Effects of Large Clostridial Cytotoxins on Activation of RBL 2H3-hm1 Mast Cells Indicate Common and Different Roles of Rac in FceRI and M1-Receptor Signaling

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ABSTRACT

Using Rho GTPases-inhibiting clostridial cytotoxins, we showed recently in RBL cells that the GTPase Rac is involved in FceRI (high-affinity receptor for IgE) signaling and receptor-mediated calcium mobilization, including influx via calcium release-activated calcium channels. Here, we studied the role of Rho GTPases in muscarinic M1 receptor signaling in RBL 2H3-hm1 cells. Clostridium difficile toxin B, which inactivates Rho, Rac, and Cdc42, and Clostridium sordelli lethal toxin, which inhibits Rac but not Rho, blocked M1-mediated exocytosis, indicating that Rac but not Rho is involved in the regulation of receptor-mediated exocytosis. Although antigen-induced FceRI stimulation caused tyrosine phosphorylation of the Rac guanine nucleotide exchange factor Vav, M1 stimulation by carbachol activated Rac independently of Vav. The Rac-inactivating toxins blocked M1 receptor-induced membrane translocation of the pleckstrin homology domain of protein kinase B, which is a phosphoinositide 3-kinase effector. The M1-induced calcium release from internal stores was not affected by toxin B; however, the subsequent calcium influx from the extracellular space was inhibited. The data suggest that besides capacitative calcium entry, the M1 signaling pathway activates further calcium entry channels with mechanisms that are not affected by the inhibition of Rac.

The low molecular mass GTPases of the Rho family (e.g., Rho, Rac, and Cdc42) are molecular switches in many cellular signaling cascades (Van Aelst and D'Souza-Schorey, 1997; Bishop and Hall, 2000). They are critically involved in the regulations of the actin cytoskeleton by extracellular signals (Kaibuchi et al., 1999) but function as switches also in various other signal processes (Nobes and Hall, 1995; Kjoller and Hall, 1999; Bokoch, 2000). Clostridial cytotoxins are established as pharmacological tools to study the function and the role of Rho GTPase proteins in signal transduction pathways. C3 transferases selectively ADP-ribosylate RhoA, RhoB, and RhoC, thereby inhibiting their biological functions (Aktories et al., 1989; Chardin et al., 1989; Paterson et al., 1990). The family of large clostridial cytotoxins inactivates small GTPases by glucosylation (Aktories and Just, 1995). Whereas Clostridium difficile toxins A and B inactivate all Rho GTPases, including Rho, Rac, and Cdc42 (Just et al., 1995), the lethal toxin from Clostridium sordelli inactivates Rac, possibly Cdc42, but not Rho (Just et al., 1996). In addition, Ras subfamily proteins (e.g., Ras, Rap, and Rap) are targets of the lethal toxin.

Using the clostridial toxins as pharmacological tools, it was shown that exocytosis of RBL 2H3-hm1 cells induced by stimulation of the high-affinity antigen receptor FceRI involves Rho GTPases (Prepens et al., 1996). In FceRI signaling in RBL cells, Rac but not RhoA seems to be involved on various levels of the signal pathway, ultimately resulting in exocytosis. Rac is essential for FceRI-mediated calcium mobilization (Djouder et al., 2000). Rac participates in phospholipase Cγ activation (Hong-Geller et al., 2001) and is involved in the phosphoinositide 3-kinase (PI3-kinase)-dependent regulation of protein kinase B (PKB/AKT/RAC) (Djouder et al., 2001). Activation of Rac by stimulation of the FceRI receptor is suggested to involve the tyrosine phosphorylation of the guanine nucleotide exchange factor Vav, a guanine nucleotide exchange factor (GEF) for Rac (Song et al., 1996; Bustelo, 2000). Moreover, Rac (or other Rho GTPases) seems to be involved in late secretory effects in RBL or mast cells induced after cell permeabilization by addition of Ca²⁺ and stable.
guanosine triphosphate derivatives (e.g., GTP[S]) (Brown et al., 1998).

RBL 2H3-hm1 cells were transfected with the muscarinic M1 receptor (Jones et al., 1991). The M1 receptor is a heptahelical receptor coupled to heterotrimeric G proteins at least including the Gq11 type (Dippel et al., 1996). Compared with the FcεRI signaling, much less is known about the signaling pathways leading to exocytosis after stimulation of the M1 receptor in RBL 2H3-hm1 cells. Therefore, we studied signaling events downstream of the M1 receptor. Using cloridri-
tial toxin as pharmacological tools to inactivate Rho GTPases, we report that activation of M1 receptor in RBL mast cells shares many similarities with FcεRI signaling, e.g., Rac but not Rho regulates M1 signaling. Similar to that reported for FcεRI signaling, PI3-kinase participates in the M1 signaling processes in a Rac-dependent manner. However, M1 receptor signaling largely differs in respect to Rac activation and calcium mobilization.

**Materials and Methods**

**Materials.** *C. difficile* toxin B (Just et al., 1995), *C. sordellii* lethal toxin (Just et al., 1996), and the C3 fusion toxin (C2IN-C3) (Barth et al., 1998) were described as prepared previously. Fura-2 acetoxy-

ethyl ester was obtained from Molecular Probes (Göttingen, Germany). Carbachol, wortmannin, LY294002, and genistein were obtained from Sigma Chemie (Deisenhofen, Germany). The glutathione S-transferase (GST)-p21-activated kinase (PAK) fusion proteins were a gift of Dr. John Collard (The Netherlands Cancer Institute, Amsterdam, Netherlands). The Rac antibody was from Transduction Labor-

atories (Hamburg, Germany), the Vav antibody was from United Biomedical, Inc. (Germany), and the p101 and p110 antibodies were kindly provided by Dr. B. Nürnberg (Institut für Physiologische Chemie II, Düsseldorf, Germany). The PH-PKB-GFP construct was kindly donated by Dr. T. Balla (National Institutes of Health, Bethesda, MD). All others reagents were of analytical grade and commercially available.

**Cell Culture.** Rat basophilic leukemia cells transfected with the human muscarinic receptor (Jones et al., 1991) (RBL 2H3-hm1) were a gift from Dr. G. Schultz (Berlin, Germany). Cells were grown in Eagle’s minimum essential medium with Earle’s salts supplemented with 15% (v/v) heat-inactivated fetal calf serum, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO₂ at 37°C. RBL 2H3-hm1 cells were detached from culture plates with SK buffer (125 mM NaCl, 1.5 mM EDTA, 5.6 mM glucose, and 10 mM HEPES, pH 7.2). After toxin treatment, cells were washed three times with 1 mM carbachol acetoxymethylester as the FcεRI receptor agonist.

**Hexosaminidase Release Assay.** Cells were seeded in 96-wells culture plates and incubated without or with toxins for the indicated times and concentrations. Hexosaminidase release was determined as described previously (Djouder et al., 2000). Briefly, the medium was removed and cells were washed twice with Tyrode’s buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl₂, 1 mM MgCl₂, 5.6 mM glucose, 10 mM HEPES, and 0.1% bovine serum albumin, pH 7.4). Incubation at 37°C with stimuli at the indicated concentrations followed for 1 h. Thereafter, aliquots (30 μl) of cells were incubated with 50 μl of 1.3 mg/ml p-nitrophenyl-N-acetyl-β-D-glucosamide in 0.1 M sodium citrate buffer (pH 4.5) at 37°C for 1 h. At the end of the incubation, 50 μl of 0.4 M glycine (pH 10.7) stop buffer was added. The total amount of hexosaminidase release was determined using 2% Triton X-100 in tyrode buffer. Absorbance was measured at 410 nm, referring to 630 nm. The values were expressed as percentage of total amount of hexosaminidase.

**Measurements of [Ca²⁺]i in Cell Suspensions.** RBL 2H3-hm1 cells were detached from culture plates with SK buffer (125 mM NaCl, 1.5 mM EDTA, 5.6 mM glucose, and 10 mM HEPES, pH 7.2). After centrifugation, cells were resuspended in serum-free minimal essential medium and loaded with fura-2 acetoxy-

ethyl ester (2.5 μM) for 45 min at 37°C. After loading, cells were washed three times with HEPES-buffered salt solution (130 mM NaCl, 5.4 mM KCl, 0.9 mM NaH₂PO₄, 0.8 mM MgSO₄, 1.8 mM CaCl₂, 10 mM glucose, and 20 mM HEPES, pH 7.4), and cell density was adjusted to 1 × 10⁶ cells/ml. Experiments were carried out at room temperature in HEPES-buffered salt solution using a PerkinElmer LS 50B spec-
trofluorometer. The fluorescence of cells suspension was examined at an excitation wavelength of 510 nm and excitation wavelengths of 340 and 380 nm, respectively. Results are presented as changes in fluo-

rescence ratio 340/380 over time.

**Measurements of [Ca²⁺]i in Attached Cells.** RBL cells were seeded on coverslips, and the intracellular Ca²⁺ was measured at room temperature 2 days later using a cell-imaging system (Till Photonics, Planegg, Germany). On the day of experiments, the control cells were incubated in medium containing fura-2 acetoxy-

ethyl ester (5 μM) for 1 h at room temperature. For paired experiments, an additional group of cells was exposed to toxin B (50 ng/ml) for 1 h for 2 to 2 h and subsequently loaded with fura-2 acetoxy-

ethyl ester as the control cells. Just before recording images, the culture medium was replaced by a bath solution with a Ca²⁺ concentration of 1.8 mM [115 mM NaCl, 1.8 mM CaCl₂, 2 mM MgCl₂, 5 mM KCl, and 10 mM HEPES, pH 7.2 (NaOH)]. Images of 10 to 35 cells/cover slips were obtained every 3 s at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm, respectively. The fluorescence ratios were calibrated in vivo as described previously (Philipp et al., 1998). Calcium mobilization was induced by bath application of 50 ng/ml TNP-OVA or 1 mM carbachol. In some experiments, TNP-OVA and carbachol were applied when the cells were bathed in a solution with Ca²⁺ concentration <10 nM [Ca₂⁺]. In other experiments, the buffer calcium was increased to 1 mM (1 mM Ca₂⁺) during the fluorescence measure-

ments. Experiments were paired by alternating Ca²⁺ measurements in control and toxin-treated coverslips. The data obtained in 4 to 10 paired experiments were pooled for statistical analysis and is given as mean ± S.E.M.

**Expression of GST-PAK-Crib Domain.** Expression of the GST-fusion proteins in BL21 cells growing at 37°C was induced by adding 0.1 mM isopropyl-β-D-thiogalactoside (final concentration) to OD₆₀₀
1.0. Two hours after induction, cells were collected and lysed by sonication in lysis buffer (50 mM Tris-HCl pH 8.0, 2 mM MgCl₂, 2.0 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 10,000 g and the supernatant was used for GST-PAK-Crib domain by affinity purification with glutathione-Sepharose (Pharmacia AB, Uppsala, Sweden). Beads loaded with GST-fusion proteins were washed twice with PBS and were used immediately for GTPase pull down experiments.

**GST-PAK-Crib Domain Pull-Down Experiments.** RBL cells were incubated with toxin B (40 ng/ml; 2 h), wortmannin (100 nM; 20 min), LY294002 (10 μM; 30 min), and genistein (100 μM; 30 min) at 37°C. Thereafter, the cells were washed twice with PBS and stimulated during 1 min at 37°C by carbachol (1 mM). After addition of 250 μl of ice-cold GST-Fish lysis buffer [10% glycerol, 50 mM Tris pH 7.4, 100 mM NaCl, 1% (v/v) Nonidet P-40, and 25 μg/ml aprotonin, pH 7.4], cells were scraped off the dishes, the detergent-soluble supernatant was recovered after centrifugation at 14,000 g for 15 min at 4°C, and the detergent-soluble supernatant was used for purification of GST-PAK-Crib domain by affinity purification using antibody against Rac (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

**Immunoprecipitation of Vav.** Cells were lysed in lysis buffer containing 50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, 1 μM microcystin-LR (BIOMOL Research Laboratories, Plymouth Meeting, PA), 1 mM sodium orthovanadate, 1 mM benzamidine, and 0.5 mM phenylmethylsulfonyl fluoride. Extracts were centrifuged at 14,000 g for 15 min at 4°C before immunoprecipitation. The detergent-soluble supernatant was gently rocked overnight at 4°C with 2 μg of anti-Vav (UBI). The immunocomplexes were isolated by using 50 μl of protein A/G Agarose (Santa Cruz Biotechnology, Inc.), and the reaction was rocked at 4°C for 2 h. The beads were collected by centrifugation at 14,000 g (5 min; 4°C), washed twice with ice-cold PBS, mixed and boiled with Laemmli buffer, and subjected to SDS-polyacrylamide gel electrophoresis (7%), followed by transfer of proteins onto nitrocellulose membranes and Western blotting using a phosphotyrosine antibody (UBI) (1:3000). Detection occurred by a chemiluminescence Western blotting system (Amersham Biosciences Inc., Braunschweig, Germany).

**Rac Regulates Hexosaminidase Release in Muscarinic M1 and FcεRI Signaling.** Recently, we reported on the involvement of small GTPases in FcεRI-mediated exocytosis of RBL 2H3-hm1 cells (Prepens et al., 1996; Djouder et al., 2000). Here, we studied the role of low molecular mass GTPases of the Rho family in muscarinic M1 receptor-induced exocytosis of RBL cells. Stimulation of hexosaminidase release in RBL 2H3-hm1 cells at increasing concentration of carbachol was inhibited by toxin B and lethal toxin but not by the chimeric toxin C2IN-C3 (Fig. 1, A and B). The maximum hexosaminidase release was observed at 1 mM carbachol. For maximal inhibition of exocytosis, we used toxin B and lethal toxin at 40 ng/ml present during 2 h of pretreatment (Fig. 1, C and D). These data indicate that similar as known for FcεRI signaling, Rac but not Rho regulates the exocytosis of RBL cells after stimulation by carbachol.

**The Guanine Nucleotide Exchange Factor Vav Is Not Involved in M1-Signaling.** Next, we addressed the question whether the Rac-GEF (guanine nucleotide exchange factor) Vav, which is activated by tyrosine phosphorylation through FcεRI receptors (Schuebel et al., 1998; Bustelo, 2000; López-Lago et al., 2000; Manetz et al., 2001), is activated by stimulation of the M1 receptor. As shown in Fig. 2, whereas TNP-OVA (50 ng/ml) caused tyrosine phosphorylation of Vav, no increase in tyrosine phosphorylation was observed after stimulation of the M1 receptor by carbachol (1 mM). As expected, toxin B had no effect on Vav activation. These data suggest that muscarinic M1 receptor signaling stimulates Rac by activating a guanine nucleotide exchange factor different from Vav.

**Toxin B and Lethal Toxin Inhibit PKB Translocation in Muscarinic M1 and FcεRI Signaling.** PK3-kinase is involved in RBL cell activation by FcεRI (Barker et al., 1995; Hirasaawa et al., 1997). We showed recently that Rac controls FcεRI-mediated activation of PKB, an effector of PK3-kinase

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**Fig. 1.** Effects of *C. sordellii* lethal toxin (40 ng/ml; 2 h), *C. difficile* toxin B (40 ng/ml; 2 h), and C3 fusion toxin (200 ng/ml C2IN-C3 and 100 ng/ml C2II; 4 h) on hexosaminidase release in RBL cells. RBL cells were treated with increasing concentrations of carbachol. B, RBL cells were treated with increasing concentrations of carbachol. C, RBL cells were treated with increasing concentrations of carbachol. D, RBL cells were treated with 40 ng/ml toxin B (a) and lethal toxin (b) for 2 h and then stimulated with 1 mM carbachol. D, RBL cells were treated with 40 ng/ml lethal toxin (b) and then stimulated with 1 mM carbachol. The release of hexosaminidase was determined as described. Data are given as means ± S.E.M., n = 4.
As an indication for PKB activation, the membrane translocation of PH-PKB-GFP can be used (Servant et al., 2000). Therefore, we tested the effects of the Rho-inactivating toxins on the M1 receptor-mediated translocation of the pleckstrin homology domain of PKB. Stimulation of the M1 receptor by carbachol (1 mM) caused a translocation of PH-PKB to the membrane. Toxin B, lethal toxin and the PI3-kinase inhibitor wortmannin (100 nM) completely inhibited the M1 receptor-mediated translocation of PH-PKB. In contrast, the RhoA-inactivating C3 chimeric toxin (C2IN-C3) had no effect on the PH-PKB membrane translocation (Fig. 3). These data indicate that Rac is involved in M1-mediated activation of the PI3-kinase effector PKB. PKB-kinase has been reported to activate Rac in various cell types (Reif et al., 1996; Vanhaesebroeck et al., 1997; Akasaki et al., 1999; Leevers et al., 1999). Therefore, we studied the effects of the PI3-kinase inhibitors wortmannin and LY294002 on the activation of Rac by a precipitation assay. This assay is based on the precipitation of the active GTP-bound Rac by the Rac-binding domain of the PAKs (Akasaki et al., 1999). Using this method, we observed that toxin B (40 ng/ml) and genistein (100 μM), a tyrosine protein kinase inhibitor, inhibited the activation of Rac induced by TNP-ovalbumin and carbachol, respectively (Fig. 4). By contrast, wortmannin (100 nM) and LY294002 (10 μM) did not block Rac activation. These findings suggest that activation of Rac is not caused by PI3-kinase but depends on tyrosine kinase activity.

Toxin B and Lethal Toxin Do Not Inhibit Calcium Response in M1 Signaling. Calcium mobilization is a prerequisite for secretion, and it has been shown recently that the calcium response is regulated by Rac and PI3-kinase in FceRI signaling (Djouder et al., 2000; Hong-Geller and Corione, 2000; Hong-Geller et al., 2001). Therefore, we addressed the question whether the Rho GTPases affect the M1 receptor-mediated calcium mobilization in RBL 2H3-hm1 cells in a similar manner. In suspended RBL cells, we tested whether the mobilization of Ca^{2+} is modified by clostridial cytotoxins. Stimulation of the M1 receptor with 1 mM carbachol caused a calcium mobilization characterized by a rapid increase in the intracellular calcium concentration followed by a plateau (Fig. 5). This type of calcium signal is believed to reflect calcium release from internal stores and calcium influx (Fig. 8). Treatment of RBL cells with toxin B, lethal toxin, and the C2IN-C3 chimeric toxin did not affect the mobilization of Ca^{2+} after stimulation with 1 mM carbachol (Fig. 5, A and B). Even stimulation of calcium mobilization by carbachol at low concentrations was not affected by the toxins (Fig. 6). In contrast to FceRI signaling (Djouder et al., 2000), our data indicate that neither Rac nor Rho is involved in regulation of
Ca\textsuperscript{2+} responses to M1 receptor stimulation. Moreover, we wanted to know whether wortmannin affects the calcium mobilization in M1 receptor signaling. Similar as found with the toxins, in suspended cells, we were not able to detect any inhibition by wortmannin in carbachol-stimulated calcium mobilization (Fig. 5C) but wortmannin inhibited Fc\textsubscript{\alpha}RI-induced calcium response in RBL mast cells (Djouder et al., 2001), and the same was shown for the PI3-kinase inhibitor LY294002 (Ching et al., 2001). The striking difference between the sensitivity of the Fc\text{RI} and M1 signaling pathway (Figs. 5 and 6) to inhibitors of Rac and Rho prompted us to analyze the effects of toxin B in single RBL cells (Fig. 7). As reported previously (Djouder et al., 2001), the majority of cells responds to stimulation with TNP-OVA with an increase of the intracellular calcium concentration seen mostly as an abrupt spike followed by a calcium plateau (Fig. 7A, left). The difference in the response of individual cells is basically reflected in the delay that may correspond to the cross-linking of Fc\text{RI}.
the individual cells build up an average signal with slow onset and sustained plateau (Fig. 7A, left). In contrast, the calcium signals induced by carbachol in individual cells show no difference in delay and, correspondingly, the average calcium signal is characterized by a fast onset (Fig. 7B, left). The average signals shown in Fig. 7, A and B (left), nicely compared with calcium signals recorded with cell suspensions after application of TNP-OVA (Prepens et al., 1996; Djouder et al., 2000) and carbachol (Figs. 5 and 6). Toxin B disrupts the calcium signals through the FceRI signaling pathway, and stimulation with TNP-OVA produces oscillations of the intracellular calcium concentration that generate a flat average calcium signal (Fig. 7A, right). Consequently, the treatment with toxin B reduced the plateau calcium concentration by about 79% when calcium signals are induced through the FceRI signaling pathway (Fig. 7A; control: 1.02 ± 0.06 μM (n = 39); toxin B: 0.21 ± 0.02 μM (n = 46), P < 0.01; time: 1 min after TNP-OVA stimulation). In contrast, both individual and average calcium signals induced by carbachol were not strongly modified by the treatment with toxin B (Fig. 7B, right), supporting the results obtained with cell suspensions (Figs. 5 and 6). During the plateau phase (Fig. 7B), the maximal calcium concentrations in control and toxin B-treated cells were 1.49 ± 0.12 μM (n = 49) and 1.35 ± 0.13 μM (n = 40), respectively. The slight difference in the plateau calcium concentration was not statistically significant. To estimate the duration of the plateau phase in individual cells, we measured the time elapsed between the beginning of stimulation with carbachol and the time point, at which the calcium concentration reached a value corresponding to 64% of the plateau calcium concentration. On average, the plateau phase lasted 84.20 ± 2.83 s (n = 42) in control cells and 71.00 ± 3.27 s (n = 30) in cells treated with toxin B. This difference in the plateau duration was statistically significant (P < 0.05) and indicated that toxin B shortened the carbachol induced calcium signals by about 13s. Nevertheless, the toxin B effect on calcium signals through the M1 signaling pathway (Fig. 7B) was not as strong as it was on calcium signals through the FceRI signaling pathway (Fig. 7A). These results are surprising because the calcium entry from the extracellular space, which is required for the maintenance of calcium signals, is strongly reduced by the toxin treatment (Djouder et al., 2000). The inhibition of Rac/Cdc42 by toxin B responsible for the reduction in calcium entry in the FceRI signaling pathway is apparently downstream from the calcium release induced by IP3, because the activation of the calcium release-activated calcium (CRAC) channels that support the calcium entry was blocked by toxin B even after intracellular dialysis of IP3 (Djouder et al., 2000). Because it is likely that the M1 and FceRI signaling pathways share calcium release mechanisms that subsequently activate capacitative calcium entry, we analyzed the effects of toxin B on calcium release and calcium entry induced by carbachol (Fig. 8). To dissect the calcium release component of calcium signals, the cells were first bathed and stimulated with carbachol in a calcium-free solution. Under these conditions, carbachol produced calcium peaks that correspond to calcium release (Fig. 8A) and were apparently not affected by toxin B (Fig. 8B). The calcium entry component was observed when calcium was introduced into the bath after carbachol stimulation and, in contrast to calcium release, we observed weak calcium entry in cells treated with toxin B (Fig. 8, A and B).

**Discussion**

As shown by several laboratories, stimulation of the transfected muscarinic M1 receptor in RBL 2H3-hm1 cells mediates several signaling events, which are known to be induced by stimulation of FceRI receptors, including exocytosis (Jones et al., 1991), PI3-kinase activation (Barker et al., 1995; Hirasewa et al., 1997; Barker et al., 1999), calcium mobilization (Choi et al., 1993), and mitogen-activated protein-kinase activation (Offermanns et al., 1994). Using toxins that selectively inactivate specific Rho GTPases, we have shown in RBL cells (Prepens et al., 1996) that both M1 receptor signaling and FceRI-mediated signaling involve Rac but not Rho to induce exocytosis. Therefore, we addressed in the present study the question whether M1 and FceRI receptors share similar pathways and same signal molecules, leading to release of inflammatory mediators.

First, we observed that M1 receptor-mediated activation of Rac does not involve the guanine nucleotide exchange factor Vav, which participates in FceRI-induced activation of Rac (Manetz et al., 2001). Vav is reportedly activated by tyrosine phosphorylation (Schuebel et al., 1998; Bustelo, 2000; López-Lago et al., 2000; Manetz et al., 2001). In our studies, M1 stimulation did not increase tyrosine phosphorylation of Vav. Therefore, in RBL cells activation of Rac mediated by M1 receptors does not involve Vav but a different guanine nucleotide exchange factor.

Next, we studied the role of PI3-kinase, which is known to activate Rac in some cell types (Reif et al., 1996; Vanhaese-
broeck et al., 1997; Akasaki et al., 1999; Leevers et al., 1999). M1 receptors are coupled to Gq proteins in RBL 2H3-hm1 cells (Dippel et al., 1996). In general, the heterotrimeric G proteins stimulate the type IB PI-3 kinase, which is made up of a p110γ catalytic subunit and a p101 regulatory subunit (Stephens et al., 1994), which are also highly expressed in RBL 2H3-hm1 cells (unpublished observation). G protein βγ subunits directly activate p101/p110γ PI-3 kinase (Stephens et al., 1994; Stoyanov et al., 1995; Leopold et al., 1998). PKB is a well known effector of PI3-kinase and is translocated to the membrane and activated by phosphorylation at residues Thr308 and Ser473 (Vanhasebroeck et al., 1997; Leevers et al., 1999). Although the activation mechanism of PKB is not entirely clear, it is generally accepted to depend on lipids formed by PI3-kinase and on the Ser/Thr kinase PKD1 (3’-phosphoinositide-dependent kinase-I). Both PKB and 3’-phosphoinositide-dependent kinase-I possess pleckstrin homology domains that bind to 3’-phosphoinositides and are responsible for membrane association. In line with the role of 3’-phosphoinositides in PKB activation, inhibition of PI3-kinase by wortmannin prevented M1 receptor mediated PH-PKB-GFP membrane translocation in RBL cells. Also, toxin B and lethal toxin completely blocked the M1-induced membrane translocation of PH-PKB-GFP, indicating an essential role of Rac and PI3-kinase in the activation of PKB in M1 receptor signaling. Our finding that C3 fusion toxin, which inactivates Rho, was without effects suggests that Rho is not essential for muscarinic-mediated activation of PKB in RBL cells. Using a pull-down assay with the Rac-binding domain of PAK, we observed that M1 receptor stimulation activates Rac similarly as known for FceRI signaling. Activation of Rac was not blocked by PI3-kinase inhibitors such as wortmannin and LY294002. In contrast, toxin B and the tyrosine kinase inhibitor genistein inhibited the activation of Rac. Taken together, these data show that PI3-kinase is not located upstream of Rac in M1 receptor signaling and not involved in activation of Rac.

Recently, we have shown that toxin B and lethal toxin inhibit the calcium mobilization induced by FceRI signaling (Djouder et al., 2000). In contrast, the toxins had no effects on calcium mobilization by stimulation of the M1 receptor in RBL 2H3-hm1 cells. Thus, Rac regulates the exocytosis but not the calcium response through the muscarinic receptor. The regulation of the calcium mobilization in FceRI signaling is not completely understood. It is known that stimulation of the FceRI receptor causes tyrosine phosphorylation and activation of phospholipase Cγ, which finally results in formation of IP3 and calcium release from internal calcium stores. In addition, PI3-kinase is apparently involved in phospholipase Cγ regulation and calcium mobilization (Scharenberg et al., 1998). In line with this notion, wortmannin inhibits calcium mobilization upon FceRI stimulation in RBL cells (Barker et al., 1995, 1999; Djouder et al., 2001). Notably, it was suggested that wortmannin has no effects on CRAC currents (Ching et al., 2001; Djouder et al., 2001). In contrast, it was proposed that PI3-kinase regulates a noncapacitative calcium entry pathway (Ching et al., 2001). What is the role of the Rho GTPase Rac and/or Cdc42 in calcium mobilization via FceRI signaling? Using transfection of dominant active and inactive GTPases, Hong-Geller and Cerione (2000) suggested a direct role of Rac/Cdc42 in regulation of phospholipase Cγ. Recently, the same group reported that activated Rac/Cdc42 reconstitutes FceRI-mediated Ca2+ mobilization in a mutant RBL mast cell line (B6A4C1) that is defective in antigen-stimulated phospholipase Cγ activation (Hong-Geller et al., 2001). Our previous results are in full agreement with the essential role of Rac/Cdc42 in calcium mobilization induced by antigen (Djouder et al., 2000). We also observed inhibitory effects of Rac/Cdc42-inactivating clodristolid cystotoxins on calcium release-activated calcium currents (ICRAC) downstream of IP3 production, suggesting an additional role of Rac/Cdc42 in calcium mobilization (Djouder et al., 2000). In the present study, we observed that toxin B inhibits the capacitative calcium entry under activation of the M1 signaling pathway. This observation is in line with the inhibitory effects of Rac/Cdc42-inactivating clodristolid cystotoxins on ICRAC. The muscarinic M1 receptor couples to Gq/11 and activates β-subtypes of phospholipase C, which produces IP3 and causes calcium mobilization. The Rho GTPase-inactivating toxins did not affect the calcium mobilization induced by activation of the M1 pathway. Because the calcium mobilization is composed of calcium release from intracellular stores and rapid calcium entry into the cytosol from the extracellular space, the important question is how calcium signals induced through the M1 pathway are maintained under blockade of capacitative calcium entry. It seems that the regulation of IP3 production differs in M1 and FceRI signaling. For example, it is well known that the IP3 production via FceRI in RBL cells is very low compared with that induced by M1 receptors stimulation. Therefore, factors such as sphingosine-1-phosphate have been proposed as additional second messengers to be involved in calcium mobilization by FceRI but not by M1 receptor stimulation (Choi et al., 1996). The different sensitivity of calcium mobilization, calcium release, and calcium entry to inhibition by toxin B reveals that different mechanisms of calcium entry are activated by the M1 and FceRI signaling pathway. It is likely that, besides calcium entry currents such as ICRAC, the M1 signaling pathway activates further calcium entry channels with activation mechanisms that are not affected by the inhibition of Rac/Cdc42.

Taken together, the data reported here by using Rho GTPase-inhibiting toxins as pharmacological tools show novel important differences in the signal transduction of regulated exocytosis via FceRI and M1 receptors and indicate that Rac has different functions in these processes.


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