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Inhibition by Toxin B of Inositol Phosphate Formation Induced by G Protein-Coupled and Tyrosine Kinase Receptors in N1E-115 Neuroblastoma Cells: Involvement of Rho Proteins

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SUMMARY

G protein-coupled receptors activate phospholipase C (PLC)-β isoforms by the α or $\beta\gamma$ subunits of G proteins, whereas growth-factor receptors activate PLC-γ isoforms by phosphorylating tyrosine residues of the enzyme. As a common substrate for PLC enzymes, phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2] may play a pivotal role in the regulation of cellular PLC activity. Because small-molecular-weight G proteins have been implicated in the synthesis of PtdIns(4,5)P_2, we studied the effect of *Clostridium difficile* toxin B, which glucosylates and thereby inactivates small G proteins of the Rho family, on receptor-stimulated PLC activity. We report here that in N1E-115 neuroblastoma cells, stimulation of inositol phosphate formation by the G protein-coupled receptor agonists bradykinin and lysophosphatidic acid and by the tyrosine kinase receptor agonist platelet-derived growth factor is largely attenuated by toxin B treatment. Furthermore, inositol phosphate production stimulated by the stable GTP analog guanosine 5′-*O*-(3-thio)-triphosphate in permeabilized N1E-115 cells was inhibited by C3 exoenzyme, which specifically inactivates Rho proteins. The inhibition by toxin B was apparently not caused by its effect on the cytoskeleton. In addition, the level of platelet-derived growth factor receptors, which was studied with immunoblotting, was unaffected by toxin B. Using exogenous PtdIns(4,5)P_2 as PLC substrate, it was found that the intrinsic enzymatic activity of PLC activated either by Ca^{2+} or by guanosine 5′-*O*-(3-thio)triphosphate was not altered by toxin B. However, toxin B decreased strongly, by up to 80%, the cellular level of PtdIns(4,5)P_2 in a concentration-dependent manner, without changing those of phosphatidylinositol and phosphatidylinositol 4-phosphate. These results, together with the recent finding that Rho family proteins can regulate phosphatidylinositol 4-phosphate 5-kinase activity, demonstrate that Rho proteins are presumably important regulators of PtdIns(4,5)P_2 synthesis and, thereby, play an integral role in the regulation of cellular signaling by PLC enzymes.

A great number of cellular responses to activation of membrane receptors use PLC in their signal transduction pathways. PLC hydrolyzes PtdIns(4,5)P_2, which results in production of inositol 1,4,5-trisphosphate and diacylglycerol, which in turn mobilizes intracellular calcium and activates protein kinase C, respectively. At least 10 distinct PLC isoforms, including 4 PLC-β, 2 PLC-γ, and 4 PLC-δ isozymes, have been characterized with biochemical and molecular biological approaches (1). Receptors that are coupled to heterotrimeric G proteins activate PLC by the α or $\beta\gamma$ subunits of certain G proteins. The isoforms of PLC activated by G protein-coupled receptors are the PLC-β isozymes. Growth factor receptors that possess intrinsic tyrosine kinase activity activate PLC-γ isoforms by phosphorylation on tyrosine residues of the enzyme (1, 2).

Although activation of PLC by receptors has been well studied, the regulatory processes involved in PLC activation in intact cells are far less understood. There is increasing evidence for the hypothesis that the supply of PtdIns(4,5)P_2 is a limiting factor for inositol phosphate production in intact cells. A PtdIns transfer protein that is involved in the transport of PtdIns from intracellular compartments to the plasma membrane for conversion to PtdIns(4,5)P_2 was recently shown to dictate the production of inositol trisphosphate through the effect on PtdIns(4,5)P_2 synthesis (3). By analogy to transport, the synthesis of PtdIns(4,5)P_2 should also have bearing on PLC activation. One would expect impairment of inositol phosphate formation induced by either G protein-

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ABBREVIATIONS: PLC, phospholipase C; GTPγS, guanosine 5′-*O*-(3-thio)triphosphate; HBSS, Hanks' balanced salt solution; LPA, lysophosphatidic acid; PDGF, platelet-derived growth factor; PtdIns, phosphatidylinositol; PtdIns4P, phosphatidylinositol 4-phosphate; PtdIns(4,5)P_2, phosphatidylinositol 4,5-bisphosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

coupled receptors or tyrosine kinase receptors whenever there are dysfunctions in the transporting and/or synthesizing systems of PtdIns(4,5)P_2, the common substrate for the different PLC isoenzymes.

PtdIns4P 5-kinase is a key enzyme in the formation of PtdIns(4,5)P_2; however, the mechanisms involved in the regulation of this kinase remain largely unclear (4). The activity of PtdIns4P 5-kinase has been shown to be regulated by GTP analogs (5), and the G proteins involved were later demonstrated to be small-molecular-weight G proteins with a molecular mass of 20 to 25 kDa (6). Recently, evidence was provided that the activity of PtdIns4P 5-kinase is stimulated by members of the Rho family of small molecular weight G proteins (7, 8). Rho proteins were first identified as essential regulators of cytoskeleton functions [e.g., formation of actin stress fibers and focal adhesion (9–11)]. However, increasing evidence suggests that the functions of Rho proteins are not restricted to cytoskeleton regulation. For example, Rho has been reported to activate phosphatidylinositide 3-kinase (12). A recent study in our laboratory showed that inactivation of Rho proteins greatly decreased receptor- and G protein-mediated stimulation of phospholipase D in human embryonic kidney cells, possibly by a decrease in cellular PtdIns(4,5)P_2 level (13), as demonstrated in mouse fibroblasts (7). To study whether Rho proteins are involved in the regulation of PLC by G protein-coupled and tyrosine kinase receptors, we investigated, in the present study, the effects of *Clostridium difficile* toxin B, a large peptide toxin (with a molecular mass of about 270 kDa) that can be taken up by cells by receptor-mediated endocytosis and that specifically glucosylates and thereby inactivates Rho-family proteins (14, 15), on inositol phosphate formation induced by activation of these receptors. The results show that toxin B efficiently inhibits inositol phosphate production stimulated by either G protein-coupled or tyrosine kinase receptors in N1E-115 neuroblastoma cells, presumably by reducing PtdIns(4,5)P_2 supply.

Experimental Procedures

Materials. *myo*-[^{3}H]inositol (24.4 Ci/mmol) and [^{3}H]PtdIns(4,5)P_2 (1–5 Ci/mmol) were purchased from Biotrend (Köhn, Germany). Unlabeled phosphoinositides, phosphatidylethanolamine, bradykinin, LPA, PDGF, and cytochalasin B were obtained from Sigma Chemical (Poole, Dorset, UK). GTPγS was purchased from Boehringer Mannheim (Mannheim, Germany). *C. difficile* toxin B and *Clostridium botulinum* C3 exoenzyme were purified as described (16, 17). Anti-PDGF-β receptor antibody was obtained from Affiniti (Exeter, UK). All other materials were from previously described sources (13, 18, 19).

Cell culture and toxin B treatment. N1E-115 neuroblastoma cells of passages 19–29 were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 100 units/ml penicillin G, and 100 μg/ml streptomycin in 5% CO_2. For experiments, cells subcultured in Dulbecco's modified Eagle's medium were grown to near confluence (150-mm or 35-mm culture dishes). For toxin B treatment, toxin B was first diluted with phosphate-buffered saline containing 1 mg/ml bovine serum albumin and then added to cell culture medium at the indicated final concentrations. Cells were exposed to toxin B-containing medium for 24 hr before experiments. Control cells received medium containing bovine serum albumin (1 mg/ml) only. The effectiveness of toxin B treatment was checked by monitoring the morphological changes (i.e., rounding-up of cells after treatment with the toxin).

Inositol phosphate formation in intact cells. Cells were labeled by incubating with medium containing *myo*-[^{3}H]inositol (1.5 μCi/ml) for 48 hr. Thereafter, the labeling medium was replaced with medium containing toxin B for 24 hr. The cells were then incubated twice for 10 min at 37° with HBSS that contained 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM D-glucose, and 15 mM HEPES, pH 7.4, supplemented with 10 mM LiCl. The drugs that were used to stimulate PLC activity were applied by incubating cells with drug-containing HBSS for 30 min at 37°. Reaction was stopped by sucking away the HBSS and adding 0.5 ml ice-cold methanol to dishes. Cells were harvested, and [^{3}H]inositol phosphates were extracted and analyzed as described before (18, 19). Protein content of dishes was measured in separate dishes by the method of Lowry, using bovine serum albumin as standard. [^{3}H]Inositol phosphate formation is expressed as cpm/mg of protein or as percent increase over basal levels.

PLC activity assay with exogenous substrate. A set of experiments was performed in which PLC activity on exogenous substrate was measured with lysates of toxin B-treated and untreated N1E-115 cells. Toxin B treatment was as described above. Cells were harvested and resuspended in buffer (135 mM KCl, 5 mM $NaHCO_3$, 5 mM EGTA, 5.6 mM D-glucose, 13.6 mM $MgCl_2$, 2 mM ATP, 10 mM LiCl, and 20 mM HEPES, pH 7.2) and then homogenized in a glass-Teflon homogenizer. Mixed lipid vesicles that contained phosphatidylethanolamine and [^{3}H]PtdIns(4,5)P_2 in a molar ratio of 2:1 were prepared by sonification in 50 mM HEPES, pH 7.0, 150 mM NaCl, and 2 mM sodium deoxycholate (20). Assays were performed for 15 min at 37° in a total volume of 70 μl, which contained neuroblastoma cell lysate (5 μg of protein), 50 μM [^{3}H]PtdIns(4,5)P_2, 100 μM phosphatidylethanolamine, 1 mM deoxycholate, 10 mM LiCl, and $CaCl_2$ to give the indicated free Ca^{2+} concentration. After stopping the reactions and phase separation, [^{3}H]inositol phosphate formation was determined in aliquots of the aqueous upper phase by liquid scintillation counting as described previously (20).

Inositol phosphate formation in digitonin-permeabilized cells. In another set of experiments, the effect of C3 exoenzyme was tested on GTPγS-stimulated PLC activity in permeabilized N1E-115 cells. For this, cells prelabeled with *myo*-[^{3}H]inositol were suspended in the buffer described in the preceding paragraph, containing 40 nM free Ca^{2+}. Assays were performed for 60 min at 37° in a total volume of 100 μl containing 1×10^6 cells, 10 μM digitonin, 50 μM NAD with and without 12 μg/ml C3 exoenzyme, and GTPγS at the indicated concentrations (see fig. 4). After stopping the reactions by adding 2 ml of chloroform:methanol (1:1, v/v), [^{3}H]inositol phosphate formation was analyzed as described above.

Analysis of cellular phosphoinositides. Cells were cultured to 90% confluence in 150-mm culture dishes. Cell labeling and toxin B treatment were the same as described above. Control and toxin B-treated cells were washed with HBSS that contained 10 mM LiCl and were incubated with the same buffer for 10 min at 37°. Thereafter, cells were harvested with 2.5 N HCl, and phase separation was performed with chloroform:methanol:concentrated HCl (200:100:0.75, v/v/v) by centrifugation for 10 min at 2,000 × *g*. An aliquot of the lipid phase (25,000 cpm) was applied onto oxalate-impregnated silica gel 60 plates (E. Merck, Darmstadt, Germany), and the plates were developed in chloroform:methanol:2.5 N ammonium hydroxide (9:7:2, v/v/v) (7). Lipids were localized by iodine staining and identified by co-migration with authentic standards. The areas corresponding to PtdIns (R_f = 0.64), PtdIns4P (R_f = 0.45) and PtdIns(4,5)P_2 (R_f = 0.25) were scraped into scintillation vials, and the radioactivity was measured by liquid scintillation counting.

Western blotting. Cells were cultured in 60-mm culture dishes to subconfluence and then serum-fasted for 3 days. Toxin B (100 pg/ml) was added for the last 24 hr. Culture dishes were washed twice and then incubated at 37° for 30 min with Hanks' buffer (138 mM NaCl, 6 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM Na_2HPO_4, 5 mM $NaHCO_3$, 5.5 D-glucose, and 20 mM HEPES, pH 7.4). Cells were harvested with 0.3 ml radioimmunoprecipitation assay cell lysis buffer (20 mM

Na_3VO_4, 100 μM phenylmethylsulfonyl fluoride, 20 μg/ml aprotinin, 20 μg/ml leupeptin, and 250 μg/ml *p*-nitrophenylphosphate) and then mixed with 0.15 ml of Laemmli gel running buffer. Protein was denatured by heating to 95° for 5 min. One hundred micrograms of protein was subjected to 6% sodium dodecyl sulfate polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membrane and visualized with Ponceau S dye solution. The membrane was then blotted with monoclonal antibodies against the PDGF-β receptor using standard procedures.

Data presentation. Results reported in the text are mean ± standard error; each independent experiment was performed in triplicate. Comparisons between means were made with either the Student *t* test or one-way analysis of variance test, whichever was appropriate. A difference was regarded as significant when $p < 0.05$.

Results

Inhibition by toxin B of PLC activation by G protein-coupled and PDGF receptors. Bradykinin and LPA, two agonists known to act on G protein-coupled receptors, and PDGF, a tyrosine kinase receptor agonist, stimulated PLC activity in N1E-115 neuroblastoma cells (Fig. 1). At the concentration of 1 μM, bradykinin and LPA increased inositol phosphate production by 82 ± 9% (four experiments) and 93 ± 22% (five experiments), respectively. An even larger increase in inositol phosphate production (252 ± 45%, five experiments) was observed upon stimulation of N1E-115 cells with 25 ng/ml PDGF. Treatment of the cells with 100 pg/ml toxin B for 24 hr greatly reduced the stimulatory effects of all three agonists studied. The increases in inositol phosphate production induced by bradykinin, LPA, and PDGF in toxin B-treated cells were only 22 ± 7% ($p < 0.01$ compared with control, untreated cells), 16 ± 6% ($p < 0.01$) and 20 ± 9% ($p < 0.001$), respectively. The basal inositol phosphate accumulation was not altered by toxin B treatment. The [^{3}H]inositol phosphate levels were 8909 ± 1014 and 8712 ± 1076 cpm/mg of protein in untreated and toxin B-treated cells, respectively ($p > 0.05$). As seen in Fig. 2, the PDGF concentration-response curve was strongly depressed by toxin B treatment. At the concentrations of 10, 25, and 50 ng/ml PDGF, production of inositol phosphates was significantly lower in toxin B-treated cells than in control, untreated cells.

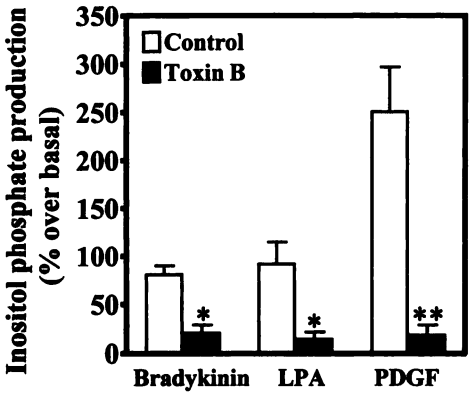

Fig. 1. Inhibition of receptor-stimulated inositol phosphate production by *C. difficile* toxin B. Cultured N1E-115 neuroblastoma cells were labeled with *myo*-[^{3}H]inositol for 48 hr, followed by 24 hr treatment without (*open bars*) and with 100 pg/ml toxin B (*filled bars*). Then, production of [^{3}H]inositol phosphates stimulated with either bradykinin (1 μM), LPA (1 μM), or PDGF (25 ng/ml) was measured as described in Experimental Procedures. Data reported are increases over basal levels of [^{3}H]inositol phosphate production (see text). Data are from 4–5 independent experiments, each performed in triplicate. *, $p < 0.01$; **, $p < 0.001$ compared with untreated cells.

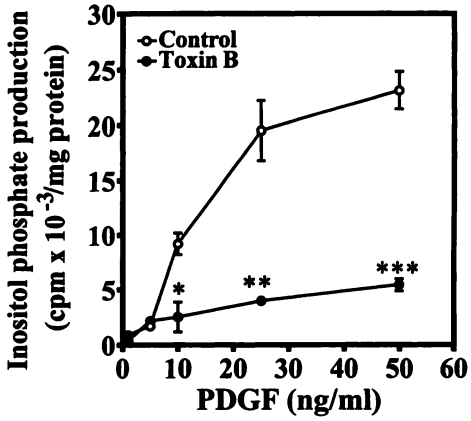

Fig. 2. Concentration-response relationship of PDGF-induced increase in inositol phosphate production in control and toxin B-treated cells. Labeling of N1E-115 neuroblastoma cells, toxin B treatment, and measurement of [^{3}H]inositol phosphate production were carried out as in Fig. 1. Increases in [^{3}H]inositol phosphate production over basal levels are indicated on the ordinate. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with untreated cells.

As with PDGF, inhibition of inositol phosphate production by toxin B was concentration-dependent. When used at a low (1 pg/ml) and high (100 pg/ml) toxin B concentration, which resulted in 53% and 77% reduction in cellular PtdIns(4,5)P_2 levels, respectively (see below), inositol phosphate production stimulated by PDGF (25 ng/ml) was reduced from 216 ± 13% in controls to 75 ± 4% and 48 ± 5% (mean ± standard error) in cells treated with 1 and 100 pg/ml toxin B, respectively.

Toxin B treatment of N1E-115 cells induced typical changes in cell morphology [i.e., cell rounding-up, as described for other cell types (14, 15)]. To test whether the effect of toxin B on PLC activation was a result of cytoskeleton disruption, we examined the effect of cytochalasin B, an agent that causes cytoskeleton disruption without involving Rho proteins, on PLC activation. Cytochalasin B (5 μg/ml) treatment of N1E-115 cells for 15 min produced similar morphological changes as toxin B (data not shown). It had no effect, however, on PLC activation induced by any of the three agonists studied. The increases in inositol phosphate production induced by bradykinin, LPA (both at 1 μM) and PDGF (25 ng/ml) were 89 ± 11%, 154 ± 5%, and 159 ± 26%, respectively, in control cells, and 106 ± 7%, 127 ± 5%, and 170 ± 7%, respectively, in cytochalasin B-treated cells ($p > 0.05$ for all three agonists, three experiments). These results rule out the possibility that the inhibitory effect of toxin B on PLC activation was related to its effect on the cytoskeleton.

Inhibition of G protein-stimulated PLC activity by C3 exoenzyme. To substantiate the involvement of Rho proteins in PLC activation, we studied whether C3 exoenzyme, which ADP-ribosylates and thereby inactivates Rho proteins (9, 10, 16), had effects on PLC activation similar to those of toxin B. For this, PLC activation by GTPγS was studied in digitonin-permeabilized N1E-115 cells with and without C3 exoenzyme (12 μg/ml) (Fig. 3). C3 exoenzyme significantly decreased ($p < 0.01$, three experiments), by 40–50%, PLC activation that had been induced by GTPγS at all concentrations used (10, 50, and 100 μM). Basal inositol

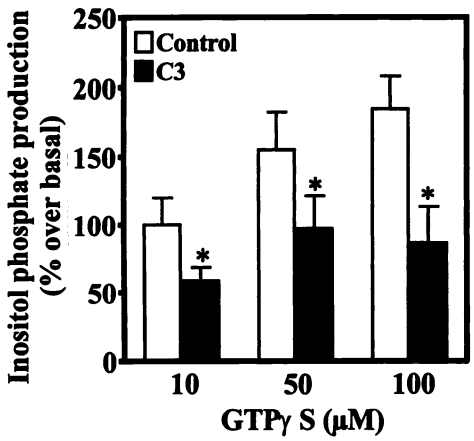

Fig. 3. Influence of C3 exoenzyme on G protein-stimulated inositol phosphate formation. Formation of [^{3}H]inositol phosphates was measured in digitonin-permeabilized N1E-115 cells prelabeled with *myo*-[^{3}H]inositol in the absence (*open bars*) and presence of 12 μg/ml C3 exoenzyme (*filled bars*) at the indicated concentrations of GTPγS as described in Experimental Procedures. *, $p < 0.01$ compared with control cells.

phosphate production was not altered by C3 exoenzyme (data not shown). These results suggest that the inhibitory effect of toxin B on receptor-stimulated inositol phosphate formation in intact cells involves Rho proteins.

Lack of toxin B effects on the intrinsic PLC activity and the PDGF receptor level. To further investigate the potential mechanism of the inhibitory effect of toxin B on PLC activation, we tested whether the intrinsic enzymatic activity of PLC was affected by toxin B treatment. This was performed in N1E-115 cell lysates. As shown in Fig. 4, PLC activities on exogenous PtdIns(4,5)P_2 induced either by Ca^{2+} or by GTPγS were similar in control lysates and in lysates of cells pretreated with 100 pg/ml toxin B for 24 hr. There were no significant differences ($p > 0.05$, three experiments) in PLC activities measured at low (10 nM) or high (100 nM) Ca^{2+} concentration between control and toxin B-treated cell lysates (Fig. 4A). Similarly, PLC activity on exogenous PtdIns(4,5)P_2 stimulated by 100 μM GTPγS was not significantly ($p > 0.05$, three experiments) affected by prior toxin B treatment (Fig. 4B). These results show clearly that the inhibitory effect of toxin B on agonist-induced PLC activity observed in intact N1E-115 cells was not caused by changes in the intrinsic enzymatic activity of the PLC isoforms.

Additionally, we studied whether the effect of toxin B on PLC activation was caused by potential loss of membrane receptors. For this, the level of PDGF receptor was measured in control and toxin B-treated cells with monoclonal antibodies against PDGF-β receptors. Fig. 5 shows the typical blot from one of four such experiments, in which the PDGF receptor signal was not different between control cells and cells treated with toxin B (100 pg/ml, 24 hr).

Reduction in PtdIns(4,5)P[infib]2 level by toxin B. Finally, we studied the effect of toxin B on cellular phosphoinositide levels. For this, N1E-115 cells prelabeled with *myo*-[^{3}H]inositol for 48 hr were treated with various concentrations of toxin B. As seen in Fig. 6, toxin B treatment, even at the highest concentration studied (500 pg/ml), did not alter the levels of PtdIns and PtdIns4P. However, the level of PtdIns(4,5)P_2 was decreased in a concentration-dependent manner by toxin B and amounted to only ~20% of control in cells treated with 100–500 pg/ml toxin B. Because PtdIns(4,5)P_2 is a common substrate for PLC isozymes, these results suggest that the effect of toxin B on inositol phosphate formation induced by different agonists results from inhibition of PtdIns(4,5)P_2 production.

Discussion

The main finding of the present study is that inactivation of small G proteins of the Rho family by *C. difficile* toxin B efficiently inhibits PLC-catalyzed inositol phosphate formation stimulated by different cell membrane receptors that either activate heterotrimeric G proteins or possess intrinsic tyrosine kinase activity. Thus, it seems reasonable to conclude that the effect of toxin B is at some common site for PLC activation by different mechanisms, probably downstream of the different receptors. This conclusion is supported by the lack of toxin B effect on the level of PDGF receptors and on the intrinsic enzymatic activity of PLC isoforms in the presence of exogenous substrate; the latter fact clearly rules out the possibility that the toxin B effect is on the enzyme itself. Thus, we suggest that the effect of toxin B is on the synthesis and, thereby, the supply of the substrate PtdIns(4,5)P_2 for PLC because of inactivation of Rho family proteins. This hypothesis is based on the following findings: First, toxin B specifically inactivates the Rho family (Rho, Rac, and Cdc42) of small G proteins (14, 15). Second, Rho family proteins bind phosphoinositide kinases (21) and stim-

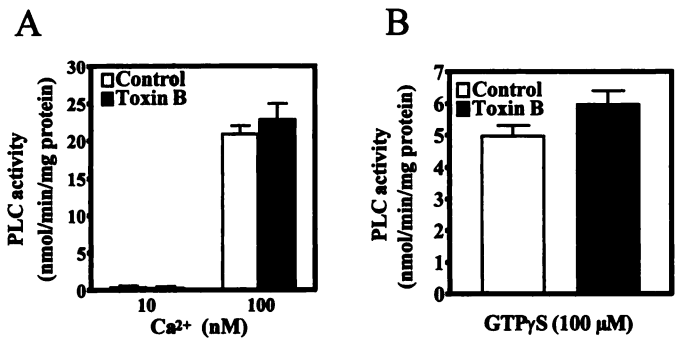

Fig. 4. Lack of toxin B effect on PLC activity measured with exogenous PtdIns(4,5)P_2 as enzyme substrate. Lysates of N1E-115 neuroblastoma cells were prepared from control cells (*open bars*) and cells treated for 24 hr with 100 pg/ml toxin B (*filled bars*). PLC activity of these lysates was measured with 50 μM [^{3}H]PtdIns(4,5)P_2 as enzyme substrate in the presence of 10 or 100 nM free Ca^{2+} (A) or 100 μM GTPγS (B) as described in Experimental Procedures. In B, basal activity measured with 40 nM free Ca^{2+} is subtracted.

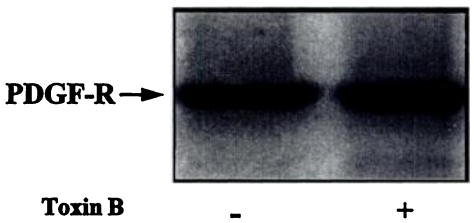

Fig. 5. Lack of toxin B effect on PDGF receptor level. Subconfluent N1E-115 neuroblastoma cells were serum-fasted for 3 days and treated for the last 24 hr without and with 100 pg/ml toxin B. After cell lysis, proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and blotted with the anti-PDGF-β receptor monoclonal antibody as described in Experimental Procedures. The signal levels of PDGF receptors (PDGF-R) were not different between control and toxin B-treated cells.

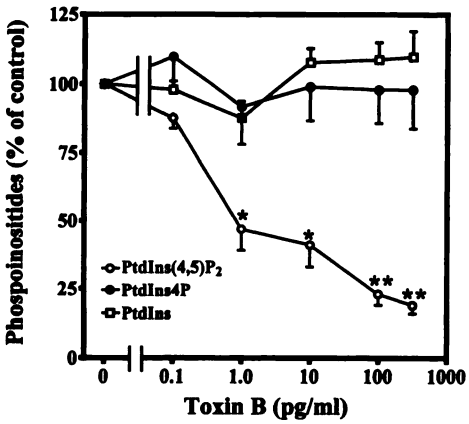

Fig. 6. Influence of toxin B on the cellular level of phosphoinositides. The levels of [^{3}H]PtdIns, [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 were determined in *myo*-[^{3}H]inositol-prelabeled N1E-115 neuroblastoma cells pretreated or not for 24 hr with toxin B at the indicated concentrations as described in Experimental Procedures. Levels of PtdIns, PtdIns4P and PtdIns(4,5)P_2 in toxin B-treated cells are expressed as % of those in control cells. In control cells, the radioactivity associated with [^{3}H]PtdIns, [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 was 5,866 ± 598, 232 ± 25, and 2,515 ± 251 cpm (three experiments), respectively, from a total of 25,000 cpm [^{3}H]phosphoinositides analyzed. *, $p < 0.05$; **, $p < 0.01$ compared with untreated cells.

ulate production of PtdIns(4,5)P_2 from PtdIns4P by PtdIns4P 5-kinase (7, 8). Third, toxin B specifically reduced the level of PtdIns(4,5)P_2 in N1E-115 cells, which correlated with the inhibition of stimulated inositol phosphate production. Finally, C3 exoenzyme that has been well established to specifically inactivate Rho proteins (RhoA, RhoB, and RhoC) efficiently inhibited PLC activation by GTPγS, which supports the involvement of Rho proteins in the effect of toxin B.

Rho proteins participate in the regulation of actin cytoskeleton functions (11). The effect of toxin B on PLC activation observed in the present study, however, is unlikely to be related to its effect on cytoskeleton, because the cytoskeleton-disrupting agent cytochalasin B had no effect on PLC activation, although it had effects on cell morphology similar to those of toxin B. The morphological changes observed are more likely to have resulted from dysfunctions of actin cytoskeleton after impaired phospholipid signaling, because phosphoinositide kinases and PtdIns(4,5)P_2 have been implicated in normal cytoskeleton functions (22). PtdIns4P and PtdIns(4,5)P_2, formed by phosphoinositide kinases, were found to bind to α-actinin and vinculin, and the levels of bound PtdIns(4,5)P_2 changed in response to PDGF stimulation in Balb/c 3T3 cells (23). Indeed, PDGF is known to induce changes in cell motility by acting on the cytoskeleton (24).

It was recently reported that Rho associates with ligand-activated PDGF type-β receptors in Balb/c-3T3 cells (25). The functional meaning of such an association, however, has been elusive. Whether the association results in activation of the Rho protein and what the components are downstream of the Rho protein are also unknown. Activation of PLC-γ by PDGF receptors depends on autophosphorylation of the receptor and the subsequent association of PLC isoforms with the receptor by virtue of the src homology (SH2) domains contained in PLC molecules (26). It has also been shown that certain signal transduction pathways initiated by PDGF receptor activation might be independent of receptor autophosphorylation (27). Taking the complexities of the signaling pathways of PDGF receptors into account, it would be of great importance to investigate the potential role of Rho proteins in PDGF receptor signaling. We are currently investigating whether the influence of toxin B, presumably by inactivating Rho proteins, on inositol phosphate formation induced by PDGF observed in the present study is related to the association of the Rho protein with PDGF receptors.

In addition to serving as a substrate for PLC and regulating cytoskeleton functions as mentioned above, PtdIns(4,5)P_2 may have other functions (1). It also serves as a substrate for phosphatidylinositide 3-kinase. Phosphatidylinositide 3-kinase has been demonstrated to be a signal transduction molecule downstream PDGF receptors (28). Evidence was recently obtained that PtdIns(4,5)P_2 also binds plekstrin homology domains and thus provides a membrane docking site for plekstrin homology-containing proteins (29). Finally, PtdIns(4,5)P_2 was found to play an important cofactor role in activation of phospholipase D (30). Thus, the broad spectrum of the biological functions of PtdIns(4,5)P_2 implicates that the control of PtdIns(4,5)P_2 synthesis by the Rho family of G proteins may have biological significance not only in the context of PLC but also in the regulation of other cellular functions in which PtdIns(4,5)P_2 is involved. Therefore, the *C. difficile* toxin B apparently provides a very useful pharmacological tool for studies related to Rho proteins and, thereby, the cellular function of PtdIns(4,5)P_2.

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