Trimeric G Proteins Control Exocytosis in Chromaffin Cells

Gα REGULATES THE PERIPHERAL ACTIN NETWORK AND CATECHOLAMINE SECRETION BY A MECHANISM INVOLVING THE SMALL GTP-BINDING PROTEIN Rho*

(Received for publication, February 26, 1997, and in revised form, May 27, 1997)

Stéphane Gasman, Sylvette Chasserot-Golaz, Michel R. Popoff‡, Dominique Aunis, and Marie-France Bader§

From the 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 and ‡Toxines Microbiennes, Institut Pasteur, 75724 Paris Cedex 15, France

Besides having a role in signal transduction, heterotrimeric G proteins may be involved in membrane trafficking events. In chromaffin cells, Gα is associated with secretory organelles and its activation by mastoparan inhibits the ATP-dependent priming of exocytosis. The effectors by which Gα controls exocytosis are currently unknown. The subplasmalemmal actin network is one candidate, since it modulates secretion by controlling the movement of secretory granules to the plasma membrane. In streptolysin-O-permeabilized chromaffin cells, activation of exocytosis produces disassembly of cortical actin filaments. Mastoparan blocks the calcium-evoked disruption of cortical actin, and this effect is specifically inhibited by antibodies against Gαo and by a synthetic peptide corresponding to the COOH-terminal domain of Gαo. Disruption of actin filaments with cytochalasin E and Clostridium perfringens iota toxin partially reverses the mastoparan-induced inhibition of secretion. Furthermore, the effects of mastoparan on cortical actin and exocytosis are greatly reduced in cells treated with Clostridium botulinum C3 exoenzyme, which specifically inactivates the small G protein Rho. We propose that the control exerted by the granule-associated Gαo on exocytosis may be related to effects on the cortical actin network through a sequence of events which eventually involves the participation of Rho.

Studies on diverse secretory cell types have highlighted the potential roles of heterotrimeric G proteins in intracellular membrane trafficking events (1–3). α and βγ subunits of G and Gα proteins have been associated with the membrane of secretory granules in various neuroendocrine cells (4–6), suggesting a role in Ca2+-regulated exocytosis. Accordingly, the participation of a plasma membrane-bound Gβγ protein in the late stages of exocytosis in mast cells has been demonstrated (7). Direct control of exocytosis by Gα and Gβγ proteins has also been described in insulin-secreting cells (8) and in chromaffin cells (6, 9, 10). Thus regulated exocytosis may represent a possible effector pathway for trimeric G proteins, although the mechanism by which this class of G proteins relates to the exocytotic machinery remains to be elucidated.

In chromaffin cells, stimulation of the secretory granule-associated Gαo by mastoparan and compounds known to stimulate late Gα subunits inhibits catecholamine secretion by selectively interfering with the ATP-dependent priming step of exocytosis (6, 9). Although the molecular machinery underlying the ATP-dependent reaction is not clearly understood, this finding predicts that the granule-bound Gαo may control an effector related to the first stages of the exocytotic pathway, presumably the recruitment of secretory granules and/or the preparation of the docking/fusion machinery. Many secretory cells display a cortical network of actin filaments that forms a physical barrier to exocytosis for the majority of secretory granules, since they are excluded from the subplasmalemmal zone (11–14). Activation of exocytosis produces disassembly of the actin network in several secretory cell types, including chromaffin cells (15, 16), mast cells (17), pancreatic acinar cells (18), and synaptosomes (19). A close correlation between the disassembly of the actin cytoskeleton, the number of secretory granules in the cortical areas and the initial rate in secretion has been also reported (14). Actin filament disassembly is not by itself a sufficient trigger to allow exocytosis to occur (15). However, rises in intracellular calcium are not capable of stimulating catecholamine release if the peripheral actin barrier has not previously been removed (20). Thus, the cortical actin network represents a dominant negative clamp, which blocks the exocytotic process and its disassembly may form part of the priming reaction.

Previous reports by several investigators have suggested possible interactions between trimeric G proteins and the actin cytoskeleton. In neutrophils, studies based on the use of mastoparan, aluminium fluoride, and pertussis toxin, which specifically ADP-ribosylates Gαi and Gβγ proteins, suggest that trimeric G proteins are closely linked to the actin organization (21–23). In mast cells, a trimeric G protein seems to participate in the reorganization of the actin cytoskeleton in response to cell activation (24). The specific association of trimeric G proteins with the actin cytoskeleton during thrombin receptor-mediated platelet activation has also been reported (25). The aim of the present work was to assess whether the cortical actin network represents a possible effector by which the granule-bound Gαo controls the exocytotic pathway in chromaffin cells. Using streptolysin-O (SLO)1-permeabilized cells, we show that the introduction of mastoparan into the cytosol inhibits the disruption of the subplasmalemmal actin network in calcium-stimulated cells. This effect can be selectively reversed by affinity-purified antibodies prepared against Gαo and by a syn-

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondance should be addressed. Tel.: 33-3-88-45-67-13; Fax: 33-3-88-60-08-06; E-mail: bader@neurochem.u-strasbg.fr.

1 The abbreviations used are: SLO, streptolysin-O; PIPES, 1,4-piperazinediethanesulfonic acid; DBH, dopamine β-hydroxylase; DTAF, dichlorotriazinyl aminofluorescein; PBS, phosphate-buffered saline; BSA, bovine serum albumin; TRITC, tetramethylrhodamine B isothiocyanate; ARF6, ADP-ribosylation factor 6.
thet peptide corresponding to the COOH-terminal sequence of Go
. Furthermore, the mastoparan-induced inhibition of secretion can be partially reversed by agents known to affect the assembly of actin and by the Clostridium botulinum C3 ADP-ribosyltransferase, which specifically inactivates the small GTPase Rho by ADP-ribosylation. Our results raise the intriguing possibility that the secretory granule-associated Go protein controls the priming step of exocytosis by modifying the actin cytoskeleton underlying the plasma membrane through a sequence of events possibly implicating Rho.

MATERIALS AND METHODS

Culture of Chromaffin Cells—Chromaffin cells were isolated from fresh bovine adrenal glands by retrograde perfusion with collagenase and purified on self-generated Percoll gradients (26). Cells were suspended in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, and containing cytosine arabinoside (10 μM), fluorodeoxyuridine (10 μM), streptomycin (50 μg/ml), and penicillin (50 units/ ml). Cells were cultured as monolayers either on 24 multiple-mm Costar plates (Costar, Cambridge, MA) at a density of 2.5 × 10⁶ cells/ well or on fibronectin-coated glass coverslips at a density of 2 × 10⁶ cells. Experiments were performed 3–7 days after plating.

Stimulation—Permeabilized Chromagen Cells—Cultured chromaffin cells were washed four times with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 0.10 mM EDTA, 11.5 mM glucose, 0.56 mM ascorbic acid, and 15 mM HEPES, pH 7.2) and twice with Ca²⁺-free Locke's solution (containing 1 mM EGTA). Cells were subsequently permeabilized for 2 min with 15 units/ml SLO (Institut Pasteur, Paris, France) in 200 μl/well Ca²⁺-free KG medium (150 mM potassium glutamate, 10 mM PIPES, pH 7.0, 5 mM nitrolotriacetic acid, 0.5 mM EGTA, 5 mM MgATP, 4.5 mM magnesium acetate, 0.2% bovine serum albumin). Extracellular fluids were then removed, and cells were incubated 10 min in 200 μl/well Ca²⁺-free KG medium in the presence of either mastoparan or GAP-43 and then indicated Go peptides, anti-Go antibodies, C3 transference, or iota toxin (1a component). Cells were subsequently stimulated for 10 minutes with KG medium containing CaCl₂. The free Ca²⁺ concentration in the KG medium was calculated by a computer program according to Flodgaard and Feron (27), kindly provided by T. Saermark, University of Copenhagen, Denmark, using the stability constants given by Sillen and Martell (28).

H Noradrenaline Release—Catecholamine stores were labeled by incubating chromaffin cells with [3H]noradrenaline (13.3 Ci/mg; Amersham Corp., Les Ulis, France) for 45 minutes in Locke's solution. Cells were then washed, permeabilized with SLO, and stimulated with calcium as described above. [3H]Noradrenaline release after stimulation was determined by measuring the radioactivity present in the incubation medium and in cells after precipitation with 10% (w/v) trichloroacetic acid. The amount of released [3H]noradrenaline is expressed as a percentage of the total radioactivity present in the cells before Ca²⁺-induced stimulation. When indicated, data are given as the net secretory values obtained by subtracting the basal release established in Ca²⁺-free KG medium from the total release measured in the KG medium containing 20 μM free calcium. Release experiments were performed in triplicate on at least two different cell preparations. In the figures that are representative of a typical experiment, data are given as the mean ± S.E. of triplicate determinations on the same cell preparation.

Antibodies—Affinity-purified antibodies against the COOH-terminal Go peptide (ANNLRCGCCGL) or Goα peptide (KNLNKLEGCL) were prepared as already described (9, 29) and their specificity against non-denatured Go protein was demonstrated. Rat polyclonal antibodies against dopamine β-hydroxylase (EC 1.14.17.1: DBH) were raised in our laboratory and their specificity previously demonstrated (30). Goat anti-rat IgG conjugated to dichlorotriazinyl aminofluorescein (DTAF) were from Chemicon International Inc.

Peptides and Proteins—Mastoparan was obtained from Sigma. Synthetic peptides were obtained from NeoSystems (Strasbourg, France): COOH-terminal Goα peptide (ANNLRCGCCGL) and COOH-terminal Goα peptide (KNLNKLEGCL) were further purified by high performance liquid chromatography and dissolved in Ca²⁺-free KG medium at 10 μM. GAP-43 was purified from a cytosolic fraction obtained from the bovine brain according to a previously published method (31).

Toxins—C. botulinum exoenzyme C3 ADP-ribosyltransferase (C3 transferase) and Clostridium perfringens iota toxin (Ia and Ib components) were prepared and purified as described (32, 33).

Immunocytochemistry and Confocal Laser Scanning Microscopy—Chromaffin cells grown on fibronectin-coated glass coverslips were washed with Locke's solution, permeabilized, and incubated for 10 minutes in Ca²⁺-free KG buffer (resting cells) or in KG buffer containing 20 μM free Ca²⁺ (stimulated cells). Cells were subsequently fixed for 15 minutes in 4% paraformaldehyde in 0.12 M sodium/potassium phosphate, pH 7.0, and then washed further 10 minutes in fixative containing 0.1% Triton X-100. Following several rinses with phosphate-buffered saline (PBS), cells were pretreated with 3% bovine serum albumin (BSA), 10% normal goat serum in PBS to reduce nonspecific staining.

To identify chromaffin cells, cells were incubated for 1 h at 37°C with antibodies against DBH diluted to 1:1200 in PBS containing 3% BSA in a moist chamber. Cells were then washed with PBS and subsequently incubated for 1 h at 37°C with rabbit anti-rat IgG diluted to DTAF diluted to 1:1200 in PBS containing 3% BSA. The transient accessibility of DBH on the plasma membrane of stimulated chromaffin cells was tested by incubating SLO-permeabilized cells for 10 minutes in Ca²⁺-free KG medium containing 20 μM free Ca²⁺ in the presence of anti-DBH antibodies diluted to 1:50. Cells were then fixed, washed, and processed for immunofluorescence labeling.

Actin filaments (F-actin) were stained by incubation with rhodamine (TRITC)-conjugated phalloidin (Sigma) at a concentration of 0.5 μg/ml in PBS for 15 min at room temperature. Coverslips were then extensively washed with PBS, rinsed with water, and mounted in Moviol 4–88 (Hoechst).

The percentage of chromaffin cells displaying an intact cortical actin network was estimated by double labeling with rhodamine-conjugated phalloidin and anti-DBH antibodies and counting 200 single-rounded DBH-positive cells per coverslip in randomly selected areas of the coverslips. Each DBH-labeled cell was classified as having either a continuous and homogeneous cortical rhodamine fluorescent ring or a disrupted one. To avoid personal bias, a single-blind method was used: the cells were examined and classified without knowing they were from control or treated preparations.

Immunofluorescence staining was monitored with a Zeiss laser scanning microscope (LSM 410) equipped with a planapo oil (63×) immersion lens (numerical aperture = 1.4). DTAF emission was excited using the argon laser 488-nm line, whereas TRITC was excited using the He/Ne laser 543-nm line. The emission signals were filtered with a Zeiss 515–565-nm filter (DTAF emission) or with a long pass 595-nm filter (TRITC signal). Cells were subjected to optical serial sectioning to produce images in the X-Y plane. Each optical section was scanned eight times to obtain an averaged image. Images were recorded digitally in a 768 × 576-pixel format. Nonspecific fluorescence was assessed by incubating cells with the secondary fluorescent antibodies and measuring the average intensity value for each fluorochrome. This value was then subtracted from all specific images.

RESULTS

Effect of Mastoparan on the Cortical Actin Network in SLO-permeabilized Chromaffin Cells—The effect of mastoparan on the peripheral actin cytoskeleton was analyzed in SLO-permeabilized chromaffin cells by confocal microscopy using rhodamine-conjugated phalloidin, which binds to filamentous but not to monomeric actin. In permeabilized cells incubated in Ca²⁺-free medium (Fig. 1A), rhodamine-phalloidin fluorescence was most intense at the cell periphery forming a continuous and homogeneous cortical ring, in agreement with the fact that in chromaffin cells the majority of actin filaments are concentrated in the subplasmalemmal region (14–16). Stimulation with 20 μM free calcium strongly reduced the binding of rhodamine-phalloïdin in the cell periphery, revealing the disruption of the cortical actin filaments (Fig. 1B). The introduction of 20 μM mastoparan into the incubation medium of permeabilized cells had no detectable effect on actin filaments in resting cells (Fig. 1C) but totally abolished the disassembly of cortical actin observed in response to a rise in cytosolic calcium (Fig. 1D).

In contrast, preincubation with mastoparan before permeabilization did not affect the Ca²⁺-evoked actin disassembly (Fig. 1, E and F), indicating that the ability of mastoparan to stabilize the actin network was related to its direct introduction into the cytoplasm through the pores created in the plasma membrane. Fig. 1G illustrates a quantitative analysis of the chromaffin cell population displaying an intact cortical actin network under resting and stimulating conditions. Stimulation
with 20 μM free Ca\(^{2+}\) strongly reduced the percentage of SLO-permeabilized cells presenting an intact fluorescent actin ring. Mastoparan inhibited the Ca\(^{2+}\)-evoked disruption of cortical actin, but only when added to the incubation medium after SLO permeabilization. Thus, the presence of mastoparan into the cytosol of permeabilized chromaffin cells stabilized the peripheral actin network and thereby prevented its disassembly upon Ca\(^{2+}\)-induced stimulation.

**Mastoparan Prevents the Ca\(^{2+}\)-evoked Disruption of Cortical Actin by Stimulating an Endogenous Trimeric G\(_o\) Protein—**

Mastoparan is a tetradecapeptide that selectively activates G\(_i\) and G\(_o\) proteins by inserting into membranes and forming an α-helix which resembles the trimeric G protein interacting domain of G protein–coupled receptors (34). On the other hand, mastoparan is an amphiphilic peptide, and its introduction into permeabilized cells has been reported to nonspecifically perturb intracellular membranes (35). To assess whether mastoparan blocked the actin network by stimulating an endogenous trimeric G protein, we attempted to antagonize the effect of mastoparan with affinity-purified antibodies prepared against G\(_o\) and G\(_i3\), and with synthetic peptides corresponding to the carboxyl terminus of G\(_o\) and G\(_i3\).

The effect of the carboxyl terminus G\(_o\) and G\(_i3\) peptides was examined because such peptides prevent the stimulation of G\(_o\) and G\(_i\) proteins by their respective receptors or by mastoparan (36, 37). Fig. 2A illustrates the effect of the synthetic G\(_o\) and G\(_i3\) peptides on the mastoparan-induced inhibition of actin disassembly. Permeabilized chromaffin cells were exposed to mastoparan in the presence or absence of G\(_o\) and G\(_i3\) peptides and subsequently stimulated with 20 μM free Ca\(^{2+}\).

The number of chromaffin cells displaying an intact cortical actin ring was estimated following rhodamine-phalloidin staining. In resting cells, neither mastoparan nor G\(_o\) peptides modified the percentage of cells having an intact cortical actin network (Fig. 2A). Mastoparan inhibited the disassembly of peripheral actin filaments in Ca\(^{2+}\)-stimulated cells, and this inhibition was unaffected by the presence of G\(_i3\) peptide. However, the stabilizing effect of mastoparan was strongly reduced in the presence of G\(_o\) peptide.

Mastoparan has been reported to activate trimeric G\(_i\) and G\(_o\) proteins by interacting with the carboxyl terminus of the α subunit (38). Thus, the effect of affinity-purified antibodies raised against the carboxyl terminus of G\(_o\) and G\(_i3\) was examined. In the absence of Ca\(^{2+}\), the percentage of cells with an intact cortical actin network was not modified by the introduction of mastoparan and anti-G\(_o\) antibodies into the cytosol of permeabilized cells (Fig. 2B). However, the anti-G\(_o\) antibodies selectively blocked the stabilizing effect of mastoparan on peripheral actin in Ca\(^{2+}\)-stimulated cells (Fig. 2B), in agreement with the results obtained with the synthetic G\(_o\) peptide. In contrast, anti-G\(_i3\) antibodies did not modify the inhibitory effect of mastoparan on Ca\(^{2+}\)-evoked disassembly of cortical actin (Fig. 2B).

These findings support the idea that mastoparan blocks the Ca\(^{2+}\)-evoked disruption of cortical actin network by stimulating an intracellular trimeric G\(_o\) protein. It is noteworthy that the G\(_o\) peptide and anti-G\(_o\) antibodies reverse at similar concentrations both the inhibitory effect of mastoparan on the ATP-dependent priming step of secretion (6) and the stabilizing effect of mastoparan on the peripheral actin cytoskeleton, suggesting a close link between these two events.

We reported previously that the “growth-associated protein” GAP-43 (neuromodulin), a neuronal protein enriched in presynaptic terminals, specifically stimulates the secretory granule-associated G\(_i\), when introduced into the cytosol of chromaffin cells and thereby inhibits the ATP-dependent priming step of Ca\(^{2+}\)-regulated secretion (31). To confirm that mastoparan inhibits the Ca\(^{2+}\)-evoked disassembly of cortical actin by activating the granule-bound G\(_o\) protein, we examined whether bovine brain GAP-43 affected actin organization like mastoparan. Permeabilized chromaffin cells were incubated in the presence of 1 μM cytosolic GAP-43 or 20 μM mastoparan and subsequently stimulated with calcium. Cells were then fixed and labeled with rhodamine-phalloidin and anti-DBH antibodies to
Cells were incubated for 10 min with the indicated peptides (100 μM) to stabilize the cortical actin network. Permeabilized chromaffin cells were subsequently fixed and labeled with rat anti-DBH antibodies detected with fluorescein anti-rat antibodies in combination with rhodamine-conjugated phalloidin. 200 DBH-positive cells were counted per coverslip and classified as having an intact or disrupted cortical rhodamine fluorescence ring. The proportion of cells displaying an intact actin network was expressed as the percentage of total counted cells. GAP-43 mimicked the stabilizing effect of mastoparan on cortical actin.

Table I

| [Ca^{2+}] | Control | Mastoparan | GAP-43 |
|-----------|---------|------------|--------|
| μM | %    | %    | %    |
| 0    | 72 ± 1.6 | 72 ± 1.4 | 73 ± 1.5 |
| 20   | 38 ± 3.1 | 69 ± 1.9 | 70 ± 3.0 |

SLO-permeabilized chromaffin cells were incubated for 10 min with calcium-free KG medium alone (control) or containing either 20 μM mastoparan or 1 μM GAP-43. Cells were subsequently incubated with calcium-free KG medium or stimulated with KG medium containing 20 μM free calcium. Cells were then fixed and labeled with rat anti-DBH antibodies. Cells with intact cortical F-actin were not further reduced by the Gα peptides nor by the anti-Gα antibodies in control cells incubated in the absence of mastoparan (data not shown).

GAP-43 did not affect the peripheral actin cytoskeleton in resting cells since the percentage of cells displaying an intact actin ring was similar in control cells and in cells incubated with either mastoparan or GAP-43. However, we found that GAP-43 mimicked the inhibitory effect of mastoparan on Ca^{2+}-evoked actin disassembly (Table I). In the presence of 1 μM cytosolic GAP-43, approximately 70% of the Ca^{2+}-stimulated cells still had an intact cortical actin ring. These results strengthen the idea that activation of the secretory granule-associated Gα protein can stabilize the cortical actin network and prevent its Ca^{2+}-induced dissociation upon cell stimulation.

**Correlation between the Mastoparan-Induced Inhibition of Secretion and the Stabilization of the Cortical Actin Network—**

Exocytosis can be visualized by immunofluorescence in living cells with anti-DBH antibodies present in the cell incubation medium (16, 30, 39). DBH, which is exclusively located on the inner face of secretory granule membranes, is exposed on the cell surface during exocytosis. Thus, the secretory activity can be evaluated by the appearance of fluorescent patches at the cell surface corresponding to DBH immunoreactivity. To correlate the exocytotic activity with the subplasmalemmal actin network organization, SLO-permeabilized cells were stimulated with 20 μM free calcium in the presence of anti-DBH antibodies. Cells were then washed rapidly, fixed, and stained with rhodamine-conjugated phalloidin. DBH immunoreactivity was detected with fluorescein-conjugated secondary antibodies. Confocal analysis indicated that fluorescent surface patches corresponding to DBH were generally observed in cells having a disrupted peripheral actin network (Fig. 3). Conversely, cells with a strong fluorescent actin ring were not labeled with DBH antibodies (Fig. 3). To probe the effect of mastoparan, the number of cells displaying a fluorescent actin ring and the percentage of cells labeled with anti-DBH antibodies were counted in randomly select areas of the coverslips. We found that mastoparan strongly reduced the Ca^{2+}-evoked exocytotic activity visualized by the appearance of fluorescent DBH surface patches (Fig. 3), in agreement with our previous results, indicating that mastoparan inhibits the secretion of catecholamines in both intact and permeabilized chromaffin cells (9, 40). Furthermore, the mastoparan-induced decrease in DBH labeling was accompanied by a parallel increase in phalloidin-staining in Ca^{2+}-stimulated cells (Fig. 3), a result indicative of a close relationship between the inhibitory effect of mastoparan on secretion and actin disassembly.

To further characterize the correlation between the effect of mastoparan on the actin cytoskeleton and the exocytotic process, we used two distinct actin filament-disrupting molecules, namely cytochalasin E and C. perfringens iota toxin, and examined whether these molecules interfere with the mastoparan-induced inhibition of Ca^{2+}-evoked secretion. At 20 μM, mastoparan inhibited Ca^{2+}-stimulated [3H]noradrenaline release in SLO-permeabilized chromaffin cells by approximately 75% (Fig. 4). Treatment with cytochalasin E or iota toxin partially reversed the mastoparan-induced inhibition of exocytosis. Preincubation of cells with 20 μM cytochalasin E reduced the maximal inhibitory effect of mastoparan to 42% (Fig. 4). Higher concentrations of cytochalasin E did not further reduce the inhibitory effect of mastoparan (data not shown). Incubation of SLO-permeabilized cells with 10 μg/ml iota toxin inhibited to a similar extent the effect of mastoparan on Ca^{2+}-evoked secretion (Fig. 4). In parallel experiments, we verified that both mastoparan and GAP-43 maximally inhibited Ca^{2+}-evoked secretion to 72% (Fig. 4).
rat anti-DBH antibodies (diluted 1:50). Cells were then washed, fixed, and stained with rhodamine-conjugated phallolidin (0.5 μg/ml) to visualize actin filaments and fluorescein-conjugated anti-rat antibodies (diluted 1:200) to reveal the exocytotic activity. A illustrates a quantitative analysis obtained by counting cells displaying either an intact cortical actin ring but no surface DBH patches (phalloidin-positive cells) or fluorescent exocytotic DBH patches, but a fragmented cortical actin network (DBH-positive cells). 200 single-rounded cells were examined per coverslips. B, represents typical images obtained in the rhodamine (phallolidin) and fluorescein (DBH) channels recorded simultaneously in the same focal plane by a double exposure procedure. Fluorescein exocytotic patches were only observed in cells displaying a reduced or fragmented rhodamine labeling in the cell periphery. Stimulation with calcium enhanced the proportion of exocytotic competent cells having a disrupted cortical actin network (DBH-positive cells). Mastoparan inhibited the cortical actin disassembly and in parallel blocked the Ca2+-evoked exocytotic activity.

Cytochalasin E and iota toxin disrupted the cortical actin network visualized by rhodamine-phalloidin staining in control and mastoparan-treated cells (data not shown). These experiments indicate that the inhibition of secretion by mastoparan is at least partially linked to the stabilization of the cortical actin network. However, mastoparan may also interfere with some other important step of the exocytotic pathway to account for the residual inhibition of secretion observed in cells having their peripheral cytoskeleton disrupted by cytochalasin E or iota toxin.

Effect of C. botulinum C3 Transferase on Mastoparan-induced Stabilization of Cortical Actin and Inhibition of Secretion—The small GTP-binding protein Rho is known to regulate the actin cytoskeleton organization (41, 42). To investigate the possible implication of Rho in the mastoparan-induced actin stabilization, we used the bacterial exoenzyme C. botulinum C3 ADP-ribosyltransferase, which specifically inactivates Rho. Chromaffin cells were permeabilized with SLO, incubated with mastoparan in the presence or absence of C3 transferase, and subsequently stimulated with calcium. Cells were then fixed and processed to visualize actin filaments. Confocal fluorescent images of resting and stimulated control cells and C3 transferase-treated cells are shown in Fig. 5A. Under resting conditions, incubation with 10 μg/ml C3 transferase generally preserved the cortical actin network, although the peripheral rhodamine-phalloidin fluorescence often appeared less dense, suggesting some fine modifications in the organization of the actin cytoskeleton. The proportion of cells displaying an intact cortical actin ring under each experimental conditions is illustrated in Fig. 5B. Treatment with C3 transferase did not modify the proportion of cells having a disrupted cortical actin ring upon Ca2+-induced stimulation. As expected, mastoparan blocked the Ca2+-evoked actin disassembly. This stabilizing effect of mastoparan was completely abolished in cells incubated with C3 transferase (Fig. 5).

We also examined the effect of C3 transferase on the mastoparan-induced inhibition of secretion. SLO-permeabilized chromaffin cells were incubated with increasing concentrations of C3 transferase in the presence or absence of mastoparan and subsequently stimulated with calcium. Treatment with C3 transferase did not significantly modify the Ca2+-evoked catecholamine release, but abolished the mastoparan-induced inhibition in a dose-dependent manner. As illustrated in Fig. 6, mastoparan inhibited secretion by 63% in control cells and by 42 and 43% in cytochalasin E- and iota toxin-treated cells, respectively.
randomly selected areas of the coverslips. In the absence of calcium, the cortical actin ring was determined by counting 200 DBH-labeled cells in the rhodamine channel. Optical sections were taken through the center of the coverslips.

identified with anti-DBH antibodies detected with fluorescein-conjugated secondary antibodies. Chromaffin cells were stained with rhodamine-conjugated phalloidin. This observation is in line with the idea that mastoparan stabilized the cortical actin cytoskeleton by controlling the granule-associated Go protein. Indeed, we found that mastoparan inhibits secretion by stabilizing the cortical actin network. To determine further the relationship between the effects of mastoparan on peripheral actin and exocytosis, we examined the effect of mastoparan in cells having their cortical actin networks disrupted by cytochalasin E or iota C3 transferase. As shown in Figs. 5 and 6, mastoparan inhibited secretion in permeabilized chromaffin cells (31). The latter effect was completely inhibited by anti-Go antibodies, suggesting that the effects of mastoparan on peripheral actin and exocytosis, we examined the effect of mastoparan in cells having their cortical actin filaments disorganized by cytochalasin E or iota C3 transferase. The aim of the present study was to identify the putative effector(s) by which the granule-associated Go protein inhibits the exocytotic response. Since the cortical actin network acts as a physical barrier to prevent granules from undergoing exocytosis (11, 12, 43), we thought that the subplasmalemmal cytoskeleton might be a possible candidate. The experiments presented here show that the introduction of mastoparan into the cytosol of permeabilized chromaffin cells prevents the calcium-evoked disruption of peripheral actin filaments. This effect was specifically reversed by the COOH-terminal peptide of Go, and affinity-purified antibodies raised against Go, indicating that mastoparan stabilized the cortical actin cytoskeleton by activating Go. Mastoparan is an amphiphilic peptide that penetrates the plasma membrane and activates associated G proteins. The fact that mastoparan stabilized actin filaments providing that the peptide penetrated into the cytoplasm implies that mastoparan stabilizes the actin cytoskeleton most likely by stimulating the secretory granule-associated Go accessible only in permeabilized cells.

We previously investigated the function(s) of trimeric G proteins in regulated secretion in chromaffin cells using mastoparan (6, 9, 10, 40). Mastoparan is a peptide from wasp venom that stimulates the GTPase of Gi and Go proteins by a mechanism that penetrates the plasma membrane and activates associated G proteins. The fact that mastoparan stabilized actin filaments providing that the peptide penetrated into the cytoplasm implies that mastoparan stabilizes the actin cytoskeleton most likely by stimulating the secretory granule-associated Go accessible only in permeabilized cells.

We found a close correlation between the inhibitory effect of mastoparan on exocytosis evaluated by the appearance of DBH immunoreactivity at the cell surface and the action of mastoparan on cortical actin filaments visualized with rhodamine-conjugated phalloidin. This observation is in line with the idea that mastoparan inhibits secretion by stabilizing the cortical actin network. To determine further the relationship between the effects of mastoparan on peripheral actin and exocytosis, we examined the effect of mastoparan in cells having their cortical actin filaments disorganized by cytochalasin E or iota C3 transferase.
**Go Controls the Cytoskeleton and Catecholamine Secretion**

Go in an activated state, an event which may also contribute to the translocation of ARF6 by maintaining the granule-bound actin cells triggered the rapid dissociation of ARF6 from secretory vesicles. These results suggest the possible involvement of Rho in the reorganization and to secretion (50–52). The exoenzyme C3 toxin, which is a useful tool for examining the cellular localization of Rho GTPases, a subgroup of the Ras superfamily of small GTP-binding proteins, represent other important modulators of actin cytoskeleton (41, 42, 48). In fibroblasts, polymerized actin is assembled into a variety of distinct structures, which have now all been shown to be controlled by members of the Rho family (49). In mast cells, Rho and Rac have been implicated in the signaling pathways that lead both to cytoskeleton reorganization and to secretion (50–52). The exoenzyme C3 from Clostridium botulinum is a useful tool for examining the cellular function of Rho, because it specifically ADP-ribosylates the protein at an asparagine residue in the putative effector domain (53, 54). We show here that the introduction of C3 trans- ferase in permeabilized chromaffin cells prevented the masto- paraman-induced stabilization of cortical actin and strongly reversed the inhibitory effect of mastoparan on secretion. These results suggest the possible involvement of Rho in the pathway by which Gs controls the peripheral actin cytoskeleton and the ATP-dependent priming step of exocytosis. Interestingly, the control of Rho-dependent actin polymerization by the alpha subunit of trimeric G proteins has been recently demonstrated in Swiss 3T3 fibroblasts (55). In mast cells, Norman et al. (24) have also suggested the occurrence of a plasma mem-

bran-associated trimeric G protein that might be able to trans- duce signals to Rho and Rac via a putative cytosolic factor. Hence, the direct effector coupling the alpha subunit of trimeric G proteins to the regulation of Rho activity remains unclear. RhoGDI might represent an attractive candidate, since its intro- duction into the cytosol inhibits exocytosis in mast cells (51, 52).

The precise mechanism by which Rho controls the organization of actin is not yet fully understood. However, use of cell- free assays and intact cell systems has shown that Rho regulates several enzymes, including phosphatidylinositol-4-phosphate 5-kinase and phosphoinositide 3-kinase (56–58), implying that Rho regulates the actin cytoskeleton through the formation of polyphosphoinositides, which are known to modulate the activity of various actin-binding proteins (59). Phosphatidylinositol (4,5)-bisphosphate decreases the actin filament severing activities of gelsolin and scinderin (60, 61), two proteins that have been found associated to the subplasmalemmal cytoskeleton in chromaffin cells (61, 62). Furthermore, recombinant scinderin facilitates exocytosis in permeabilized chromaffin cells, an effect that can be blocked by phosphatidylinositol (4,5)-bisphosphate (63). These observations suggest that Rho may stabilize the cortical actin network in chromaffin cells by controlling the level of phosphatidylinositol (4,5)- bisphosphate and thereby modulating the actin-severing activ- ity of scinderin and/or gelsolin. Since phosphatidylinositol-4-phosphate 5-kinase has been identified among the cytosolic proteins involved in the ATP-dependent priming reaction of exocytosis (64), it is tempting to speculate that the Rho-depend- ent synthesis of phosphatidylinositol (4,5)-bisphosphate is the link integrating the granule-bound Gs with the regulation of the cortical actin network, a scheme that might represent some of the biochemical reactions underlying the priming of exocytosis in neuroendocrine cells.

The intracellular regulatory mechanism of Gs activation and inactivation in resting and stimulated chromaffin cells remains elusive. Cytosolic GAP-43 is an attractive candidate, since the protein is a major substrate for protein kinase C, binds calmod- ulin, and is therefore sensitive to variations in cytosolic calcium. Although the introduction of GAP-43 modulates the exo- cytotic response in both adrenergic and noradrenergic permeabilized cells (31), its apparent absence in adrenergic cells (65) raises the question of its general function in neuroen- docrine cells. Alternatively, novel putative endogenous regula- tors of trimeric G proteins have been discovered recently. These newly identified proteins interacting either at the level of the alpha subunit (66) or with the beta-gamma complex (67) may well represent potential partners for the secretory granule-associated Gs during the exocytotic process. We previously proposed that Gs acts essentially as an inhibitory clamp preventing the priming of secretory granules and the ongoing of exocytosis in resting cells. The present data indicating that stimulation of the granule-bound Gs results in actin filament stabilization suggest that Gs could also play an active function in the terminal phase of exocytosis by facilitating the rapid re-assembly of cortical actin filaments at the site of fusion between granule and plasma membranes. In line with this hypothesis, it is interesting to note that active Rho has recently been described as a key regulator of the association of actin filaments with the plasma membrane (68). Further characterization of the identity and calcium sensitivity of the upstream regulators controlling the activation/inactivation cycle of Gs in stimulated chromaffin cells is now required to provide a more detailed picture of the exocytotic stages involving the participation of the secretory granule-bound Gs protein.

**Acknowledgments**—We gratefully acknowledge Danièle Thiersé for her expert technical assistance and Dr. Nicolas Vitale for preliminary

---

[Note: The provided text is a direct transcription of the document, including formatting and layout.]
experiments and stimulating discussions. We thank Dr. Nancy Grant for revising the manuscript.

REFERENCES

1. Barr, F. A., Leyte, A., and Huntting, W. B. (1992) Trends Cell Biol. 2, 91–94
2. Helms, J. B. (1995) FEBS Lett. 369, 84–88
3. Nürnberg, B., and Ahnert-Hilger, G. (1996) FEBS Lett. 389, 61–65
4. Ahnert-Hilger, G., Schäfer, T., Spicher, K., Grund, C., Schultz, G., and Wiedemann, R. (1994) Eur. J. Cell Biol. 65, 38–43
5. Konrad, R. J., Young, R. A., Record, R. D., Smith, R. M., Butkerait, P., Manning, D., Jarett, L., and Wolf, B. A. (1995) J. Biol. Chem. 270, 24981–24984
6. Vitale, N., Genese, M., Chasserot-Golaz, S., Aunis, D., and Bader, M. F. (1996) Eur. J. Neurosci. 8, 1275–1285
7. Aridor, M., Rajmilevich, G., Beaven, M. A., and Sagi-Eisenberg, R. (1993) Science 260, 1569–1572
8. Bernstein, B. W., and Bamburg, J. R. (1985) J. Neurosci. 5, 2565–2569
9. Burgoyne, R. D., Morgan, A., and O'Sullivan, A. J. (1989) J. Biol. Chem. 264, 159–163
10. Vitale, N., Gensse, M., Chasserot-Golaz, S., Aunis, D., and Bader, M. F. (1996) J. Cell Biol. 134, 1005–1015
11. Cheek, T. R., and Burgoyne, R. D. (1987) J. Biol. Chem. 262, 1569–1572
12. Aunis, D., and Bader, M. F. (1988) Science 241, 832–835
13. Chasseras-Golaz, S., Vitale, N., Nagot, I., Delouche, B., Dirrig, S., Pradel, L. A., Henry, J. P., Aunis, D., and Bader, M. F. (1996) J. Cell Biol. 133, 1217–1236
14. Bader, M. F., Thierse, D., Aunis, D., and Bader, M. F. (1996) Cell. Mol. Neurobiol. 17, 71–87
15. Machesky, L. M., and Hall, A. (1996) Trends Cell Biol. 6, 304–310
16. Jungermann, J., Lerch, M. M., Weidenbach, H., Lutz, M. P., Kruger, B., and Manning, D., Eberle, M., Lemke, H. D., and Aktories, K. (1992) FEBS Lett. 302, 287–296
Trimeric G Proteins Control Exocytosis in Chromaffin Cells: Go REGULATES THE PERIPHERAL ACTIN NETWORK AND CATECHOLAMINE SECRETION BY A MECHANISM INVOLVING THE SMALL GTP-BINDING PROTEIN Rho
Stéphane Gasman, Sylvette Chasserot-Golaz, Michel R. Popoff, Dominique Aunis and Marie-France Bader

J. Biol. Chem. 1997, 272:20564-20571.
doi: 10.1074/jbc.272.33.20564

Access the most updated version of this article at http://www.jbc.org/content/272/33/20564

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 67 references, 35 of which can be accessed free at http://www.jbc.org/content/272/33/20564.full.html#ref-list-1