ROLE OF RHO FAMILY GTP-BINDING PROTEINS IN IgE RECEPTOR-MEDIATED PHOSPHOLIPASE D ACTIVATION IN MAST CELLS

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ABSTRACT

To investigate the role of Rho family proteins in antigen-mediated phospholipase D (PLD) activation in cultured rat basophilic leukemia (RBL-2H3) mucosal mast cells, we used two toxins, *Clostridium difficile* toxin B (toxin B) and *Clostridium botulinum* C3 toxin (C3 toxin), which inhibit Rho family proteins by monoglucosylation and ADP-ribosylation, respectively. Pretreatment with toxin B caused rounding-out of RBL-2H3 cells, strong inhibition of antigen-induced PLD activation, and also a complete blockage of serotonin secretion. By contrast, C3 toxin was without effect on both PLD activation and serotonin secretion, although RhoA was markedly ADP-ribosylated. Recombinant ADP-ribosylation factor (Arf) stimulated the PLD activity in isolated membranes in a dose-dependent manner, and 4 β -phorbol 12-myristate 13-acetate (PMA) pretreatment of cells potentiated this recombinant Arf effect. The recombinant Arf- and PMA-stimulated PLD activities were partially inhibited by pretreatment with toxin B but not by C3 toxin. Stimulation of RBL cells with antigen induced translocation to membranes of factors involving PLD activation, e.g. protein kinase C (PKC) (α , β 2, δ , ϵ) isozymes, Cdc42 and Arf, but not RhoA. These results suggest that the membrane-translocation of Cdc42 plays a major role in antigen-induced PLD activation in RBL cells and also that the translocated Arf and PKCs exert a co-operative effect for PLD activation.

Abbreviations: PLD, phospholipase D; RBL, rat basophilic leukemia; Toxin B, *Clostridium difficile* Toxin B; C3 toxin, *Clostridium botulinum* C3 toxin; Arf, ADP-ribosylation factor; PMA, 4 β -phorbol 12-myristate 13-acetate; PKC, protein kinase C; Fc ϵ RI, high affinity IgE Fc receptor; PA, phosphatidic acid; DG, 1,2-diacylglycerol; G protein, GTP-binding proteins; GST, glutathione S-transferase; DNP, 2,4-dinitrophenyl; MEM, minimum essential medium; FBS, fetal bovine serum; serotonin, 5-hydroxytryptamine; NAD, nicotinamide adenine dinucleotide; GTP γ S, guanosine 5'-O-(3-thiotriphosphate); PBut, phosphatidylbutanol; EGTA, O,O'-bis(2-aminoethyl) ethyleneglycol-N,N,N',N'-tetraacetic acid; DTT, dithiothreitol; IPs, inositol phosphates; SDS, sodium dodecyl sulfate; CD, cytochalasin D; PI-PLC, phosphoinositide-specific phospholipase C

Upon antigen stimulation, cross-linking of receptor-IgE complexes induces release of chemical mediators such as histamine, serotonin, leukotrienes and prostaglandins. Rat basophilic leukemia (RBL) cells share many of the properties of mucosal mast cells and are widely used as a model for the study of high affinity IgE Fc receptor (Fc ϵ RI)-mediated signaling events. Aggregation of Fc ϵ RI leads to activation of various signal-transducing phospholipases (1, 16, 23). Hydrolysis of phosphatidylcholine by phospholipase D (PLD) produces phosphatidic acid (PA), which can be fur-

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ther converted to 1,2-diacylglycerol (DG) by phosphohydrolase (3, 9). Since both PA and DG have been shown to act as second messengers (2, 10, 29), PLD has been believed to play an important role in cellular signaling. Recently, there has been increasing evidence that PLD is activated by several regulatory factors (10). For example, two small GTP-binding proteins (G protein), ADP-ribosylation factor (Arf) and Rho family, mainly RhoA were shown to be the potent factors for PLD activity in various cell types (2, 10, 29).

On the other hand, the Rho family proteins, Rho, Rac and Cdc42 are involved in the formation of actin stress fibers, lamellipodia and filopodia, respectively (12, 30, 44). In association with such cytoskeletal reorganization, these proteins partake in regulation of smooth muscle contraction, transcription and cell proliferation. It has also been shown that protein kinases, lipid kinases and phospholipases are implicated in the Rho signaling pathway (7, 33, 44). Recently, Prepens *et al.* (37) indicated that Cdc42, but not RhoA was a principal factor in FcεRI-mediated serotonin secretion in RBL cells by using two bacterial toxins. It has been known that *Clostridium difficile* toxins A and B block the functions of Rho family proteins (Rho, Rac and Cdc42) by monoglucosylation at Thr³⁷ (17, 18) and *Clostridium botulinum* C3 toxin (C3 toxin) blocks Rho by ADP-ribosylation at Asn⁴¹ (17). Our previous study has demonstrated that antigen-mediated PLD activation in RBL cells was suppressed by toxin B (34), suggesting that Rho family proteins are involved in the PLD activating process.

The present study was designed to gain further insight into the mechanism for the antigen-stimulated PLD activation in RBL cells by using two bacterial toxins (toxin B and C3 toxin). The results presented here have suggested that Cdc42 rather than Rho A plays a major role in antigen-stimulated PLD activation in RBL cells.

MATERIALS AND METHODS

Materials

Toxin B purified from *C. difficile* VPI 10463 was kindly supplied by Dr David M. Lyster (VPI Corporate Research Center, VA). *E. coli* bearing the glutathione *S*-transferase (GST)-C3 toxin plasmid was a kind gift from Dr Alan Hall (University College London, U.K.). *E. coli* bearing Arf1 plasmid was a kind gift from Dr Joel Moss (National Institutes of Health, MD). Monoclonal mouse anti-2,4-

dinitrophenyl (DNP) IgE was obtained from Seikagaku Kogyo (Tokyo, Japan). *Ascaris suum* antigen coupled with 2,4-dinitrophenyl (DNP-*Ascaris* antigen) was prepared as described previously (16). Eagle's minimum essential medium (MEM), penicillin and streptomycin were obtained from Gibco Laboratories (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Irvine Scientific (Santa Ana, CA). [¹⁴C]5-Hydroxytryptamine (serotonin) (50 μCi/mmol), [³²P]nicotinamide adenine dinucleotide (NAD) (30 Ci/mmol), and Aquasol-2 were purchased from Dupont-New England Nuclear (Boston, MA). [9,10-³H]Palmitic acid (52.4 Ci/mmol), *myo*-[2-³H]inositol (18.2 Ci/mmol), UDP-[U-¹⁴C]glucose (0.3 Ci/mmol), the enhanced chemiluminescence system and anti-rabbit IgG horseradish peroxidase-coupled secondary antibodies were from Amersham International (Buckinghamshire, U.K.). Dowex AG1-X8 (200–400 mesh, formate form) was obtained from Bio-Rad Laboratories (Richmond, CA). 4β-Phorbol 12-myristate 13-acetate (PMA) was from Sigma (St. Louis, MO). Guanosine 5'-*O*-(3-thiotriphosphate) (GTPγS) was from Boehringer Mannheim GmbH (Mannheim, Germany). Silica gel LK6D plates were from Whatmann (Clifton, NJ). Antibodies against small G proteins (RhoA, Rac1, Cdc42Hs) and PKC isozymes were from Santa Cruz Biotechnology (Santa Cruz, CA). Phosphatidylbutanol (PBut) standard was obtained from Avanti (Albaster, AL). All other reagents used were of the highest analytical grade.

Cell Culture

Monolayer cultures of RBL cells were maintained in MEM supplemented with 10% heat-inactivated FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 95% air/5%CO₂ at 37°C (1, 23). For detection of translocation to membranes of PKC isozymes and G proteins, cells were incubated in MEM with 1% heat-inactivated FBS, penicillin and streptomycin for 12 h before stimulation.

Assay of Glucosylation and ADP-Ribosylation Activities

Glucosylation of proteins by toxin B in RBL cell lysates was performed by the method described by Just *et al.* (17), with minor modifications. For measurement of glucosylation, RBL cells were pretreated with 5 ng/ml toxin B for 4 h, and then lysed

by sonication. The lysates (1 mg protein/ml) were incubated with 1 μ g/ml toxin B in the presence of 30 μ M UDP-[14 C]glucose at 37°C for 40 min.

Unlike toxin B, C3 toxin can be hardly incorporated into RBL cells. Therefore, we used the scrape-loading method which has been used to incorporate macromolecules into adherent cells (26). RBL cells were gently scraped from dishes by rubber policemen in the presence of 2 μ g/ml C3 toxin in MEM and then incubated for 12 h (24). During the incubation time, the cells touched the dish again. The cells thus treated with C3 toxin were scraped and sonicated for measurement of ADP-ribosylation of Rho, and the lysates (1 mg protein/ml) were incubated with 10 μ g/ml C3 toxin in the presence of 10 μ M [32 P]NAD at 37°C for 30 min. Aliquots mixed in Laemmli's sample buffer were subjected to electrophoresis on 13% polyacrylamide gel. Incorporations of UDP-[14 C]glucose and [32 P]NAD were measured by a bioimaging analyzer (Fuji FUJIX BAS 2000) and autoradiography, respectively.

Assay of PLD Activity

Intact cells The PLD activity in response to antigen was determined by measuring the formation of [3 H]PBut in the presence of butanol (0.3%, v/v) as described previously (19). RBL cells (1.5×10^6 cells/35-mm dishes) were labeled with [3 H]palmitic acid (2 μ Ci/dish) for 12 h. For sensitization, anti-DNP IgE (0.3 μ g/ml) was incubated for 2 h before stimulation. After removal of the radiolabeling medium, the dishes were rinsed three times and equilibrated in Tyrode-Hepes solution (134 mM NaCl, 12 mM NaHCO₃, 2.9 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 0.36 mM NaH₂PO₄, 5.6 mM glucose, 10 mM Hepes and 0.1% BSA; pH 7.4). After this preincubation in Tyrode-Hepes solution containing 0.3% butanol for 5 min, cells were stimulated with 100 ng/ml DNP-*Ascaris* antigen in the presence of butanol (0.3%) for 20 min.

The reactions were stopped by removing medium, followed by the immediate addition of 1 ml of ice-cold PBS/methanol (2:5, v/v) mixture to the cultured dishes. Cells were gently scraped with rubber policemen from the dishes with an additional 1 ml of PBS/methanol mixture, and then transferred to a tube containing 0.7 ml of chloroform. The lipid extraction was performed according to the method of Bligh and Dyer (4). [3 H]PBut was separated on silica gel LK6D thin-layer plate in a solvent system of the upper phase of ethyl

acetate/2, 2,4 - trimethylpentane / acetic acid / water (13:2:3:10, v/v) (22). The area corresponding to [3 H]PBut was scraped off the plate and the radioactivity was determined in a liquid scintillation counter (Beckmann LS 6500).

Isolated membranes RBL cells (2×10^6 cells/100 mm dish) were labeled with [3 H]palmitic acid (5 μ Ci/dish) for 12 h. After removal of the radiolabeling medium, the dishes were rinsed three times in Tyrode-Hepes solution. In some experiments, the cells were treated with PMA (100 nM) for 15 min and then scraped in 10 ml of ice-cold PBS containing 1 mM *O,O'*-bis (2-aminoethyl) ethyleneglycol-*N,N,N',N'*-tetraacetic acid (EGTA). After centrifugation, the control and PMA-treated cells were resuspended in ice-cold buffer A (25 mM Hepes, pH 7.4, 100 mM KCl, 3 mM NaCl, 5 mM MgCl₂, 0.5 mM phenylmethylsulfonyl fluoride, 5 mM dithiothreitol (DTT), 10 μ g/ml leupeptin and 1 mM EGTA) and were lysed by N₂ cavitation. The membranes from PMA-untreated (control membranes) and PMA-treated (PMA-membranes) cells were collected by centrifugation at $100,000 \times g$ for 30 min and were resuspended in the buffer A. Control and PMA-membranes (40 μ g) were incubated at 37°C for 30 min in 100 μ l of the reaction buffer (25 mM Hepes, pH 7.4, 100 mM KCl, 3 mM NaCl, 6 mM MgCl₂, 5 mM DTT, 1 mM EGTA, 1 mM ATP, 0.3% butanol and 1 μ M Ca²⁺) in the presence of 100 μ M GTP γ S and 5 μ M recombinant Arf. The lipid extraction and determination of radioactivity in [3 H]PBut were performed as described above.

Serotonin Release

RBL cells were labeled with [14 C]serotonin (10 nCi/ 1.5×10^5 cells) for 12 h at 37°C. For sensitization, anti-DNP IgE (0.3 μ g/ml) was incubated for 2 h before stimulation. After removal of the radiolabeling medium, the dishes were rinsed three times and equilibrated in Tyrode-Hepes solution for 5 min at 37°C. The cells were then stimulated for 20 min with 100 ng/ml DNP-*Ascaris* antigen. Incubation was stopped by removing the medium, followed by addition of 0.2 ml of ice-cold PBS with 1% TritonX-100. The radioactivities in the medium and the cells (TritonX-100 extract) were determined in a liquid scintillation counter (Beckmann LS 6500, Fullerton, CA) in 5 ml of Aquasol-2. [14 C]Serotonin release was determined as percent of the radioactivity in the medium to the total radioactivity.

Fig. 1 Morphological changes of RBL-2H3 cells treated with *C. difficile* toxin B, *C. botulinum* C3 toxin and cytochalasin D. Cells were incubated with 5 ng/ml of toxin B for 4 h (B) or with 10 μ M of cytochalasin D for 1 h (D). For incorporation of C3 toxin, RBL cells were gently scraped from dishes by rubber policeman in the presence of the medium containing 2 μ g/ml C3 toxin and further incubated in the same medium for 12 h (C). The control cells (A) were incubated without toxins for 12 h. Similar results were obtained with two additional and different cell preparations and showed at $\times 100$ of original magnification.

Measurement of Phosphoinositide Hydrolysis

[3 H]Inositol phosphates were separated by anion-exchange column chromatography (5). RBL cells (1.5×10^6 cells/dish) in 35 mm dishes were labeled with [3 H]myo-inositol (1 μ Ci/ml) in inositol-free MEM for 12 h. For sensitization, anti-DNP IgE (0.3 μ g/ml) was incubated for 2 h before stimulation. After removal of the radiolabeling medium, the dishes were rinsed three times and equilibrated in Tyrode-Hepes solution. After this preincubation in Tyrode-Hepes solution containing 10 mM LiCl for 15 min, the cells were stimulated with antigen for 10 min. The reactions were terminated by the addition of 0.5 ml of ice-cold 10% perchloric acid.

The cells were scraped and pH was adjusted to 7.4 with 1.53 M KOH solution containing 75 mM Hepes. After being kept on ice for 1 h, the formed KClO_4 was sedimented by centrifugation at $1,600 \times g$ for 10 min at 4°C. The resulting supernatant was diluted with 10 ml of water and applied onto a Dowex 1-X8 column (200–400 mesh). Inositol phosphates (IPs) were eluted with an ammonium formate gradient for radioactivity measurement.

Translocation to Membranes of Protein Kinase C (PKC) Isozymes and Low Molecular Weight G Proteins

To examine translocation to membranes of PKC

Fig. 2 Glucosylation and ADP-ribosylation of Rho family proteins by toxin B and C3 toxin in RBL-2H3 cells. RBL-2H3 cells were pretreated with 5 ng/ml toxin B for 4 h. For incorporation of C3 toxin, RBL cells were gently scraped from dishes by rubber policeman in the presence of the medium containing 2 μ g/ml C3 toxin and then incubated in the same medium for 12 h. After cell lysis, incorporation of UDP-[14 C]glucose or [32 P]NAD was measured as described in the 'Materials and Methods'. The results were representative of three different experiments.

isozymes and low molecular weight G proteins, RBL cells stimulated with antigen were lysed by N_2 cavitation in buffer A and the membrane fractions were collected by centrifugation at $100,000 \times g$ for 30 min. The membranes (10 μ g protein for PKC and 50 μ g protein for G proteins) were resuspended in Laemmli's sample buffer (20) and subjected to electrophoresis on 8% and 13% sodium dodecyl sulfate (SDS)-polyacrylamide gels for PKC and G proteins, respectively. The separated proteins were electrotransferred onto polyvinylidene difluoride membranes and the membranes were probed with specific antibodies to PKC isozymes and G proteins. Detection was performed with the enhanced chemiluminescence system according to the manufacturer's protocol. The density of protein bands was measured with a densitometer (Densitograph for the Macintosh ver.3.02, ATTO).

Expression and Purification of Recombinant Proteins

Recombinant GST-C3 toxin was expressed as GST fusion proteins in *E. coli* and purified on glutathione-Sepharose beads as described (31). Recombinant Arf was expressed in *E. coli* and purified by column chromatographies on DEAE-Sepharcel and Sephacryl S-300 columns as described (14). Purified GST-C3 toxin and Arf showed a single band on Coomassie-stained SDS-

polyacrylamide gels, respectively.

RESULTS

Effects of Toxin B and C3 Toxin on Cell Morphology and Rho Family Proteins

To delineate the implication of Rho family proteins in the antigen-mediated signaling process in RBL cells, we examined the effects of two toxins, toxin B and C3 toxin. RBL cells were markedly responsive to toxin B, which blocks Rho family proteins (Rho, Cdc42, Rac) by monoglucosylation. When added to the culture medium, morphological changes were induced. Cell shape changes were initiated 2 h after addition of toxin B (5 ng/ml), followed by distinct rounding-out at 4 h (Fig. 1B). Since C3 toxin specific for Rho was difficult to enter the RBL cells, the method of scrape-loading was used in the presence of C3 toxin (2 μ g/ml). This treatment with C3 toxin caused similar morphological changes (Fig. 1C). Rho family proteins have been known to be associated with actin polymerization (30). Treatment of RBL cells with cytochalasin D (CD) (10 μ M, 1 h), which causes depolymerization of the actin cytoskeleton (8), also induced similar rounding-out of cells (Fig. 1D).

It has been known that toxin B glucosylates Rho family proteins (Rho, Cdc42, Rac) (17), whereas C3 toxin ribosylates Rho. Our previous study indicated that RhoA and Cdc42, but not Rac1 were present in RBL cells (34). In control cells without

Fig. 3 Effects of toxin B, C3 toxin and CD on antigen-induced PLD activation (A) and serotonin secretion (B) in RBL-2H3 cells. (A) The [³H]palmitic acid-labeled cells were treated with 5 ng/ml toxin B for last 4 h or scrape-loaded in the presence of 2 μ g/ml C3 toxin for 12 h or treated with 10 μ M cytochalasin D for last 1 h. The treated cells were then stimulated with 100 ng/ml antigen in the presence of 0.3% butanol for 20 min. [³H]PBut formation was determined as described under the 'Materials and Methods'. (B) RBL-2H3 cells were labeled with [¹⁴C]5-HT for 12 h, and treated with 5 ng/ml toxin B for last 4 h or scrape-loaded in the presence of 2 μ g/ml C3 toxin for 12 h or treated with 10 μ M cytochalasin D for last 1 h. The cells were then stimulated with 100 ng/ml antigen for 20 min. [¹⁴C]5-HT release (%) was determined as described under the 'Materials and Methods'. Each point represents the mean $\pm$ SD of two experiments, each performed in duplicate.

toxin B treatment, there was marked incorporation of UDP-[¹⁴C]glucose into the protein band of M_r ~22 kDa which was cross-reacted with anti-RhoA and Cdc42 (Fig. 2). However, by the pretreatment of cells with toxin B (5 ng/ml), the incorporation of UDP-[¹⁴C]glucose into the same protein band was greatly inhibited, indicating that these proteins were endogenously glucosylated by the toxin B pretreatment. There was marginal incorporation of [³²P]NAD into the M_r ~22 kDa proteins in lysate from cells which had been treated with C3 toxin (2 μ g/ml) for 12 h, indicating that approximately 80% of RhoA was endogenously ribosylated by the C3 toxin pretreatment (Fig. 2).

Effects of Toxin B, and C3 Toxin on Antigen-Mediated PLD Activity and Serotonin Secretion

We have previously shown that toxin B suppressed antigen-induced [³H]PBut formation in RBL cells. In the current study, further experiments to delineate the preferential contribution of Rho family proteins in antigen-mediated PLD activation were

performed by using two toxins, toxin B and C3 toxin. When control RBL cells labeled with [³H]palmitic acid were stimulated with antigen (100 ng/ml) in the presence of 0.3% butanol, [³H]PBut formation, a potential parameter of PLD activity, was rapidly increased within first 2 min, reaching a plateau at 10 min and remained unchanged for 30 min (data not shown). However, the treatment with 5 ng/ml toxin B for 4 h caused a marked inhibition of antigen-mediated PLD activity (Fig. 3A), suggesting the involvement of Rho family proteins in antigen-mediated PLD activation in RBL cells. In contrast, pretreatment of C3 toxin (2 μ g/ml, 12 h) failed to suppress the PLD activity. Different effects of toxin B and C3 toxin were also observed in the antigen-stimulated serotonin secretion (Fig. 3B). Secretion was completely inhibited by toxin B, but not affected by C3 toxin.

As described above, CD pretreatment of RBL cells at 10 μ M induced similar cell rounding-out as observed in the toxin treatments. This pretreatment rather potentiated (1.4-fold) antigen-mediated PLD activity and had no effect on antigen-stimulated serotonin secretion (Fig. 3).

Effects of Toxin B on Antigen-Induced Inositolphosphate Formation

It has been reported that PLD is activated downstream of the phosphoinositide-specific phospholipase C (PI-PLC)/PKC activation (45, 46). To test this possibility, we have examined the effect of toxin B on PLC activity in RBL cells. When *myo*-[³H]inositol-labeled RBL cells were stimulated with antigen (100 ng/ml) in the presence of 10 mM LiCl, IPs formation was increased 3.8-fold (4.3% of total radioactivity) within 10 min of incubation (data not shown). However, toxin B pretreatment for 4 h showed a small reduction in the antigen-induced IPs formation (Fig. 4A). In sharp contrast, toxin B greatly suppressed the PLD activation (Fig. 4B).

Effect of Recombinant Arf on PLD Activity in Isolated Membranes

As another small molecular weight G protein, Arf is well accepted to be cofactor for PLD activation. Recently, Arf-dependent PLD (hPLD1) has been cloned and was shown to be synergistically activated by Rho family proteins and PKC (13). Our previous study in permeabilized RBL cells indicated that GTP γ S and PMA synergistically stimulated PLD activity (34). We have extended the previous work to further characterize the PLD activity. In this study, the effects of Arf were examined in the isolated membranes. Cells were pretreated with PMA (100 nM) for 15 min to induce PKC translocation to membranes and then membranes were isolated (PMA-membranes). Similarly membranes were prepared from PMA-untreated control cells (control membranes). The PLD activity was greatly increased by addition of GTP γ S alone (without Arf) in PMA-membranes. Addition of recombinant Arf enhanced the GTP γ S-dependent PLD activity in both control and PMA-membranes, suggesting the involvement of Arf and PKC for PLD activation (Fig. 5).

Effects of Toxin B, C3 Toxin on GTP γ S-Dependent PLD Activity in Membranes

To assess the involvement of Rho family protein in the membrane-associated PLD activity of RBL cells, the effects of two toxins were examined (Fig. 6). The GTP γ S-dependent PLD activity in control membranes was enhanced by addition of recombinant Arf (5 μ M). In the PMA-membranes, addi-

Fig. 4 Dose-dependent inhibition of antigen-induced IPs formation (A) and PLD activation by toxin B (B). (A) RBL-2H3 cells labeled with [³H]*myo*-inositol were preincubated with the indicated concentrations of toxin B for 4 h prior to stimulation. After preincubation in Tyrode-Hepes solution containing 10 mM LiCl for 15 min, the labeled cells were stimulated with 100 ng/ml antigen for 10 min in the presence of 10 mM LiCl. [³H]IPs formation was measured as described in the 'Materials and Methods'. (B) After preincubation with the indicated concentrations of toxin B for 4 h, [³H]palmitic acid-labeled cells were incubated in Tyrode-Hepes solution containing 0.3% butanol for 5 min and then stimulated with 100 ng/ml antigen for 20 min in the presence of 0.3% butanol. [³H]PBut formation was determined as described under the 'Materials and Methods'. Each point represents the mean $\pm$ SD of two experiments, each performed in duplicate.

tion of recombinant Arf caused a considerable elevation of the GTP γ S-dependent PLD activity. However, pretreatment of the membranes with toxin B (1 μ g/ml) for 30 min reduced the recombinant Arf-mediated PLD activation in control and PMA-membranes. In contrast, the C3 toxin treatment (10 μ g/ml for 30 min) failed to suppress these membrane PLD activities but rather slightly enhanced.

Membrane Translocation of Small Molecular Weight G Proteins and PKC Isozymes in Antigen-Stimulated RBL-2H3 Cells

Recent studies have demonstrated that most of the activating factors of PLD exist in cytosol in unstimulated cells and translocate to membranes when cells are stimulated with agonists (11, 15). To examine membrane translocation of small molecular G proteins and PKC isozymes in antigen-

Fig. 5 Effect of Arf on GTP γ S-induced PLD activation in RBL-2H3 cell membranes. [3 H]Palmitic acid-labeled RBL-2H3 cells were pretreated with buffer (○) or PMA (100 nM for 15 min) (●) and lysed by N₂ cavitation. The membranes from PMA-untreated (control-membranes) and PMA-treated (PMA-membranes) cells were collected by centrifugation at 100,000 $\times g$ for 30 min. Both membranes (40 μ g) were stimulated with the indicated concentrations of recombinant Arf in the presence of 100 μ M GTP γ S at 37°C for 30 min. [3 H]PBut was measured as described under the 'Materials and Methods'. Each point represents the mean $\pm$ SD of two experiments, each performed in duplicate.

stimulated RBL cells, the membrane fractions were isolated from stimulated and unstimulated cells with antigen for 15 min. Western blotting analysis of PKC (α , β_2 , δ , ϵ) isozymes and G proteins revealed that these proteins were markedly increased in the membranes after antigen-stimulation (Fig. 7). The membrane-associated PKC δ was most considerably increased after antigen-stimulation. Cdc42 and Arf were also redistributed to membranes by antigen-stimulation, but RhoA was much less translocated.

DISCUSSION

In the present study, we have demonstrated that Rho family proteins were involved in the antigen-mediated PLD activation pathway in RBL cells by using two toxins, toxin B and C3 toxin. Toxin B monoglucosylates Rho, Rac and Cdc42 (17, 18),

Fig. 6 Effects of toxin B and C3 toxin on the membrane-associated PLD activity in RBL-2H3 cells. The [3 H]palmitic acid-labeled cells were pretreated with buffer or PMA (100 nM for 15 min) and lysed by N₂ cavitation. The membranes prepared from PMA-untreated (control-membranes) and PMA-treated (PMA-membranes) cells were treated with buffer (□), 1 μ g/ml toxin B (▨) or 10 μ g/ml C3 toxin (■) for 30 min and stimulated with recombinant Arf (10 μ M) in the presence of GTP γ S (100 μ M) for 30 min. Each point represents the mean $\pm$ SD of two experiments, each performed in duplicate.

whereas C3 toxin ADP-ribosylates Rho (6). Thus, the toxins impair the functions of these G proteins. In contrast to C3 toxin, toxin B is feasible to enter RBL cells and induced disruption of the actin cytoskeleton at a very low concentration (5 ng/ml). The mechanism of cytotoxicity is well correlated with the observation that both toxins lead to disassembly of the actin filament network (27). Members of the Rho family proteins regulate the organization of the actin cytoskeleton (12); Rho controls the assembly of actin stress fibers and focal adhesion complexes (44), and Cdc42 stimulates formation of filopodia (30). It has been reported that toxin B and CD exert similar effects on the actin organization and assembly in various cells (8, 35). However, in contrast to strong inhibition of the antigen-induced PLD activation by toxin B, the pretreatment with CD exerted a rather stimulatory effect in RBL cells. The difference in the effects of toxin B and CD was consistent with other works shown in the carbachol-stimulated PLD activation

Fig. 7 Translocation of PKC isozymes and small molecular weight G proteins. Unstimulated ① and antigen-stimulated for 15 min ② RBL-2H3 cells were lysed by N_2 cavitation in the lysis buffer. The membrane fractions were collected by centrifugation at $100,000 \times g$ for 30 min and resuspended in the lysis buffer. The membrane fractions mixed in Laemmli's sample buffer were subjected to electrophoresis on 8 and 13% SDS-polyacrylamide gels for PKC and G proteins, respectively, and electrotransferred onto polyvinylidene difluoride membranes. The membranes were probed with specific antibodies for PKC isozymes or G proteins. Detection was performed with the ECL system according to the manufacture's protocol. The density of protein bands was measured with a densitometer.

in HEK cells (40) and antigen-stimulated serotonin secretion in RBL cells (37). Our present study suggested that antigen-induced PLD activation in RBL cells is mediated by the action of Rho family proteins independent of actin disassembly.

It has been reported that Rho A activates phosphatidylinositol-4-phosphate 5-kinase which synthesizes phosphatidylinositol 4,5-bisphosphate (PIP_2) (41). Several recent studies have indicated that PIP_2 is an essential cofactor for PLD activity (7, 38). Therefore, it could be speculated that inhibition of Rho family proteins by toxin B could lead to a decrease in the PIP_2 level *in vivo*. In HEK cells, toxin B inhibited carbachol-induced PLD activation and PI-PLC activation by reducing PIP_2 production (39). However, this may not be true with RBL cells. Toxin B showed only a small inhibitory effect (30% inhibition) on antigen-mediated PI-PLC activation, whereas it caused almost complete inhibition of PLD activation. Therefore, it was conceivable that Rho family protein-dependent PLD activation did not occur downstream of PI-PLC activation and also that the PIP_2 level was not largely decreased in toxin B-treated RBL cells. Indeed, inhibition of PLD activation by toxin B was not recovered by addition of PIP_2 (data not shown).

Several *in vitro* experiments have demonstrated that members of Rho family proteins were differentially implicated in PLD activation depending on

the cell type (25, 32, 42, 43). Usage of two toxins, toxin B and C3 toxin, was of advantage to understand the preferential contribution of Rho family proteins. For example, the inhibitory effect of toxin B on $GTP\gamma S$ -induced PLD activation was nearly equivalent to that induced by C3 toxin in permeabilized HEK cells (40), suggesting that Rho plays a principal role. On the other hand, antigen-induced serotonin secretion in RBL cells was inhibited by toxin B, but not by C3 toxin, suggesting that Cdc42 rather than RhoA takes a main part (37). Our observations obtained here with these two toxins also indicated that Rho family proteins were involved in PLD activation in RBL cells. Toxin B caused a nearly complete inhibition of either the antigen-mediated PLD activation or Arf-induced PLD activation, whereas the treatment with C3 toxin, even though at least 80% of RhoA was ADP-ribosylated, failed to diminish the antigen-stimulated PLD activation and did not inhibit Arf-induced PLD activation. These results led us to assume that Cdc42 rather than RhoA takes a greater part in the antigen-mediated PLD activation in RBL cells. Recently, it was reported that agonist-induced PLD activation was due to the translocation to membranes of G proteins (11, 15). Indeed, it was shown that translocation to membranes of Cdc42 but not RhoA occurred upon antigen-stimulation of RBL cells.

We have previously demonstrated that the treat-

ment of RBL cells with Brefeldin A, a potent inhibitor of Arf activity, caused an inhibition of the antigen-induced PLD activation, suggesting the possibility of Arf implication (28). In this study, we demonstrated that the GTP γ S-induced PLD activation was stimulated by recombinant Arf in isolated membranes of RBL cells and the membrane translocation of Arf was induced by antigen-stimulation. Furthermore, the stimulatory effect of recombinant Arf was partially inhibited by toxin B pretreatment but not by C3 toxin, suggesting that Arf-induced PLD activation was dependent on Cdc42.

In addition to these G proteins, protein kinase C has also been known to be involved in PLD activation (10). Previously, we have shown that Rho A and PKC α synergistically activated the GTP γ S-dependent PLD in HL60 cell membranes (32, 33). In permeabilized RBL cells, GTP γ S and PMA also synergistically activated PLD (34). Here we demonstrated that PLD activation by Arf in PMA-membranes was partially inhibited by toxin B, but not by C3 toxin, thus suggesting that PKC exerts a co-operative action in the Cdc42-dependent PLD activation pathway in RBL cells. Furthermore, recent study suggested that the PLD/PA phosphohydrolase process is necessary for secretion (21, 36). We also suggested in this study that Cdc42 may play an important role in secretion *via* PLD activation in RBL cells.

In summary, the results presented here suggested that Cdc42 would play a principal role in the antigen-induced PLD activation. However, to delineate the precise mechanisms, further extensive work including the reconstitution system using the purified proteins is required.

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