes ATP-independent secretion in permeabilized chromaffin cells presumably through the stimulation of phospholipase A_2 . These findings extend the previous observations showing that a protein of the G_o family acts as an inhibitory control at an ATP-dependent step in secretion (Ohara-Imaizumi et al., 1992; Vitale et al., 1993) and suggest the sequential involvement of two distinct trimeric G-proteins in the exocytotic pathway in adrenal chromaffin cells.

MATERIALS AND METHODS

Culture of chromaffin cells

Chromaffin cells were isolated from bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generating gradients (Bader et al., 1986). They were suspended in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal-calf serum containing streptomycin (50 $\mu\text{g}/\text{ml}$) and penicillin (50 units/ml). Growth of non-chromaffin cells was inhibited by the addition of cytosine arabinoside (10 μM) and 5-fluorodeoxyuridine (10 μM) to the culture medium. Cells were grown on 24 multiple 16 mm Costar plates (Cambridge MA, U.S.A.) at a density of 2.5×10^5 cells/well and maintained at 37 °C in a humidified atmosphere containing 5% $\text{CO}_2/95\%$ air. Release experiments were carried out at 37 °C on 3–7-day-old cultures.

Streptolysin-O (SLO) and tetanus toxin

SLO (Institut Pasteur, Paris, France) was activated with 4 mM dithiothreitol, purified by precipitation with 75%-satd. $(\text{NH}_4)_2\text{SO}_4$ and stored as a suspension in 75%-satd. $(\text{NH}_4)_2\text{SO}_4$

solution at 4 °C. The haemolytic activity of the toxin was determined with a 2.5% rabbit erythrocyte suspension. The dilution of toxin haemolysing 50% of erythrocytes (EC_{50}) was estimated and the reciprocal of this value was taken as the number of haemolytic units/ml of the non-diluted toxin solution.

Tetanus toxin (purity greater than 2000 Lf/mg of protein) was purchased from Calbiochem (San Diego, CA, U.S.A.).

[^3H]Noradrenaline release from permeabilized chromaffin cells

Chromaffin cells loaded with [^3H]noradrenaline were washed four times with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl_2 , 1.2 mM MgCl_2 , 1.2 mM KH_2PO_4 , 11 mM glucose, 0.56 mM ascorbic acid and 15 mM Hepes, pH 7.5) and twice with Ca^{2+} -free Locke's solution (containing 1 mM EGTA). Cells were subsequently permeabilized for 2 min with SLO (15 units/ml) in 200 μl of Ca^{2+} -free KG medium [150 mM glutamate (potassium salt), 10 mM Pipes (pH 7.0), 5 mM nitrilotriacetic acid, 0.5 mM EGTA, 4.5 mM magnesium acetate, 5 mM MgATP and 0.2% BSA] or in MgATP-free Ca^{2+} -free KG medium [150 mM glutamate (potassium salt), 10 mM Pipes (pH 7.2) 5 mM nitrilotriacetic acid, 0.5 mM EGTA, 3.8 mM magnesium acetate and 0.2% BSA] as indicated in the Figure and Table legends. Extracellular fluids were then removed and cells were preincubated in 200 μl of Ca^{2+} -free KG medium or MgATP-free Ca^{2+} -free KG medium with mastoparan or the compound to be tested as indicated in legends. Cells were subsequently stimulated for 10 min with 200 μl of KG medium (in the presence or absence of 5 mM MgATP) containing various amounts of CaCl_2 , to yield the indicated free Ca^{2+} concentrations, and magnesium acetate, to maintain a final free Mg^{2+} concentration of 1 mM. The exact free Ca^{2+} and Mg^{2+} concentrations in the KG medium were calculated as described by Flodgaard and Fleron (1974) using the stability constants given by Sillen and Martell (1971). Release of [^3H]noradrenaline is expressed as the percentage of total radioactivity present in the cells before Ca^{2+} -induced stimulation. The basal release was established by incubating cells for 10 min with Ca^{2+} -free KG medium.

Arachidonic acid release

Cultured chromaffin cells grown on 24-well 16 mm Costar plates at a density of 2.5×10^5 cells/well were incubated for 5 h with culture medium (500 $\mu\text{l}/\text{well}$) containing 0.2 μCi of [^{14}C]arachidonic acid/ml (Amersham; 53 mCi/mmol). Cells were then washed four times with Locke's solution and twice with Ca^{2+} -free Locke's solution and permeabilized for 2 min with SLO (15 units/ml) in Ca^{2+} -free MgATP-free KG medium. Subsequently cells were preincubated for 6 min in Ca^{2+} -free MgATP-free KG medium in the presence or absence of 7,7-dimethyl-5,8-eicosadienoic acid (DEDA) and then stimulated for 1 min in the presence or absence (basal release) of 20 μM free Ca^{2+} in MgATP-free KG medium with or without mastoparan. [^{14}C]Arachidonic acid release after stimulation was determined by measuring the radioactivity present in the incubation medium after centrifugation for 5 min at 12000 g and in cells after solubilization in 1% Triton X-100. Basal [^{14}C]arachidonic acid release estimated in the absence of Ca^{2+} (73 ± 5.7 pmol/ 10^6 cells) represented $0.96 \pm 0.08\%$ of the total radioactivity present in cells before stimulation assessed from the amount released plus the amount remaining in the cells.

Presentation of the data

[^3H]Noradrenaline- and [^{14}C]arachidonic acid-release experiments were performed on at least two different cell preparations.

In the Figures and tables which are representative of a typical experiment, data are given as the mean $\pm$ S.E.M. of three determinations on the same cell preparation. Error bars smaller than the point symbols were not drawn.

RESULTS

Effect of mastoparan on catecholamine secretion from SLO-permeabilized chromaffin cells

Table 1 shows the effects of mastoparan on Ca^{2+} -evoked catecholamine secretion from SLO-permeabilized chromaffin cells. Two types of experiments were designed. Cells were either permeabilized, incubated with mastoparan and subsequently stimulated with $20 \mu\text{M}$ free Ca^{2+} in MgATP-free media or permeabilized, incubated with mastoparan and stimulated with Ca^{2+} in media containing 5 mM MgATP. As has been previously described by us (Vitale et al., 1993), mastoparan inhibited catecholamine release when chromaffin cells were stimulated with Ca^{2+} in the presence of MgATP (Table 1). The continuous presence of MgATP during permeabilization and during the

preincubation with mastoparan was not required to observe the inhibitory effect on secretion. The incubation period with mastoparan was the crucial parameter as mastoparan inhibited secretion only when present within the cells before the cells were challenged with Ca^{2+} and MgATP (results not shown).

When cells were permeabilized and incubated in MgATP-free permeabilizing medium, Ca^{2+} alone was unable to cause significant catecholamine release (Table 1). However, Ca^{2+} was able to trigger secretion in the absence of MgATP when cells were preincubated with mastoparan before stimulation (Table 1) or stimulated in the presence of mastoparan (Table 2). This stimulatory effect of mastoparan on catecholamine secretion was not observed in cells that were stimulated with Ca^{2+} in the presence of MgATP even though they had been permeabilized and preincubated with mastoparan in MgATP-free media. Moreover, a certain delay (4–6 min) between permeabilization and stimulation was required in the absence of added MgATP to observe the stimulatory effect of mastoparan (results not shown). This delay was probably necessary to allow the exit of intracellular MgATP from the permeabilized cells. The stimulatory effect of mastoparan was also not observed when cells were preincubated with mastoparan and MgATP and stimulated with Ca^{2+} in the absence of MgATP (results not shown). Thus mastoparan promoted secretion in conditions in which Ca^{2+} alone was totally unable to trigger exocytotic release, i.e. in the total absence of MgATP. Note that in all experiments performed in the absence of MgATP, the concentration of Mg^{2+} was maintained at 1 mM , indicating that the absence of MgATP species was the actual requirement for mastoparan to stimulate secretion. The presence of Ca^{2+} was also absolutely necessary for mastoparan-evoked MgATP-independent secretion, as mastoparan did not provoke catecholamine release when Ca^{2+} was omitted from the stimulation period (Table 1).

To determine whether mastoparan was able to stimulate a MgATP-independent component of the exocytotic machinery, we examined the effect of tetanus toxin on mastoparan-induced MgATP-independent catecholamine release. Tetanus toxin is a potent neurotoxin that specifically blocks the exocytotic release of neurotransmitters and neurohormones (Habermann and Dreyer, 1986; Schiavo et al., 1992), including catecholamine secretion from chromaffin cells (Penner et al., 1986; Ahnert-Hilger et al., 1989; Bittner et al., 1989). Incubation of permeabilized chromaffin cells with reduced tetanus toxin strongly inhibited the MgATP-independent secretion evoked by Ca^{2+} and mastoparan (Table 2). When the toxin was inactivated, inhibition of mastoparan-induced secretion was not observed, confirming the specificity of the tetanus toxin effect (Table 2). Tetanus toxin did not modify the basal release estimated in the absence of Ca^{2+} or in the presence of Ca^{2+} without mastoparan (Table 2). These observations suggest that the catecholamine release triggered by mastoparan and Ca^{2+} in the absence of MgATP is likely to be due to the stimulation of a component of the exocytotic machinery that does not require ATP rather than to an artificial lysis of the secretory granules.

Figure 1 shows the effects of various mastoparan concentrations on catecholamine secretion from SLO-permeabilized chromaffin cells. Cells were permeabilized, incubated with mastoparan and stimulated with $20 \mu\text{M}$ free Ca^{2+} in the presence or absence of 5 mM MgATP. Confirming our previous observations (Vitale et al., 1993), mastoparan produced a dose dependent inhibition of MgATP-dependent Ca^{2+} -evoked secretion with a mean inhibitory dose, ID_{50} , of approximately $10 \mu\text{M}$. In the absence of MgATP, mastoparan triggered the release of catecholamines with a mean stimulatory concentration of $14 \mu\text{M}$. Both stimulatory and inhibitory effects of mastoparan required the presence

Table 1 Effect of mastoparan on Ca^{2+} -evoked catecholamine release from SLO-permeabilized chromaffin cells in the presence or absence of MgATP

Chromaffin cells were permeabilized for 2 min with SLO (15 units/ml) in Ca^{2+} -free KG medium in the presence or absence of 5 mM MgATP as indicated. Cells were then preincubated for 10 min in Ca^{2+} -free KG medium in the presence or absence of 5 mM MgATP with or without $20 \mu\text{M}$ mastoparan. Cells were subsequently stimulated in the presence or absence of $20 \mu\text{M}$ free Ca^{2+} in KG medium containing 5 mM MgATP or in MgATP-free KG medium. [^3H]Noradrenaline release is expressed as the percentage of total radioactivity present in the cells before stimulation.

[Ca^{2+}] (μM)	[^3H]Noradrenaline release (%)			
	ATP-free		ATP (5 mM)	
	Control	Mastoparan	Control	Mastoparan
0	4.2 $\pm$ 0.1	4.5 $\pm$ 0.1	4.4 $\pm$ 0.1	5.0 $\pm$ 0.1
20	4.5 $\pm$ 0.1	12.3 $\pm$ 0.1	26.5 $\pm$ 0.4	8.6 $\pm$ 0.2

Table 2 Effect of tetanus toxin on mastoparan-induced secretion in the absence of MgATP

Chromaffin cells were exposed for 2 min to 15 units/ml SLO in Ca^{2+} -free MgATP-free KG medium. Cells were then preincubated for 6 min in Ca^{2+} -free MgATP-free KG medium containing 1 mM dithiothreitol and when indicated either $1 \mu\text{M}$ tetanus toxin (TTX) or $1 \mu\text{M}$ TTX previously heated for 15 min to 95°C . Cells were subsequently stimulated for 10 min with Ca^{2+} -free MgATP-free KG medium or with MgATP-free KG medium containing $20 \mu\text{M}$ free Ca^{2+} in the presence or absence of $20 \mu\text{M}$ mastoparan.

	[^3H]Noradrenaline release (%)		
	Untreated	TTX	Heat-treated TTX
Zero Ca^{2+}	5.1 $\pm$ 0.3	6.1 $\pm$ 0.1	5.4 $\pm$ 0.1
$20 \mu\text{M}$ Ca^{2+}	5.5 $\pm$ 0.2	6.5 $\pm$ 0.2	5.6 $\pm$ 0.1
Zero Ca^{2+} + mastoparan	5.8 $\pm$ 0.3	6.6 $\pm$ 0.3	5.8 $\pm$ 0.5
$20 \mu\text{M}$ Ca^{2+} + mastoparan	11.7 $\pm$ 0.2	7.2 $\pm$ 0.4	12.2 $\pm$ 0.2

Figure 1 Effects of various mastoparan concentrations on secretion in the presence or absence of MgATP

Chromaffin cells were permeabilized for 2 min with SLO in Ca^{2+} -free KG medium in the presence (■, □) or absence (●, ○) of 5 mM MgATP. Cells were then incubated with the indicated concentrations of mastoparan for 10 min in Ca^{2+} -free KG medium containing 5 mM MgATP (■, □) or for 6 min in Ca^{2+} -free MgATP-free KG medium (●, ○). Extracellular fluids were subsequently removed and cells were stimulated for 10 min in the presence (■, ●) or absence (□, ○) of 20 μM free Ca^{2+} with (■, ●) or without (□, ○) 5 mM MgATP.

Table 3 Effect of mastoparan, mastoparan analogues and AlF_4^- on secretion from SLO-permeabilized cells in the absence of MgATP

Chromaffin cells were treated for 2 min with SLO in Ca^{2+} -free MgATP-free KG medium. After removal of extracellular fluids, cells were incubated for 6 min in Ca^{2+} -free MgATP-free KG medium and subsequently stimulated for 10 min in the presence or absence of 20 μM free Ca^{2+} in MgATP-free KG medium (control) or in MgATP-free KG medium containing 20 μM mastoparan, 10 μM Mas7, 20 μM Mas17 or 20 mM NaF plus 50 μM AlCl_3 (AlF_4^-) as indicated. AlF_4^- , mastoparan and the active analogue Mas7 induced MgATP-independent secretion of [^3H]noradrenaline in the presence of Ca^{2+} . The inactive analogue Mas17 was unable to produce secretion in the absence of ATP.

[Ca^{2+}] (μM)	[^3H]Noradrenaline release (%)				
	Control	Mastoparan	Mas7	Mas17	AlF_4^-
0	6.0 ± 0.1	6.0 ± 0.1	6.2 ± 0.2	5.6 ± 0.1	5.9 ± 0.1
20	6.2 ± 0.2	11.8 ± 0.2	11.5 ± 0.2	5.7 ± 0.1	11.6 ± 0.1

of Ca^{2+} , as mastoparan had no effect on basal catecholamine release measured in the absence of Ca^{2+} (Figure 1).

We also compared the effects of Mas7, a mastoparan analogue highly active in stimulating heterotrimeric G-proteins (Higashijima et al., 1990), and Mas17, an inactive analogue (Higashijima et al., 1990), on catecholamine secretion in the absence of MgATP. As illustrated in Table 3, Mas7 mimicked the stimulatory effect of mastoparan but Mas17 had no effect. Similarly AlF_4^- , which selectively activates heterotrimeric but not monomeric G-proteins (Higashijima et al., 1991; Kahn, 1991), triggered MgATP-independent secretion in the presence of Ca^{2+} (Table 3). Neither Mas7 nor AlF_4^- stimulated basal release in the absence of Ca^{2+} .

Figure 2 Effect of various Ca^{2+} concentrations on mastoparan-induced secretion in the absence of MgATP

Chromaffin cells were exposed for 2 min to 15 units/ml SLO in Ca^{2+} -free MgATP-free KG medium. Cells were then preincubated for 6 min in Ca^{2+} -free MgATP-free KG medium and subsequently stimulated for 10 min with increasing Ca^{2+} concentrations in MgATP-free KG medium in the presence (●) or absence (○) of 20 μM mastoparan.

Taken together these results indicate that several activators of heterotrimeric G-proteins, such as mastoparan, mastoparan peptide analogues and AlF_4^- , affect the exocytotic process at two different stages: they block a MgATP-dependent step (Vitale et al., 1993; this report) and, as shown here, they stimulate a Ca^{2+} -dependent element when MgATP is omitted. The fact that the absence of MgATP was a strict requirement to observe this stimulatory effect of mastoparan on catecholamine secretion suggests that the Ca^{2+} -dependent element sensitive to G-protein activators occurs after the MgATP-dependent step in the exocytotic process.

Ca^{2+} and ATP dose-response curves for the effects of mastoparan on secretion in permeabilized chromaffin cells

Figure 2 illustrates the calcium dose-response curve for mastoparan-induced secretion. Cells were permeabilized, incubated and stimulated with various Ca^{2+} concentrations in the absence of MgATP. Ca^{2+} at concentrations up to 100 μM was unable to produce secretion in the absence of MgATP. Mastoparan promoted MgATP-independent secretion over the range of Ca^{2+} concentrations tested. Half-maximal stimulation occurred at 20 μM free Ca^{2+} (Figure 2). The peptide had no effect on basal release estimated in the absence of Ca^{2+} indicating that the target of mastoparan might be a G-protein involved in a Ca^{2+} -dependent step of the secretory process.

In order to characterize further the activity of mastoparan in permeabilized cells, we examined its effect on Ca^{2+} -evoked secretion in the presence of increasing concentrations of MgATP. As shown in Figure 3, Ca^{2+} alone was able to trigger secretion only when the concentration of MgATP was above 100 μM . However, in the presence of mastoparan, Ca^{2+} produced sustained secretion from 0 to 100 μM MgATP. Above 100 μM MgATP, mastoparan inhibited the MgATP-dependent secretion, and was more effective as the MgATP concentration increased

Figure 3 Effects of various MgATP concentrations on the effect of mastoparan on secretion in SLO-permeabilized chromaffin cells

Chromaffin cells labelled with [^3H]noradrenaline were permeabilized for 2 min with SLO in Ca^{2+} -free KG medium in the absence of MgATP. Cells were then preincubated for 10 min in Ca^{2+} -free MgATP-free KG medium in the presence (■, □) or absence (●, ○) of 20 μM mastoparan. Cells were subsequently stimulated for 10 min with KG medium containing the indicating concentrations of MgATP in the presence (■, ●) or absence (□, ○) of 20 μM free Ca^{2+} .

Table 4 Effect of mastoparan on secretion from cells preincubated with metabolic inhibitors

Chromaffin cells labelled with [^3H]noradrenaline were washed for 40 min in modified Locke's solution (glucose and KH_2PO_4 were omitted) in the presence or absence of metabolic inhibitors (4 $\mu\text{g}/\text{ml}$ antimycin A and 30 mM 2-deoxy-D-glucose). Cells were then incubated for 10 min in modified Ca^{2+} -free Locke's solution in the presence or absence of metabolic inhibitors as indicated and subsequently permeabilized for 2 min with SLO in Ca^{2+} -free MgATP-free KG medium. After a 6 min incubation period in Ca^{2+} -free MgATP-free KG medium, cells were stimulated for 10 min with 50 μM free Ca^{2+} in the presence or absence of 20 μM mastoparan in MgATP-free KG medium. The basal release was determined in Ca^{2+} -free MgATP-free KG medium in the presence or absence of 20 μM mastoparan.

[Ca^{2+}] (μM)	[^3H]Noradrenaline release (%)			
	Untreated		2-Deoxy-D-glucose + antimycin A	
	Control	Mastoparan	Control	Mastoparan
0	5.7 $\pm$ 0.4	6.6 $\pm$ 0.4	6.5 $\pm$ 0.4	7.3 $\pm$ 0.4
50	6.1 $\pm$ 0.3	15.5 $\pm$ 0.4	7.1 $\pm$ 0.2	7.3 $\pm$ 0.3

(Figure 3). In other words, mastoparan had dual effects on Ca^{2+} -evoked exocytosis in chromaffin cells: it inhibited MgATP-dependent secretion at high MgATP concentration and promoted MgATP-independent secretion when the cytosolic concentration of MgATP was too low to support secretion. More importantly, the stimulatory effect of mastoparan was never observed when MgATP-dependent secretion occurred, suggesting that mastoparan may activate two different G-proteins that probably act in series in the exocytotic machinery.

Effect of metabolic inhibitors on MgATP-independent secretion induced by mastoparan

In diverse secretory cell types, it has been reported that ATP acts before Ca^{2+} in the pathway of regulated secretion (Vilmart-Seuwen et al., 1986; Holz et al., 1989; Howell et al., 1989; Lumpert et al., 1990; Hay and Martin, 1992). Thus intact chromaffin cells are primed by intracellular ATP so that immediately after permeabilization there is a component of secretion that is independent of exogenous ATP (Holz et al., 1989; Bittner and Holz, 1992b). In order to determine whether mastoparan stimulated the release of granules through a MgATP-independent secretory pathway or the release of granules that were maintained in a primed state by cytosolic ATP before permeabilization, we used metabolic inhibitors. Holz et al. (1989) have previously shown that metabolic inhibitors rapidly deplete chromaffin cells of cytosolic ATP and that the MgATP-independent component of exocytosis disappears in poisoned cells. Table 4 illustrates the effect of metabolic inhibitors on mastoparan-induced ATP-independent secretion. Depletion of cytosolic ATP was assessed by permeabilizing cells for 10 min in MgATP-free medium in the presence or absence (basal release) of 50 μM free Ca^{2+} . Basal release in unpoisoned cells was slightly lower than in metabolically inhibited cells, being $13.8 \pm 0.3\%$ and $16.7 \pm 0.7\%$ respectively. In the absence of exogenous MgATP, Ca^{2+} produced $23.1 \pm 0.7\%$ secretion in unpoisoned cells but was unable to support secretion ($15.4 \pm 0.6\%$) in metabolically inhibited cells, confirming the depletion of cytosolic ATP. If the mastoparan-induced ATP-independent secretion from SLO-permeabilized cells depends on a prior reaction involving MgATP in intact cells, then mastoparan-evoked secretion should not occur in cells pretreated with metabolic inhibitors. As predicted, no MgATP-independent secretion was detectable in metabolically inhibited cells stimulated with Ca^{2+} and mastoparan (Table 4). In contrast, unpoisoned cells underwent significant MgATP-independent secretion when stimulated with Ca^{2+} and mastoparan (Table 4). These results strongly suggest that mastoparan stimulates a step of the exocytotic machinery that may correspond to the ATP-primed state described by Holz et al. (1989) and Bittner and Holz (1992b) in chromaffin cells and by others in different secretory cell types (Vilmart-Seuwen et al., 1986; Howell et al., 1989; Lumpert et al., 1990; Hay and Martin, 1992).

Effect of non-hydrolysable GTP analogues on MgATP-dependent and MgATP-independent secretion from permeabilized chromaffin cells

To strengthen the hypothesis that mastoparan stimulates two different G-proteins involved in MgATP-dependent and MgATP-independent steps of exocytosis, we examined the effects of different non-hydrolysable analogues of GTP that can discriminate between G-proteins. Figure 4(a) shows the effects of GTP[S] and p[NH]ppG on MgATP-independent secretion. Both analogues triggered release in the presence of Ca^{2+} but, at concentrations below 20 μM , p[NH]ppG was twice as effective as GTP[S]. In contrast, when cells were stimulated in the presence of MgATP (Figure 4b), the inhibiting effect of p[NH]ppG on Ca^{2+} -evoked catecholamine release was only 50% of that obtained with GTP[S]. Thus GTP analogues mimicked the dual effects of mastoparan on Ca^{2+} -evoked secretion by inhibiting exocytosis in the presence of MgATP and stimulating MgATP-independent release. The different potencies observed with the two GTP analogues are in line with the possible involvement of two distinct G-proteins in MgATP- and Ca^{2+} -dependent stages in the exocytotic pathway.

Figure 4 Effect of non-hydrolysable analogues of GTP on secretion from SLO-permeabilized cells in the presence or absence of MgATP

(a) Effects of GTP[S] and p[NH]ppG on secretion in the absence of MgATP. Chromaffin cells were permeabilized in the absence of MgATP, preincubated for 6 min in Ca²⁺-free MgATP-free KG medium and then stimulated for 10 min in the presence (■, ●) or absence (□, ○) of 20 μM free Ca²⁺ in MgATP-free KG medium containing the indicated concentrations of either GTP[S] (●, ○) or p[NH]ppG (■, □). p[NH]ppG was more potent than GTP[S] in promoting Ca²⁺-activated [³H]noradrenaline release in the absence of MgATP. (b) Effects of GTP[S] and p[NH]ppG on Ca²⁺-evoked secretion in the presence of MgATP. Cells were permeabilized with SLO in Ca²⁺-free KG medium containing 5 mM MgATP and then preincubated for 10 min with various concentrations of GTP[S] (●, ○) or p[NH]ppG (■, □) in Ca²⁺-free KG medium containing 5 mM MgATP. Subsequently, cells were stimulated for 10 min in the presence (■, ●) or absence (□, ○) of 20 μM free Ca²⁺ in KG medium containing 5 mM MgATP. p[NH]ppG was less effective in inhibiting MgATP-dependent Ca²⁺-evoked secretion than GTP[S].

Figure 5 Effect of PTX on the dual effects of mastoparan on secretion from permeabilized chromaffin cells

(a) Effect of PTX on mastoparan-induced MgATP-independent catecholamine secretion. Chromaffin cells loaded with [³H]noradrenaline were incubated for 4 h in the presence or absence of 1 μg/ml PTX as indicated. Cells were then permeabilized with SLO in Ca²⁺-free MgATP-free KG medium, incubated for 6 min in Ca²⁺-free MgATP-free KG medium and subsequently stimulated for 10 min in the presence (■) or absence (□) of 20 μM free Ca²⁺ in MgATP-free KG medium. The presence (MP) or absence (control) of 20 μM mastoparan during the stimulation period is indicated. Pretreatment with PTX abolished completely the mastoparan-induced secretion observed in the absence of MgATP. (b) Influence of PTX on the inhibitory effect of mastoparan on MgATP-dependent secretion. Cells loaded with [³H]noradrenaline were incubated for 24 h in the presence or absence of 1 μg/ml PTX as indicated. Cells were permeabilized and preincubated for 10 min in the presence or absence of 20 μM mastoparan in Ca²⁺-free KG medium containing 5 mM MgATP. Subsequently, cells were stimulated for 10 min with (■) or without (□) 20 μM free Ca²⁺ in KG medium containing 5 mM MgATP. PTX treatment was unable to reverse the inhibitory effect of mastoparan on Ca²⁺-triggered secretion in the presence of 5 mM MgATP.

Effect of pertussis toxin (PTX)

In an attempt to determine whether the mastoparan-stimulated G-proteins involved in the exocytotic process in chromaffin cells are PTX substrates, we compared the PTX sensitivity of the mastoparan effects on MgATP-independent and -dependent secretion. As previously reported (Vitale et al., 1993), we found that the inhibitory effect of mastoparan on Ca^{2+} -evoked secretion in the presence of MgATP was resistant to PTX even after prolonged incubation periods with the toxin (Figure 5b). Similarly the inhibitory effect of GTP[S] on MgATP-dependent secretion was also found to be insensitive to PTX treatment (Ahnert-Hilger et al., 1992; Sontag et al., 1992). In contrast, as illustrated in Figure 5(a), the mastoparan-induced secretion observed in the absence of MgATP was highly sensitive to PTX, an observation that provides additional evidence for the involvement of two distinct heterotrimeric G-proteins in the exocytotic machinery in chromaffin cells.

Effect of mastoparan on arachidonic acid release in SLO-permeabilized chromaffin cells

We examined the possible intracellular pathways involved in the mastoparan stimulation of exocytosis in the absence of exogenous MgATP. Mastoparan has a wide variety of cellular actions which have been attributed to the activation of heterotrimeric G-proteins (Argiolas and Pisano, 1983; Yokokawa et al., 1989; Aridor et al., 1990; Bueb et al., 1990; Ozaki et al., 1990). For instance, mastoparan stimulates the activity of phospholipase A_2 and phospholipase C (Argiolas and Pisano, 1983; Okano et al., 1985; Norgauer et al., 1992), two enzymes known to be regulated by heterotrimeric G-proteins (Axelrod et al., 1988; Cockcroft,

Table 5 Effect of mastoparan, DEDA and PTX on [^{14}C]arachidonic acid release in resting and stimulated permeabilized chromaffin cells

Chromaffin cells were loaded for 5 h with [^{14}C]arachidonic acid in culture medium in the presence or absence of PTX (1 $\mu\text{g}/\text{ml}$; 5 h). Cells were then washed, permeabilized with SLO in Ca^{2+} -free MgATP-free KG medium and preincubated for 6 min in Ca^{2+} -free MgATP-free KG medium in the presence or absence of 75 μM DEDA or 500 μM neomycin, as indicated. Cells were subsequently stimulated for 1 min in the presence or absence of 20 μM free Ca^{2+} and 20 μM mastoparan as indicated. The basal Ca^{2+} -independent [^{14}C]arachidonic acid release (73 ± 5.7 pmol/ 1×10^6 cells) represented $0.96 \pm 0.08\%$ of the total [^{14}C]arachidonic acid present in the cells before stimulation. Mastoparan increased the release of [^{14}C]arachidonic acid in the presence of Ca^{2+} . Both PTX and DEDA reversed totally this stimulatory effect of mastoparan.

	[^{14}C]Arachidonic acid release (pmol/ 10^6 cells)			
	Control	+ Neomycin	+ PTX	+ DEDA
Zero Ca^{2+}	73.0 ± 5.7	71.6 ± 2	75.1 ± 2.1	69.7 ± 0.9
20 μM Ca^{2+}	75.8 ± 6.3	69.7 ± 1.4	72.6 ± 1.1	69.7 ± 0.6
Zero Ca^{2+}	73.6 ± 3.2	71.0 ± 0.2	73.4 ± 0.7	73.4 ± 2.3
+ mastoparan				
20 μM Ca^{2+}	140.1 ± 2.9	140 ± 0.8	73.9 ± 5.9	80.1 ± 1.5
+ mastoparan				

1992). Because arachidonic acid, the principal fatty acid liberated by phospholipase A_2 , is thought to have an important function in the exocytotic machinery, we examined the possible relationship between arachidonate generation and mastoparan-stimulated exocytosis. Table 5 shows the effect of mastoparan on arachidonic acid release in permeabilized chromaffin cells. In the

Figure 6 Effect of DEDA, a phospholipase A_2 inhibitor, and neomycin, a phospholipase C inhibitor on mastoparan-evoked catecholamine release in the absence of MgATP

(a) Effect of neomycin on catecholamine secretion induced by mastoparan in the absence of MgATP. Chromaffin cells were permeabilized for 2 min with SLO in Ca^{2+} -free MgATP-free KG medium. Cells were then preincubated for 6 min in Ca^{2+} -free MgATP-free KG medium in the presence or absence of 500 μM neomycin. Cells were subsequently stimulated for 10 min in the presence or absence of 20 μM mastoparan and 500 μM neomycin as indicated in MgATP-free Ca^{2+} -free KG medium (□) or in MgATP-free KG medium containing 20 μM free Ca^{2+} (■). (b) Effect of DEDA on mastoparan-induced catecholamine secretion. Chromaffin cells were permeabilized with SLO in Ca^{2+} -free MgATP-free KG medium and then incubated for 6 min in Ca^{2+} -free MgATP-free KG medium containing the indicated concentrations of DEDA. Cells were stimulated for 10 min in the presence (■) or absence (□) of 20 μM free Ca^{2+} in MgATP-free KG medium containing 20 μM mastoparan and various concentrations of DEDA. DEDA produced a dose-dependent inhibition of mastoparan-induced secretion in the absence of MgATP.

presence of mastoparan, the amount of [^{14}C]arachidonic acid released into the medium increased almost 2-fold when cells were stimulated with Ca^{2+} in the absence of MgATP. Pretreatment with PTX did not significantly modify the basal [^{14}C]arachidonic acid release measured in the absence of Ca^{2+} , nor did it affect the [^{14}C]arachidonic acid release evoked by 20 μM free Ca^{2+} (Table 5). However, PTX completely abolished the mastoparan-stimulated [^{14}C]arachidonic acid release, which is consistent with the possibility that mastoparan enhanced the formation of arachidonate in chromaffin cells by activating a PTX-sensitive heterotrimeric G-protein.

Effect of phospholipases A_2 and C inhibitors on mastoparan-stimulated secretion in the absence of MgATP

In chromaffin cells, the release of arachidonic acid has been ascribed to either activation of phospholipase A_2 (Petit et al., 1992) or sequential action of phospholipase C and diacylglycerol lipase (Rindlisbacher et al., 1990). As G-proteins are involved in activation of both phospholipase C and phospholipase A_2 , we used specific inhibitors to determine the actual pathway by which mastoparan stimulates arachidonic acid release. Neomycin, which inhibits phospholipase C by binding phosphatidylinositols (Schacht, 1976; Cockcroft and Gomperts, 1985), did not antagonize the stimulatory effect of mastoparan on [^{14}C]arachidonic acid release (Table 5) and only slightly reduced the mastoparan-dependent catecholamine release observed in the absence of MgATP (Figure 6a). In contrast, DEDA, a well-characterized inhibitor of intracellular phospholipase A_2 (Lister et al., 1989), inhibited mastoparan-stimulated catecholamine secretion in a dose-dependent manner, reaching complete inhibition at 75 μM (Figure 6b). Similarly, DEDA at 75 μM totally blocked the effect of mastoparan on [^{14}C]arachidonic acid release in permeabilized chromaffin cells (Table 5). Similar results were obtained with quinacrine, an antimalarial agent known to inhibit phospholipase A_2 , although millimolar concentrations were required (results not shown). These data emphasize the close relationship between mastoparan-induced arachidonate generation and MgATP-independent secretion and suggest the possibility that mastoparan may stimulate the MgATP-independent step of the exocytotic pathway in chromaffin cells by acting on a G-protein coupled to phospholipase A_2 and the release of arachidonic acid.

DISCUSSION

The results presented here demonstrate that the effects of two activators of trimeric G-proteins, namely mastoparan and AIF_4^- , on Ca^{2+} -evoked secretion from permeabilized chromaffin cells is markedly dependent on the presence of MgATP in the incubation medium. Mastoparan and fluoride trigger MgATP-independent catecholamine secretion but inhibit secretion evoked by Ca^{2+} when MgATP is present. Similarly, p[NH]ppG and GTP[S] produce stimulatory and inhibitory effects on MgATP-independent and MgATP-dependent secretion respectively, but with different sensitivities. The stimulatory effect of mastoparan on catecholamine secretion in the absence of MgATP can be inhibited by PTX pretreatment, indicating that the G-protein involved belongs to the heterotrimeric $\text{G}_{i/o}$ family. In contrast, the inhibitory effect of mastoparan on ATP-dependent secretion is resistant to PTX. Taken together, these findings suggest the involvement of two distinct heterotrimeric G-proteins in the exocytotic machinery in chromaffin cells.

With the development of permeabilized cell systems to study exocytosis, it has been established that MgATP is necessary for

optimal secretion in a number of cell types including chromaffin cells (Knight and Baker, 1982; Brooks and Trembl, 1983; Dunn and Holz, 1983; Bader et al., 1986), pituitary cells (Ronning and Martin, 1986) and insulin-secreting cells (Vallar et al., 1987). The biochemical events responsible for ATP-dependent Ca^{2+} -activated secretion have not been elucidated. However, some progress has been made in ordering the ATP- and Ca^{2+} -dependent steps relative to one another. Using digitonin-permeabilized chromaffin cells (Holz et al., 1989; Bittner and Holz, 1992b), Holz and collaborators observed that intact cells are primed by intracellular ATP so that immediately after permeabilization there is a component of secretion that is independent of exogenous ATP. They suggest that MgATP acts before Ca^{2+} to maintain a 'primed' state of the secretory apparatus. Similarly, in PC12 cells (Hay and Martin, 1992) and *Paramecium* (Vilmart-Seuwen et al., 1986; Lumpert et al., 1990), it has been concluded that ATP is required to prime the exocytotic process but is not required for the final stages of exocytotic event. We have previously investigated the effect of mastoparan and other activators of heterotrimeric G-proteins on Ca^{2+} -evoked MgATP-dependent secretion in intact and permeabilized chromaffin cells (Vitale et al., 1993). Our results, together with those reported by Ohara-Imaizumi et al. (1992), suggest that an inhibitory G_o -protein, possibly located on the membrane of secretory granules, is involved in an ATP-dependent phase of exocytosis in chromaffin cells. Here, we show that mastoparan and other activators of heterotrimeric G-proteins trigger exocytosis in the absence of added MgATP. The fact that mastoparan is unable to produce ATP-independent secretion in cells depleted of ATP by incubation with metabolic inhibitors suggests that mastoparan triggers the release of a subpopulation of granules that are primed by cytosolic ATP before permeabilization. In other words, the putative Ca^{2+} -dependent step that occurs after the ATP-dependent priming step may be an event that requires not only Ca^{2+} , but also GTP to activate a G-protein. Holz et al. (1989) reported that it takes a few minutes for the ATP-primed state to decay after permeabilization with digitonin, an observation that we have confirmed using SLO-permeabilized chromaffin cells (results not shown). We would like to suggest that this rapid decay may actually reflect the rapid release of cytosolic GTP, as, in the presence of GTP analogues or mastoparan, secretion can be measured up to 25 min after permeabilization in the absence of exogenous ATP. Another secretory system in which the nucleotide requirement has been examined in some detail is the SLO-permeabilized mast cell. Howell et al. (1989) found that the final stages of the secretory pathway in mast cells require both Ca^{2+} and GTP analogues, and ATP is only necessary to maintain the responsiveness of the secretory apparatus. The present results indicating that the final steps of exocytosis in chromaffin cells also require the dual involvement of Ca^{2+} and GTP suggest that regulated secretory granules may be released through a common exocytotic machinery in endocrine and neuroendocrine cells.

An intriguing observation is that the stimulatory effect of mastoparan on secretion is not simply ATP-independent but seems to be inhibited by millimolar concentrations of MgATP (see Figure 3). Thus it appears that ATP is involved in priming exocytosis but there is a later ATP-inhibitable event. A similar observation has been reported in mast cells (Tatham and Gomperts, 1989) and in *Paramecium* (Vilmart-Seuwen et al., 1986) and this has been interpreted as evidence that a phosphatase is involved at a late step in exocytosis in these secretory cell types. Indeed, in *Paramecium* the dephosphorylation of a 65 kDa protein is correlated with the activation of exocytosis (Ziesenis and Plattner, 1985). We and others have previously reported that ATP[S] inhibits exocytosis in permeabilized chromaffin cells

(Brooks et al., 1984; Bader et al., 1986). Taken together, one interpretation may be that phosphorylation is needed to prime exocytosis but dephosphorylation of an unknown regulator protein may comprise the late step in the exocytotic pathway as suggested from the work on mast cells and *Paramecium*.

The Ca^{2+} sensitivities of the ATP-dependent and the ATP-independent stages of exocytosis have been examined by Bittner and Holz (1992b). They reported that low concentrations of Ca^{2+} seem to be optimal for modulating the ATP-dependent priming reaction, whereas higher Ca^{2+} concentrations are more effective at triggering the final step of the exocytotic pathway. We show here that half-maximal MgATP-independent secretion evoked by mastoparan is achieved at 20 μM free Ca^{2+} . In contrast, half-maximal MgATP-dependent secretion occurs at 2 μM free Ca^{2+} (Vitale et al., 1993). Thus our results confirm that the early MgATP-dependent event in the exocytotic machinery requires at least 10-fold less Ca^{2+} than the late MgATP-independent step. These observations are in line with the recent findings on chromaffin and pituitary cells indicating that exocytosis comprises kinetically distinct phases with specific Ca^{2+} -dependences ranging from 1 to 300 μM (Augustine and Neher, 1992; Neher and Zucker, 1993; Thomas et al., 1993; Von Rüden and Neher, 1993).

We examined the possible intracellular pathways by which mastoparan is able to stimulate the ATP-independent component in the exocytotic machinery. Mastoparan can be expected to interact with many trimeric G-proteins in the intracellular environment, including for example, G α which regulates phospholipase C and the generation of diacylglycerol and inositol phosphates in many different kinds of cell (Cockcroft, 1987). Indeed we have previously demonstrated that GTP analogues are able to stimulate exocytosis in permeabilized chromaffin cells by activating protein kinase C-linked events presumably through the stimulation of G α and the production of diacylglycerol (Bader et al., 1989). However, the mastoparan-induced secretion observed here is unlikely to be related to the activation of protein kinase C because the stimulatory effect of mastoparan required the absence of exogenous MgATP and was also observed in cells where protein kinase C had been down-regulated by prolonged incubation with active phorbol esters (results not shown). Moreover, mastoparan can induce MgATP-independent exocytosis under conditions in which the G α -linked activation of phospholipase C is prevented by neomycin, indicating that mastoparan must involve some other cellular component. This could be G β , the hypothetical G-protein that activates phospholipase A_2 and has been shown to be a substrate for PTX in many different cell types (Cockcroft, 1992).

To test this hypothesis, we investigated the effect of mastoparan on arachidonate release in chromaffin cells and found that mastoparan significantly enhanced the generation of arachidonic acid in a PTX-sensitive manner. Furthermore, inhibitors of phospholipase A_2 blocked, in a similar range of concentrations, both the stimulatory effect of mastoparan on arachidonate release and the mastoparan-induced catecholamine secretion in the absence of MgATP. These observations lead us to propose that phospholipase A_2 may be the intracellular effector by which the mastoparan-activated G-protein is able to stimulate an ATP-independent step in exocytosis.

Chromaffin cells possess three types of phospholipase A_2 ; a lysosomal, a chromaffin-granule-bound and a cytoplasmic form (Petit et al., 1992). The cytoplasmic Ca^{2+} -regulated form of phospholipase A_2 has been suggested as a candidate for a role in the exocytotic machinery, being translocated to the cell membrane on stimulation (Channon and Leslie, 1990; Cockcroft, 1992; Petit et al., 1992). Our observation that the stimulatory

effect of mastoparan on MgATP-independent secretion is lost by prolonged incubation after permeabilization (> 30 min) may be explained by the release from the cytosol of the intracellular effector, i.e. the cytosolic form of phospholipase A_2 which can escape through the pores created in the plasma membrane in permeabilized cells. An interesting finding is the Ca^{2+} dependence of mastoparan-stimulated arachidonate release which paralleled the ATP-independent catecholamine secretion. The intracellular effector is thus likely to be a Ca^{2+} -regulated protein. Indeed, to access its substrate, the cytoplasmic phospholipase A_2 has to be translocated from the cytosol to membranes and this translocation is known to be Ca^{2+} -dependent (Cockcroft, 1992). However, Ca^{2+} *per se* is only a weak activator of phospholipase A_2 , as seen here by the low amount of arachidonate release induced by elevated Ca^{2+} concentrations in permeabilized chromaffin cells. Mastoparan stimulates arachidonic acid release through a PTX-sensitive G-protein provided that Ca^{2+} is also present. In other words, Ca^{2+} is probably necessary to translocate phospholipase A_2 from the cytosol to the membrane but activation of phospholipase A_2 requires in addition the trigger of a PTX-sensitive G-protein.

The stimulus to secretion in many cell types frequently results in the activation of phospholipase A_2 and the generation of arachidonic acid (Roberts et al., 1979; Naor and Catt, 1981; Frye and Holz, 1984; Cockcroft, 1992). The question thus arises whether the activation of phospholipase A_2 is an essential step in the pathway leading to exocytosis, or whether it is a parallel process not strictly necessary for secretion. To date no clear answer to this question has been obtained, as the findings concerning the possible role of phospholipase A_2 and arachidonate in exocytosis are rather controversial. Arachidonic acid stimulates secretion from anterior pituitary cells (Chang et al., 1986), GH $_3$ cells (Kolesnick et al., 1984), pancreatic β -cells (Metz, 1988; 1991) and adrenal chromaffin cells (Rindlisbacher et al., 1990), and is capable of interacting with second-messenger systems by stimulating phospholipase C (Zeitler and Handwerker, 1985) and protein kinase C (Koda et al., 1989) and mobilizing intracellular Ca^{2+} (Axelrod et al., 1988). Furthermore, arachidonic acid has been shown to induce membrane-membrane fusion between isolated chromaffin granules (Creutz, 1981), and so could be directly involved in the exocytotic fusion of granule and plasma membranes. Release of arachidonic acid accompanies exocytosis in both intact (Frye and Holz, 1984) and permeabilized (Frye and Holz, 1985; Sontag et al., 1991) chromaffin cells, and inhibition of arachidonate release with phospholipase A_2 inhibitors blocks exocytosis (Frye and Holz, 1985). On the other hand, Morgan and Burgoyne (1990) described circumstances in which it was possible to obtain exocytosis without measurable release of arachidonate. The idea that arachidonic acid could facilitate exocytosis by acting as a potent fusogen was reiterated again in a recent publication (Prabhathi et al., 1993). In this study, botulinum neurotoxin type A was found to inhibit both secretion and the associated release of arachidonate in PC12 cells, but agents that increase cytoplasmic arachidonic acid could protect against the toxin effect on secretion.

The results described here suggest that phospholipase A_2 coupled to a PTX-sensitive G-protein may be an essential component of the late Ca^{2+} -dependent stage of exocytosis in chromaffin cells. Phospholipase A_2 hydrolyses fatty acids from the *sn*-2-position of membrane phospholipids, thus generating increases in non-esterified arachidonate and lysophospholipids. As mentioned previously, arachidonic acid has been shown to induce membrane fusion between isolated chromaffin granules (Creutz, 1981) and between chromaffin granules and plasma-membrane vesicles (Karli et al., 1990) and so could be involved

in the exocytotic fusion step. Furthermore, annexin II, which possesses properties that suggest a key role in the regulation of the exocytotic response (Ali, S. M. et al., 1989; Sarafian et al., 1991), requires arachidonic acid to promote *in vitro* aggregation and fusion of secretory granules in physiological Ca^{2+} concentrations (Drust and Creutz, 1988). It is interesting to note also that the *N*-ethylmaleimide-sensitive protein that seems to be implicated in many intracellular fusion processes (Rothman and Orci, 1992) requires long-chain fatty acyl-CoA to promote fusion of transport vesicles with Golgi cisternae (Pfanner et al., 1990). Alternatively, the essential event for the fusion process may actually be the local increase in lysophospholipids in the plasma membrane that leads to perturbation of lipid bilayers. The fact that exogenous arachidonic acid (up to 300 μM) is unable to restore exocytosis in cells pretreated with phospholipase A_2 inhibitors (V. Ricchiuti and M. F. Bader, unpublished work) together with the observation that exogenous arachidonic acid cannot replace mastoparan to trigger MgATP -independent secretion (results not shown) may favour the latter proposition.

In conclusion, our results suggest the involvement of two distinct heterotrimeric G-proteins in the exocytotic pathway in chromaffin cells. In agreement with the model proposed by Holz and collaborators (Bittner and Holz, 1992a,b), secretory granules first undergo an ATP-dependent priming step which requires low Ca^{2+} and a final fusion step which requires high Ca^{2+} but is ATP-independent. We propose that free granules are under the control of a G_o -protein which exerts an inhibitory influence, thereby preventing the priming reaction (Ohara-Imaizumi et al., 1992; Vitale et al., 1993). Thus the priming reaction requires, not only ATP and low Ca^{2+} , but also the inhibition of this G_o -protein (Vitale et al., 1993). Primed granules can then undergo fusion in the presence of high Ca^{2+} . The present data suggest that the fusion reaction depends on the stimulation of a second trimeric G-protein possibly coupled to the production of arachidonic acid.

We thank Dr. Tsutomu Higashijima for his generous gift of mastoparan analogues and Dr. Nancy Grant for revising the manuscript. N.V. is a recipient of a fellowship from the Ministère de la Recherche et de l'Espace and D.T. is supported by the Université Louis Pasteur.

REFERENCES

- Ahnert-Hilger, G., Bader, M. F., Bhakdi, S. and Gratzl, M. (1989) *J. Neurochem.* **52**, 1751–1758.
- Ahnert-Hilger, G., Wegenhorst, U., Stecher, B., Spicher, K., Rosenthal, W. and Gratzl, M. (1992) *Biochem. J.* **284**, 321–326.
- Ali, N., Milligan, G. and Evans, W. H. (1989) *Biochem. J.* **261**, 905–912.
- Ali, S. M., Geisow, M. J. and Burgoyne, R. D. (1989) *Nature (London)* **340**, 313–315.
- Argiolas, A. and Pisano, J. J. (1983) *J. Biol. Chem.* **258**, 13697–13702.
- Aridor, M., Traub, L. M. and Sagi-Eisenberg, R. (1990) *J. Cell Biol.* **111**, 909–917.
- Audigier, Y., Nigam, S. K. and Blobel, G. (1988) *J. Biol. Chem.* **263**, 16352–16357.
- Augustine, G. J. and Neher, E. (1992) *J. Physiol. (London)* **450**, 247–271.
- Axelrod, J., Burch, R. M. and Jelsema, C. L. (1988) *Trends Neurosci.* **11**, 117–123.
- Bader, M. F., Thiersé, D., Aunis, D., Ahnert-Hilger, G. and Gratzl, M. (1986) *J. Biol. Chem.* **261**, 5777–5783.
- Bader, M. F., Sontag, J. M., Thiersé, D. and Aunis, D. (1989) *J. Biol. Chem.* **264**, 16426–16434.
- Balch, W. E. (1990) *Trends Biochem. Sci.* **15**, 473–477.
- Balch, W. E. (1992) *Curr. Opin. Biol.* **2**, 157–160.
- Barr, F. A., Leyte, A., Muller, S., Pfeuffer, T., Toozé, S. A. and Huttner, W. B. (1991) *FEBS Lett.* **294**, 239–243.
- Barr, F. A., Leyte, A. and Huttner, W. B. (1992) *Trends Cell Biol.* **2**, 91–94.
- Bittner, M. A. and Holz, R. W. (1992a) *J. Biol. Chem.* **267**, 16226–16229.
- Bittner, M. A. and Holz, R. W. (1992b) *J. Biol. Chem.* **267**, 16219–16225.
- Bittner, M. A., Habig, W. H. and Holz, R. W. (1989) *J. Neurochem.* **53**, 966–968.
- Brooks, J. C. and Tremblé, S. (1983) *J. Neurochem.* **40**, 468–473.
- Brooks, J. C., Tremblé, S. and Brooks, M. (1984) *Life Sci.* **35**, 569–574.
- Bueb, J. L., Mousli, M., Rouot, B. and Landry, Y. (1990) *Mol. Pharmacol.* **38**, 816–822.
- Chang, J. P., Greuter, J. and Catt, K. J. (1986) *Biochem. Biophys. Res. Commun.* **134**, 134–139.
- Channon, J. Y. and Leslie, C. C. (1990) *J. Biol. Chem.* **265**, 5409–5413.
- Cockcroft, S. (1987) *Trends Biochem. Sci.* **12**, 75–78.
- Cockcroft, S. (1992) *Biochim. Biophys. Acta* **1113**, 135–160.
- Cockcroft, S. and Gomperts, B. D. (1985) *Nature (London)* **314**, 534–536.
- Colombo, M. I., Mayorga, L. S., Casey, P. J. and Stahl, P. D. (1992) *Science* **255**, 1695–1697.
- Creutz, C. E. (1981) *J. Cell Biol.* **91**, 247–256.
- Donaldson, J. G., Kahn, R. A., Lippincott-Schwartz, J. and Klausner, R. D. (1991) *Science* **254**, 1197–1199.
- Drust, D. S. and Creutz, C. E. (1988) *Nature (London)* **331**, 88–91.
- Dunn, L. A. and Holz, R. W. (1983) *J. Biol. Chem.* **258**, 4989–4993.
- Ercolani, L. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4635–4639.
- Flodgaard, H. and Fleron, P. (1974) *J. Biol. Chem.* **249**, 3465–3474.
- Frye, R. A. and Holz, R. W. (1984) *J. Neurochem.* **43**, 146–150.
- Frye, R. A. and Holz, R. W. (1985) *J. Neurochem.* **44**, 265–273.
- Gilman, A. G. (1987) *Annu. Rev. Biochem.* **56**, 615–649.
- Goda, Y. and Pfeiffer, S. R. (1989) *FASEB J.* **3**, 2488–2494.
- Gorvel, J. P., Chavrier, P., Zerial, M. and Gruenberg, J. (1991) *Cell* **64**, 915–925.
- Gruenberg, J. and Clague, M. J. (1992) *Curr. Opin. Cell Biol.* **4**, 593–599.
- Habermann, E. and Dreyer, F. (1986) *Curr. Top. Microbiol. Immunol.* **129**, 93–179.
- Hay, J. C. and Martin, T. F. J. (1992) *J. Cell Biol.* **119**, 139–151.
- Higashijima, T., Uzu, S., Nakajima, T. and Ross, E. M. (1988) *J. Biol. Chem.* **263**, 6491–6494.
- Higashijima, T., Burnier, J. and Ross, E. M. (1990) *J. Biol. Chem.* **265**, 14176–14186.
- Higashijima, T., Graziano, M. P., Suga, H., Kainosho, M. and Gilman, A. G. (1991) *J. Biol. Chem.* **266**, 3396–3401.
- Holz, R. W., Bittner, M. A., Peppers, S. C., Senter, R. A. and Eberhard, D. A. (1989) *J. Biol. Chem.* **264**, 5412–5419.
- Howell, T. W., Kramer, I. M. and Gomperts, B. D. (1989) *Cell. Signal.* **1**, 157–163.
- Kahn, R. (1991) *J. Biol. Chem.* **266**, 15595–15597.
- Karli, U. O., Schäffer, T. and Burger, M. M. (1990) *Proc. Natl. Acad. Sci. U.S.A.* **87**, 5912–5915.
- Knight, D. E. and Baker, P. F. (1982) *J. Membr. Biol.* **68**, 107–140.
- Koda, Y., Wada, A., Yanagihara, N., Uezono, Y. and Isumi, F. (1989) *Neuroscience* **29**, 495–502.
- Kolesnick, R. N., Musacchio, L., Thaw, C. and Gershengorn, M. C. (1984) *Am. J. Physiol.* **246**, E458–E462.
- Lister, M. D., Glaser, K. B., Ulevitch, R. J. and Dennis, E. A. (1989) *J. Biol. Chem.* **264**, 8520–8528.
- Lumpert, C. J., Kersken, H. and Plattner, H. (1990) *Biochem. J.* **269**, 639–645.
- Metz, S. A. (1988) *Diabetes* **37**, 1453–1469.
- Metz, S. A. (1991) *Diabetes* **40**, 1565–1573.
- Morgan, A. and Burgoyne, R. (1990) *Biochem. J.* **271**, 571–574.
- Naor, Z. and Catt, K. J. (1981) *J. Biol. Chem.* **256**, 2226–2229.
- Neher, E. and Zucker, R. S. (1993) *Neuron* **10**, 21–30.
- Norgauer, J., Eberle, M., Lemke, H. D. and Aktories, K. (1992) *Biochem. J.* **282**, 393–397.
- Ohara-Imaizumi, M., Kameyama, K., Kawae, N., Takeda, K., Muramatsu, S. and Kumakura, K. (1992) *J. Neurochem.* **58**, 2275–2284.
- Okano, Y., Takagi, H., Tohmatsu, T., Nakashima, S., Kuroda, Y., Saito, K. and Nozawa, Y. (1985) *FEBS Lett.* **188**, 363–366.
- Ozaki, Y., Matsumoto, Y., Yatomi, Y., Higashihara, M., Kariya, T. and Kume, S. (1990) *Biochem. Biophys. Res. Commun.* **170**, 779–785.
- Penner, R., Neher, E. and Dreyer, F. (1986) *Nature (London)* **324**, 76–78.
- Petit, K., Miserez, B., De Block, J., Van Dessel, G. and De Potter, W. (1992) *Biochim. Biophys. Acta* **1125**, 150–156.
- Pfanner, N., Glick, B. S., Arden, S. R. and Rothman, J. E. (1990) *J. Cell Biol.* **110**, 955–961.
- Prabhathi, R., Berman, J. D., Middleton, W. and Brendle, J. (1993) *J. Biol. Chem.* **268**, 11057–11064.
- Rindlisbacher, B., Sidler, M. A., Galatioto, L. E. and Zahler, P. (1990) *J. Neurochem.* **54**, 1247–1252.
- Roberts, L. J., Lewis, R. A., Oates, J. A. and Austen, K. F. (1979) *Biochim. Biophys. Acta* **575**, 185–192.
- Ronning, S. A. and Martin, T. F. J. (1986) *J. Biol. Chem.* **261**, 7834–7839.
- Rothmann, J. E. and Orci, L. (1992) *Nature (London)* **355**, 409–415.
- Sarafian, T., Pradel, L. A., Henry, J. P., Aunis, D. and Bader, M. F. (1991) *J. Cell Biol.* **114**, 1135–1147.
- Schacht, J. (1976) *J. Neurochem.* **27**, 1119–1124.
- Schiavo, G., Benfenati, F., Poulain, B., Rossetto, O., Polverino de Laureto, P., DasGupta, B. R. and Montecucco, C. (1992) *Nature (London)* **359**, 832–835.

- Sillen, L. G. and Martell, A. E. (1971) *Stability Constants of Metal Ion Complexes*, Suppl. I Chemical Society, London
- Sontag, J. M., Thiersé, D., Rouot, B., Aunis, D. and Bader, M. F. (1991) *Biochem. J.* **274**, 339–347
- Sontag, J. M., Aunis, D. and Bader, M. F. (1992) *Eur. J. Neurosci.* **4**, 98–101
- Stow, J. L., De Almeida, J. B., Narula, N., Holtzman, E. J., Ercolani, L. and Ausiello, D. A. (1991) *J. Cell Biol.* **114**, 1113–1124
- Tatham, P. E. and Gomperts, B. D. (1989) *Biosci. Rep.* **9**, 99–109
- Taylor, C. W. (1990) *Biochem. J.* **272**, 1–13
- Thomas, P., Wong, J. G. and Almers, W. (1993) *EMBO J.* **12**, 303–306
- Toutant, M., Aunis, D., Bockaert, J., Homberger, V. and Rouot, B. (1987) *FEBS Lett.* **215**, 339–344
- Vallar, L., Biden, T. J. and Wollheim, C. B. (1987) *J. Biol. Chem.* **262**, 5049–5056
- Vilmart-Seuwen, J., Kersken, H., Strürzl, R. and Plattner, H. (1986) *J. Cell Biol.* **103**, 1279–1288
- Vitale, N., Mukai, H., Rouot, B., Thiersé, D., Aunis, D. and Bader, M. F. (1993) *J. Biol. Chem.* **268**, 14715–14723
- Von Rüden, L. and Neher, E. (1993) *Science* **262**, 1061–1065
- Weingarten, R., Ransnäs, L., Mueller, H., Sklar, L. A. and Bokoch, G. M. (1990) *J. Biol. Chem.* **265**, 11044–11049
- Yokokawa, N., Komatsu, M., Takeda, T., Aizawa, T. and Yamada, T. (1989) *Biochem. Biophys. Res. Commun.* **158**, 712–716
- Zeitler, P. and Hadwiger, S. (1985) *Mol. Pharmacol.* **28**, 549–554
- Zieseniss, E. and Plattner, H. (1985) *J. Cell Biol.* **101**, 2028–2035