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

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d) The abbreviations used are: C., Clostridium; C2 toxin, Clostridium botulinum C2 toxin consisting of the enzyme component C2I and the binding component C2II; C2IN-C3, C3 fusion toxin consisting of C3 ADP-ribosyltransferase and the N-terminal part of component I of Clostridium botulinum C2 toxin; [Ca^{2+}]_i, cytoplasmic free calcium; TNP-OVA, trinitrophenyl-conjugated ovalbumin; FceRI, high affinity receptor for IgE; I_{CRAC}, calcium release-activated calcium current; IgE, immunoglobulin E; IP_3, inositol 1,4,5-triphosphate; lethal toxin, Clostridium sordellii lethal toxin; PAGE, polyacrylamide gel electrophoresis; RBL 2H3-hm1, 2H3-hm1 subline of rat basophilic leukemia cells. exoenzyme; PKB, protein kinase B; PH-PKB-GFP, pleckstrin homology domain of PKB tagged with GFP; PI3-kinase, phosphoinositide 3-kinase

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Abstract

Using Rho GTPases-inhibiting clostridial cytotoxins, we showed recently in RBL cells that the GTPase Rac is involved in FcεRI-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 sordellii* 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. While antigen-induced FcεRI 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 (PKB), which is a PI3-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; Bokoch, 2000; Kjoller and Hall, 1999). Clostridial cytotoxins are established as pharmacological tools to study the function and the role of Rho GTPases 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 sordellii* inactivates Rac, possibly Cdc42 but not Rho (Just et al., 1996). In addition Ras subfamily proteins (e.g., Ras, Ral 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 (FcεRI) involves Rho GTPases (Prepens et al., 1996). In FcεRI signaling in RBL cells, Rac but not RhoA appears to be involved on various levels of the signal pathway finally resulting in exocytosis. Rac is essential for FcεRI-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 FcεRI receptor is suggested to involve the tyrosine phosphorylation of the guanine nucleotide exchange factor Vav, a GEF for Rac (Bustelo, 2000; Song et al., 1996). Moreover, Rac (or other Rho GTPases) appears to be involved in late secretory effects in RBL or mast cells.
induced after cell permeabilization by addition of Ca$^{2+}$ and stable guanosine triphosphate derivatives (e.g., GTP[S]) (Brown et al., 1998).

RBL 2H3-hm1 cells are 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 G$_{q/11}$ type (Dippel et al., 1996). As compared to 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 clostridial 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 as 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

Clostridium difficile toxin B (Just et al. 1995), Clostridium sordellii lethal toxin (Just et al., 1996), and the C3 fusion toxin (C2IN-C3) (Barth et al., 1998) were prepared as described. Fura-2 acetoxymethylester was obtained from Molecular Probes (Göttingen, Germany). Carbachol, wortmannin, LY294002 and genistein were obtained from Sigma (Deisenhofen, Germany). The glutathione S-transferase (GST)-PAK fusion proteins were a gift of Dr. John Collard (Amsterdam, Netherlands). The Rac antibody was from Transduction Laboratories (Germany), the Vav antibody was from UBI (Germany) and the p101 and p110γ antibodies were kindly provided by Dr. B. Nürnberg (Düsseldorf, Germany). 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), a gift from Dr. G. Schultz, Berlin / Dr. P. Jones, Burlington, USA) 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); no trypsin was used in order to avoid a partial destruction of membrane receptors.

Treatment with toxins

RBL cells were treated with C. difficile toxin B (40 ng/ml, 2-4 h), C. sordellii lethal toxin (40 ng/ml, 2-4 h), C. botulinum C2 toxin (200 ng/ml C2I and 100 ng/ml C2II, 4 h) or C. limosum C3 fusion toxin (100 ng/ml C2II and 200 ng/ml C2IN-C3, 4 h) for the indicated times and
concentrations. After toxin treatment, cells were washed with the appropriate buffer and used for the assays. To compare the effects of toxins, paired experiments were carried out on control and toxin-treated cells that were grown under identical conditions. Additionally, the experiments were repeated with cells of at least two independent passages.

Transfection with PH-PKB-GFP construct

RBL 2H3-hm1 cells were transfected with 15 µg of the PH-PKB-GFP by electroporation at 240 V and 950 µf and RBL cells were seeded on glass coverslips. PH-PKB-GFP proteins were expressed overnight. The transfected RBL cells were starved for another night in MEM medium in the presence of 5% FCS. Thereafter, transfected RBL cells were incubated without or with wortmannin (100 nM, 20 min), toxin B (40 ng/ml, 2 h), lethal toxin (40 ng/ml, 2 h) and the chimeric C3-fusion toxin (100 ng/ml C2II and 200 ng/ml C2IN-C3, 4 hours). Then, RBL cells were stimulated during 15 min with 1 mM carbachol. For confocal microscopy, transfected RBL cells were fixed with 4% formaldehyde and translocation of the PH-PKB-GFP construct to the membrane after 1 mM carbachol-stimulation was analyzed using a Zeiss Axiovert microscope 135 TV equipped with BioRad MRC 1024 ES.

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 (Djouder et al., 2000). Briefly, the medium was removed, and cells were washed 2 times with tyrode-buffer (130 mM NaCl, 5 mM KCl, 1.4 mM CaCl2, 1 mM MgCl2, 5.6 mM glucose, 10 mM HEPES and 0.1% BSA, 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 percent of total amount of hexosaminidase.

**Measurements of \([\text{Ca}^{2+}]_i\) 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). Following centrifugation, cells were resuspended in serum-free MEM medium and loaded with fura-2 acetoxymethylester (2.5 µM) for 45 min at 37°C. After loading, cells were washed three times with HEPES-buffered salt solution (HBS) (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 1.10^6 cells/ml. Experiments were carried out at room temperature in HBS using a Perkin Elmer LS 50B spectrofluorimeter. The fluorescence of cells suspension was examined at an emission wavelength of 510 nm and excitation wavelengths 340 nm and 380 nm, respectively. Results are presented as changes in fluorescence ratio 340/380 over time.

**Measurements of \([\text{Ca}^{2+}]_i\) in attached cells**

RBL cells were seeded on cover-slips and the intracellular \(\text{Ca}^{2+}\) was measured at room temperature 2 days later using an cell-imaging system (Till Photonics, Planegg, Germany). In the day of experiments, the control cells were incubated in medium containing fura-2 acetoxymethylester (5 µM) for 1 hour at room temperature. For paired experiments, an additional group of cells were exposed to toxin B (50 ng/ml) for 1-2 hour and subsequently loaded with fura-2 acetoxymethylester as the control cells. Just before recording images, the culture medium was replaced by a bath solution with a \(\text{Ca}^{2+}\) concentration of 1.8 mM (115 mM NaCl, 1.8 mM CaCl2, 2 mM MgCl2, 5 mM KCl, 10 mM HEPES, pH 7.2 (NaOH)). Images of 10-35 cells per cover-slip were obtained every 3 s at an emission wavelength of 510 nm and excitation wavelengths of 340 nm and 380 nm, respectively. The fluorescence
ratios were calibrated in vivo as previously described (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 a Ca$^{2+}$ concentration <10 nM (0 Ca$_o$: 115 mM NaCl, 0.5 mM EGTA, 2 mM MgCl$_2$, 5 mM KCl, 10 mM HEPES, pH 7.2 (NaOH)). Subsequently, the Ca$^{2+}$ concentration in this bath solution was increased to 1 mM (1 mM Ca$_o$) during the fluorescence measurements. Experiments were paired by alternating Ca$^{2+}$ measurements in control and toxin-treated cover-slips. The data obtained in 4 -10 paired experiments was pooled for statistical analysis and is given as mean ± SEM.

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 IPTG (final concentration) at OD$_{600}$ 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 MgCl$_2$, 2.0 mM dithiothreitol, 10% glycerol, and 1 mM phenylmethylsulfonyl fluoride). The lysate was centrifuged at 10,000 x g and the supernatant was used for purification of GST-PAK-Crib domain by affinity purification with glutathione-Sepharose (Pharmacia). 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, 2 mM MgCl$_2$, and 25 µg/ml aprotinin, pH 7.4), cells
were scraped off the dishes and the detergent-soluble supernatant was recovered after centrifugation at 14000 g for 15 min at 4°C and GTP-Rac proteins were immunoprecipitated at 4°C for 1 h with 20 µl of GST-PAK fusion protein. The complexes were washed 3 times with ice-cold PBS, resuspended and boiled with Laemmli buffer. Bound Rac proteins were detected by Western blotting using antibody against Rac (Santa Cruz).

*Immunoprecipitation of Vav*

Cells were lysed in lysis buffer, containing 50 mM Tris-HCl, pH 7.4, 1 % NP-40, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 40 mM β-glycerophosphate, 1 µM microcystin-LR (Biomol), 1 mM sodium orthovanadate, 1 mM benzamidine and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Extracts were centrifuged at 14000 g for 15 min at 4°C prior to immunoprecipitation. The detergent-soluble supernatant was gently rocked overnight at 4°C with 2 µg of anti-Vav (UBI). The immunocomplexes were isolated by adding 50 µl of protein A/G Agarose (Santa-Cruz) and the reaction was rocked at 4°C for 2 h. The beads were collected by centrifugation at 14000 g (5 min, 4°C), washed twice with ice-cold PBS, mixed and boiled with Laemmli buffer and subjected to SDS-PAGE (7%), followed by transfer of proteins onto nitrocellulose membranes and Western blotting using a phosphotyrosine antibody (UBI) (1:3000). Detection occurred by a chemiluminescence (ECL) Western blotting system (Amersham, Braunschweig).
Results

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. 1A and 1B). The maximum hexosaminidase release was observed at 1 mM carbachol. For maximal inhibition of exocytosis, we employed toxin B and lethal toxin at 40 ng/ml present during 2 h of pretreatment (Fig.1C and 1D). 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 (Bustelo, 2000; López-Lago et al., 2000; Manetz et al., 2001; Schuebel et al., 1998), 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

PI3-kinase is involved in RBL cell activation by FcεRI (Barker et al., 1995; Hirasawa et al., 1997). We showed recently that Rac controls FcεRI-mediated activation of PKB, an effector of PI3-kinase (Djouder et al., 2001). As an indication for PKB activation the membrane translocation of the pleckstrin homology domain of PKB tagged with GFP (PH-PKB-GFP) can be utilized (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. By 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. PI3-kinase has been reported to activate Rac in various cell types (Vanhaesebroeck et al., 1997; Leevers et al., 1999; Akasaki et al., 1999; Reif et al., 1996). 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 (RBD) of the p21-activated kinases PAK (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 (Hong-Geller and Cerione, 2000; Hong-Geller et al., 2001; Djouder et al., 2000). 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 (see 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. 5A 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 are involved in regulation of Ca\(^{2+}\) 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 FceRI-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 FceRI and M1 signaling pathway (Fig. 5, 6) to inhibitors of Rac and Rho prompted us to analyze the effects of toxin B in single RBLs cells (Fig. 7). As reported previously (Djouder et al., 2001), the majority of cells respond 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 FcεRI. The various delays in the response of the individual cells build up an average signal with slow onset and sustained plateau (Fig. 7A, left). By 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. 7A and B (left) nicely compared to calcium signals recorded with cell suspensions after application of TNP-OVA (Prepens et al., 1996; Djouder et al., 2000) and carbachol (Fig. 5 and 6). Toxin B disrupts the calcium signals through the FcεRI 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 FcεRI 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). By 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 (Fig. 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. In order 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 FcεRI 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 FcεRI signaling pathway is apparently downstream from the calcium release induced by IP₃, because the activation of the CRAC channels that support the calcium entry was blocked by toxin B even after intracellular dialysis of IP₃ (Djouder et al., 2000). Since it is likely that the M1 and FcεRI 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). In order 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, by contrast to calcium release, we observed weak calcium entry in cells treated with toxin B (Fig. 8A and B). Thus, although the dissected calcium entry is blocked by toxin B (Fig. 8), the calcium mobilization induced by carbachol (Fig. 5, 6, 7) is not sensitive to toxin 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 FcεRI receptors, including exocytosis (Jones et al., 1991), PI3-kinase activation (Barker et al., 1995; Barker et al., 1999; Hirawasa et al., 1997), calcium mobilization (Choi et al., 1993) and MAP-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 FcεRI-mediated signaling involve Rac but not Rho to induce exocytosis. Therefore, we addressed in the present study the question whether M1- and FcεRI 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 FcεRI-induced activation of Rac (Manetz et al., 2001). Vav is reportedly activated by tyrosine phosphorylation (Bustelo, 2000; López-Lago et al., 2000; Manetz et al., 2001; Schuebel et al., 1998). 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 (Vanhaesebroeck et al., 1997; Leevers et al., 1999; Akasaki et al., 1999; Reif et al., 1996). 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 PI3-kinase (Stephens et al., 1994; Stoyanov et al., 1995;
PKB is a well-known effector of PI3-kinase, and is translocated to the membrane and activated by phosphorylation at residues Thr308 and Ser473 (Leevers et al., 1999; Vanhaesebroeck et al., 1997). 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 PDK1 (3'-phosphoinositide-dependent kinase-I). Both PKB and PDK1 possess PH domains which 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 like wortmannin and LY294002. By 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). By 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 FcεRI stimulation in RBL cells (Djouder et al., 2001; Barker et al., 1995; Barker et al., 1999). Notably, it was suggested that wortmannin has no effects on CRAC currents (Djouder et al., 2001; Ching et al., 2001). By contrast, it was proposed that the PI3-kinase regulates a non-capacitative calcium entry pathway (Ching et al., 2001). What is the role of the Rho GTPase Rac and/or Cdc42 in calcium mobilization via FcεRI signaling? Using transfection of dominant active and inactive GTPases, Hong-Geller and Cerione suggested a direct role of Rac/Cdc42 in regulation of phospholipase Cγ (Hong-Geller and Cerione, 2000). Recently the same group reported that activated Rac/Cdc42 reconstitutes FcεRI-mediated Ca^{2+} 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 clostridial cytotoxins on calcium release–activated calcium currents (I_{CRAC}) 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 clostridial cytotoxins on calcium release–activated calcium currents (I_{CRAC}). 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. Since 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 appears that
the regulation of IP3 production differs in M1- and FcεRI-signaling. For example, it is well known that the IP3 production via FcεRI in RBL cells is very low as compared to that induced by M1 receptors stimulation. Therefore, factors like sphingosine-1-phosphate have been proposed as additional second messengers to be involved in calcium mobilization by FcεRI 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 FcεRI signaling pathway. It is likely that, besides calcium entry currents like I_{CRAC}, 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 FcεRI and M1 receptors and indicate that Rac have different functions in these processes.
References


Figure Legends

Figure 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. A. RBL cells were treated without (control, □) and with toxin B (∇, 40 ng/ml, 2 h), lethal toxin (▲, 40 ng/ml, 2 h), B. with C3 fusion toxin (□, 200 ng/ml C2IN-C3 and 100 ng/ml C2II, 4 h) and then stimulated by increasing concentrations of carbachol. C. RBL cells were treated with increasing concentrations of toxin B (∇) and lethal toxin (▲) for 2 h and then stimulated with 1 mM carbachol. D. RBL cells were treated with 40 ng/ml of toxin B (∇) and 40 ng/ml lethal toxin (▲) for the indicated times 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.

Figure 2. Effects of toxin B (40 ng/ml, 2 h) on tyrosine phosphorylation of the guanine nucleotide exchange factor Vav. RBL cells primed overnight with IgE (0.3 µg/ml) are stimulated during 2 min with 1 mM carbachol or 50 ng/ml TNP-OVA. Vav was immunoprecipitated from whole lysates followed by Western blot analysis and probed with a phosphotyrosine antibody (4G10 UBI). Shown is one representative blot. The experiment was repeated four times with similar results.

Figure 3. Confocal fluorescence microscopy of the pleckstrin homology domain of PKB (PH-PKB-GFP).

RBL cells were transfected with PH-PKB-GFP and then starved in 5% FCS overnight. Thereafter, cells were treated with inhibitors and stimulated with 1 mM carbachol for 15 min. Controls (A; B for carbachol), PI3-kinase inhibitor wortmannin (100 nM, 20 min, C and D), toxin B (40 ng/ml for 2 h, E and F for carbachol), lethal toxin (40 ng/ml for 2 h, G and H) and
chimeric toxin C2IN-C3 (200 ng/ml, plus C2II (100 ng/ml) for 4 h, I and J). The experiment was repeated three times with similar results.

**Figure 4.** Effects of PI3-kinase inhibitors, genistein and toxin B on Rac activation. RBL cells were stimulated by 1 mM carbachol or 50 ng/ml TNP-OVA in RBL cells primed overnight with IgE (0.3 µg/ml). Effects of wortmannin (100 nM, 20 min), LY294002 (10 µM, 30 min), genistein (100 µM, 30 min) and toxin B (40 ng/ml, 2 h) on precipitation of GTP-bound Rac by the Rac-binding domain of PAK. Bound GTP-Rac was analyzed on Western blots using an anti-Rac antibody. The experiment was repeated four times with similar results.

**Figure 5.** Influences of toxins on Ca$^{2+}$ responses of RBL cells stimulated with 1 mM carbachol. Cells were treated with toxin B (40 ng/ml), lethal toxin (40 ng/ml) for 2 h (A), C3 fusion toxin (100 ng/ml C2IN-C3 and 200 ng/ml C2II) for 4 h (B) and wortmannin (100 nM) for 20 min (C). RBL cells primed with IgE were treated with toxin B and lethal toxin (each 40 ng/ml) for 2 h and stimulated with 50 ng/ml TNP-ovalbumin (D). Shown is one representative experiment. The experiment was repeated six times with similar results.

**Figure 6.** Influences of toxin B and lethal toxin (both 40 ng/ml, 2 h) on Ca$^{2+}$ responses of RBL cells stimulated with a decrease of carbachol (CCH) at concentrations of 1; 0.1; 0.01, and 0.001 mM carbachol. Shown is one representative experiment. The experiment was repeated three times with similar results.

**Figure 7.** Effect of toxin B on calcium mobilization in single RBL cells. A. RBL cells were primed with 0.3 µg/ml TNP-IgE overnight and calcium signaling was initiated by the addition of 50 ng/ml TNP-ovalbumin (TNP-OVA). B. The calcium mobilization was induced by application of 1 mM carbachol (CCH). The effects of toxin B on calcium signals of single
RBL cells were assayed in paired experiments with control cells (Control) and with cells treated with toxin B (ToxB, 50 ng/ml, 1-2 h exposure). In each experiment, the calcium signals of 10-35 cells were obtained simultaneously. Each panel shows representative calcium signals of 20 cells and the mean response of all cells (bold line; A, Control, n=39, ToxB, n=46; B, control, n=49, ToxB, n=40).

Figure 8. Ca\(^{2+}\) release and capacitative Ca\(^{2+}\) entry in single RBL cells treated with toxin B. The cells were exposed to an extracellular Ca\(^{2+}\) free solution (0 Ca\(_o\)) and the Ca\(^{2+}\) release was induced by the application of 1 mM carbachol (CCH). Subsequently, the capacitative Ca\(^{2+}\) entry was initiated by increasing the extracellular Ca\(^{2+}\) concentration to 1 mM (1 mM Ca\(_o\)).

A. Time course of the intracellular Ca\(^{2+}\) concentration (Ca\(_i\)) in representative paired experiments with control and toxin B treated cells (Control, left; ToxB, right). B. Peak Ca\(_i\) values in 0 Ca\(_o\) and in 1 mM Ca\(_o\) from control cells (n=97, black bars) and from cells treated with toxin B (n=104, white bars) measured in experiments as shown in A. Peak values in 1 mM Ca\(_o\) were 50\% smaller in cells treated with toxin B compared to control cells (\#P<0.05).
Fig 2

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Fig 3

+ 1 mM carbachol

Control

Wortmannin

Toxin B

Lethal toxin

C3-C2IN
Fig 4

Control  Wortmannin  LY294002  Genistein  Toxin B  Control  Wortmannin  LY294002  Genistein  Toxin B  Control  Wortmannin  LY294002  Genistein  Toxin B

\[ + 1 \text{ mM carbachol} \quad + 50 \text{ ng/ml TNP-OVA} \]

\[ \text{Rac-GTP} \]
Fig. 5
Fig. 7

A

Control

ToxB

$C_{ai} (\mu M)$

TNP-OVA

$C_{ai} (\mu M)$

CCH

Time (min)

B
**Fig. 8**

A

Control

ToxB

\[ \text{Ca}_i (\mu M) \]

\[
\begin{array}{c}
\text{CCH} \\
0 \text{ Ca}_o & 1 \text{ mM Ca}_o \\
\end{array}
\]

\[
\begin{array}{c}
0 \text{ Ca}_o & 1 \text{ mM Ca}_o \\
\end{array}
\]

B

\[
\begin{array}{c}
\text{peak Ca}_i (\mu M) \\
0 & 0.5 & 1 & 1.5 \\
0 \text{ Ca}_o & 1 \text{ mM Ca}_o \\
\end{array}
\]

*