

Inhibition of Receptor Signaling to Phospholipase D by *Clostridium difficile* Toxin B

ROLE OF Rho PROTEINS*

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Rho proteins have been reported to activate phospholipase D (PLD) in *in vitro* preparations. To examine the role of Rho proteins in receptor signaling to PLD, we studied the effect of *Clostridium difficile* toxin B, which glucosylates Rho proteins, on the regulation of PLD activity in human embryonic kidney (HEK) cells stably expressing the m3 muscarinic acetylcholine receptor (mAChR). Toxin B treatment of HEK cells potently and efficiently blocked mAChR-stimulated PLD. In contrast, basal and phorbol ester-stimulated PLD activities were not or only slightly reduced. Cytochalasin B and *Clostridium botulinum* C2 toxin, mimicking the effect of toxin B on the actin cytoskeleton but without involving Rho proteins, had no effect on mAChR-stimulated PLD. Toxin B did not alter cell surface mAChR number and mAChR-stimulated binding of (guanosine 5'-O-(thio)triphosphate (GTP γ S)) to G proteins. In addition to mAChR-stimulated PLD, toxin B treatment also inhibited PLD activation by the direct G protein activators, AIF $_1$ and GTP γ S, studied in intact and permeabilized cells, respectively. Finally, *C. botulinum* C3 exoenzyme, which ADP-ribosylates Rho proteins, mimicked the inhibitory effect of toxin B on GTP γ S-stimulated PLD activity. In conclusion, the data presented indicate that toxin B potently and selectively interferes with receptor coupling mechanisms to PLD, and furthermore suggest an essential role for Rho proteins in receptor signaling to PLD.

Stimulation of PLD,¹ hydrolyzing the major membrane phospholipid, phosphatidylcholine, to phosphatidic acid and choline, has been reported in a wide range of cell types in response to many hormones, neurotransmitters, and growth factors (1, 2). However, the components and mechanisms involved in receptor signaling to PLD are only poorly defined, and may even be distinct for different receptors and cellular systems. Besides the receptor themselves, Ca²⁺, protein kinase C, tyrosine ki-

nases, and GTP-binding proteins have been shown to be involved in the regulation of cellular PLD activity (1, 2). We have recently shown that in HEK cells, stably expressing the human m3 mAChR, various mechanisms, including Ca²⁺, protein kinase C, tyrosine phosphorylation, and GTP-binding proteins, can lead to PLD activation (3). Coupling of mAChR to PLD in these cells is largely independent of concomitant mAChR-mediated activation of phospholipase C but rather involves a mechanism sensitive to tyrosine kinase inhibitors. On the other hand, direct activation of protein kinase C by the phorbol ester PMA leads to a strong PLD response, which is additive to that caused by agonist-activated mAChR and directly activated G proteins, suggesting that in this cell type PLD activity can be stimulated by at least two distinct mechanisms (3).

Recently, evidence has been provided that small molecular weight G proteins, notably ADP-ribosylation factors (4, 5) and members of the Rho protein family (6–10), can stimulate PLD activity in *in vitro* preparations. The present study, therefore, was designed to examine whether Rho proteins are involved in regulation of PLD activity in HEK cells as well and in particular whether Rho proteins may play distinct roles in mAChR signaling to PLD and stimulation of PLD activity by directly activated protein kinase C. For this purpose, we used the cytotoxin B of *Clostridium difficile*, which enters cells by receptor-mediated endocytosis (11). This toxin has recently been reported to monoglucosylate members of the Rho protein family, thereby inactivating these proteins and finally resulting in disorganization of the actin cytoskeleton (12, 13). For comparison, we studied in permeabilized cells the effect of *Clostridium botulinum* C3 exoenzyme, which also inactivates Rho proteins, but by a distinct mechanism, namely ADP-ribosylation (14). We report here that toxin B treatment of HEK cells potently and selectively abolishes mAChR-stimulated PLD, suggesting an essential role for Rho proteins in receptor signaling to PLD.

EXPERIMENTAL PROCEDURES

Materials—Cytochalasin B was from Sigma. [³H]Oleic acid (10 Ci/mmol), [³H]NMS (79.5 Ci/mmol), [³²P]NAD (800 Ci/mmol), and [³⁵S]GTP γ S (1344 Ci/mmol) were obtained from DuPont NEN. *C. difficile* toxin B was purified as described (15). *C. botulinum* C2 toxin and C3 exoenzyme were kind gifts from Drs. I. Just and K. Aktories (Universität Freiburg, Freiburg, Germany). All other materials were from previously described sources (3, 16).

Cell Culture—Culture conditions of HEK cells stably expressing the human m3 mAChR were as reported in detail before (3). For experiments, cells subcultured in Dulbecco's modified Eagle's medium/F-12 medium were grown to near confluence (175-cm² culture flasks or 35-mm culture dishes).

Toxin B Treatment and Assay of PLD Activity in Intact Cells—For measurement of intact cell PLD activity, cellular phospholipids were labeled by incubating nearly confluent monolayers of cells for 20–24 h with [³H]oleic acid (2 μ Ci/ml) in growth medium. To exclude the possibility that toxin B treatment interferes with the incorporation of radio-

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¹ The abbreviations used are: PLD, phospholipase D; G protein, guanine nucleotide-binding protein; GTP γ S, guanosine 5'-O-(thio)triphosphate; HBSS, Hanks' balanced salt solution; HEK, human embryonic kidney; mAChR, muscarinic acetylcholine receptor; NMS, N-methylscopolamine; PMA, phorbol 12-myristate 13-acetate; PtdEtOH, phosphatidylethanol; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate.

activity into phospholipids, the labeling medium was replaced before the cells were treated with toxin B. Thereafter, cells were detached from the culture flasks, resuspended twice in HBSS, 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. The PLD activity assay was carried out for 60 min at 37 °C in a total volume of 200 μl containing 100 μl of cell suspension (1×10^6 cells), 400 mM ethanol, and the indicated stimulatory agents. Stop of the reaction and isolation of labeled phospholipids and the specific PLD product [^3H]PtdEtOH were as described before (3). Formation of [^3H]PtdEtOH is expressed as percentage of total labeled phospholipids. As reported before (17), PLD activity assays were generally performed both in the absence and presence of ethanol, and radioactivity associated with authentic PtdEtOH on TLC plates in the absence of ethanol was subtracted as unspecific background from the values obtained in the presence of ethanol. Thus, basal [^3H]PtdEtOH accumulation is due to PLD activity. Notably, significant basal PLD activity in intact HEK cells was only detected when studied in suspension, while when measured in cell monolayers no or only minimal basal [^3H]PtdEtOH formation could be observed (3, 16, 17).

Assay of PLD Activity in Permeabilized Cells—Cells prelabeled with [^3H]oleic acid (2 $\mu\text{Ci}/\text{ml}$) and pretreated or not with toxin B were detached from the culture flasks, washed twice by centrifugation and resuspension in phosphate-buffered saline, and resuspended in assay buffer, 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. The assay of PLD activity was carried out for 60 min at 37 °C in a total volume of 200 μl , containing 100 μl of the cell suspension (1×10^6 cells), 10 μM digitonin, and 400 mM ethanol as well as the test agents. Effects of C3 exoenzyme on basal and GTP γS -stimulated PLD activities in digitonin-permeabilized cells (1×10^6 cells) were assayed (total volume 100 μl) in the same buffer as above, containing additionally 50 μM NAD without and with 12 $\mu\text{g}/\text{ml}$ C3 exoenzyme.

mAChR Binding Assay—HEK cells pretreated or not with toxin B were washed twice with ice-cold HBSS. Thereafter, cell surface mAChRs were measured by specific binding of the membrane-impermeant mAChR antagonist, [^3H]NMS (2 nM), to intact cells in HBSS in a total volume of 1 ml for 4 h at 4 °C as described before (16).

GTP γS Binding to Permeabilized HEK Cells—HEK cells pretreated or not with toxin B and washed twice with HBSS were permeabilized with digitonin. Thereafter, specific binding of [^3S]GTP γS to permeabilized adherent HEK cells was determined as described before (16).

ADP-ribosylation of Rho Proteins by C3 Exoenzyme—C3-catalyzed [^{32}P]ADP-ribosylation of Rho proteins in lysates of control and toxin B-pretreated HEK cells with C3 exoenzyme (0.3 $\mu\text{g}/\text{ml}$) and [^{32}P]NAD (1 μM) and analysis of [^{32}P]ADP-ribosylated proteins by SDS-polyacrylamide gel electrophoresis and autoradiography were carried out as described (12).

Data Presentation—Data shown are mean \pm S.D. ($n = 3$) from one experiment, repeated as indicated in the figure legends.

RESULTS

Pretreatment of m3 mAChR-expressing HEK cells with *C. difficile* toxin B caused a time- and concentration-dependent inhibition of mAChR-stimulated PLD activity. [^3H]PtdEtOH accumulation induced by carbachol (1 mM) was inhibited by up to 90% in [^3H]oleic acid-prelabeled cells pretreated for increasing periods of time with as low as 10 pg/ml toxin B (Fig. 1A). Half-maximal inhibition was observed after approximately 10 h treatment with this toxin concentration (Fig. 1A). In contrast, stimulation of PLD activity by the phorbol ester PMA (0.1 μM) was only slightly reduced and only upon prolonged pretreatment with 10 pg/ml toxin B (Fig. 1B). When pretreated for 24 h, mAChR-stimulated PLD activity was half-maximally inhibited with 3 pg/ml toxin B (Fig. 2A) and completely abolished upon treatment with 50 pg/ml toxin B (see Fig. 4). In contrast, PMA-stimulated PLD activity was not altered by treatment with up to 30 pg/ml toxin B for 24 h (Fig. 2B) and was reduced by about 25% with 50 pg/ml toxin B (data not shown). Basal [^3H]PtdEtOH accumulation was not affected by toxin B treatment of HEK cells, even when treated with a rather high toxin B concentration (1 ng/ml) (data not shown). These data suggested that toxin B potently and selectively interferes with the signal transduction pathway of m3 mAChR to PLD.

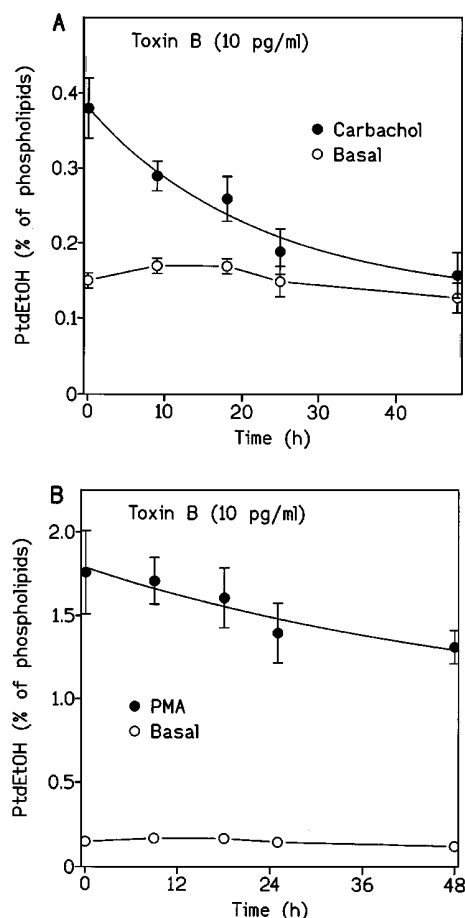


FIG. 1. Influence of toxin B on mAChR- and PMA-stimulated PLD activities in HEK cells: time course studies. m3 mAChR-expressing HEK cells prelabeled with [^3H]oleic acid were treated for the indicated periods of time with 10 pg/ml *C. difficile* toxin B, followed by measurement of basal PLD activity (○) and PLD activities stimulated by 1 mM carbachol (●) (A) or 0.1 μM PMA (●) (B) as described under "Experimental Procedures." Data are representative of two similar experiments.

As observed in other cell types (11–13), treatment of HEK cells with toxin B potently induced rounding-up of the cells (data not shown), indicating disruption of the actin cytoskeleton. To exclude the possibility that inhibition of receptor-mediated PLD response by toxin B was merely due to its effect on the cytoskeleton, cytochalasin B and *C. botulinum* C2 toxin were used as controls. Both agents, by distinct mechanisms, cause depolymerization of actin, but notably without involving Rho proteins (18, 19). As shown in Fig. 3, neither cytochalasin B (5 $\mu\text{g}/\text{ml}$, 15 min) nor C2 toxin (20 ng/ml component I plus 40 ng/ml component II, 24 h), both causing similar morphological changes (rounding-up) of HEK cells as toxin B, had any effect on mAChR-stimulated PLD activity.

To study at which level toxin B interferes with receptor signaling to PLD, several possibilities were considered. First, cell surface mAChR number was determined by binding of the membrane-impermeant mAChR ligand [^3H]NMS, at a receptor-saturating concentration (2 nM), to intact cells. Second, receptor coupling to G proteins was measured as agonist-stimulated binding of [^3S]GTP γS to permeabilized adherent HEK cells (16). Treatment of HEK cells with toxin B (50 pg/ml, 24 h) affected neither mAChR surface number nor receptor coupling to G proteins. In control and toxin B-treated cells, [^3H]NMS binding was 0.65 ± 0.06 and 0.78 ± 0.01 pmol/dish, respectively. Basal and carbachol (1 mM)-stimulated [^3S]GTP γS binding amounted to 150 ± 5 and 258 ± 8 fmol/mg protein,

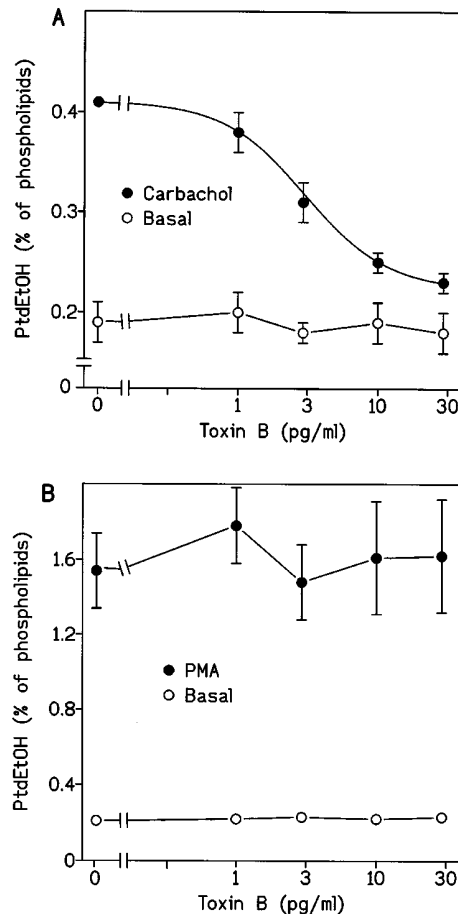


FIG. 2. **Influence of toxin B on mAChR- and PMA-stimulated PLD activities: concentration dependence.** m3 mAChR-expressing HEK cells prelabeled with [3 H]oleic acid were treated for 24 h with the indicated concentrations of toxin B, followed by measurement of basal PLD activity (\circ) and PLD activities stimulated by 1 mM carbachol (\bullet) (A) or 0.1 μ M PMA (\bullet) (B). Data are representative of four similar experiments.

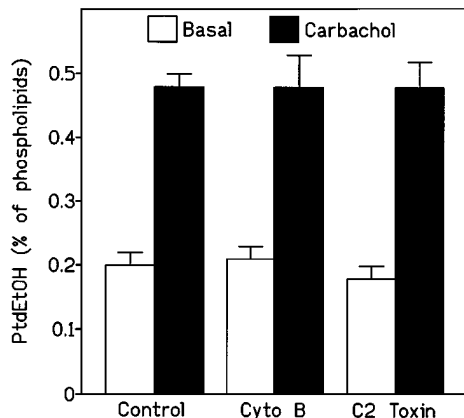


FIG. 3. **Lack of effect of cytochalasin B and C2 toxin on mAChR-stimulated PLD activity.** m3 mAChR-expressing HEK cells prelabeled with [3 H]oleic acid were treated without (Control) and with cytochalasin B (Cyto B, 5 μ g/ml, 15 min) or C2 toxin (20 ng/ml component I plus 40 ng/ml component II, 24 h), followed by measurement of basal and carbachol (1 mM)-stimulated PLD activities. Data are representative of three similar experiments.

respectively, in control cells, and the corresponding numbers in toxin B-treated cells were 138 ± 4 and 242 ± 1 fmol/mg protein, respectively.

m3 mAChR-induced PLD activation in HEK cells is apparently mediated by pertussis toxin-insensitive G proteins (20).

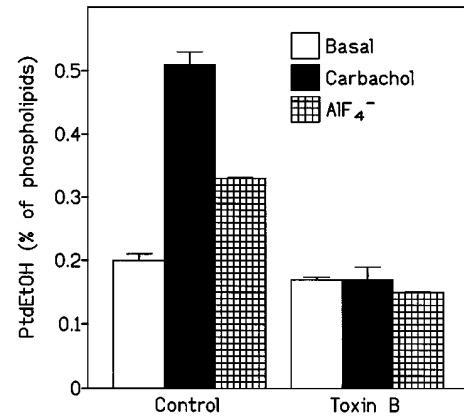


FIG. 4. **Influence of toxin B on mAChR- and AIF $_4^-$ -stimulated PLD activities.** In m3 mAChR-expressing HEK cells prelabeled with [3 H]oleic acid and treated for 24 h without (Control) and with 50 pg/ml toxin B, formation of [3 H]PtdEtOH was determined in the absence (Basal) and presence of carbachol (1 mM) or AIF $_4^-$ (10 mM NaF plus 10 μ M AlCl $_3$). Data are representative of three similar experiments.

Therefore, we studied whether toxin B treatment also affects PLD activation caused by direct activation of heterotrimeric G proteins. As shown in Fig. 4, similar to carbachol-induced stimulation, stimulation of PLD activity in intact HEK cells by AIF $_4^-$, a direct activator of heterotrimeric G proteins, was completely abolished by prior toxin B treatment (50 pg/ml, 24 h).

In permeabilized HEK cells, PLD activity can be stimulated by the stable GTP analog GTP γ S, apparently by activating small molecular weight G proteins (3, 21), and by the phorbol ester PMA, which stimulation is additive to that caused by GTP γ S (3). In control digitonin-permeabilized cells, GTP γ S (100 μ M) and PMA (0.1 μ M) increased [3 H]PtdEtOH accumulation by about 4–5-fold. Pretreatment of [3 H]oleic acid-prelabeled intact cells with toxin B (24 h), followed by digitonin permeabilization and measurement of PLD activity, caused a concentration-dependent reduction of the response to GTP γ S (Fig. 5A). Half-maximal inhibition was observed at about 5 pg/ml toxin B, and treatment with 30 pg/ml toxin B decreased GTP γ S-stimulated PtdEtOH formation by about 80%. Similar to data obtained in intact cells, basal PLD activity and stimulation by the phorbol ester PMA in permeabilized HEK cells were not affected by this pretreatment with toxin B (Fig. 5B).

To corroborate the hypothesis that inactivation of Rho proteins is responsible for the toxin B effects on PLD activity, we studied the effect of C3 exoenzyme on GTP γ S-stimulated PLD activity in digitonin-permeabilized cells. C3 exoenzyme had no effect on basal [3 H]PtdEtOH accumulation (Fig. 6). However, stimulation of PLD activity by GTP γ S was strongly reduced. [3 H]PtdEtOH accumulation induced by 10 μ M GTP γ S was virtually abolished, and the stimulatory effect of 100 μ M GTP γ S, the maximally effective concentration, was reduced by about 60%.

Finally, we studied whether Rho proteins in HEK cells are affected by toxin B treatment. For this, C3-catalyzed incorporation of [32 P]ADP-ribose into Rho proteins was determined in lysates of HEK cells pretreated without and with toxin B (50 pg/ml, 24 h). As reported before in other cell types, glucosylation of Rho by toxin B prevents subsequent ADP-ribosylation by C3 exoenzyme (12, 13). As illustrated in Fig. 7, incorporation of [32 P]ADP-ribose into Rho proteins, using [32 P]NAD as substrate and fresh C3 exoenzyme, was reduced by C3 pretreatment of permeabilized HEK cells by about 80%. A similar, at least 80% reduction in C3-catalyzed [32 P]ADP-ribosylation of Rho proteins was also observed in lysates of cells pretreated with toxin B, indicating that endogenous Rho proteins in HEK cells were affected by prior toxin B treatment.

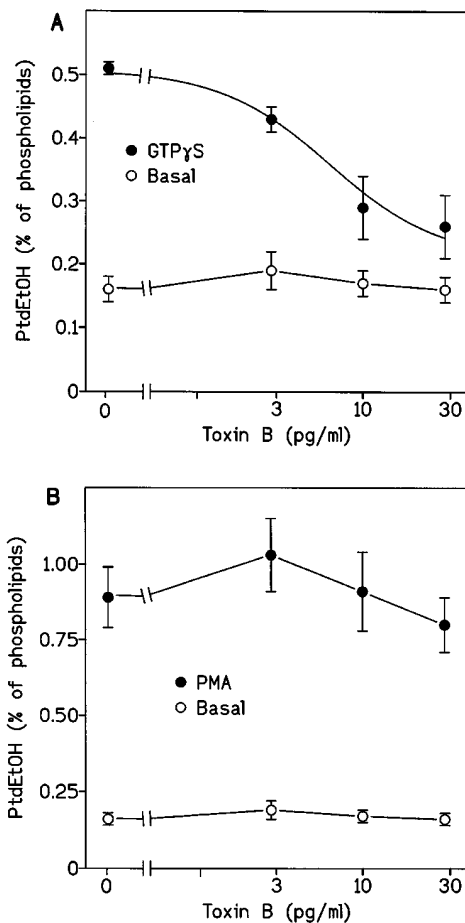


FIG. 5. Influence of toxin B on GTP γ S- and PMA-stimulated PLD activities in permeabilized HEK cells. [3 H]Oleic acid-prelabeled HEK cells were treated for 24 h with toxin B at the indicated concentrations. Thereafter, formation of [3 H]PtdEtOH was determined in digitonin-permeabilized cells without (Basal, ○) and with 100 μ M GTP γ S (●) (A) or 0.1 μ M PMA (●) (B) as described under "Experimental Procedures." Data are representative of four similar experiments.

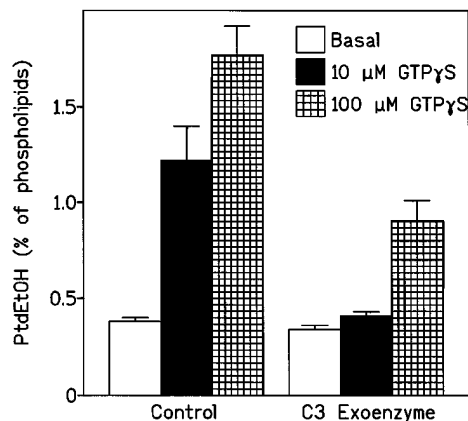


FIG. 6. Inhibition of GTP γ S-stimulated PLD activity by C3 exoenzyme. In [3 H]oleic acid-prelabeled and digitonin-permeabilized HEK cells, formation of [3 H]PtdEtOH was determined in the absence (Control) and presence of 12 μ g/ml C3 exoenzyme without (Basal) and with 10 μ M or 100 μ M GTP γ S as detailed under "Experimental Procedures." Data are representative of at least three similar experiments.

DISCUSSION

In the present study, we examined the involvement of Rho proteins in stimulation of PLD activity in HEK cells stably expressing the human m3 mAChR subtype. To study the role of Rho proteins in G protein-coupled receptor signaling to PLD,

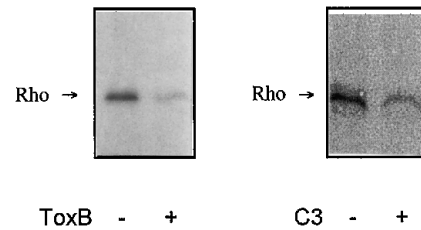


FIG. 7. C3-catalyzed ADP-ribosylation of Rho proteins in HEK cell lysates pretreated with toxin B or C3 exoenzyme. C3-catalyzed [32 P]ADP-ribosylation of Rho proteins was determined in lysates of control and toxin B (ToxB)-pretreated intact cells (50 pg/ml, 24 h) and for comparison in lysates of digitonin-permeabilized HEK cells pretreated without and with C3 exoenzyme (12 μ g/ml, 60 min). Phosphorimage data (Molecular Dynamics) of the SDS-polyacrylamide gel electrophoresis are shown. Other labeled bands were not detected.

an intact cellular system was necessary, since receptor stimulation of PLD activity has as yet not been reported in cell-free preparations. *C. botulinum* C3 exoenzyme inactivates Rho proteins; however, it does not enter intact cells or does so only poorly (14), making it an unsuitable agent to study the role of Rho proteins in receptor coupling to PLD. Recently, *C. difficile* toxin B, known to enter cells by receptor-mediated endocytosis and to induce disruption of the actin cytoskeleton in a manner similar to micro-injected C3 exoenzyme, has been shown to specifically inactivate Rho proteins in intact cells, apparently by causing monoglucosylation of these proteins (12, 13). We demonstrate here that toxin B treatment of HEK cells potently and efficiently blocks mAChR-stimulated PLD activity. For example, in HEK cells pretreated for 24 h, half-maximal inhibition of carbachol-stimulated PtdEtOH formation was observed with only 3 pg/ml, and complete inhibition upon treatment with 50 pg/ml toxin B. On the other hand, under similar treatment conditions, stimulation of PLD activity by the phorbol ester PMA, both in intact and permeabilized HEK cells, was not or only slightly reduced, and basal activity was not affected at all. These data indicate that toxin B does not modify PLD enzyme(s) itself and support the view that distinct signaling pathways leading to PLD activation exist in HEK cells. Furthermore, the differential sensitivity of PLD stimulation by the G protein-coupled mAChR and the PMA-activated protein kinase C may reflect the presence of different PLD isoenzymes in this cell type as suggested by studies in other cellular systems (9, 22).

Control experiments with cytochalasin B and *C. botulinum* C2 toxin demonstrated that the inhibitory effect of toxin B treatment is not an event secondary to the destruction of the actin cytoskeleton. Furthermore, toxin B treatment had no effects on mAChR number and location and on receptor activation of G proteins. Finally, toxin B treatment also abolished stimulation of PLD activity by AIF $_4^-$, directly activating heterotrimeric G proteins. Together, these findings indicate that the cytotoxin interferes with the signaling pathway to PLD somewhere downstream of the receptor-activated heterotrimeric G proteins. This conclusion was further substantiated by the finding that toxin B treatment also potently reduced stimulation of PLD activity by the stable GTP analog GTP γ S in permeabilized HEK cells. This inhibitory effect on GTP γ S-induced PLD stimulation was mimicked by C3 exoenzyme added to permeabilized HEK cells. We have no obvious explanation for the reported ineffectiveness of C3 to inhibit GTP γ S-induced PLD stimulation in neutrophil and liver plasma membranes (6, 7), it may be due to the use of different cellular systems. Finally, it is demonstrated that toxin B treatment of HEK cells apparently affects endogenous Rho proteins, thereby largely reducing C3-catalyzed incorporation of ADP-ribose. Toxin B has been reported to monoglucosylate Rho A, Rac 1, and Cdc42,

while C3 exoenzyme ADP-ribosylates Rho A, B, and C (13, 14). The combined analysis with both tools working on distinct members of the Rho protein family thus suggests that toxin B causes its effects on receptor signaling to PLD in HEK cells, most likely by an action on Rho A proteins.

PtdIns(4,5)P₂ is apparently an essential co-factor for PLD activation (23, 24) and is absolutely required for ADP-ribosylation factor regulation of PLD activity (4, 22). Synthesis of PtdIns(4,5)P₂ in fibroblast cells has recently been reported to be stimulated by activated Rho (25), although more recent data suggest that stimulation of phosphatidylinositol-4-phosphate 5-kinase by Rho A is rather indirect (26). In light of these data, it is tempting to speculate that mAChR-mediated PLD activation in HEK cells involves both ADP-ribosylation factor (21) and Rho A proteins, the former stimulating PLD activity rather directly, while Rho A may be required for the supply of the PLD co-factor, PtdIns(4,5)P₂. As recently reported in U937 cells, stimulation of PLD activity by protein kinase C is apparently in part independent of PtdIns(4,5)P₂ as a cofactor (24). This finding is consistent with the data presented herein that, in comparison to mAChR-stimulated PLD, stimulation of PLD activity by phorbol ester-activated protein kinase C exhibited a largely reduced sensitivity to toxin B.

In conclusion, we demonstrate that *C. difficile* toxin B, known to inactivate Rho proteins, potently and efficiently blocks mAChR signaling to PLD in HEK cells, making an essential role of Rho proteins in this receptor action likely. On the other hand, stimulation of PLD activity by protein kinase C was markedly less sensitive to toxin B, suggesting the presence of distinct regulatory mechanisms and/or PLD isoenzymes.

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REFERENCES

1. Billah, M. M. (1993) *Curr. Opin. Cell Biol.* **5**, 114–123
2. Exton, J. H. (1994) *Biochim. Biophys. Acta* **1212**, 26–42
3. Schmidt, M., Hüwe, S. M., Fasselt, B., Homann, D., Rümenapp, U., Sandmann, J., and Jakobs, K. H. (1994) *Eur. J. Biochem.* **225**, 667–675
4. Brown, H. A., Gutowski, S., Moonaw, C. R., Slaughter, C., and Sternweis, P. C. (1993) *Cell* **75**, 1137–1144
5. Cockcroft, S., Thomas, G. M. H., Fensome, A. M., Geny, B., Cunningham, E., Gout, I., Hiles, I., Totty, N. F., Truong, O., and Hsuan, J. J. (1994) *Science* **263**, 523–526
6. Bowman, E. P., Uhlinger, D. J., and Lambeth, J. D. (1993) *J. Biol. Chem.* **268**, 21509–21512
7. Malcolm, K. C., Ross, A. H., Qui, R.-G., Symons, M., and Exton, J. H. (1994) *J. Biol. Chem.* **269**, 25951–25954
8. Singer, W. D., Brown, H. A., Bokoch, G. M., and Sternweis, P. C. (1995) *J. Biol. Chem.* **270**, 14944–14950
9. Siddiqi, A. R., Smith, J. L., Ross, A. H., Qiu, R.-G., Symons, M., and Exton, J. H. (1995) *J. Biol. Chem.* **270**, 8466–8473
10. Deleted in proof
11. Ciesielski-Treska, J., Ulrich, G., Rihn, B., and Aunis, D. (1989) *Eur. J. Cell Biol.* **48**, 191–202
12. Just, I., Fritz, G., Aktories, K., Giry, M., Popoff, M. R., Boquet, P., Hegenbarth, S., and von Eichel-Streiber, C. (1994) *J. Biol. Chem.* **269**, 10706–10712
13. Just, I., Selzer, J., Wilm, M., von Eichel-Streiber, C., Mann, M., and Aktories, K. (1995) *Nature* **375**, 500–503
14. Aktories, K., and Just, I. (1993) in *GTPases in Biology I* (Dickey, B. F., and Birnbaumer, L., eds), pp. 87–112, Springer-Verlag, Berlin
15. Von Eichel-Streiber, C., Harperath, U., Bosse, U., and Hadding, U. (1987) *Microb. Pathog.* **2**, 307–318
16. Schmidt, M., Fasselt, B., Rümenapp, U., Bienek, C., Wieland, T., Van Koppen, C. J., and Jakobs, K. H. (1995) *J. Biol. Chem.* **270**, 19949–19956
17. Sandmann, J., Peralta, E. G., and Wurtman, R. J. (1991) *J. Biol. Chem.* **266**, 6031–6034
18. Cooper, J. A. (1987) *J. Cell Biol.* **105**, 1473–1478
19. Aktories, K., Bärman, M., Ohishi, I., Tsuyama, S., Jakobs, K. H., and Habermann, E. (1986) *Nature* **322**, 390–392
20. Offermanns, S., Wieland, T., Homann, D., Sandmann, J., Bombien, E., Spicher, K., Schultz, G., and Jakobs, K. H. (1994) *Mol. Pharmacol.* **45**, 890–898
21. Rümenapp, U., Geiszt, M., Wahn, F., Schmidt, M., and Jakobs, K. H. (1995) *Eur. J. Biochem.* **234**, 240–244
22. Massenburg, D., Han, J.-S., Liyanage, M., Patton, W., Rhee, S. G., Moss, J., and Vaughan, M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11718–11722
23. Liscovitch, M., Chalifa, V., Pertile, P., Chen, C.-S., and Cantley, L. C. (1994) *J. Biol. Chem.* **269**, 21403–21406
24. Pertile, P., Liscovitch, M., Chalifa, V., and Cantley, L. C. (1995) *J. Biol. Chem.* **270**, 5130–5135
25. Chong, L. D., Traynor-Kaplan, A., Bokoch, G. M., and Schwartz, M. A. (1994) *Cell* **75**, 1137–1144
26. Talias, K. F., Cantley, L. C., and Carpenter, C. L. (1995) *J. Biol. Chem.* **270**, 17656–17659