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Rac and Phosphatidylinositol 3-Kinase Regulate the Protein Kinase B in FcεRI Signaling in RBL 2H3 Mast Cells[1]

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Rac and Phosphatidylinositol 3-Kinase Regulate the Protein Kinase B in FcεRI Signaling in RBL 2H3 Mast Cells[1]

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FcεRI signaling in rat basophilic leukemia cells depends on phosphatidylinositol 3-kinase (PI3-kinase) and the small GTPase Rac. Here, we studied the functional relationship among PI3-kinase, its effector protein kinase B (PKB), and Rac using inhibitors of PI3-kinase and toxins inhibiting Rac. Wortmannin, an inhibitor of PI3-kinase, blocked FcεRI-mediated tyrosine phosphorylation of phospholipase Cγ, inositol phosphate formation, calcium mobilization, and secretion of hexosaminidase. Similarly, *Clostridium difficile* toxin B, which inactivates all Rho GTPases including Rho, Rac and Cdc42, and *Clostridium sordellii* lethal toxin, which inhibits Rac (possibly Cdc42) but not Rho, blocked these responses. Stimulation of the FcεRI receptor induced a rapid increase in the GTP-bound form of Rac. Whereas toxin B inhibited the Rac activation, PI3-kinase inhibitors (wortmannin and LY294002) had no effect on activation of Rac. In line with this, wortmannin had no effect on tyrosine phosphorylation of the guanine nucleotide exchange factor Vav. Wortmannin, toxin B, and lethal toxin inhibited phosphorylation of PKB on Ser^{473}. Similarly, translocation of the pleckstrin homology domain of PKB tagged with the green fluorescent protein to the membrane, which was induced by activation of the FcεRI receptor, was blocked by inhibitors of PI3-kinase and Rac inactivation. Our results indicate that in rat basophilic leukemia cells Rac and PI3-kinase regulate PKB and suggest that Rac is functionally located upstream and/or parallel of PI3-kinase/PKB in FcεRI signaling. *The Journal of Immunology*, **2001, 166: 1627–1634.**

In rat basophilic leukemia (RBL[3] 2H3) cells, cross-linking of the FcεRI mediates cell activation which finally results in degranulation and release of granule content (1, 2). Several signal transduction pathways are involved in this process, including tyrosine kinases Lyn and Syk, activation of phospholipase Cγ1, subsequent increase in the intracellular calcium by mobilization of calcium stores, and calcium influx through calcium release-activated calcium current channels (3–6). Recently, it was shown that phosphatidylinositol 3-kinase (PI3-kinase) is also essential for activation in RBL cells (7, 8). PI3-kinase is involved in the regulation of a number of cellular functions, including secretion, cytoskeleton organization, proliferation, differentiation, and apoptosis (9, 10). Major products of PI3-kinase are phosphatidylinositol (PI) 3-phosphate (PI(3)P), PI 3,4-bisphosphate (PI(3,4)P_2), and PI 3,4,5-trisphosphate (PI(3,4,5)P_3) which interact with FYVE- and pleckstrin homology domains to modulate protein-protein and protein-lipid interactions. One established target of PI3-kinase is protein kinase B (PKB), also termed RAC kinase or protein kinase Akt (11). PKB is activated by phosphorylation at Thr^{308} and Ser^{473} which are located in the so-called activation loop and C terminus of the kinase, respectively (12, 13). Whereas the kinase responsible for the phosphorylation of Ser^{473} is still unknown, phosphorylation of the A-loop site occurs by phosphoinositide-dependent protein kinase-1 (PDK1). Activation of PKB is paralleled by its membrane translocation as shown recently by activation of leukocytes using the pleckstrin homology domain of the protein kinase B, tagged with a green-fluorescent protein GFP (14).

The Rho GTPases, including Rho, Rac, and Cdc42, are critical regulators of the actin cytoskeleton and participate in several signaling events (15–17). Recent studies indicate that Rho GTPases are also involved in activation of RBL cells (18–21). For example, stable expression of dominant negative Rac and Cdc42 in RBL cells inhibited FcεRI-mediated degranulation (22), and RhoGDI, a guanine nucleotide dissociation inhibitor that was shown to block the biological activity of Rho GTPases, inhibited regulated exocytosis from RBL cells (23).

Rho GTPases are the specific targets of various bacterial protein toxins, which are used as pharmacological tools to analyze the functional roles of the GTPases in diverse signal processes (24). *Clostridium difficile* toxins B inactivate Rho GTPases by glucosylation at Thr^{37} (RhoA, -B, and -C) and Thr^{35} (Rac and Cdc42), respectively (25). The lethal toxin of *Clostridium sordellii* glucosylates and inactivates Rac, possibly Cdc42, but not Rho (26, 27). In addition, Ras subfamily proteins (e.g., Ras, Ral, and Rap) are glucosylated by the lethal toxin. Finally, *Clostridium botulinum* C3 exoenzyme and related C3-like transferases, including the

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[3] Abbreviations used in this paper: RBL cells, rat basophilic leukemia cells; 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 *C. botulinum* C2 toxin; $[Ca^{2+}]_i$, cytoplasmic free calcium; Crib, Cdc42/Rac interactive binding; TNP-OVA, trinitrophenyl-conjugated OVA; toxin B, *Clostridium difficile* toxin B; lethal toxin, *Clostridium sordellii* lethal toxin; IP, inositol phosphate; IP_3, inositol 1,4,5-triphosphate; PH, pleckstrin homology domain; PKB, protein kinase B; PAK, p21-activated protein kinase; RBD, Rac-binding domain; PDK1, phosphoinositide-dependent protein kinase-1; GFP, green-fluorescent protein; PLCγ1, phospholipase Cγ1; MAP, mitogen-activated protein; ERK, extracellular signal-related kinase; PI, phosphatidylinositol; PI(3)P, PI 3-phosphate; PI(3,4)P_2, PI 3,4-bisphosphate; PI(3,4,5)P_3, PI 3,4,5-trisphosphate.

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C3 chimeric toxin C2IN-C3, selectively ADP-ribosylate RhoA, -B, and -C at Asn^{41} and inhibit their functions (28–31). Therefore, the findings that *C. difficile* toxin B and *C. sordellii* lethal toxin but not C3-like toxins inhibit FcεRI-mediated degranulation suggested a crucial role of Rac/Cdc42 but not of Rho isoforms in FcεRI signaling in RBL cells (32, 33).

Several studies suggest that Rho GTPases and PI3-kinase functionally interact (34, 35). In porcine aortic endothelial cells, it was shown that PDGF by a stably expressed PDGF receptor stimulates the increase in GTP-Rac via activation of PI3-kinase to regulate the actin cytoskeleton (36). Moreover, Rac is activated by stimulation of the FMLP and leukotriene B_4 receptors in neutrophils. This effect is blocked by the PI3-kinase inhibitors wortmannin and LY294002 (37). The guanine nucleotide exchange factor Vav, which activates Rac (38), is activated by tyrosine phosphorylation. The PI3-kinase substrate $PI(4,5)P_2$ inhibits the phosphorylation and activation of Vav, whereas the PI3-kinase product $PI(3,4,5)P_3$ enhances phosphorylation and activation of the nucleotide exchange factor (39). In contrast, GTP-bound Rac and Cdc42 reportedly bind to the p85 subunit of PI3-kinase, possibly activating the lipid kinase (40). Moreover, it was shown that PI3-kinase acts downstream of Rac/Cdc42 in the signaling of integrin-mediated cell motility and invasiveness (41).

To obtain more insights into the functional interactions of PI3-kinase, its effector PKB, and Rho GTPases, we studied the effects of inhibitors of PI3-kinase (wortmannin and LY294002) and of Rho GTPases (various bacterial toxins) on FcεRI-mediated RBL cell activation. Furthermore, we used a construct of the pleckstrin homology (PH) domain of PKB tagged with GFP (14) to follow the membrane translocation of PKB. Here, we report that inhibition of PI3-kinase impairs FcεRI-signaling including degranulation, inositol phosphate (IP) accumulation, calcium mobilization, and PKB phosphorylation and translocation; however, inhibitors of PI3-kinase did not block activation of Rac by FcεRI stimulation. Rac-inactivating toxins blocked the above mentioned FcεRI-signaling including PKB phosphorylation and translocation. Our results indicate that in RBL cells Rac and PI3-kinase regulate PKB and suggest that Rac is functionally located upstream and/or parallel of PI3-kinase/PKB in FcεRI signaling.

Materials and Methods

Materials

Cell culture medium and FCS were obtained from PAN Systems, (Aidenbach, Germany). *C. difficile* toxin B (42), *C. sordellii* lethal toxin (26), *C. botulinum* C2 toxin (43), and the *C. botulinum* transferase C3 fusion toxin (C2IN-C3) (31) were prepared as described recently. Trinitrophenyl conjugated to OVA (TNP-OVA; 50 ng/ml) and IgE (0.3 μg/ml) were kindly donated by Dr. A. Hoffmann (Paul-Ehrlich Institute, Langen, Germany). fura-2 acetoxymethyl ester was obtained from Molecular Probes (Göttingen, Germany). *myo*-[2-^{3}H]-Inositol was purchased from NEN (Bad Homburg, Germany). The GST-p21-activated protein kinase (PAK) fusion proteins were a gift of Dr. J. Collard (Amsterdam, The Netherlands). The anti-phosphotyrosine (clone 4G10), anti-Vav, and anti-phospholipase Cγ1 (PLCγ1) Abs were from Upstate Biotechnology (Lake Placid, NY). The Rac Ab was from Transduction Laboratories (Lexington, KY). The anti-Ser^{473} PKB_{9271} and the anti-PKB_{9272} Abs were from New England Biolabs (Schwalbach, Germany). Phosphorylated p42 and p44 mitogen-activated protein (MAP) kinases were detected with the monoclonal anti-diphosphorylated Ab from Sigma (St. Louis, MO). p42 (extracellular signal-related kinase (ERK)-2) was detected with the polyclonal Ab C14 (Santa Cruz Biotechnology, Santa Cruz, CA). The PH-PKB-GFP construct was kindly donated by Dr. T. Balla (Bethesda, MD). Wortmannin and LY294002 were from Sigma. All other reagents were of analytical grade and commercially available.

Cell culture

RBL cells transfected with the human muscarinic receptor (44) (RBL 2H3 m1, a gift from Dr. G. Schultz, Berlin, Germany, and Dr. P. Jones, Burlington, MA) were grown in Eagle's MEM with Earle's salts supplemented with 15% (v/v) heat-inactivated FCS, 4 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere of 5% CO_2 at 37°C. RBL 2H3 m1 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 to avoid a partial destruction of membrane receptors. Cells were incubated overnight with 0.3 μg/ml anti-TNP IgE before Ag stimulation experiments.

Treatment with toxins

Subconfluent cells were preloaded with anti-TNP-OVA IgE overnight. Thereafter, the medium was changed, and the cells were treated with *C. difficile* toxin B and *C. sordellii* lethal toxin for the indicated times and concentrations. After toxin treatment, cells were washed with the appropriate buffer and used for the assays. Viability of cells were checked by trypan blue exclusion, indicating more than 90% viable cells. The toxins did not induce any unspecific release of hexosaminidase.

Transfection with PH-PKB-GFP construct

RBL cells were transfected with 15 μg of the PH-PKB-GFP by electroporation at 240 V and 950 μF, and 30 h later coverslips were placed in MEM medium (overnight) in the presence of 5% FCS and loaded with 0.3 μg/ml anti-TNP IgE before Ag stimulation.

Microscopy

RBL cells were seeded on glass coverslips in 24-wells culture plates and incubated without or with wortmannin (100 nM, 20 min) and toxin B (40 ng/ml, 2 h). The coverslips were mounted onto glass slides using PBS/glycerol (1:1) and immediately subjected for microscopy using a Zeiss Axiovert microscope. Photographs were taken at ×480 magnification.

For confocal microscopy, transfected RBL cells were fixated with 4% formaldehyde and translocation of the PH-PKB-GFP construct to the membrane after 50 ng/ml TNP-OVA-stimulation were analyzed using a Zeiss Axiovert microscope 135 TV equipped with Bio-Rad MRC 1024 ES.

Hexosaminidase release assay and measurement of [^{3}H]IP formation

For hexosaminidase release, cells were seeded in 96-wells culture plates and incubated without or with inhibitors (toxins, wortmannin) for the indicated times and concentrations. Hexosaminidase release was determined as described (45). The values are expressed as percent of total amount of hexosaminidase which was determined using 2% Triton X-100 in Tyrode's buffer.

For the measurement of IP formation, cells were seeded into 24-well plates, grown to subconfluence, and labeled with [^{3}H]inositol (3 μCi/ml) in serum-free medium for 20 h. IP formation was measured as described (46). When wortmannin (100 nM, 20 min) and toxin were used in experiments, cells were pretreated at the indicated concentrations and times during labeling with [^{3}H]inositol.

Measurements of [Ca^{2+}] in cell suspensions

RBL 2H3 m1 cells were detached from culture plates with SK buffer (125 mM NaCl, 1.5 mM EDTA, 5.6 mM glucose, 10 mM HEPES, pH 7.2). After centrifugation, cells were resuspended in serum-free MEM medium and loaded with fura-2 acetoxymethyl ester (2.5 μM) for 45 min at 37°C. After loading, cells were washed three times with HEPES-buffered saline, and cell density was adjusted to 1×10^6 cells/ml. Experiments were conducted at room temperature in HEPES-buffered saline using a Perkin-Elmer LS 50B spectrofluorometer (Perkin-Elmer, Norwalk, CT). The fluorescence of cell suspensions was examined at an emission wavelength of 510 nm and excitation wavelengths of 340 and 380 nm, respectively. Results are presented as changes in fluorescence ratio 340/380 over time.

Measurements of [Ca^{2+}] in attached cells

RBL cells were seeded on coverslips, and the intracellular Ca^{2+} was measured at room temperature 2 days later using an cell-imaging system (Till Photonics, Planegg, Germany). On the day of the experiments, the cells were incubated in medium containing fura-2 acetoxymethyl ester (5 μM) for 1 h at room temperature. Subsequently, the culture medium was replaced by a bath solution with a Ca^{2+} concentration of 4.5 mM (115 mM NaCl, 4.5 mM $CaCl_2$, 2 mM $MgCl_2$, 5 mM CsCl, 10 mM HEPES, pH 7.2 (NaOH)). Images of 20–35 cells per coverslip 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 previously described (47) Experiments were paired by alternating Ca^{2+} measurements in control and wortmannin treated coverslips.

Expression of GST-PAK-Cdc42/Rac interactive binding (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) 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 DTT, 10% glycerol, and 1 mM PMSF). The lysate was centrifuged at 10,000 × *g*, and the supernatant was used for purification of the GST-PAK-Crib domain by affinity purification with glutathione-Sepharose (Pharmacia, Piscataway, NJ). Beads loaded with GST-fusion proteins were washed twice with PBS and used immediately for GTPase pull down experiments.

GST-PAK-Crib domain pull down experiments

RBL cells (1×10^6) primed with anti-TNP-OVA-IgE (0.3 μg/ml) overnight and 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 were washed twice with PBS and stimulated during 1 min at 37°C by TNP-OVA (50 ng/ml). After addition of 250 μl 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), 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 GTP-Rac proteins were immunoprecipitated at 4°C, 1 h with 20 μl GST-PAK fusion protein, respectively. The complexes were washed three times with ice-cold PBS, resuspended, and boiled with Laemmli buffer. Bound Rac proteins were detected by Western blotting using anti-Rac Ab (Santa Cruz Biotechnology).

Immunoprecipitation of PLCγ1 and 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 (pH 7.4), 1 mM sodium *o*-vanadate, 1 mM benzamidine, and 0.5 mM PMSF. 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 anti-PLCγ1 or anti-Vav (Upstate Biotechnologies). Immunocomplexes were captured by adding 50 μl protein A/G agarose (Santa Cruz Biotechnology), 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, boiled with Laemmli buffer, and subjected to SDS-PAGE (7%), followed by transfer of proteins onto nitrocellulose membranes and Western blotting using a phosphotyrosine Ab (Upstate Biotechnologies) (1:3000). Detection occurred by an ECL Western blotting system (Amersham Pharmacia Biotech, Piscataway, NJ).

p42/p44 MAP kinase and PKB assays

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 (pH 7.4), 1 μM microcystin-LR (Biomol), 1 mM sodium *o*-vanadate, 1 mM benzamidine, and 0.5 mM PMSF. Extracts were centrifuged at 14,000 × *g* for 15 min at 4°C before electrophoresis and Western blotting. Phosphorylated p42 and p44 MAP kinases were detected with the monoclonal antidiphosphorylated Ab (Sigma). p42 (ERK-2) was detected with the polyclonal Ab C14 (Santa Cruz Biotechnology). Detection of activated PKB was performed with Abs directed against phosphorylated Ser^{473} (New England Biolabs). Protein kinases were visualized by the ECL system (Amersham Pharmacia Biotech).

Results

PI3-kinase is essential for cytoskeleton regulation

PI3-kinase and Rho GTPases, especially Rac and Cdc42, are important players in FcεRI-signaling. Because PI3-kinase activates Rho proteins in some cell types (9, 39) but is located downstream of the GTPases in other signaling systems (41), we were prompted to study the role and relationship of Rho GTPases and PI3-kinase in RBL cells. It is well known that Rho GTPases are involved in regulation of the actin cytoskeleton (15). This appears to be also true for PI3-kinase in RBL cells. Treatment of RBL cells with wortmannin (100 nM), a potent inhibitor of PI3-kinase, caused major morphological changes that were mainly characterized by shrinking of the cell body and reduction in cell spreading (Fig. 1*B*).

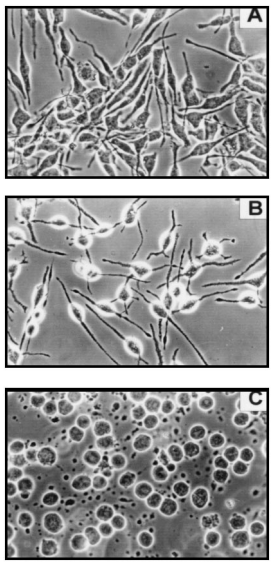

FIGURE 1. Effects of wortmannin on cytoskeleton. RBL cells were seeded on glass coverslips in 24-well culture plates and incubated without (*A*) or with wortmannin (100 nM, 20 min (*B*)) and toxin B (40 ng/ml, 2 h (*C*)). The coverslips were mounted on glass slides using PBS-glycerol (1:1) and immediately subjected for microscopy. Magnification, ×480.

For comparison, we used *C. difficile* toxin B, which is known to inactivate all Rho GTPases including Rho, Rac, and Cdc42 by glucosylation (25). Treatment of RBL cells with toxin B (40 ng/ml) for 2 h caused a different phenotype characterized by a complete rounding up of cells (Fig. 1*C*).

PI3-kinase regulates the degranulation, the calcium response, and IP formation in FcεRI signaling

Rac regulates the secretion of RBL cells (22, 48). Recently, it has been shown that the glucosylating clostridial cytotoxins (e.g., toxin B) completely inhibit the degranulation of RBL cells after stimulation of the FcεRI receptor (32). Fig. 2 shows the effect of wortmannin and toxin B on the hexosaminidase release of primed RBL cells stimulated with increasing concentrations of TNP-OVA. Wortmannin (100 nM) almost completely inhibited hexosaminidase secretion (Fig. 2*A*). Treatment with toxin B and lethal toxin abrogated secretion at concentrations of 40 ng/ml (Fig. 2*B*). Note that lethal toxin glucosylates Rac (to a minor extent Cdc42) but not Rho (26), indicating the role of Rac in secretion of RBL cells.

The effects of wortmannin, toxin B, and lethal toxin on calcium mobilization are shown in Fig. 2, *C* and *D*. Both agents blocked the calcium mobilization in RBL cells stimulated by TNP-OVA. Toxin B and lethal toxin were somehow more active than wortmannin. Treatment of RBL cells with wortmannin (100 nM) caused 50% inhibition of TNP-OVA-induced calcium mobilization (Fig. 2*C*), whereas toxin B and lethal toxin reduced calcium mobilization by ~80% (Fig. 2*D*). This inhibition by wortmannin of calcium mobilization observed in cell suspensions appears to reflect the disruption of calcium signals in individual cells (Fig. 3). Basically, the calcium signals of cells treated with wortmannin were delayed and exhibit repetitive elevations (spikes) above resting levels (Fig. 3*B*). This pattern contrasts with the frequently observed steady state elevations (plateaus) of intracellular calcium in control cells (Fig. 3*A*). Recently PI3-kinase has been suggested to affect calcium mobilization by regulating PLCγ1 (49). Therefore, we determined the effects of wortmannin and toxin B on IP formation. Both agents inhibited IP formation to a similar extent (not shown). Because total IP formation was significantly increased

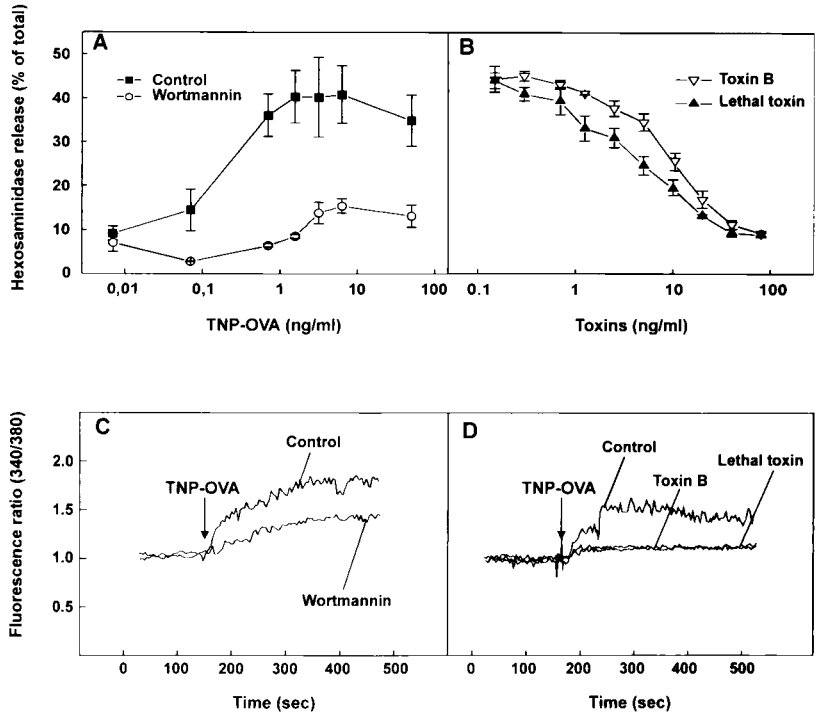

FIGURE 2. Effects of wortmannin, *C. sordellii* lethal toxin, *C. difficile* toxin B on hexosaminidase release in RBL cells. *A*, RBL cells were primed with 0.3 μg/ml TNP-IgE overnight, treated without (control (■)) and with wortmannin (○, 100 nM, 20 min) and then stimulated by increasing concentrations of TNP-OVA. *B*, Primed RBL cells were treated with increasing concentrations of toxin B (▽) and lethal toxin (▲) for 2 h and then stimulated with 50 ng/ml TNP-OVA. The release of hexosaminidase was determined as described. Data are given as SEM; $n = 4$. Shown is one representative experiment repeated four times with similar results. Influences of wortmannin and toxins on Ca^{2+} responses of RBL cells primed with anti-TNP-IgE. Cells were treated with wortmannin (100 nM, for 20 min (*C*)), toxin B (40 ng/ml, 2 h (*D*)), and lethal toxin (40 ng/ml, 2 h (*D*)) and stimulated by 50 ng/ml TNP-OVA.

only at rather high concentrations of Ag under the conditions used, we tested the effects of the toxins on tyrosine phosphorylation of PLCγ1. Clostridial cytotoxins toxin B (inactivating Rho, Rac, and Cdc42) and lethal toxin (inactivating Rac) (each 40 ng/ml), wortmannin (100 nM), and LY294002 (10 μM), but not the fusion toxin C2IN-C3 which inactivates Rho but not Rac and Cdc42, completely inhibited phosphorylation of PLCγ1 (Fig. 4). Taken together, our results demonstrate that PLCγ1 is regulated by PI3-kinase, in line with a recent report by Barker et al. (50)

PI3-kinase does not regulate Rac in FcεRI signaling

To obtain more insights into the localization of Rac protein in the signaling pathway of the FcεRI receptor, we studied the activation of Rac using a PAK-binding assay. The assay is based on the coprecipitation of the active GTP-bound Rac with the Rac-binding domain of the p21-activated kinases PAK. Using GST-PAK-Rac-binding domain (37, 51), we observed that neither wortmannin (100 nM) nor LY294002 (10 μM) inhibited the activation of Rac induced by FcεRI (Fig. 5*A*). In contrast, genistein (100 μM) inhibited this Rac activation by ~50%. The expression level of Rac was the same in untreated and treated RBL cells (not shown). Accordingly, in TNP-OVA-stimulated RBL cells, we did not observe any wortmannin and LY294002-induced changes in the tyrosine phosphorylation of the guanine nucleotide exchange factor Vav, which activates Rac (Fig. 5*B*). Both findings indicate that activation of Rac is not dependent on PI3-kinase.

Rac and PI3-kinase regulate the Ser^{473} phosphorylation of PKB in FcεRI signaling

To further address the question of how PI3-kinase and Rac signaling pathways are interconnected, we tested the effects of the Rac-inactivating toxin B on 3-phosphoinositide metabolism. However, FcεRI-stimulated 3-phosphoinositide formation measured in whole lipid extracts of RBL cells was too marginal to provide unequivocal results (not shown). Instead, we studied the phosphorylation of PKB. Activation of PI3-kinase leads to phosphorylation of PKB at Ser^{473}. As shown in Fig. 6*A*, activation of the FcεRI by TNP-OVA increased the phosphorylation of PKB in a time-dependent manner to reach the maximal phosphorylation after stimulation for 20 min. Wortmannin (100 nM) abolished this phosphorylation, indicating that PI3-kinase regulates effectively PKB in RBL cells (Fig. 6*B*). Also, toxin B and the *C. sordellii* lethal toxin completely inhibited this activating phosphorylation (Fig. 6, *C* and *D*). By contrast, the fusion toxin C2IN-C3, which specifically inactivates Rho but not Rac or Cdc42, did not affect PKB phosphorylation (Fig. 6, *E* and *F*). In addition, the actin-ADP-ribosylating *C. botulinum* C2 toxin, which depolymerizes F-actin (43, 52), had no effects on PKB phosphorylation, indicating that a redistribution of the cytoskeleton is not responsible for the inhibition of phosphorylation caused by toxin B or lethal toxin.

Rac and PI3-kinase regulate the translocation of PKB to the plasma membrane in FcεRI signaling

We used a construct of the pleckstrin homology domain of PKB tagged with GFP (PH-PKB-GFP) (14) to follow the membrane translocation of PKB. Stimulation by TNP-OVA caused a translocation of PH-PKB-GFP to the membrane indicated by an increase of PH-PKB-GFP at the membrane and a decrease in the cytosol. In line with the phosphorylation study, wortmannin, toxin B, and lethal toxin completely inhibited the translocation of PH-PKB-GFP to the membrane in unstimulated cells as well as in TNP-OVA-stimulated cells. Again the C2IN-C3 fusion toxin had no effect on the PH-PKB-GFP membrane translocation (Fig. 7).

Rho GTPase protein and PI3-kinase do not regulate the phosphorylation of MAP kinase in FcεRI signaling

To show that the effects of the toxins used were specific, we studied the FcεRI-induced activation of the MAP kinases ERK-1 and ERK-2. Wortmannin, toxin B, C2 toxin, and C2IN-C3 fusion toxin had no effect on the FcεRI-stimulated MAP kinase pathway (Fig. 8). By contrast, lethal toxin treatment completely inhibited the MAP kinase activation (Fig. 8*B*). In addition to Rac, lethal toxin modifies Ras subfamily GTPases (26). Taken together, the effects of toxins suggest that Rac and PI3-kinase regulate PKB but not

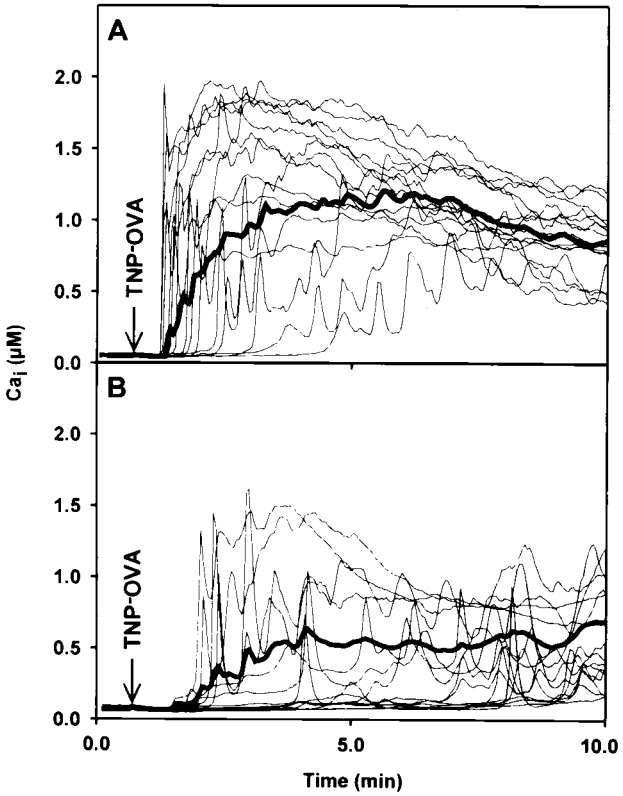

FIGURE 3. Disruption of intracellular calcium signals by wortmannin. RBL cells were primed with 0,3 μg/ml TNP-IgE for 2 days, and calcium signaling was initiated by the addition of 50 ng/ml TNP-OVA (TNP-OVA). The effects of wortmannin on calcium signals were assayed in paired experiments with control cells (*A*) and with cells treated with wortmannin (100 nM, 20 min (*B*)). In each experiment, the calcium signals of 20–35 cells were obtained simultaneously. Each panel shows representative calcium signals of 15 cells and the mean response of all cells (bold line; *A*, $n = 35$; *B*, $n = 31$). Similar results were obtained in at least three paired experiments.

MAP kinase. This regulation occurs most likely independently of the actin cytoskeleton because depolymerization of the actin cytoskeleton by C2 toxin had no effect on PKB phosphorylation.

Discussion

In RBL 2H3 cells, the GTPases of the Rho subfamily essentially regulate responses stimulated by FcεRI including redistribution of the actin cytoskeleton (16), calcium mobilization (18), degranulation (22), and JNK activation (15). PI3-kinase activity also appears to be required for these signaling events (7, 53–55). Therefore, we studied the relationship among PI3-kinase, its effector PKB, and Rac in RBL cells by using various toxins as pharmacological tools. Wortmannin and LY294002 are potent and highly specific inhibitors of PI3-kinase. Rho GTPases were inactivated by various toxins including toxin B, lethal toxin, and C3 toxin. In agreement with a central role of PI3-kinase in FcεRI signaling, wortmannin caused changes in the morphology of RBL cells, decreased calcium mobilization and secretion. Similarly, Rho GTPase-inhibiting toxins (toxin B and lethal toxin but not C3 toxin) blocked FcεRI-mediated activation of RBL cells, including calcium mobilization and degranulation. Because lethal toxin modifies Rac (in vitro also Cdc42) but not Rho (26), the findings indicate that Rac but not Rho is involved in these events. It has been reported that PLCγ1 is regulated by PI3-kinase. Accordingly, phosphorylation of PLCγ1, which appears to be a key event in PLCγ activation, was inhibited

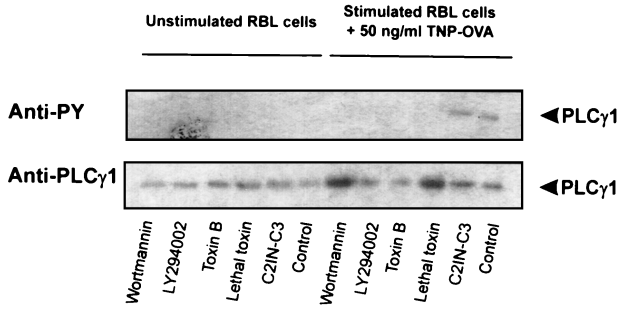

FIGURE 4. Effects of toxin B and lethal toxin (each 40 ng/ml, 2 h), C2IN-C3 (200 ng/ml) + C2II (100 ng/ml, 4 h), the PI3-kinase inhibitors wortmannin (100 nM, 20 min) and LY294002 (10 μM, 30 min) on tyrosine phosphorylation of PLCγ1. RBL cells primed overnight with IgE were stimulated by 50 ng/ml TNP-OVA. PLCγ1 was immunoprecipitated from RBL cell lysates with anti-PLCγ1 IgG, separated on SDS-PAGE, and analyzed by Western blotting using anti-phosphotyrosine Ab (anti-PY). Equal loading was tested by probing the membranes with anti-PLCγ1. Shown is one representative blot. The experiment was repeated four times with similar results.

by the PI3-kinase inhibitor wortmannin and LY294002 and also by Rac-inactivating toxins (toxin B and lethal toxin) but not by C3 toxin. In addition, we observed that toxin B and wortmannin partially inhibited IP formation in FcεRI signaling (not shown). Thus, PI3-kinase and the GTPase Rac appear to regulate similar signaling processes. However, the mechanisms underlying the effects of toxin B, lethal toxin, and wortmannin may be different. For example, wortmannin reproducibly increased the delay of calcium mobilization, a finding also reported recently (50). Toxin B dramatically inhibits calcium mobilization (as shown here) but does not

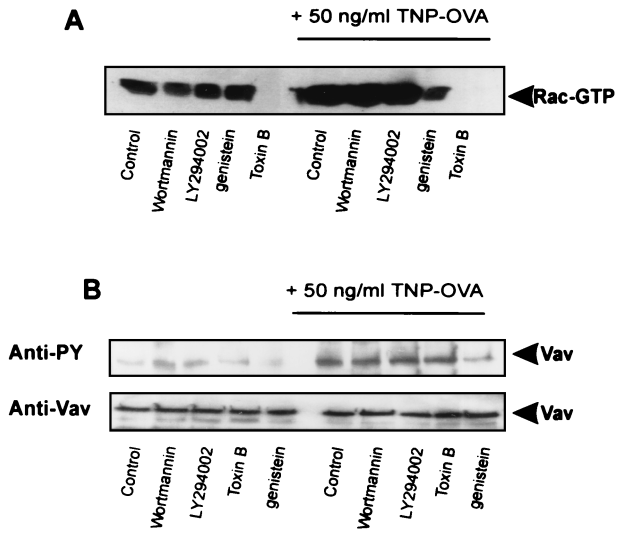

FIGURE 5. Effects of PI3-kinase inhibitors, genistein, and toxins on Rac activation. Immunoprecipitation of GTP-bound Rac and tyrosine phosphorylation of Vav. RBL cells primed overnight with IgE were stimulated by 50 ng/ml TNP-OVA. *A*, Effects of wortmannin (100 nM, 20 min), LY294002 (10 μM, 30 min), 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 Abs against Rac. *B*, 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 tyrosine phosphorylation of the guanine nucleotide exchange factor Vav. Vav was immunoprecipitated from whole lysates followed by Western blot analysis and probed with a phosphotyrosine Ab (anti-PY). The experiment was repeated four times with similar results.

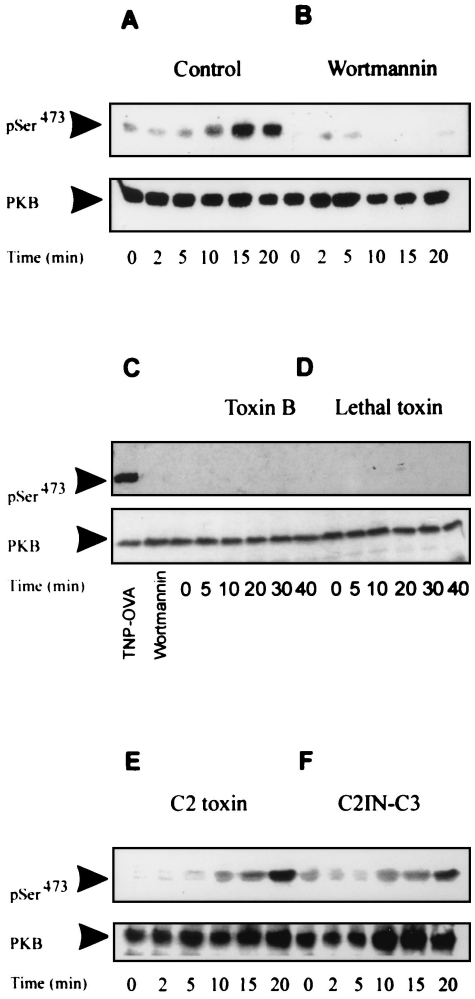

FIGURE 6. Influence of wortmannin and toxins on phosphorylation of PKB on Ser[473]. RBL cells primed overnight with IgE and treated without (*A*) or with wortmannin (100 nM, 20 min (*B*)), toxin B (40 ng/ml, 2 h (*C*)), lethal toxin (40 ng/ml, 2 h (*D*)), C3 fusion toxin (200 ng/ml C2IN-C3 and 100 ng/ml C2II, for 4 h (*E*)) and C2 toxin (200 ng/ml C2I and 100 ng/ml C2II, for 4 h (*F*)). Primed RBL cells were stimulated with 50 ng/ml TNP-OVA for the times (min) given. Cell lysates were separated on SDS-PAGE and subjected to Western blot analysis. Phosphorylation of PKB was detected using the anti-pSer[473]-specific Ab. Blots were stripped and reprobed for total PKB. The experiment was repeated four times with similar results.

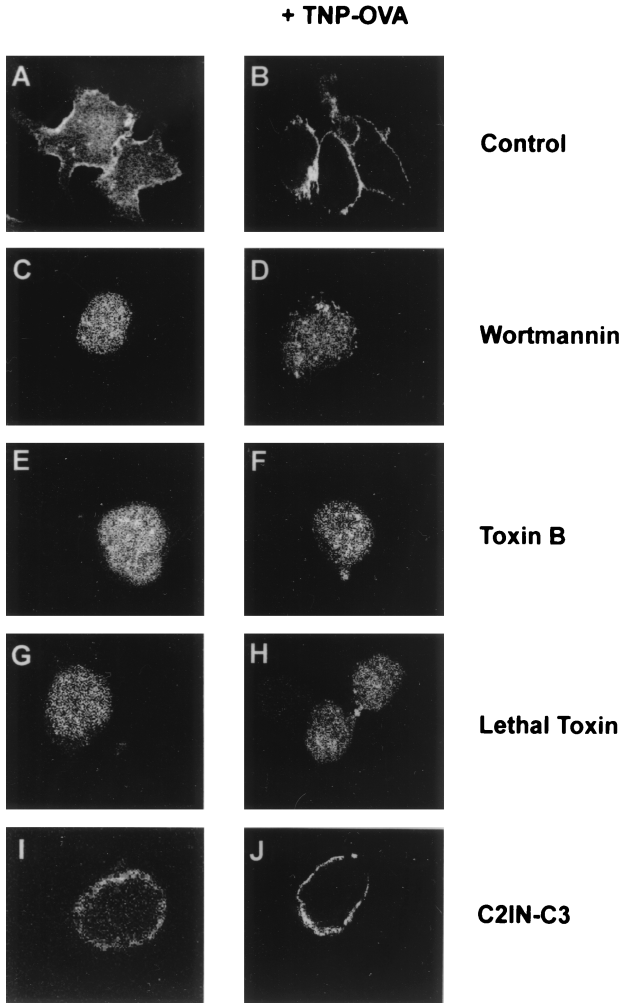

FIGURE 7. Confocal microscopy of the pleckstrin homology domain of PKB. A construct of the pleckstrin homology domain of PKB tagged with GFP (PH-PKB-GFP) (14) was used to follow the membrane translocation of PKB. RBL cells were transfected with PH-PKB-GFP and then starved in 5% FCS overnight. Thereafter, cells were treated with inhibitors and stimulated with 50 ng/ml TNP-OVA for 20 min (*right*) or remained unstimulated (*left*). *A* and *B*, Control; *C* and *D*, PI3-kinase inhibitor wortmannin (100 nM, 20 min); *E* and *F*, toxin B (40 ng/ml for 2 h; *G* and *H*, lethal toxin (40 ng/ml for 2 h; *I* and *J*, fusion toxin C2IN-C3 (200 ng/ml, plus C2II (100 ng/ml) for 4 h. The experiment was repeated three times with similar results.

cause major delay in calcium signals (33). In preliminary experiments, we did not find indication for inhibition of the capacitative calcium entry by wortmannin (unpublished observation). By contrast, we showed recently that toxin B and lethal toxin strongly inhibit the capacitative calcium entry in RBL cells (33). Moreover, the findings that the Rac-inactivating toxins blocked IP_3-induced calcium influx in a patch clamp configuration suggest a direct involvement of the GTPase in calcium release-activated calcium current (33).

Several reports describe the PI3-kinase-dependent activation of Rac in different cell types. To gain more insight into the possible relationship between the PI3-kinase and Rac signaling, we measured the activity of Rac, using a coprecipitation assay for the active GTPase. The assay exploits the interaction of GTP-bound Rac with the Rac-binding domain of PAK (56, 57). Neither wortmannin nor LY294002 inhibited activation of Rac induced by FcεRI cross-linking. However, toxin B and genistein inhibited the activation of Rac. Inhibition of Rac activation by toxin B was stronger than that by genistein and is explained by toxin B-induced glucosylation of Rac. Inhibition by genistein indicate that protein tyrosine kinases participate in activation of Rac. In mast cells, the guanine nucleotide exchange factor Vav is involved in Rac activation (58). Vav was suggested to participate in early signaling events downstream of the Src tyrosine kinase Syk, which causes the tyrosine phosphorylation of Vav, inducing its translocation to the plasma membrane and the activation of Rho GTPases (59). In agreement with the results of the PI3-kinase inhibitors on GTP-Rac coprecipitation, we did not observe an inhibition of the tyrosine phosphorylation of Vav by wortmannin or LY294002. However, genistein inhibited the Vav phosphorylation by ~50%, indicating that tyrosine kinases are involved in the regulation of Vav. Thus, our results suggest that PI3-kinase is not involved in the Vav-mediated activation of Rac by the FcεRI. This finding appears to be contradictory to a recent report showing that PI3-kinase products (e.g., PIP_3) directly activate Vav and promote its

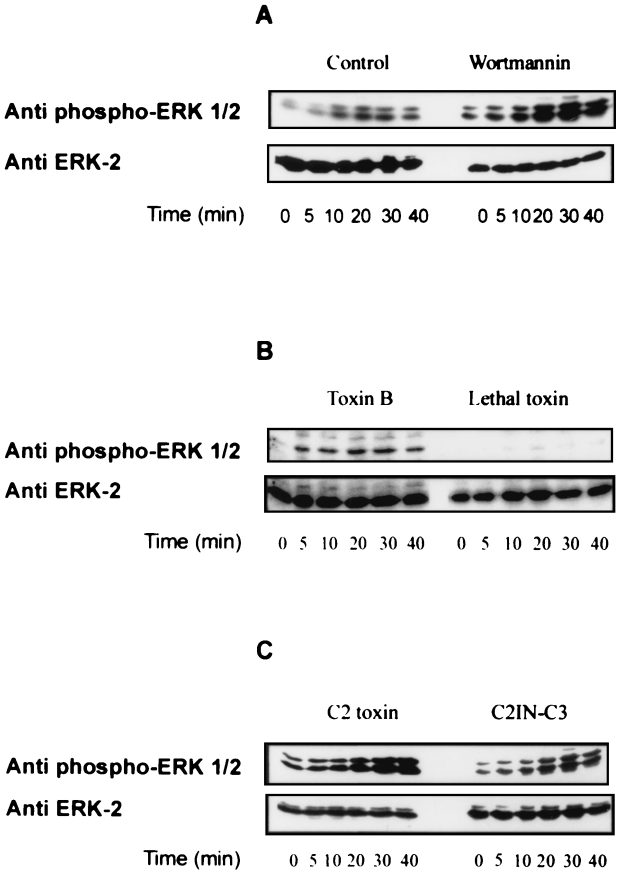

FIGURE 8. Phosphorylation of p42/p44 MAP kinase. Whole cell lysates were analyzed on Western blots using an anti-diphospho-ERK1/2 Ab. Equal loading was assessed by reprobing the membranes with a specific anti-ERK-2 IgG. RBL cells were primed overnight with IgE and then treated without (control (*A*)) or with wortmannin (100 nM, 20 min (*A*)), toxin B (40 ng/ml, 2 h (*B*)), lethal toxin (40 ng/ml, 2 h (*B*)), C3 fusion toxin (200 ng/ml C2IN-C3 and 100 ng/ml C2II, for 4 h (*C*)), and C2 toxin (200 ng/ml C2I and 100 ng/ml C2II, for 4 h (*C*)). RBL cells were stimulated with 50 ng/ml TNP-OVA for the times given. The experiment was repeated three times with similar results.

tyrosine phosphorylation (39). However, these effects of PI3-kinase products on Vav were shown in in vitro assays and may not apply to RBL cells.

From the present data, we conclude that in FcεRI-stimulated RBL 2H3 cells the activation of PI3-kinase is not a prerequisite for the activation of Rac. Although several studies show that Rac is activated by PI3-kinase, and many studies give evidence for a control of PI3-kinase by Rac, our findings are in line with the recent observation that in a murine T cell line inhibition of Rho GTPases by *C. difficile* toxin B attenuates PI3-kinase activity (60). By contrast, in CD5-stimulated T-cells, it was shown that Vav and Rac function downstream of PI3-kinase, i.e., inhibition by wortmannin could be overcome by overexpression of constitutively active Vav or Rac (61). In bone marrow-derived mast cells (BMMC), it was shown that PI3-kinase is involved in activation of the Rac/Jun kinase pathway (62). In these cells, however, the Rac/Jun kinase pathway was also activated by Kit using a PI3-kinase independent pathway. Thus, signal transduction and the cross-talk between PI3-kinase and Rac appear to depend on the cell type and on the receptor type investigated.

To obtain more insights into the functions of PI3-kinase and Rac in FcεRI-signaling, we tested the activation of PKB. PKB is a well-known effector of PI3-kinase, and is translocated to the membrane and activated by phosphorylation at residues Thr^{308} and Ser^{473} (63). 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. Both PKB and PDK1 possess PH domains which bind to 3′-phosphoinositides and are responsible for membrane association. In line with the decisive role of 3′-phosphoinositides in PKB activation, inhibition of PI3-kinase by wortmannin or LY294002 prevented PKB phosphorylation of Ser^{473} in RBL cells. Moreover, the inhibitors of the lipid kinase prevented the FcεRI-mediated PH-PKB-GFP-membrane translocation. Also toxin B and lethal toxin completely blocked the membrane translocation of PH-PKB-GFP and the activating phosphorylation of PKB, indicating an essential role of Rac and PI3-kinase in the activation of PKB in FcεRI signal transduction. Recently, a role of Rho GTPases in PKB translocation has been shown in chemoattractant receptor signaling in neutrophils (14). However, the specific Rho GTPases responsible for the PKB translocation was not identified. Our finding that C3 fusion toxin which inactivates RhoA, B, and C, was without effects and lethal toxin, which does not inhibit Rho, blocked PKB activation, suggest that Rho isoforms are not essential for FcεRI-mediated activation of PKB in RBL cells. Moreover, the observation that C2 toxin which depolymerizes F-actin, did not affect the phosphorylation of PKB indicates that changes in the actin cytoskeleton induced by Rho GTPases are not responsible for inhibition of PKB activation. A more recent report is noteworthy, showing that in mast cells derived from Rac2-deficient mice, phosphorylation of PKB by stem cell factor (SCF) was blocked indicating a role of Rac2 in PKB activation (64). However, in this report no conclusion upon the localization of Rac2 in the signaling to PKB was possible. Our data indicate that in RBL cells Rac is located upstream and/or parallel of PI3-kinase in FcεRI-signaling, because inhibition of PI3-kinase by wortmannin did not affect Rac activation and, in contrast, toxin B and lethal toxin blocked PKB translocation and phosphorylation. Our findings do not rule out that Rac controls PKB phosphorylation by a mechanism that is independent of PI3-kinase. For example, it was shown that hyperosmotic stress leads to dephosphorylation of PKB without changing PI3-kinase activity most likely by decreasing the phosphatase-dependent conversion of PIP_3 to $PI(3,4)P_2$ (65). However, we demonstrate here that in RBL cells PI3-kinase is essential to propagate Rac-mediated activation of PKB upon FcεRI cross-linking.

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