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A role for Rho in receptor- and G protein-stimulated phospholipase C

Reduction in phosphatidylinositol 4,5-bisphosphate by *Clostridium difficile* toxin B

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Abstract Receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins) activate phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2)-hydrolyzing phospholipase C (PLC) enzymes by activated α or free $\beta\gamma$ subunits of the relevant G proteins. To study whether low molecular weight G proteins of the Rho family are involved in receptor signalling to PLC, we examined the effect of *Clostridium difficile* toxin B, which glucosylates and thereby inactivates Rho proteins, on the regulation of PLC activity in human embryonic kidney (HEK) cells stably expressing the m3 muscarinic acetylcholine receptor (mAChR) subtype. Toxin B treatment of HEK cells did not affect basal PLC activity, but potently and efficiently inhibited mAChR-stimulated inositol phosphate formation. PLC activation by the endogenously expressed thrombin receptor and by the direct G protein activators, AlF_4^- and guanosine 5′-[γ-thio]triphosphate (GTPγS), studied in intact and permeabilized cells, respectively, were also inhibited by toxin B treatment. C3 exoenzyme, which ADP-ribosylates Rho proteins, mimicked the inhibitory effect of toxin B on GTPγS-stimulated PLC activity. Finally, both toxin B and C3 exoenzyme significantly reduced, by 40 to 50%, the total level of PtdIns(4,5)P_2 in HEK cells, without affecting the levels of phosphatidylinositol and phosphatidylinositol 4-phosphate. Accordingly, when PLC activity was measured with exogenous PtdIns(4,5)P_2 as enzyme substrate, Ca^{2+}- as well as GTPγS- or AlF_4^--stimulated PLC activities were not altered by prior toxin B treatment. In conclusion, evidence is provided that toxin B and C3 exoenzyme, apparently by inactivating Rho proteins, inhibit G protein-coupled receptor signalling to PLC, most likely by reducing the cellular substrate supply.

Key words Muscarinic receptor · Phospholipase C · Rho · *Clostridium difficile* toxin B · C3 exoenzyme · Phosphatidylinositol 4,5-bisphosphate

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Introduction

Hydrolysis of phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P_2) by phospholipase C (PLC), generating the two second messengers, inositol 1,4,5-trisphosphate ($InsP_3$) and diacylglycerol, is one of the major signalling pathways of cell surface receptors (for recent reviews see Berridge 1993; Divecha and Irvine 1995). Among the receptors linked to PLC stimulation, the seven transmembrane helix receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins) constitute the largest group. These G protein-coupled receptors stimulate PLC isozymes of the PLC-β subtype, apparently by activated α subunits of the pertussis toxin-insensitive G_q class of G proteins or by free $\beta\gamma$ dimers of pertussis toxin-sensitive G_i proteins (for recent reviews see Smrcka and Sternweis 1993; Lee and Rhee 1995). Reconstitution of purified proteins supports the general assumption that only three components are required for efficient PLC activation by G protein-coupled receptors, the receptor, the relevant heterotrimeric G protein and a PLC-β isozyme (Berstein et al. 1992).

In intact cells, however, receptor signalling to PLC appears to be more complex, particularly considering the supply of the PLC substrate, PtdIns(4,5)P_2. This highly acidic phosphoinositide constitutes even in resting cells only a very minor part of membrane phospholipids and also of total phosphoinositides (Carpenter and Cantley 1996; Divecha and Irvine 1995; Lee and Rhee 1995). Upon cell stimulation, the turnover of PtdIns(4,5)P_2 can

rise dramatically (Divecha and Irvine 1995; Lee and Rhee 1995), suggesting that an unopposed phosphoinositide synthesis is needed and essential to maintain efficient PtdIns(4,5)P_2-dependent signalling pathways. Indeed, two proteins involved in the (re)synthesis of PtdIns(4,5)P_2, namely CDP-diacylglycerol synthase and phosphatidylinositol (PtdIns) transfer protein, have recently been shown to be indispensable for PLC activation by G protein-coupled receptors in intact cells (Cunningham et al. 1995; Wu et al. 1995).

Activated RhoA, a Ras-related low molecular weight G protein, has recently been reported to stimulate synthesis of PtdIns(4,5)P_2 in mouse fibroblast cells (Chong et al. 1994). Inactivation of Rho proteins by microinjected C3 exoenzyme, which specifically ADP-ribosylates Rho proteins (Aktories and Just 1993), blunted the calcium mobilization in response to thrombin and platelet-derived growth factor (Chong et al. 1994). Furthermore, constitutively active Rac1, another member of the Rho protein family, has recently been demonstrated to stimulate PtdIns(4,5)P_2 synthesis in permeabilized human platelets (Hartwig et al. 1995). These data raise the possibility that G proteins of the Rho family are involved in PLC signalling. Therefore, in the present study, we examined the effects of toxin B of *Clostridium difficile* (von Eichel-Streiber et al. 1987, 1992) on cellular phosphoinositide level and PLC-catalyzed inositol phosphate formation. This toxin, which in contrast to C3 exoenzyme readily enters intact cells (Ciesielski-Treska et al. 1989), has recently been shown to specifically inactivate Rho family G proteins by a distinct covalent modification, i.e., monoglucosylation, finally resulting in disorganisation of the cytoskeleton (Just et al. 1994, 1995a). As cellular model, we used human embryonic kidney (HEK) cells stably expressing the human m3 muscarinic acetylcholine receptor (mAChR) subtype and exhibiting a robust PLC response (Peralta et al. 1988; Schmidt et al. 1994, 1995b). The m3 mAChR activates in these cells $G_{q/11}$ proteins and stimulates PLC in a pertussis toxin-insensitive manner (Offermanns et al. 1994). We recently reported that toxin B inhibits m3 mAChR signalling to phospholipase D (PLD) in these cells (Schmidt et al. 1996). We demonstrate here that toxin B treatment decreases the cellular level of PtdIns(4,5)P_2 and inhibits receptor- and G protein-stimulated inositol phosphate formation in HEK cells and that this inhibitory effect is mimicked by C3 exoenzyme.

Materials and methods

Materials. UDP-[^{14}C]glucose (300 mCi/mmol) was from DuPont-New England Nuclear and *myo*-[^{3}H]inositol (24.4 Ci/mmol) from BIOTREND. Unlabeled phosphoinositides, phosphatidylethanolamine and thrombin were from Sigma. *Clostridium difficile* toxin B was purified as described (von Eichel-Streiber et al. 1987). All other materials were from previously described sources (Schmidt et al. 1994, 1995a, 1995b, 1996; Just et al. 1994).

Cell culture. Culture conditions of HEK cells stably expressing human mAChRs were as reported in detail before (Schmidt et al. 1994; Offermanns et al. 1994). For experiments, cells subcultured in DMEM/F-12 medium were grown to near confluence (175 cm^2 culture flasks).

Toxin B treatment and assay of PLC activity in intact cells. For measurements of intact cell PLC activity, cellular phospholipids were labeled by incubating nearly confluent monolayers of cells for 20–24 h with [^{3}H]inositol (1 μCi/ml) in growth medium. Then, the labeling medium was replaced before the cells were treated for 24 h or the indicated periods of time without and with toxin B at the indicated concentrations. Thereafter, the cells were detached from the culture flasks, resuspended twice in Hank's balanced salt solution, containing 118 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM D-glucose, buffered at pH 7.4 with 15 mM HEPES, at a cell concentration of approximately 1×10^7 cells/ml. The PLC assays were carried out with 1×10^6 cells for 60 min at 37°C ([^{3}H]inositol phosphates) or for 15 s at 37°C ([^{3}H]InsP_3) in a total volume of 200 μl containing 10 mM LiCl and the indicated stimulatory agents. Stop of the reactions, extraction and analysis of [^{3}H]inositol phosphates were carried out as described before (Schmidt et al. 1994, 1995a). Formation of [^{3}H]inositol phosphates and [^{3}H]InsP_3 is given as cpm per 10^6 cells.

Assay of PLC activity in permeabilized cells. Prior to permeabilization, [^{3}H]inositol-prelabeled cells were treated for 24 h without and with toxin B at the indicated concentrations. Thereafter, the cells were detached from the culture flasks, pelleted by centrifugation and resuspended in buffer A, containing 135 mM KCl, 5 mM $NaHCO_3$, 5 mM EGTA, 4 mM $MgCl_2$, 2 mM ATP, 1.5 mM $CaCl_2$ (corresponding to 40 nM free Ca^{2+}), 5.6 mM D-glucose and 20 mM HEPES, pH 7.2, at a cell concentration of 1×10^7 cells/ml. The assays of PLC activity were carried out for 60 min at 37°C in a total volume of 200 μl, containing 100 μl of the cell supension (1×10^6 cells), 10 μM digitonin, 10 mM LiCl and the indicated stimulatory agents. Effects of C3 exoenzyme on PLC activities in digitonin-permeabilized cells were assayed in the same buffer as above, containing additionally 50 μM NAD without and with 12 μg/ml C3 exoenzyme.

PLC activity assay with exogenous substrate. Mixed phospholipid vesicles containing phosphatidylethanolamine and [^{3}H]PtdIns(4,5)P_2 in a molar ratio of 2:1 were dried and resuspended in 50 mM HEPES, 150 mM NaCl, 2 mM sodium deoxycholate, pH 7.0, followed by sonication on ice (Camps et al. 1990). Unlabeled HEK cells pretreated or not with toxin B were detached from the culture flasks, resuspended in buffer A, and homogenized in a glass Teflon homogenizer. Assays were performed for 15 min at 37°C in a total volume of 70 μl containing HEK cell lysate (5 μg protein), 50 μM [^{3}H]PtdIns(4,5)P_2 (10,000 cpm), 100 μM phosphatidylethanolamine, 1 mM deoxycholate, 10 mM LiCl and the indicated stimulatory agents in buffer A. After stop of the reactions and phase separation, [^{3}H]inositol phosphate accumulation was determined in aliquots of the aqueous upper phase by liquid scintillation counting as described before (Camps et al. 1990).

Phosphoinositide analysis. Cellular phosphoinositides were labeled with [^{3}H]inositol, followed by treatment of intact cells for 24 h without and with toxin B (50 or 500 pg/ml) or of digitonin-permeabilized cells for 60 min without and with C3 exoenzyme (12 μg/ml) in the presence of 50 μM NAD, using the above mentioned incubation buffers. Thereafter, the phosphoinositides of approximately 6×10^7 cells were extracted with 3 ml 2.4 M HCl and 10 ml chloroform:methanol:concentrated HCl (200:100:0.75). After centrifugation, the lower phase containing the phosphoinositides was collected. There was no difference in total [^{3}H]inositol incorporation into phosphoinositides between control and toxin B- or C3 exoenzyme-treated cells. An aliquot of the lipid phase (100,000 cpm and 50,000 cpm from intact and permeabilized cells, respectively) was applied onto oxalate-impregnated silica gel 60 plates (E. Merck), and the plates were developed in chloroform:acetone:methanol:acetic acid:H_2O (40:15:13:12:8). Lipids were localized by iodine staining and identified by migration with authentic standards. The areas corresponding to the phosphoinositides, PtdIns (R_f = 0.47), phosphatidylinositol 4-phosphate (PtdIns4P, R_f = 0.37) and PtdIns(4,5)P_2 (R_f = 0.31), were

scraped into scintillation vials, and the radioactivity was measured by liquid scintillation counting.

Glucosylation of Rho proteins. [^{14}C]Glucosylation of Rho proteins in HEK cell lysates by toxin B (1 μg/ml) and 30 μM UDP-[^{14}C]glucose (500 nCi), and analysis of [^{14}C]glucosylated proteins by SDS-polyacrylamide gel electrophoresis and autoradiography were carried out as described before (Just et al. 1995a).

$[Ca^{2+}]_i$ measurements. Intracellular free Ca^{2+} ($[Ca^{2+}]_i$) was determined in m3 and m2 mAChR-expressing HEK cells with the fluorescent indicator dye Fura-2 in a Hitachi F2000 spectrofluorometer exactly as previously described (Schmidt et al. 1995a).

Data presentation. Data shown are mean±SD (n = 3) from one experiment, repeated as indicated in the figure legends.

Results

To study the effect of toxin B on PLC signalling, phosphoinositides of the m3 mAChR-expressing HEK cells were labeled to equilibrium with [^{3}H]inositol, followed by toxin B treatment and subsequent measurement of receptor-stimulated inositol phosphate formation. PLC stimulation induced by the mAChR agonist carbachol, used at a maximally effective concentration (1 mM), was inhibited by toxin B in a time- and concentration-dependent manner (Fig. 1). When pretreated with 10 pg/ml toxin B, the agonist response was reduced by 50% after about 20 h and by about 80% after 48 h treatment. When pretreated for 24 h with various toxin B concentrations, the carbachol-induced PLC stimulation was half-maximally attenuated at a toxin B concentration of about 3 pg/ml and reduced by about 80% at 30 pg/ml. At 500 pg/ml toxin, mAChR-stimulated inositol phosphate formation was reduced by 91±0.9% (mean ± SEM, n = 3 independent experiments, data not shown). Analysis of individual inositol phosphate species, particularly of the immediate PLC product, $InsP_3$, indicated that toxin B treatment inhibited the production of $InsP_3$ to a similar extent as that of total inositol phosphates. For example, after treatment with 50 pg/ml toxin B for 24 h, carbachol-stimulated $InsP_3$ accumulation, which was maximal after 15 s stimulation (Schmidt et al. 1995a), was reduced from an 120–150% increase in controls to only about 60% in cells pretreated with toxin B (Fig. 1C).

As reported before (Schmidt et al. 1996), treatment of HEK cells with toxin B potently induced rounding-up of the cells, indicating disruption of the actin cytoskeleton. However, similar as observed for m3 mAChR-stimulated PLD activity (Schmidt et al. 1996), treatment of HEK cells with cytochalasin B (5 μg/ml, 15 min) or *Clostridium botulinum* C2 toxin (20 ng/ml component I plus 40 ng/ml component II, 24 h), two agents also causing cytoskeleton disruption but not inactivating Rho proteins, had no effect on mAChR-stimulated inositol phosphate formation (data not shown). Additionally, toxin B treatment of HEK cells did not cause mAChR internalization or down-regulation and also did not interfere with receptor coupling to heterotrimeric G proteins (Schmidt et al. 1996). Furthermore, toxin B treatment did not only attenuate PLC stimulation

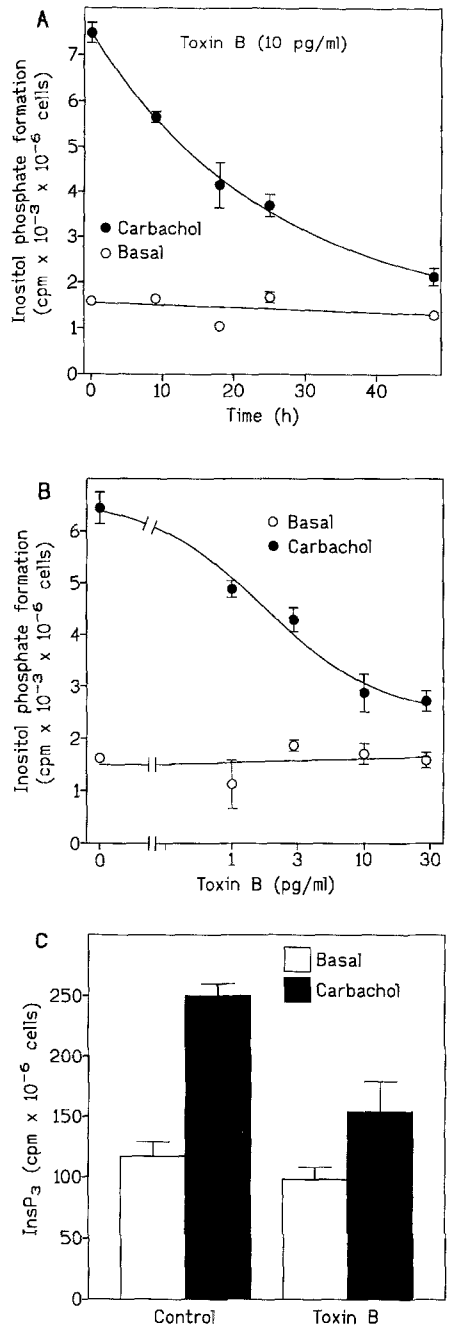

Fig. 1 Influence of toxin B on mAChR-stimulated PLC activity. Time course and concentration response studies. m3 mAChR-expressing HEK cells prelabeled with [^{3}H]inositol were pretreated with 10 pg/ml toxin B for the indicated periods of time (**A**) or for 24 h with the indicated concentrations of toxin B (**B**), followed by measurement of basal (○) and carbachol (1 mM)-stimulated (●) formation of [^{3}H]inositol phosphates as described in "Materials and methods". In **C**, basal and carbachol (1 mM)-stimulated [^{3}H]$InsP_3$ accumulation was studied for 15 s in control and toxin B-treated cells (50 pg/ml, 24 h). Data are representative of three separate experiments

by agonist-activated m3 mAChR. Stimulation of inositol phosphate formation by the thrombin receptor endogenously expressed in HEK cells as well as by the m2 mAChR also stably expressed in HEK cells (Peralta et al. 1988) was similarly attenuated by prior cell treatment with

toxin B (Table 1). Thus, the target affected by and responsible for PLC inhibition by toxin B is apparently downstream of the heptahelical receptors.

Therefore, the effect of toxin B treatment on G protein-stimulated PLC activity was studied. Direct activation of heterotrimeric G proteins by AlF_4^- and the stable GTP analogue, guanosine 5'-[γ-thio]triphosphate (GTPγS), increased PLC activity in HEK cells. PLC stimulation by AlF_4^- in intact HEK cells (2.5-fold) was almost completely abolished by toxin B pretreatment (50 pg/ml, 24 h) (Table 1). Furthermore, treatment of intact HEK cells for 24 h with toxin B resulted in a concentration-dependent reduction in GTPγS-stimulated inositol phosphate formation, measured in permeabilized [^{3}H]inositol-prelabeled cells (Fig. 2). Half-maximal inhibition of the GTPγS (100 µM) response (approximately 4-fold stimulation) was observed at about 10 pg/ml toxin B, and treatment with 30 pg/ml toxin B decreased GTPγS-stimulated PLC activity by about 75%. At 500 pg/ml toxin B, inositol phosphate formation stimulated by GTPγS was reduced by 87±2.0% (mean ± SEM, $n = 3$ independent experiments, data not shown). Neither in intact nor in permeabilized HEK cells, unstimulated inositol phosphate formation was affected by toxin B.

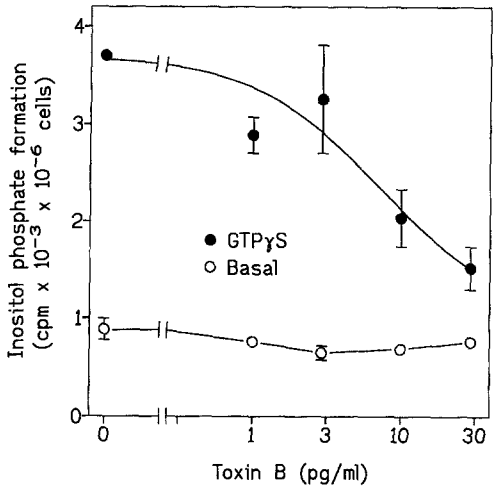

Fig. 2 Influence of toxin B on GTPγS-stimulated PLC activity. HEK cells prelabeled with [^{3}H]inositol were treated for 24 h with toxin B at the indicated concentrations. Thereafter, basal (○) and GTPγS (100 µM)-stimulated (●) formation of [^{3}H]inositol phosphates was measured in digitonin-permeabilized cells as described in "Materials and methods". Data are representative of three separate experiments

Table 1 Effect of toxin B on m2 mAChR-, thrombin- and AlF_4^--stimulated PLC activities. m2 and m3 mAChR-expressing HEK cells prelabeled with [^{3}H]inositol were treated for 24 h without and with 50 pg/ml toxin B, followed by measurement of carbachol (1 mM)-stimulated PLC activity in m2 mAChR-expressing cells and of thrombin (2 U/ml)- and AlF_4^- (10 mM NaF plus 10 µM $AlCl_3$)-stimulated PLC activities in m3 mAChR-expressing cells as described in "Materials and methods". Basal [^{3}H]inositol phosphate accumulation amounted to 1.18±0.07 and 2.8±0.08×10^3 cpm/10^6 cells in m2 and m3 mAChR-expressing HEK cells, respectively, and was not affected by toxin B treatment in either cell type. Data given as stimulated, i.e., total minus basal, [^{3}H]inositol phosphate formation are from one representative experiment, repeated at least three times

		Stimulated inositol phosphate formation (cpm×10^3 per 10^6 cells)	
		Control	Toxin B
m2 mAChR cells	Carbachol	1.79±0.03	0.59±0.07
m3 mAChR cells	Thrombin	2.40±0.31	0.90±0.29
	AlF_4^-	4.20±0.19	0.70±0.09

By measuring C3-catalyzed ADP-ribosylation of Rho proteins, we recently provided indirect evidence that endogenous Rho proteins in HEK cells are glucosylated by toxin B (Schmidt et al. 1996). A similar inhibitory effect was observed when the toxin B-catalyzed glucosylation of Rho proteins was measured directly. In lysates of untreated HEK cells, toxin B induced incorporation of [^{14}C]glucose into two substrate proteins (Fig. 3), of which the upper one (22 kDa) was identified as RhoA and the lower one as Cdc42Hs by immunoblotting with specific antibodies (data not shown), as described for *Clostridium difficile* toxin A-catalyzed glucosylation of Rho family G proteins in RBL cells (Just et al. 1995b). Pretreatment of intact HEK cells with toxin B (50 pg/ml, 24 h) reduced the toxin B-catalyzed [^{14}C]glucosylation of Rho proteins by at least 80%.

To substantiate the hypothesis that inactivation of Rho proteins is responsible for the toxin B-induced inhibition of inositol phosphate formation, we studied the effect of C3 exoenzyme on the GTPγS-induced PLC stimulation in permeabilized HEK cells. For this, [^{3}H]inositol-prelabeled HEK cells were permeabilized with digitonin in the presence of NAD (50 µM) without and with C3 exoenzyme (12 µg/ml). As shown in Fig. 4, C3 exoenzyme had no effect on basal PLC activity. However, GTPγS (100 µM)-stimulated inositol phosphate formation was reduced by about 50%.

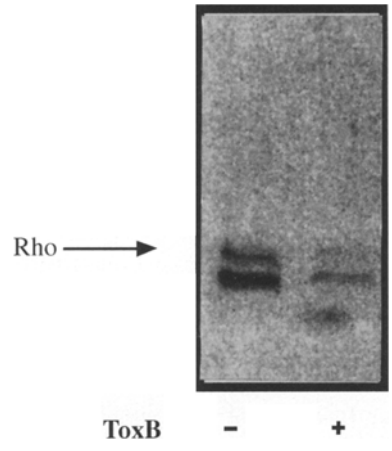

Fig. 3 Toxin B-catalyzed glucosylation of Rho proteins in HEK cells. Toxin B-catalyzed [^{14}C]glucosylation of Rho proteins was determined in lysates of control and toxin B (ToxB)-pretreated cells (50 pg/ml, 24 h) as described in "Materials and methods". PhosphorImager data (Molecular Dynamics) of the SDS-polyacrylamide gel are shown

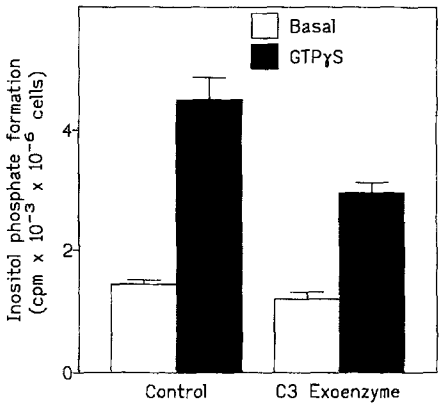

Fig. 4 Inhibition of GTPγS-stimulated PLC activity by C3 exoenzyme. In [^{3}H]inositol-prelabeled and digitonin-permeabilized HEK cells, basal and GTPγS (100 μM)-stimulated formation of [^{3}H]inositol phosphates was measured in the absence (control) and presence of 12 μg/ml C3 exoenzyme as detailed in "Materials and methods". Data are characteristic of three similar experiments

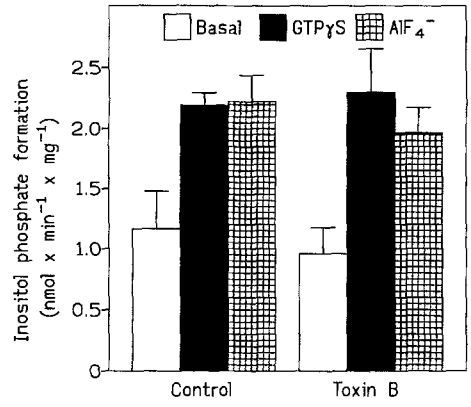

Fig. 6 Influence of toxin B on basal and G protein-stimulated PLC activities using exogenous enzyme substrate. HEK cells were pretreated for 24 h without (control) and with 500 pg/ml toxin B. In homogenates of these cells, formation of [^{3}H]inositol phosphates from exogenous [^{3}H]PtdIns(4,5)P_2 was determined as described in "Materials and methods" in the absence and presence of GTPγS (100 μM) or AlF_4^- (10 mM NaF plus 10 μM $AlCl_3$) as indicated. Data are representative of at least three similar experiments

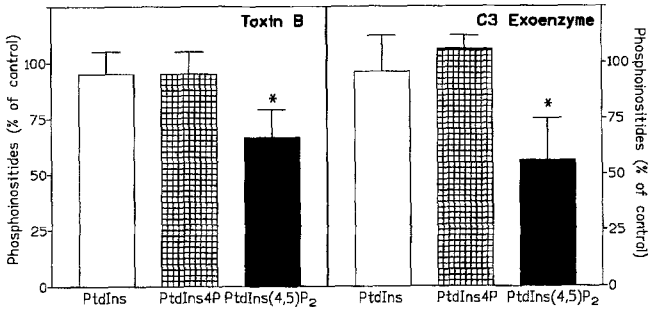

Fig. 5 Effects of toxin B and C3 exoenzyme on the level of phosphoinositides in HEK cells. The level of [^{3}H]phosphoinositides was quantified in m3 mAChR-expressing HEK cells prelabeled with [^{3}H]inositol and subsequently treated without and with 50 pg/ml toxin B (intact cells, *left panel*) or 12 μg/ml C3 exoenzyme (permeabilized cells, *right panel*) as described in "Materials and methods". Data are given as % of control, i.e., in cells not treated with toxin B or C3 exoenzyme. From the total [^{3}H]phosphoinositides analyzed (100,000 cpm and 50,000 cpm in intact and permeabilized cells, respectively), the radioactivity associated with [^{3}H]PtdIns, [^{3}H]PtdIns4P and [^{3}H]PtdIns(4,5)P_2 in control intact cells was 65.0±2.8×10^3 cpm, 4.0±0.3×10^3 cpm and 2.4±0.2×10^3 cpm, respectively, and 30.0±0.3×10^3 cpm, 1.6±0.2×10^3 cpm and 1.6±0.3×10^3 cpm, respectively, in control permeabilized cells. Data are from three separate experiments. *, significantly different (P <0.01) from untreated controls

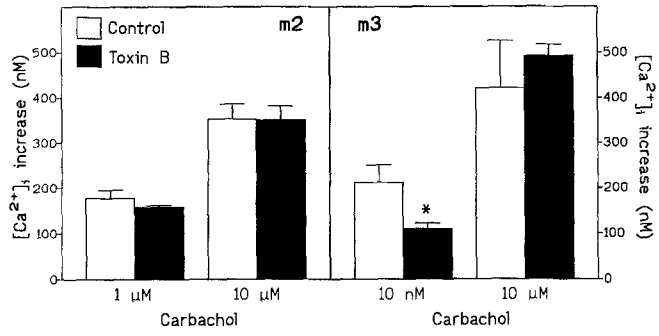

Fig. 7 Influence of toxin B on mAChR-induced calcium signalling in HEK cells. Maximal $[Ca^{2+}]_i$ increases in response to the indicated concentrations of carbachol were determined in m2 (*left panel*) and m3 (*right panel*) mAChR-expressing HEK cells pretreated (*filled columns*) or not (*open columns*) for 24 h with 50 pg/ml toxin B.
*, significantly different (P <0.01) from untreated controls. Data are representative of three similar experiments

Since toxin B and C3 exoenzyme exhibited similar inhibitory effects on receptor and G protein signalling to either PLD (Schmidt et al. 1996) or PLC, it was tempting to speculate that toxin B and C3 exoenzyme reduce the cellular level of PtdIns(4,5)P_2, acting as substrate and cofactor for PLC and PLD, respectively. Therefore, [^{3}H]inositol-prelabeled HEK cells were treated with toxin B, followed by extraction and quantification of the labeled phosphoinositide species. Toxin B had no effect on the levels of [^{3}H]PtdIns and [^{3}H]PtdIns4P compared to untreated controls (Fig. 5). However, the level of [^{3}H]PtdIns(4,5)P_2 was significantly reduced, and amounted to only about 65% of that of untreated controls after 24 h treatment with 50 pg/ml toxin B. At 500 pg/ml toxin B, the level of [^{3}H]PtdIns(4,5)P_2 was reduced by 49±0.8% (mean ± SEM, n = 3 independent experiments, data not shown). Similar to toxin B, C3 exoenzyme (12 μg/ml, 60 min) treatment of permeabilized cells did not significantly alter the levels of [^{3}H]PtdIns and [^{3}H]PtdIns4P, whereas the level of [^{3}H]PtdIns(4,5)P_2 was significantly reduced by about 40% (Fig. 5).

If the fall in cellular PtdIns(4,5)P_2 level caused by toxin B treatment is responsible for the observed PLC inhibition, inositol phosphate formation should not be reduced by toxin B when PLC activity is measured with exogenous PtdIns(4,5)P_2 as enzyme substrate. As demonstrated in Fig. 6, in lysates of cells pretreated for 24 h with a maximally effective concentration of toxin B (500 pg/ml), both basal PLC activity and PLC stimulation by GTPγS or AlF_4^-, using exogenous PtdIns(4,5)P_2 as PLC substrate, were similar as in lysates of control, untreated cells.

Furthermore, PLC activation by Ca^{2+} (1 μM) was also not altered by toxin B pretreatment (data not shown).

Finally, to study a consequence of toxin B-induced reduction in inositol phosphate formation, we measured $[Ca^{2+}]_i$ changes in response to carbachol in m2 and m3 mAChR-expressing HEK cells. As reported before, carbachol increases $[Ca^{2+}]_i$ in m2 and m3 mAChR-expressing HEK cells with quite distinct potencies, with EC_{50} values of about 7 μM and 30 nM, respectively (Schmidt et al. 1995a). As illustrated in Fig. 7, toxin B treatment (50 pg/ml, 24 h), although largely reducing PLC activation (see Table 1), had no effect on $[Ca^{2+}]_i$ increases induced by carbachol used at concentrations (1 and 10 μM) around it's EC_{50} value in m2 mAChR-expressing HEK cells. In m3 mAChR-expressing HEK cells, toxin B did not affect $[Ca^{2+}]_i$ increase induced by a maximally effective concentration of carbachol (10 μM). However, calcium signalling induced by a half-maximally effective carbachol concentration (10 nM) was significantly reduced, by about 50%, in m3 mAChR-expressing HEK cells pretreated with toxin B.

Discussion

A large variety of early and late cellular responses in eukaryotic cells, such as secretion, smooth muscle contraction, sensory perception, neuronal signalling as well as cell growth and transformation, are initiated upon the activation of PLC, catalyzing the hydrolysis of the minor membrane phospholipid PtdIns(4,5)P_2 (Berridge 1993; Divecha and Irvine 1995). PLC stimulation by G protein-coupled receptors apparently requires only three components, the receptor, a heterotrimeric G protein and a PLC-β enzyme, as demonstrated in many in vitro studies (Berstein et al. 1992; Smrcka and Sternweis 1993; Lee and Rhee 1995). In intact cells, however, two additional proteins, i.e., CDP-diacylglycerol synthase and PtdIns transfer protein, both involved in early steps of PtdIns(4,5)P_2 synthesis, have recently been demonstrated to be essential for receptor and G protein-mediated signalling to PLC (Cunningham et al. 1995; Wu et al. 1995). As activated RhoA and Rac1, members of the Rho family of low molecular weight G proteins, have recently been shown to stimulate synthesis of PtdIns(4,5)P_2 by PtdIns4P 5-kinase (Chong et al. 1994; Hartwig et al. 1995), we hypothesized that Rho family G proteins are involved in PLC signalling as well.

Rho proteins can be inactivated by C3 exoenzyme and by the cytotoxin B from *Clostridium difficile*, which in contrast to C3 exoenzyme readily enters intact cells (Ciesielski-Treska et al. 1989; Just et al. 1994). While C3 exoenzyme ADP-ribosylates and thereby inactivates RhoA, RhoB and RhoC, inactivation of Rho proteins (RhoA, RhoB, RhoC, Rac1, Rac2 and Cdc42) by toxin B is apparently caused by monoglucosylation of a threonine residue located in the putative effector domain of these G proteins (Aktories and Just 1993; Just et al. 1995a). Using these two Rho-inactivating "toxins", evidence is provided that Rho proteins are involved in receptor and G protein signalling to PLC, most likely by regulating the cellular PLC substrate supply. First, toxin B treatment of intact HEK cells potently and efficiently attenuated PLC-catalyzed inositol phosphate formation stimulated by either agonist-activated heptahelical receptors (m3 and m2 mAChR and thrombin receptors) or directly activated heterotrimeric G proteins. This inhibition cannot be attributed to a loss or reduced activity of individual components, i.e., receptor, G protein and PLC, involved in receptor signalling to PLC, or the cytoskeleton disruption caused by toxin B. Second, treatment of permeabilized HEK cells with C3 exoenzyme mimicked the inhibitory effect of toxin B on G protein-stimulated inositol phosphate formation. Third, as demonstrated herein by directly measuring toxin B-catalyzed glucosylation and reported before by measuring C3-catalyzed ADP-ribosylation (Schmidt et al. 1996), endogenous Rho proteins are glucosylated by toxin B treatment of intact HEK cells. Fourth, toxin B treatment of intact HEK cells significantly reduced, by up to 50%, the total level of PtdIns(4,5)P_2 in HEK cells, without altering the levels of PtdIns and PtdIns4P. A similarly reduced level of PtdIns(4,5)P_2, without change in those of PtdIns and PtdIns4P, was observed upon treatment of permeabilized HEK cells with C3 exoenzyme. That the actually measured PtdInsP_2 indeed is PtdIns(4,5)P_2, and not PtdIns(3,4)P_2, is supported by the well-known finding that PtdIns(4,5)P_2 comprises more than 98% of the total cellular PtdInsP_2 level (Lips et al. 1989; Divecha and Irvine 1995). Finally, when instead of endogenous substrate, hydrolysis of exogenously added PtdIns(4,5)P_2 was measured, PLC-catalyzed inositol phosphate formation was not affected by prior toxin B treatment. Under this condition, toxin B neither reduced Ca^{2+}-stimulated activity, an index of total cellular PLC activity, nor PLC activity stimulated by the direct G protein activators, AlF_4^- and GTPγS. Thus, based on the available information about inactivation of specific members of the Rho protein family by toxin B and C3 exoenzyme and the data reported herein, it is feasible to assume that toxin B and C3 exoenzyme interfere with receptor- and G protein-mediated signalling to PLC in HEK cells by inactivation of Rho proteins (RhoA, RhoB or RhoC), resulting in reduced PtdIns(4,5)P_2 synthesis. Considering that only part of the total cellular PtdIns(4,5)P_2 is accessible to PLC and particularly to G protein-coupled receptor-stimulated PLC (Lee and Rhee 1995), the up to 50% reduction in total PtdIns(4,5)P_2 level can apparently fully account for the larger, up to 90% reduction in receptor-stimulated inositol phosphate formation in toxin B-treated cells.

mAChR-mediated calcium signalling in HEK cells was differentially affected by toxin B treatment. Although largely inhibiting m2 mAChR-stimulated inositol phosphate production, toxin B did not affect the m2 AChR-induced increase in $[Ca^{2+}]_i$, which notion is in agreement with the recently reported, at least partial PLC independence of calcium signalling by m2 mAChR in HEK cells (Schmidt et al. 1995a). In contrast, the m3 mAChR-induced $[Ca^{2+}]_i$ increase was significantly reduced by toxin B treatment, although only when studied at a half-maxi-

mally effective carbachol concentration (10 nM), but not at a maximally effective concentration (10 μM). This may be explained by the fact that the potencies of carbachol for causing increase in $[Ca^{2+}]_i$ and inositol phosphate formation differ by about two orders of magnitude (the respective EC_{50} values are 30 nM and 2 μM, respectively) (Schmidt et al. 1995a). Thus, the residual increase in inositol phosphate formation caused by 10 μM carbachol in toxin B-treated cells apparently suffices for a full calcium response to m3 mAChR activation. The calcium data, in addition, indicate that toxin B-treated HEK cells are not generally deteriorated, but can respond quite normally to extracellular signals.

In addition to acting as PLC substrate, polyphosphoinositides are apparently involved in an increasing number of cellular functions, such as activation of PLD, vesicular traffic, and the assembly and disassembly of the cytoskeleton (Burgoyne 1994; Janmey 1994; Liscovitch and Cantley 1995). Thus, the fall in cellular PtdIns(4,5)P_2 level caused by toxin B and C3 exoenzyme reported herein apparently does not only explain the reduced PLC-catalyzed inositol phosphate formation, but also perfectly fits and explains the concomitant inhibition of receptor- and G protein-stimulated PLD activity in HEK cells (Schmidt et al. 1996) and the apparent role PtdIns(4,5)P_2 plays as an essential cofactor for PLD activity (Liscovitch et al. 1994; Pertile et al. 1995). Furthermore, the similar alterations of the actin cytoskeleton caused by toxin B and microinjected C3 exoenzyme (Just et al. 1994; Paterson et al. 1990) may be explained, at least in part, by a fall in the cellular level of PtdIns(4,5)P_2, serving as an anchor phospholipid of actin-binding proteins (Janmey 1994). In conclusion, the data presented herein indicate that the cytotoxin B of *Clostridium difficile* is an extremely potent and very efficient agent to interfere with receptor signalling to PLC in intact cells. This cytotoxin will most likely be a powerful tool for our understanding of other signalling pathways and cellular functions as well, which also involve or depend on phosphoinositides.

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