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Involvement of Rho GTPases in calcium-regulated exocytosis from adrenal chromaffin cells

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SUMMARY

The Rho GTPase family, including Rho, Rac and Cdc42 proteins, is implicated in various cell functions requiring the reorganization of actin-based structures. In secretory cells, cytoskeletal rearrangements are a prerequisite for exocytosis. We previously described that, in chromaffin cells, the trimeric granule-bound Go protein controls peripheral actin and prevents exocytosis in resting cells through the regulation of RhoA. To provide further insight into the function of Rho proteins in exocytosis, we focus here on their intracellular distribution in chromaffin cells. By confocal immunofluorescence analysis, we found that Rac1 and Cdc42 are exclusively localized in the subplasmalemmal region in both resting and nicotine-stimulated cells. In contrast, RhoA is associated with the membrane of secretory granules. We then investigated the effects of clostridial toxins, which differentially impair the function of Rho GTPases, on the subplasmalemmal actin network and catecholamine secretion. *Clostridium difficile* toxin B, which inactivates Rho, Rac and Cdc42, markedly altered the distribution of peripheral actin filaments. Neither *Clostridium botulinum* C3 toxin, which selectively ADP-ribosylates Rho, nor *Clostridium sordellii* lethal toxin, which inactivates Rac, affected cortical actin, suggesting that Cdc42 plays a specific role in the organization of subplasmalemmal actin. Indeed, toxin B strongly reduced secretagogue-evoked catecholamine release. This effect on secretion was not observed in cells having their actin cytoskeleton depolymerized by cytochalasin E or *Clostridium botulinum* C2 toxin, suggesting that the inhibition of secretion by toxin B is entirely linked to the disorganization of actin. *C. sordellii* lethal toxin also inhibited catecholamine secretion, but this effect was not related to the actin cytoskeleton as seen in cells pretreated with cytochalasin E or C2 toxin. In contrast, C3 exoenzyme did not affect secretion. We propose that Cdc42 plays an active role in exocytosis by coupling the actin cytoskeleton to the sequential steps underlying membrane trafficking at the site of exocytosis.

Key words: Secretion, Actin filament, Clostridial toxin, Secretory granule, Catecholamine

INTRODUCTION

Rho proteins are small GTPases that belong to the Ras superfamily and consist of the Rho, Rac and Cdc42 subfamilies. Rho proteins have been implicated in a number of cellular functions requiring a reorganization of actin based-structures (Takai et al., 1995; Symons, 1996; Hall, 1998). 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: Rho regulates the formation of stress fibers and cell adhesion (Ridley and Hall, 1992), Rac is involved in membrane ruffling and formation of lamellipodia (Ridley et al., 1992) and Cdc42 participates in receptor-induction of microspikes (Kozma et al., 1995). At the molecular level, Rho proteins represent switches in various intracellular pathways through the regulation of lipid kinases, like phosphatidylinositol 4-phosphate 5-kinase (Chong et al., 1994) and phosphoinositide 3-kinase (Zhang et al., 1993), and serine-threonine protein kinases, such as Rho kinase (Leung et al., 1996) and protein kinase N (Amano et al., 1996).

In secretory cells, cytoskeletal rearrangements are a prerequisite for exocytosis, enabling docking and fusion of secretory granules with the plasma membrane (Linstedt and Kelly, 1987; Sontag et al., 1988; Norman et al., 1994; Vitale et al., 1995). Indeed, it has been recently reported that GTP-activated Rac and Cdc42 can induce exocytosis in permeabilized mast cells (Brown et al., 1998). A role for Rac and Cdc42 has also been described in the late steps of calcium-induced insulin secretion from pancreatic β cells (Kowluru et al., 1997). Both of these studies rule out an active role for Rho itself in the exocytotic machinery. On the other hand, we recently described that the secretory granule-associated trimeric Go protein inhibits exocytosis in chromaffin cells by stabilizing the cortical actin network through a sequence

of events that involves RhoA and a granule-bound phosphatidylinositol 4-kinase activity (Gasman et al., 1997, 1998).

The present study was undertaken to further characterize the role of Rho and Rho-related proteins in the exocytotic machinery of chromaffin cells, using clostridial toxins that specifically, but differentially, inactivate Rho GTPases. *Clostridium difficile* toxin B monoglucosylates the threonine residue at position 35 in Rac and Cdc42 and threonine 37 in Rho (Just et al., 1995; Aktories, 1997). *Clostridium sordellii* lethal toxin glucosylates threonine 35 in Rac. It does not modify any other protein of the Rho subfamily but affects also Rap and Ral GTPases (Popoff et al., 1996). *Clostridium botulinum* exoenzyme C3 specifically inactivates Rho through ADP-ribosylation at the asparagine 41 residue (Aktories et al., 1989). Glucosylation or ADP-ribosylation in the effector domain of the various Rho, Rac and Cdc42 isoforms disrupts their interaction with downstream effectors and thereby inactivates the intracellular pathways controlled by these GTPases. Here, we have examined the effects of these three toxins on cortical actin cytoskeleton and catecholamine secretion in cultured chromaffin cells in order to identify the candidate GTPases involved in the cascade of events leading to secretion. Our results support a different role for RhoA and Cdc42 in the organization of the actin subplasmalemmal network and the recruitment of secretory granules for exocytosis.

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-generating Percoll gradients (Bader et al., 1986). Cells were cultured as monolayers either on 24 multiple 16 mm Costar plates (Costar, Cambridge, MA) at a density of 2.5×10^5 cells/well to measure catecholamine secretion, or on 35 mm Costar plates at a density of 1.25×10^6 cells for ADP-ribosylation assay, or on fibronectin-coated glass coverslips at a density of 2×10^5 cells for immunocytochemistry. Experiments were performed 3-7 days after plating.

[^{3}H]noradrenaline release

Catecholamine stores were labeled before or after toxin treatment (see legends to figures) by incubating chromaffin cells with [^{3}H]noradrenaline (14.7 Ci/mmol; NEN Du Pont de Nemours, Les Ulis, France) for 30-45 minutes. Cells were then washed four times with Locke's solution (140 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 0.01 mM EDTA, 11 mM glucose, 0.56 mM ascorbic acid, and 15 mM Hepes, pH 7.2). The cellular content of [^{3}H]noradrenaline was 15 nCi/10^6 cells and was not significantly modified by the toxin treatment. To trigger exocytosis, cells were subsequently stimulated for 10 minutes with Locke's solution containing either 10 μM nicotine or 59 mM K^+. Alternatively, cells were permeabilized for 2 minutes with 15 units/ml streptolysin-O (SLO) in 200 μl/well Ca^{2+}-free KG medium (150 mM potassium glutamate, 10 mM Pipes, pH 7.0, 5 mM nitrilotriacetic acid, 0.5 mM EGTA, 5 mM Mg^{2+}-ATP, 4.5 mM magnesium acetate, 0.2% bovine serum albumin) and then stimulated for 10 minutes with KG medium containing 20 μM free Ca^{2+} (150 mM potassium glutamate, 10 mM Pipes, pH 7.0, 5 mM nitrilotriacetic acid, 0.5 mM EGTA, 5 mM Mg^{2+}-ATP, 3.75 mM magnesium acetate, 1.14 mM $CaCl_2$, 0.2% bovine serum albumin). [^{3}H]noradrenaline release after stimulation was determined by measuring the radioactivity present in the incubation medium and in cells after precipitation with 10% (v/v) trichloroacetic acid. Net secretory values were obtained by subtracting the basal release established in the absence of secretagogue.

$[Ca^{2+}]_i$ measurements

Cells grown on glass coverslips coated with collagen were placed in an observation chamber and washed twice with Locke's solution. Cells were then loaded by incubation with 4 μM of the fluorescent Ca^{2+} indicator penta-acetoxymethyl ester of indo 1 (indo 1/AM, Molecular Probes, Eugene, OR) and 0.02% Pluronic F-127 (Molecular Probes, Eugene, OR) in Locke's solution for 30 minutes at 37°C. Cells were subsequently washed with Locke's solution and maintained at room temperature in the dark prior to fluorescence measurements. Nicotine (100 μM) was applied with glass micropipettes (Eppendorf, Hamburg, Germany) positioned near the cell using an hydraulic micro-manipulator. The fluorescence measured from a field slightly larger than the size of the cell was monitored using a Nikon Diaphot inverted microscope fitted for dual emission microspectrofluorometry measurements, as described in detail elsewhere (Mollard et al., 1989). The excitation wavelength was set at 355 nm and emission was simultaneously recorded at 405 nm (yielding F_{405}) and 480 nm (yielding F_{480}) using two parallel photomultipliers (P1, Nikon). Single photon currents were converted to voltage signals, which were divided on line by a monolithic laser trimmed two-quadrant divider, giving the ratio $R=F_{405}/F_{480}$. This ratio, which represents variations in cytosolic $[Ca^{2+}]_i$, was simultaneously traced on a pen recorder. The mean background fluorescence recorded with separate unloaded cells was subtracted. Signals were digitized and their amplitude was estimated by integrating the surface area below the current curve using the NIH Image computer program.

Immunocytochemistry and confocal laser scanning microscopy

Chromaffin cells grown on fibronectin-coated glass coverslips were fixed for 15 minutes in 4% paraformaldehyde in 0.12 M sodium/potassium phosphate, pH 7.0, and for a 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% (v/v) normal goat serum in PBS to reduce nonspecific staining. Cells were then incubated for 2 hours at 37°C with mouse monoclonal antibodies against Rac1 (diluted to 1:50) or against RhoA (diluted 1:10) in PBS containing 3% BSA. Cells were then washed and incubated in PBS containing Cy3-labelled anti-mouse secondary antibodies (1:2000) and 3% BSA for 1 hour. Actin filaments were stained by incubation with rhodamine (TRITC)-conjugated phalloidin (Sigma) at a concentration of 0.5 μg/ml in PBS for 15 minutes. Coverslips were extensively washed with PBS, rinsed with water and mounted in Mowiol 4-88 (Hoechst).

Stained cells were monitored using the Zeiss laser scanning microscope (LSM 410 invert) with a planapo oil immersion lens (63×, NA=1.4). TRITC and Cy3 were excited using the He/Ne laser (543 nm). The emission signal was filtered with a 595 nm long pass filter. Non-specific fluorescence was assessed as described (Chasserot-Golaz et al., 1996).

ADP-ribosylation assay with recombinant C3 enzyme

Chromaffin cells (2.5×10^6 cells per experimental condition) were preincubated for 18 hours with 20 μg/ml *C. botulinum* C3 ADP-ribosyltransferase. Cells were then collected and centrifuged. ADP-ribosylation was performed with 15 μg membrane-bound proteins in 20 mM Hepes, pH 7.5, 10 mM thymidine, 1 mM DTT, 1 mM EDTA, 5 mM $MgCl_2$, 1 mM ATP, 100 μM GTP, 0.5 μCi [^{32}P]NAD (0.15 μM; 30 Ci/mmol, 2 mCi/ml, NEN Life Science Products). The reaction was carried out for 30 minutes at 37°C in the presence of 5 μg/ml C3 ADP-ribosyltransferase in a final volume of 120 μl. Proteins were then precipitated with 10% (v/v) trichloroacetic acid, centrifuged and dissolved in sample buffer for SDS-polyacrylamide gel separation.

Labeled proteins were analyzed by autoradiography with a Bio-Imaging Analyzer FUJIX BAS1000 (Fuji, Tokyo, Japan).

Antibodies and toxins

Monoclonal antibodies against Rac1 were from Transduction Laboratories. Monoclonal antibodies againts RhoA and rabbit polyclonal antibodies against Cdc42 were from Santa Cruz Biotechnology. Anti-Rap (monoclonal), anti-RalA (monoclonal) and anti-RalB (rabbit polyclonal) antibodies were from Transduction Laboratories. Anti-chromogranin A antibodies (rabbit polyclonal) were prepared in our laboratory. Goat anti-mouse antibodies conjugated to Cy3 and goat anti-rabbit antibodies conjugated to Cy3 were purchased from Amersham (Les Ulis, France).

Clostridium botulinum exoenzyme C3 ADP-ribosyltransferase and *Clostridium botulinum* C2 toxin (C2I and C2II components) were purified as described (Popoff and Boquet, 1988; Popoff et al., 1991). *Clostridium difficile* toxin B (von Eichel-Streiber et al., 1987) and *Clostridium sordellii* lethal toxin (Popoff, 1987) were prepared and purified according to previously published methods.

RESULTS

Subcellular localization of RhoA, Rac1 and Cdc42 in cultured chromaffin cells

We examined the intracellular distribution of RhoA, Rac1 and Cdc42 in cultured chromaffin cells by immunofluorescence and confocal microscopy. Chromaffin cells were identified with anti-chromogranin A antibodies which specifically label chromaffin secretory granules. Using monoclonal anti-Rac1 antibodies and Cy3-conjugated anti-mouse antibodies, we found that Rac1 was restricted to the subplasmalemmal region in resting chromaffin cells (Fig. 1A). Similarly, Cdc42 immunoreactivity was essentially detected in the cell periphery (Fig. 1C). Stimulation with 10 μM nicotine did not modify the staining pattern corresponding to Rac1 (Fig. 1B) or Cdc42 (Fig. 1D), indicating that Rac1 and Cdc42 are apparently not redistributed during the exocytotic reaction. Hence, actin is probably not the attachment point of Rac1 and Cdc42 since stimulation with nicotine triggers an important reorganization of actin filaments in the subplasmalemmal space (Chasserot-Golaz et al., 1998).

In contrast, immunostaining with RhoA antibodies revealed a punctuate pattern of fluorescence over the whole cell body (Fig. 1E), in agreement with our previous observations that most of the cellular RhoA is associated with the membrane of secretory organelles in chromaffin cells (Gasman et al., 1998). After several days in culture, chromaffin cells are known to develop cellular extensions and neurites in which secretory granules can be individually visualized at the optical level. Double-labeling experiments with anti-RhoA (Fig. 1G) and anti-chromogranin A antibodies (Fig. 1H) in 7-day-old cultured chromaffin cells confirmed that almost all secretory granules were labeled with the anti-RhoA antibodies. We also compared the distribution of RhoA immunoreactivity in resting and nicotine-stimulated chromaffin cells (Fig. 1F), but found no apparent modification in the punctuate staining pattern, suggesting that RhoA remains associated with the membrane of secretory granules during the course of exocytosis.

Effect of clostridial cytotoxins on cortical actin network in chromaffin cells

To investigate the possible implication of Rho-related proteins in the actin cytoskeleton organization in chromaffin cells, we made use of *C. difficile* toxin B, *C. sordellii* lethal toxin and *C. botulinum* C3 exoenzyme, which modify and inactivate specific subsets of Rho GTPases (Table 1). Chromaffin cells were treated with either 0.5 μg/ml toxin B (18 hours), 20 μg/ml exoenzyme C3 (18 hours) or 1 μg/ml lethal toxin (6 hours), fixed and then incubated with rhodamine-conjugated phalloidin to visualize filamentous but not monomeric actin.

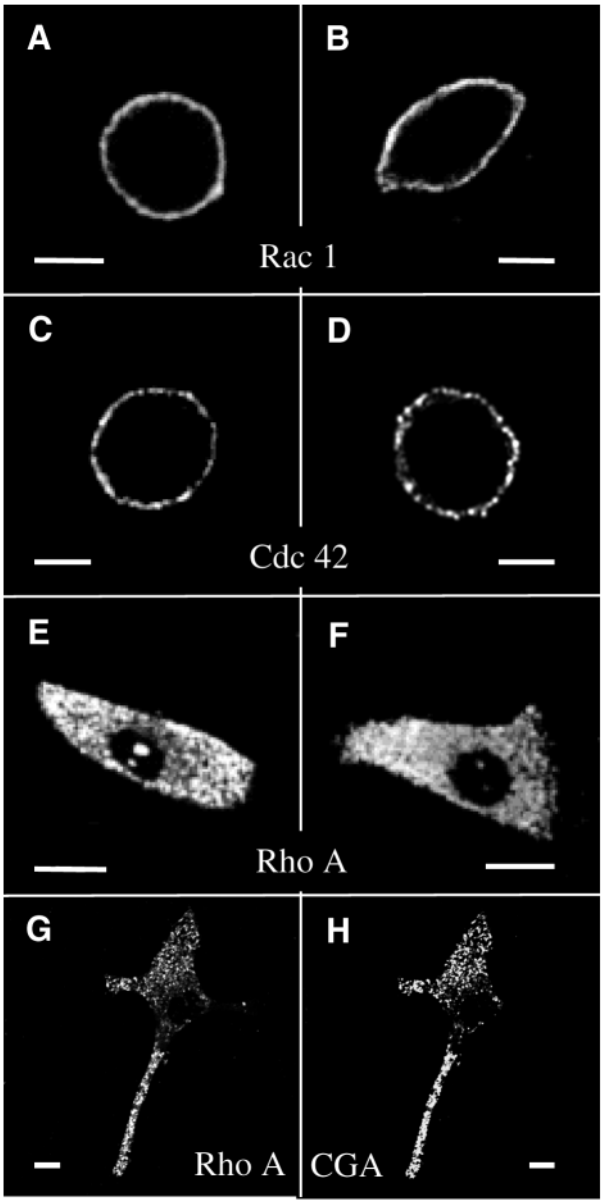

Fig. 1. Intracellular distribution of Rac1, Cdc42 and RhoA in cultured chromaffin cells. Chromaffin cells in culture for 3 days were maintained under resting conditions (A,C,E) or stimulated with 10 μM nicotine (B,D,F). Confocal immunofluorescent images were obtained by labeling resting and stimulated chromaffin cells with either anti-Rac1, anti-Cdc42 or anti-RhoA antibodies visualized with Cy3-conjugated secondary antibodies. (G,H) A 7-day-old cultured chromaffin cell double stained with anti-RhoA antibodies revealed with Cy3-conjugated antibodies and anti-chromogranin A antibodies detected with Cy2-conjugated secondary antibodies. Sections were taken with the minimum pinhole size in the plane of the nuclei using excitation and emission filtering. Scale bars, 5 μm.

Table 1. Bacterial protein toxins used in the present study

Toxin/exoenzyme	Protein substrate	Cosubstrate	Modification	Effect
C. botulinum exoenzyme C3	Rho A-C	NAD	ADP-ribosylation of Asn 41 in the effector region	Inactivation of Rho signalling
C. difficile toxin B	Rho, Rac, Cdc 42	UDP-glucose	Glucosylation of Thr37 (Rho) or Thr35 (Rac, Cdc42) in the effector region	Inactivation of Rho, Rac, Cdc42 signalling
C. sordellii lethal toxin	Rac, Ras, Ral, Rap	UDP-glucose	Glucosylation of Thr35 (Rac) in the effector region	Inactivation of Rac signalling. Inhibition of Rap and Ral
C. botulinum C2	Actin G	NAD	ADP-ribosylation of Arg177	Disassembly of actin filaments

Confocal images of control and toxin-treated cells are shown in Fig. 2. In untreated chromaffin cells, rhodamine-phalloidin fluorescence was most intense at the cell periphery, forming a continuous and homogeneous cortical ring, in agreement with the fact that in secretory cells the majority of actin filaments are generally concentrated in the subplasmalemmal region (Fig. 2). Incubation with toxin B, which glucosylates Rho, Rac and Cdc42 (Aktories, 1997), reduced the binding of rhodamine-phalloidin in the cell periphery and triggered the appearance of fluorescent patches in the cytosol (Fig. 2), revealing an important redistribution of the actin filaments. In contrast, exposure to C3 exoenzyme, which ADP-ribosylates Rho (Aktories, 1997), or lethal toxin, which glucosylates Rac (Popoff et al., 1996; Just et al., 1996; Hofmann et al., 1996), had no apparent effect on the distribution of rhodamine-phalloidin fluorescence (Fig. 2), although some minor modifications in the organization of the actin network cannot be excluded. Thus, the cortical actin ring is apparently not affected by the selective inactivation of Rho by C3 or Rac by lethal toxin. On the other hand toxin B, which also inactivates Cdc42, produced a profound modification of actin distribution, suggesting that the subplasmalemmal Cdc42 is likely to play an important function in the maintainance of the peripheral actin filaments in chromaffin cells.

Effect of clostridial toxins on secretagogue-evoked catecholamine secretion

Next, we investigated the effects of toxin B, exoenzyme C3 and lethal toxin on secretagogue-evoked catecholamine release in chromaffin cells. As illustrated in Fig. 3, preincubation of cultured chromaffin cells with toxin B induced a dose-dependent inhibition of the secretory response evoked by either nicotine or 59 mM K^+ (Fig. 3A). The maximal effect (approximately 70% inhibition) was observed by pretreating cells with 1 μg/ml of toxin B for 18 hours and the mean inhibitory dose, ID_{50}, was approximately 0.35 μg/ml. Lethal toxin produced a similar dose-dependent inhibition of catecholamine secretion in cells stimulated with 10 μM nicotine or 59 mM K^+ (Fig. 3B). We then assessed the secretory activity from chromaffin cells previously incubated for 18 hours with increasing concentrations of C3 toxin. Exposure to C3 exoenzyme did not affect significantly nicotine or potassium-induced catecholamine secretion (Fig. 3C). We verified that the 18 hour period in the presence of C3 toxin was sufficient to ADP-ribosylate intracellular Rho. As illustrated in Fig. 4, the incorporation of [^{32}P]NAD catalyzed by C3 toxin was markedly reduced (>90 %) in proteins collected from cells that had been pre-exposed for 18 hours to C3 toxin, indicating that under these experimental conditions most Rho endogenous to chromaffin cells had been ADP-ribosylated. Thus, the activation of a Rho-dependent pathway probably does not represent a critical step for exocytosis in stimulated chromaffin cells.

To confirm that toxin B and lethal toxin affect the secretory mechanisms at a step distal to the rise of cytosolic calcium, we examined first the effect of toxin B and lethal toxin on calcium-evoked catecholamine secretion from streptolysin-O-permeabilized cells. Both toxins inhibited to a similar extent (ID_{50} of 0.35-0.40 μg/ml) the release of catecholamines from permeabilized cells in response to elevated calcium in the incubation medium (data not shown). Fig. 5 illustrates the effect of toxin B and lethal toxin on the cytosolic Ca^{2+} concentration estimated with the fluorescent Ca^{2+} probe, indo 1. Representative traces of $[Ca^{2+}]_i$ transients in individual chromaffin cells stimulated with nicotine are depicted. In untreated cells, a local application of nicotine (100 μM) elicited

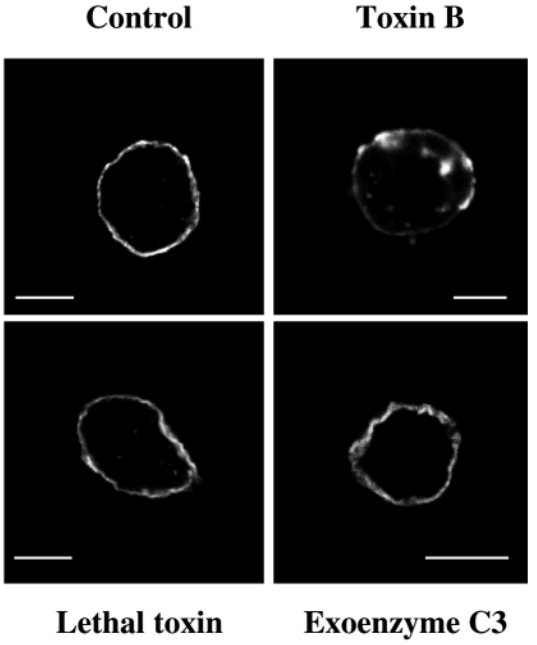

Fig. 2. Effect of clostridial cytotoxins on the subplasmalemmal actin network in chromaffin cells. Chromaffin cells were maintained in culture medium in the absence of toxin (Control) or incubated for 18 hours in culture medium in the presence of either 0.5 μg/ml *C. difficile* toxin B (Toxin B) or 20 μg/ml *C. botulinum* exoenzyme C3 (Exoenzyme C3), or exposed for 6 hours to 1 μg/ml *C. sordellii* lethal toxin (Lethal toxin) in culture medium. Cells were then fixed and labeled with rhodamine-conjugated phalloidin to visualize filamentous actin. Confocal fluorescent images were taken through the center of the nucleus. Scale bars, 5 μm.

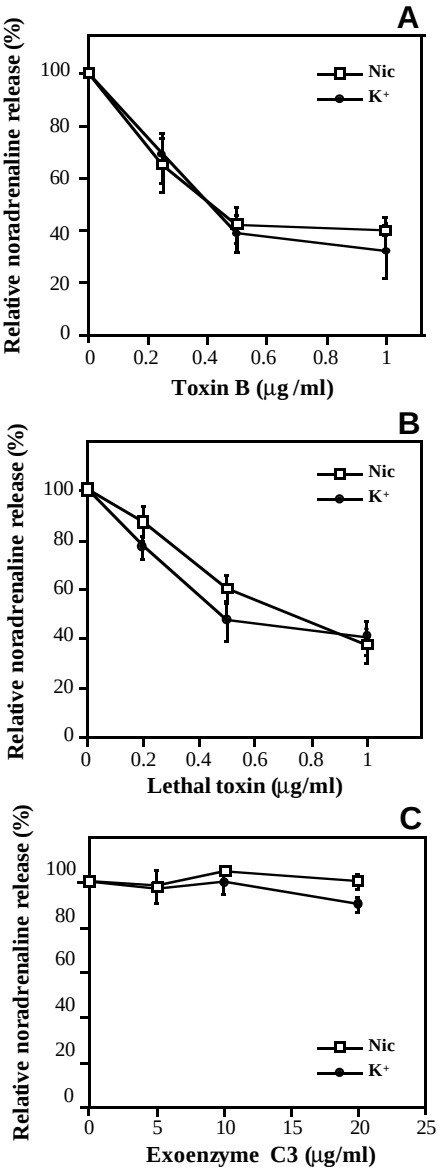

Fig. 3. Effect of clostridial cytotoxins on secretagogue-evoked catecholamine release in chromaffin cells. (A) Cells were preincubated for 45 minutes with [^{3}H]noradrenaline and then exposed for 18 hours in culture medium to the indicated concentrations of *C. difficile* toxin B. (B) Chromaffin cells were exposed for 6 hours to the indicated concentrations of *C. sordellii* lethal toxin and then incubated for 30 minutes with [^{3}H]noradrenaline. (C) [^{3}H]noradrenaline-labeled chromaffin cells were exposed for 18 hours in culture medium to the indicated concentrations of *C. botulinum* exoenzyme C3. Cells were subsequently stimulated for 10 minutes with either 10 μM nicotine (Nic) or a 59 mM depolarizing concentration of potassium (K^+). Results are expressed relative to the net [^{3}H]noradrenaline release obtained from control cells incubated in the absence of toxins.

within a few seconds a rapid and transient rise in $[Ca^{2+}]_i$ (Fig. 5). In agreement with previous reports (Kao and Schneider, 1986; Ohta et al., 1996; Herrington et al., 1996), the decay of cytosolic calcium to resting levels occured on a time scale of minutes and appeared to proceed by at least two components

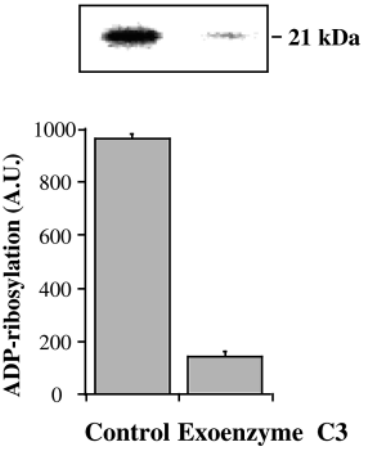

Fig. 4. *C. botulinum* exoenzyme C3-catalyzed ADP-ribosylation of Rho in chromaffin cells. Chromaffin cells were incubated for 18 hours in the presence (Exoenzyme C3) or absence (Control) of 20 μg/ml exoenzyme C3. Cells were then collected and centrifuged. ADP-ribosylation of the lysates was performed with [^{32}P]NAD^+ and 5 μg/ml exoenzyme C3. Labeled proteins were subsequently resolved by SDS-gel electrophoresis and detected by autoradiography. Pretreatment of intact cells with exoenzyme C3 reduced by more than 90% the amount of endogenous Rho available for subsequent ADP-ribosylation in vitro. A.U., arbitrary units.

(Fig. 5). No significant differences of the nicotine-evoked $[Ca^{2+}]_i$ transients were observed in cells pretreated either for 18 hours with toxin B or for 6 hours with lethal toxin (Fig. 5). Both the size of the initial peak and the duration of the two decay phases displayed some cell-to-cell variability, but there was no correlation with the toxin treatment. Furthermore, the amplitude of the signals, quantified by integrating the area below the current curve, was not significantly modified in cells exposed to toxin B or lethal toxin compared to control cells (Fig. 5). Taken together, these data indicate that toxin B and lethal toxin inhibit catecholamine secretion at a step that occurs after the mobilization of cytosolic calcium, most likely by affecting directly some element(s) of the exocytotic machinery.

The time course of nicotine-stimulated catecholamine secretion in toxin B- and lethal toxin-treated chromaffin cells is presented in Fig. 6. Both toxins inhibited to a similar extent (50-60% inhibition) the maximal secretory response achieved after 5 min of stimulation. However the inhibitory effect induced by lethal toxin was immediate whereas the effect of toxin B was delayed and only observed after 1 min of stimulation (Fig. 6). This observation suggests that the two toxins may interfere with distinct targets in the sequence of events underlying exocytosis.

Correlation between the effects of toxin B and lethal toxin on cortical actin cytoskeleton and catecholamine secretion

To further evaluate the relationship between the effects of *C. difficile* toxin B on the actin cytoskeleton and the exocytotic process, we used two actin-depolymerizing molecules, namely C2 toxin from *C. botulinum* or cytochalasin E, and examined whether these molecules interfere with the toxin B-induced inhibition of secretion. Chromaffin cells were pretreated for 3 hours with 5 nM C2 toxin, subsequently incubated with toxin

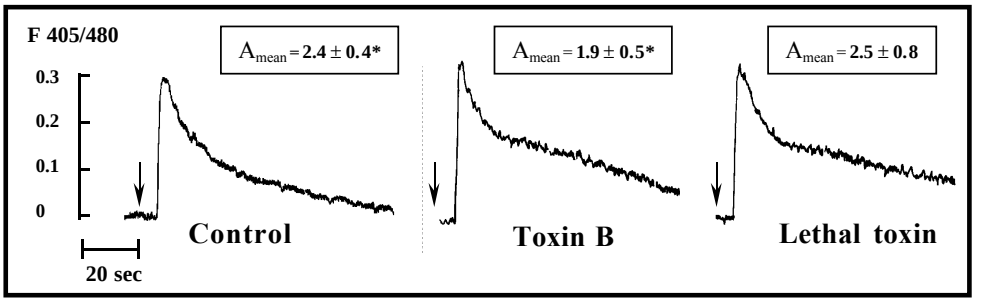

Fig. 5. Effect of *C. difficile* toxin B and *C. sordellii* lethal toxin on $[Ca^{2+}]$ transients in nicotine-stimulated chromaffin cells. Chromaffin cells maintained in culture medium (Control) or preincubated for 18 hours with 1 μg/ml toxin B (Toxin B) or for 6 hours with 1 μg/ml lethal toxin (Lethal toxin) were loaded with Indo 1. Cells were stimulated with a 10 second pulse application of 100 μM nicotine (arrow). The ratio F_{405}/F_{480} measured in individual chromaffin cells was taken as an index of $[Ca^{2+}]_i$ variation. For each experimental condition, the traces shown are representative of 20 individual cell responses recorded in the same dish. Similar results were obtained in two separate experiments. The mean amplitude (A_{mean}) of the signals estimated by integrating the surface area below the current curve was not significantly different between control and toxin-treated cells. **P*>0.05 when tested by Student's test.

B and then stimulated with 59 mM K^+. At 1 μg/ml, toxin B inhibited K^+-stimulated catecholamine secretion by 74% (Fig. 7A). Preincubation of cells with C2 toxin completely abolished this inhibitory effect of toxin B (Fig. 7A). Similarly, preincubation of chromaffin cells with 20 μM cytochalasin E strongly reduced the inhibitory effect of toxin B on catecholamine secretion (Fig. 7A). These experiments indicate that the inhibition of secretion by *C. difficile* toxin B is likely to be linked to the modification of the actin cytoskeleton organization induced by toxin B in chromaffin cells.

In contrast, pretreatment of chromaffin cells with C2 toxin or cytochalasin E did not significantly modify the inhibitory effect of lethal toxin on secretagogue-evoked catecholamine secretion (Fig. 7B). Thus, lethal toxin inhibited the exocytotic process in chromaffin cells through a pathway that was apparently not related to the organization of the cortical actin cytoskeleton. It is tempting to speculate that lethal toxin may evoke this distinct cellular effect through the inactivation of a GTPase that is not a target for toxin B, for instance by interfering with a Rap- and/or a Ral-dependent cellular pathway.

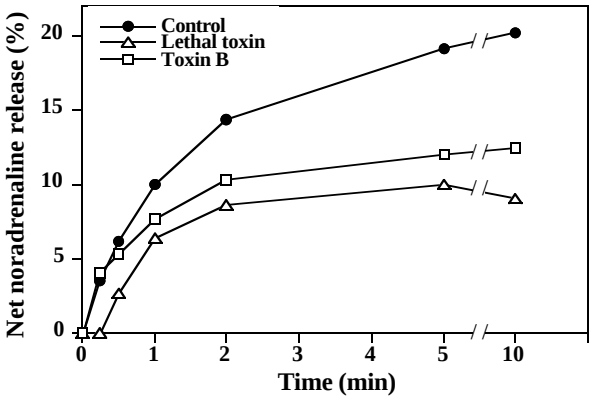

Fig. 6. Time course of nicotine-evoked catecholamine secretion in clostridial cytotoxin-treated chromaffin cells. Cells were either preincubated for 45 minutes with [^{3}H]noradrenaline and then exposed for 18 hours in culture medium to 1 μg/ml *C. difficile* toxin B or treated for 6 hours with 1 μg/ml *C. sordellii* lethal toxin and then incubated with [^{3}H]noradrenaline. Cells were then rapidly washed and stimulated for the indicated periods of time with 10 μM nicotine. Data given as mean values of duplicate determinations are representative of two separate experiments.

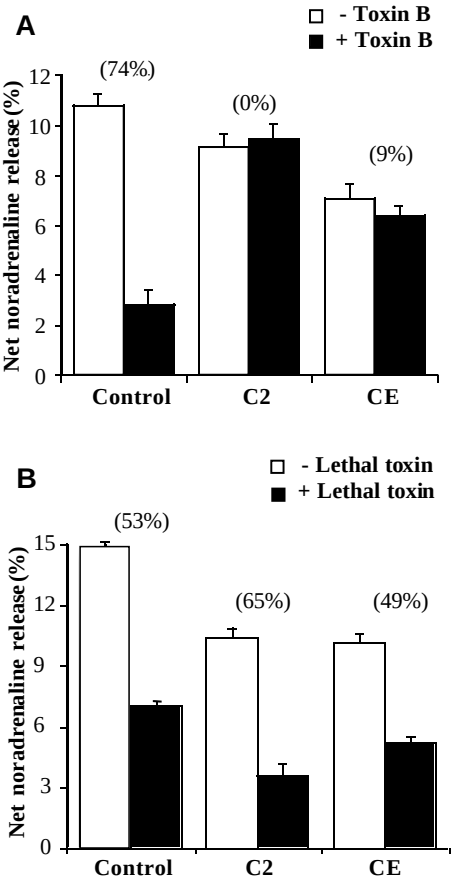

Fig. 7. Effect of cytochalasin E and *C. botulinum* C2 toxin on toxin B- and lethal toxin-induced inhibition of catecholamine secretion. [^{3}H]noradrenaline-labeled chromaffin cells were preincubated with either 5 nM C2 toxin for 3 hours (C2) or 20 μM cytochalasin E for 1 hour (CE) and then either (A) incubated for 18 hours with or without 1 μg/ml toxin B in the presence of C2 toxin or cytochalasin E or (B) exposed for 6 hours with or without 1 μg/ml lethal toxin in the presence of C2 toxin or cytochalasin E. Cells were then washed and stimulated for 10 minutes with 59 mM K^+. Basal release was estimated in the absence of secretagogue and subtracted to obtain the net noradrenaline release. Values in brackets represent the percentage of inhibition induced by toxin B or lethal toxin for each experimental condition. Depolymerization of the cortical actin network strongly reduced the toxin B-induced inhibition of catecholamine secretion but did not significantly modify the inhibitory effect of lethal toxin.

Presence and localization of Ral and Rap in chromaffin cells

Fig. 8A illustrates a western blot analysis of a total cell homogenate prepared from cultured chromaffin cells using anti-Ral and anti-Rap antibodies. Both RalA and RalB were present in substantial amounts among the chromaffin cell proteins. In contrast, the immunosignal for Rap was hardly detectable. The intracellular distribution of Ral proteins was further investigated by immunocytochemistry and confocal microscopy. We found that RalA was essentially located in the subplasmalemmal region whereas RalB displayed a punctuate pattern of distribution over the whole cell body exept the nucleus (Fig. 8B). Note that the anti-Rap antibodies failed to specifically label chromaffin cells, confirming the low level of expression or the absence of Rap in chromaffin cells. To probe the eventual association of RalB with chromaffin granules, double-labeling experiments were performed with antibodies against RalB and chromogranin A. However, the two proteins were not colocalized, as revealed by the cytofluorogram representing the pixels measured in the RalB and chromogranin A images (data not shown), indicating that RalB is not associated to secretory granules but probably to some other intracellular membrane-bound compartment in chromaffin cells.

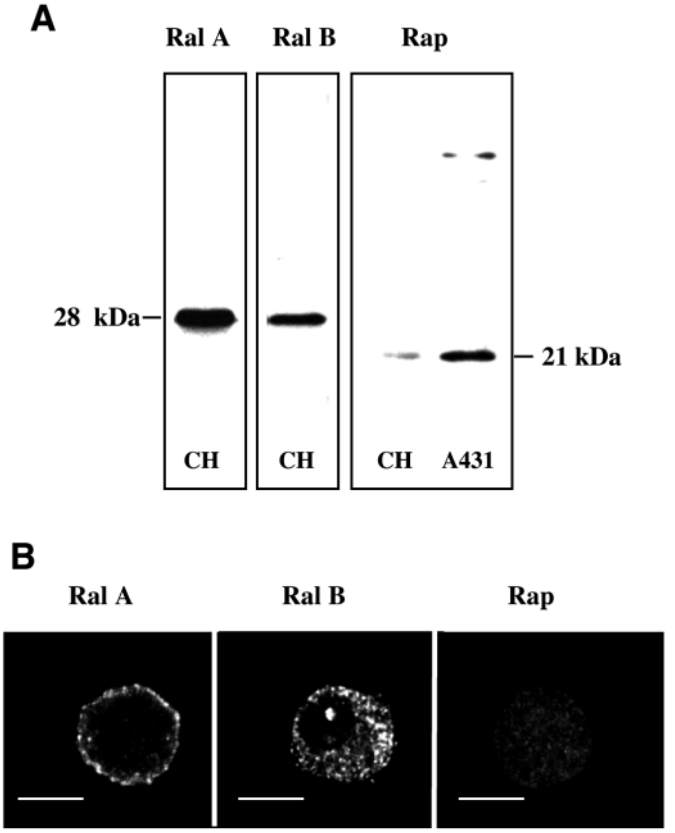

Fig. 8. Immunodetection and immunocytochemical localization of Ral and Rap in cultured chromaffin cells. (A) Proteins (10 μg) from chromaffin cell (CH) or human epidermoid carcinoma cell (A431) lysates were subjected to gel electrophoresis and immunodetection on nitrocellulose sheets using anti-RalA, anti-RalB and anti-Rap antibodies. Blots were developed with secondary antibodies coupled to horseradish peroxidase and immunoreactive bands were revealed with the ECL system. (B) Confocal immunofluorescent images obtained by labeling chromaffin cells with either anti-RalA, anti-RalB or anti-Rap antibodies vizualized with Cy3-conjugated secondary antibodies. Sections were taken with the minimum pinhole size in the plane of the nuclei using excitation and emission filtering. Scale bars, 10 μm.

DISCUSSION

Exocytotic fusion of secretory granules with the plasma membrane requires calcium and ATP. Using permeabilized cell models, it has been possible to establish that exocytosis consists of sequential ATP-dependent and -independent phases of release (Vitale et al., 1994; Bittner and Holz, 1992; Hay and Martin, 1992). The ATP-independent step has been attributed to the fusion of docked granules (Neher and Zucker, 1993; Parsons et al., 1995), whereas the ATP-dependent component has been assumed to include the so-called priming reactions allowing the recruitment and docking of secretory granules with the plasma membrane. Investigating the functions of trimeric G proteins in chromaffin cell exocytosis, we previously described that the activation of a secretory-granule-associated Go protein blocks the ATP-dependent step of secretion by stabilizing the cortical actin network (Vitale et al., 1993; Gasman et al., 1997). This observation suggested that the priming of exocytosis is related at least in part to cytoskeletal rearrangements in the subplasmalemmal region. Indeed it is now widely accepted that peripheral actin filaments require a specific reorganization to enable the movement and docking of secretory granules with the plasma membrane (Sontag et al., 1988; Burgoyne et al., 1989; Koffer et al., 1990; Zhang et al., 1995; Vitale et al., 1995). We attempted to identify the putative effector(s) by which the granule-bound Go inhibits the priming of exocytosis and found that the control exerted by Go on the actin cytoskeleton is related to the activation of a downstream RhoA protein associated with the membrane of secretory granules (Gasman et al., 1998).

The aim of the present study was to probe the actual role of members of the Rho family in the exocytotic pathway in chromaffin cells by use of clostridial toxins that selectively modify specific GTP-binding proteins. We found that *C. difficile* toxin B, which specifically catalyzes the glucosylation of Rho, Rac and Cdc42, strongly reduced nicotine- and high potassium-evoked secretion from intact chromaffin cells and inhibited calcium-triggered release from permeabilized cells. Moreover, toxin B markedly altered the distribution of actin filaments visualized with rhodamine-conjugated phalloidin, suggesting an important modification of the subplasmalemmal cytoskeletal architecture. We found a close correlation between the toxin B-induced inhibition of exocytosis and its action on cortical actin filaments, since treatment with toxin B had no effect in cells with their actin filaments depolymerized by previous incubation with cytochalasin E or C2 toxin. These results suggest that toxin B-mediated inactivation of any of the three GTPases (Rho, Rac or Cdc42) interferes with the actin reorganization required for exocytosis. Neither *C. botulinum* C3, which selectively ADP-ribosylates Rho, nor *C. sordellii* lethal toxin, which glucosylates Rac, were able to modify the rhodamine-phalloidin staining in chromaffin cells, suggesting that inactivation of Rho or Rac was not sufficient to disorganize the subplasmalemmal actin network. In view of the subcellular localization of Cdc42 in the subplasmalemmal region, we propose that Cdc42 plays a key regulatory role in the actin rearrangements related to exocytosis.

Electron microscopic analysis of chromaffin cells has shown that granules are trapped by actin filaments near the plasma membrane. A close correlation between the disassembly of the actin cytoskeleton, the number of docked granules and the initial rate in secretion has been described (Vitale et al., 1995). Actin filament disassembly alone is not sufficient to trigger exocytosis (Lelkes et al., 1986; Sontag et al., 1988; Burgoyne et al., 1989), neither are rises in cytosolic calcium if the peripheral actin barrrier has not been previously removed (Zhang et al., 1995). On the other hand, addition of agents that completely depolymerize actin filaments inhibit agonist-induced exocytosis in HIT insulin-secreting cells (Li et al., 1994). These findings suggest a dual role for actin filaments: one inhibiting secretory granule movement by a trapping mechanism and a second that positively stimulates exocytosis. Indeed, in mast cells, activation of secretion triggers not only the disassembly of cortical actin, but also the appearance of actin filaments that seem to provide a structural support for degranulation (Norman et al., 1994). To what extent secretion in chromaffin cells requires de novo actin polymerization and the possible regulatory mechanisms underlying this event remain to be investigated. It is tempting to speculate that active Cdc42 supports exocytosis by inducing specific actin-based structures. Accordingly, the active participation of Cdc42 in the exocytotic reaction in pancreatic β cells (Kowluru et al., 1997) and mast cells (Brown et al., 1998) has been recently described.

We previously proposed that Go-stimulated RhoA stabilizes actin filaments at the secretory granule membrane and thereby exerts an inhibitory clamp on exocytosis in resting cells. This model implied that the exocytotic reaction requires a transient inactivation of Go/RhoA, excluding the active participation of RhoA in the molecular events underlying docking and fusion. Indeed, in agreement with our previous observations (Gasman et al., 1997), we found here that inactivation of RhoA by *C. botulinum* does not modify secretagogue-evoked secretion in chromaffin cells even under conditions that caused large ADP-ribosylation of Rho. Furthermore, despite the evidence pointing to a key regulatory function for Rho-related GTPases in secretion in mast cells (Brown et al., 1998), basophilic leukemia cells (Prepens et al., 1996) and pancreatic β cells (Kowluru et al., 1997), the active role of Rho itself in the exocytotic machinery has been excluded in these secretory cell models. Rho may be needed in resting cells to connect secretory granules with the actin cytoskeleton and restrict granule movement, thus preventing any uncontrolled secretory responses. Alternatively, a rapid reassembly of cortical actin filaments during the terminal phase of exocytosis and/or the early step(s) of endocytosis may be required. In view of its granular location, RhoA represents an attractive candidate capable of stimulating the actin filament repolymerization specifically at the site where the granule has fused with the plasma membrane.

Treatment of chromaffin cells with *C. sordellii* lethal toxin strongly reduced secretory responses without affecting the apparent distribution of cortical actin filaments. In addition, preincubation of cells with cytochalasin E or C2 toxin did not modify lethal toxin-induced inhibition of secretion, indicating that lethal toxin is able to block the exocytotic process independently of effects on actin filaments. Lethal toxin is known to glucosylate and inactivate Rac and two other related members of the Ras GTPase superfamily, Rap and Ral (Popoff et al., 1996; Hofmann et al., 1996). Rap has been identified in the membrane of secretory granules in neutrophils (Maridonneau-Parini and de Gunzburg, 1992) and parotid acinar cells (D'Silva et al., 1997; 1998) and a potential role for Rap in the regulation amylase secretion has been recently proposed (D'Silva et al., 1998). However, attempts to detect Rap in chromaffin cells by immunoreplica analysis and confocal immunofluorescence failed. Alternatively, RalA appears to be associated constitutively with a phospholipase D (Jiang et al., 1995; Luo et al., 1997). Although RalA binding itself does not activate phospholipase D, it does enhance phospholipase D activation by the ARF GTPase (Kim et al., 1998). We recently described that an ARF6-dependent phospholipase D located at the plasma membrane is required for the exocytotic reaction in chromaffin cells (Caumont et al., 1998). In view of the presence of RalA in the subplasmalemmal region, it is tempting to consider RalA as a candidate involved in the cooperative regulation of phospholipase D in the course of exocytosis in chromaffin cells.

To conclude, our results indicate that the actin cytoskeletal organization underlying membrane trafficking at the site of exocytosis is under the combined control of two members of the Rho family. In resting chromaffin cells, the granule-bound RhoA maintains actin filaments in the vicinity of the secretory granule, most likely by modulating the activity of a granule-associated phosphatidylinositol 4-kinase. In stimulated cells, the subplasmalemmal Cdc42 plays an active role in the actin rearrangements preceeding catecholamine secretion. A future challenge will be to dissect the effector pathway integrating Cdc42 to the actin architecture required for the exocytotic reaction in chromaffin cells.

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