Activation of m3 muscarinic acetylcholine receptor (mAC1R), stably expressed in human embryonic kidney (HEK)-293 cells, leads to phospholipase D (PLD) stimulation, a process apparently involving Rho GTPases, as shown by studies with Clostridium botulinum C3 exoenzyme and Clostridium difficile toxin B (TcdB). Direct activation of protein kinase C (PKC) by phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), also induces PLD stimulation, which is additive to the mAC1R action and which is only poorly sensitive to inactivation of Rho proteins by TcdB. To study whether Ras-like GTPases are involved in PLD regulation, we studied the effects of the TcdB variant TcdB-1470 and Clostridium sordellii lethal toxin (TcsL), known to inactivate Rac and some members of the Ras protein family, on PLD activities. TcdB-1470 and TcsL did not affect basal PLD activity and PLD stimulation by mAC1R or direct G protein activation. In contrast, PMA-induced PLD stimulation was inhibited by TcdB-1470 and TcsL in a time- and concentration-dependent manner, without alteration in immunologically detectable PKC isoform levels. In membranes of HEK-293 cells pretreated with TcdB-1470 or TcsL, basal and stable GTP analog-stimulated PLD activities measured with exogenous phosphatidylcholine, in the presence or absence of phosphatidylinositol 4,5-bisphosphate, were not altered. In contrast, pretreatment with TcdB-1470 and TcsL, but not TcdB, strongly reduced PMA-stimulated PLD activity. The addition of recombinant Rac1, serving as glycosylation substrate for TcdB, TcsL, and TcdB-1470, did not restore PLD stimulation by PMA. Furthermore, PMA-stimulated PLD activity, suppressed by prior treatment with TcdB-1470 or TcsL, was not rescued by the addition of recombinant Ras (RasG12V) or Rap proteins, acting as glycosylation substrates for TcsL only (Ras) or TcdB-1470 and TcsL (Rap). In contrast, the addition of recombinant Rac proteins (RacA and RacB), glycosylation substrates for TcsL and TcdB-1470, but not for TcdB, to membranes of TcdB-1470- or TcsL-treated cells fully restored PLD stimulation by PMA without altering the strict MgATP dependence of PMA-induced PLD stimulation. RalA-mediated restoration of PMA-stimulated PLD activity in membranes of TcsL-treated cells was not enhanced by coaddition of RasG12V. In conclusion, the data presented indicate that TcdB-1470 and TcsL selectively interfere with phorbol ester stimulation of PLD and suggest an essential role of Ral proteins in PKC signaling to PLD in HEK-293 cells.

Activation of phospholipase D (PLD)\(^1\) by tyrosine kinase receptors and receptors coupled to heterotrimeric guanine nucleotide-binding proteins (G proteins) has been reported in a wide range of cell types in response to various hormones, neurotransmitters, and growth factors. Stimulation of PLD, hydrolyzing the major membrane phospholipid, phosphatidylcholine (PtdCho), to phosphatidic acid and choline, has been implicated in different physiological processes, such as cell growth and differentiation as well as membrane trafficking (1–6).

Despite the recent cloning and identification of different PLD isozymes (i.e., PLD1a, PLD1b, and PLD2 (7–11)), the exact mechanisms linking cell surface receptors to PLD stimulation are largely unknown. Various small molecular weight GTPases of different families are apparently involved in the regulation of PLD activity in different cellular systems. Members of the ADP-ribosylation factor (ARF) family have been demonstrated to mediate stimulation of PLD activity by the stable GTP analog GTP\(\gamma\)S, and G protein-coupled and growth factor receptors (7, 12–19). In addition to and apparently synergistic with ARF, PLD activity is stimulated by GTP\(\gamma\)S-activated Rho proteins (7, 20–26). Members of the Rho protein family have been shown to mediate PLD stimulation by G protein-coupled and growth factor receptors as well (27–29). Furthermore, Ral proteins, Ras-like GTPases, can apparently also mediate PLD stimulation. Stimulation of PLD activity induced by v-Src in Balb/c and NIH 3T3 fibroblasts was shown to be mediated by

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\(^1\) The abbreviations used are: PLD, phospholipase D; ARF, ADP-ribosylation factor; G protein, guanine nucleotide-binding protein; GST, glutathione S-transferase; GTP\(\gamma\)S, guanosine 5’-O-(3-thiotriophosphate; HEK, human embryonic kidney; mAC1R, muscarinic acetylcholine receptor; PBS, phosphate-buffered saline; PKC, protein kinase C; c-FRC, conventional PKC; a-FRC, atypical PKC; PMA, phorbol 12-myristate 13-acetate; PtdE0H, phosphatidylethanol; PtdIns(4,5)P\(_2\), phosphatidylinositol 4,5-bisphosphate; TcdB, C. difficile toxin B; TcdB-1470, C. difficile toxin B-1470; TcsL, C. sordellii lethal toxin; PtdCho, phosphatidylcholine; PAGE, polyacrylamide gel electrophoresis.
Ras and RalA, the latter acting apparently downstream of Ras (30). Finally, Ras itself has been reported to activate PLD in different cell types (31–33). Thus, GTPase cascades are apparently not only involved in cell proliferation and cytoskeleton organization, but they may also participate in PLD activation (34).

Another pathway for PLD stimulation involves isoforms of the protein kinase C (PKC) family, specifically members of the classical forms of PKC, as shown by studies in human neurophilis, in membranes of porcine brain, and with purified PLD (7, 35, 36). In various cellular systems, ARF and Rho proteins seem to be involved in PLD stimulation by PKC. Furthermore, activation of PLD by Ras proteins can apparently involve PKC-dependent and PKC-independent pathways, depending on cell type and/or PLD isozyme (32). Finally, synergism between PLD stimulation by GTPases and phorbol-ester-activated PKC has been demonstrated in several systems (35–39) and very recently also with purified PLD isoforms (7, 9).

In human embryonic kidney (HEK)-293 cells, stably expressing the m3 muscarinic acetylcholine receptor (mACHr), various signal transduction components, including GTPases, PKC, tyrosine kinases, and phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2), are apparently involved in PLD activation. We have recently shown that mACHr-mediated PLD stimulation in these cells involves small molecular weight GTPases of both the ARF and Rho protein families (17, 27). As shown in studies with Clostridium difficile toxin B (TcdB), which inactivates Rho, Rac, and Cdc42, and with the selective Rho-inactivating Clostridium botulinum C3 exoenzyme, Rho proteins apparently regulate the supply of the essential PLD cofactor, PtdIns(4,5)P2 (40, 41). Direct activation of PKC by phorbol esters, such as phorbol 12-myristate 13-acetate (PMA), also induces marked PLD stimulation in HEK-293 cells, which is additive to the mACHr action and apparently does not involve ARF (17, 42, 43). Furthermore, PMA stimulation of PLD in HEK-293 cells was by far less sensitive to inactivation of Rho proteins by TcdB than mACHr stimulation (27). To examine whether Ras-like G proteins are involved in PLD stimulation in HEK-293 cells, we studied the effects of Clostridium sordellii lethal toxin (TcsL) and the variant TcdB, TcdB-1470, which induce similar cytopathic effects (44, 45), on regulation of PLD activities. TcsL has recently been shown to inactivate Rac and some members of the Ras protein family (Ras, Rap, and Ral) by monoglucosylation (46–48). We demonstrate here that TcsL and TcdB-1470, which glucosylates Rac, Rap, and Ral GTPases, potently and selectively inhibit PMA-induced PLD stimulation in HEK-293 cells and that this inhibition is selectively reversed by Ral proteins, suggesting an essential role for Ral GTPases in PKC signaling to PLD in HEK-293 cells.

**EXPERIMENTAL PROCEDURES**

**Materials**—[^H]Oleic acid (10 Ci/mmol), 1-palmitoyl-2-[9,10,14C]palmitoyl-glycerophospholipids ([14C]PtdCho, 37.5 Ci/mmol) and UDP-[14C]glucose (256 mCi/mmol) were obtained from NEF Life Science Products. PtdCho, phosphatidylethanolamine, and p-amino- benzidine were from Sigma. PtdIns(4,5)P2 was from Boehringer Mannheim, and glutathione-Sepharose was from Pharmacia. TcdB (from strain 10463), TcdB-1470, and TcsL (from strains 82 and 1522, exhibiting identical glucosylation patterns) were purified as described (44, 49, 50). Antibodies against conventional PKC (c-PKC) (a, 82 kDa; b, 80 kDa; g, 80 kDa) and atypical PKC (a-PKC) (ζ, 72 kDa; η, 74 kDa) isoforms and Rac proteins were purchased from Santa Cruz Biotechnology. Antibodies against RalA, RalB, Rap, and Ras were from Transduction Laboratories. All other materials were from previously described sources (17, 27, 40–43).

**Cell Culture**—Culture conditions of HEK-293 cells stably expressing the m3 mACHr were as reported in detail before (27, 40). For experiments, cells subcultured in Dulbecco’s modified Eagle’s medium/F-12 medium were grown to near confluence (170- to 75-cm² culture flasks or 145-mm culture dishes).

**Toxin 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 µCi/ml) in growth medium. To exclude the possibility that the toxins interfere with the incorporation of radioactivity into cellular phospholipids, the labeling medium was changed 6 h before the indicated periods of time without and with TcsL or TcdB-1470 at the indicated concentrations. Thereafter, cells were detached from the culture flasks, resuspended twice in Hank’s balanced salt solution, containing 118 mM NaCl, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 5 mM d-glucose, buffered at pH 7.4 with 15 mM HEPES, 7.5 mM KH, PO4, 1.5 mM Na2HPO4, 30 mM NaHCO3, and 400 mM ethanol, and the indicated stimulatory agents. Stop of the reaction and isolation of labeled phospholipids and the specific PLD product, [3H]phosphatidylethanolamine ([14H]PtdEtOH), were as described before (27, 40). The formation of [3H]PtdEtOH is expressed as percentage of total labeled phospholipids.

**Assay of PLD Activity in Permeabilized Cells**—Prior to permeabilization, [3H]Oleic acid-prelabeled cells were treated for 24 h without and with TcdB-1470 at the indicated concentrations. Thereafter, the cells were detached from the culture flasks, washed twice with centrifugation and resuspension in PBS (137 mM NaCl, 2.7 mM KCl, 0.9 mM CaCl2, 0.5 mM MgCl2, 6.5 mM Na2HPO4, 30 mM NaHCO3, 137 mM glucose), and pelleted, washed once in PBS, recentrifuged, and resuspended in homogenization buffer, containing 1 mM EDTA, 1 mM diithiothreitol, 250 mM sucrose, 3 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 1 mM leupeptin, 1 µg/ml soybean trypsin inhibitor, and 20 mM Tris-HCl, pH 7.5. The cells were homogenized by nitrogen cavitation and pelleted by centrifugation for 10 min at 1000 × g. Crude membranes were prepared from the resulting supernatant by centrifugation for 60 min at 50,000 × g. The membranes were resuspended in a buffer containing 1 mM EDTA, 1 mM dithiothreitol, and 20 mM Tris-HCl, pH 7.5, and stored at −80 °C.

**Assay of PLD Activity in Membranes—**PLD activity in HEK-293 cell membranes was measured by two distinct assays as described in detail before (12, 51) with some modifications. The assay in the presence of detergents (51) was performed for 60 min at 30 °C in an incubation medium, containing 200 µCi [3H]PtdCho (about 250,000 cpm/assay), 50 mM HEPES, pH 7.0, 1 mM EGTA, 1 mM sodium oleate, 1 mM sodium cholate, and 2% (by volume) ethanol. In the detergent-free assay (12), [3H]PtdCho was mixed with PtdIns(4,5)P2 in a molar ratio of 8:1, dried, and resuspended in 50 mM HEPES, pH 7.5, 3 mM Na2HPO4, 10 mM KH2PO4, 1 mM EDTA, and 1 mM dithiothreitol, followed by sonication on ice. The assays were performed for 60 min at 37 °C in a total volume of 100 µl, containing 200 µCi [3H]PtdCho (about 500,000 cpm/assay), 25 µM PtdIns(4,5)P2, 80 mM KCI, 3 mM MgCl2, 2 mM CaCl2, 3 mM EGTA, 1 mM dithiothreitol, 50 mM HEPES, pH 7.5, and 2% (by volume) ethanol. The reactions started by the addition of membranes (200 µg of protein) were stopped by adding 2 ml of chloroform/methanol (1:1) and 1 ml of H2O, followed by analysis of [3H]PtdEtOH formed (27, 40).

**Glucosylation of G Proteins**—[^14C]Glucosylation of recombinant GTPases or GTPases in HEK-293 cell lysates by TcdB, TcsL, or TcdB-1470 and analysis of [14C]-glucosylated proteins by SDS-PAGE and autoradiography were carried out as described before (46, 48), with the following modifications. HEK-293 cells, grown to near confluence on 145-mm culture dishes, were treated without and with TcsL (100 ng/ml) or TcdB-1470 (300 µg/ml) for 24 h. Thereafter, the cells were detached from the culture flasks, washed once with ice-cold PBS, and resuspended in lysis buffer, containing 2 mM MgCl2, 0.1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 1 mM dithiothreitol, 5 mM GDP, and 50 mM HEPES, pH 7.5, followed by sonication on ice. HEK-293 cell lysates were prepared for the glucosylation reaction for 10 min at 1000 × g. The protein concentration was adjusted to 1 mg/ml. [14C]Glucosylation was performed for 45 min at 37 °C in a total volume of 30 µl containing 30 µM UDP-[3H]glucose (100 nCi), 0.1 mM dithiothreitol, 5 mM GDP, 2 mM MgCl2, and 50 mM HEPES, pH 7.5, as well as TcdB (1 µg/ml), TcdB-1470 (2 µg/ml), or TcsL (2 µg/ml). The reactions were started by the addition of 10 µl of HEK-293 cell lysate.
(100 μg of protein) or recombinant GTPases (2 μg of protein). The reactions were terminated by the addition of SDS-PAGE sample buffer and 5-min incubation at 95 °C. Thereafter, 14C-glucosylated proteins were separated by SDS-PAGE on 12.5% (mass/volume) acrylamide gels and visualized by autoradiography, using a Kodak Biomax MS film in combination with Transcreen-LE (46, 48).

**Purification of Recombinant GTPases**—The cDNAs of hRap1A, hRap2A, and hRap2B were inserted into the pGEX-2T vector (52). Fusion proteins containing an NH2-terminal glutathione S-transferase (GST) domain were induced in Escherichia coli by adding 1 mM isopropyl-1-thio-β-D-galactopyranoside to the cultures. The bacteria were sonicated on ice in buffer A, containing 50 mM NaCl, 5 mM MgCl2, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 50 mM Tris-HCl, pH 7.5, and the crude membrane fractions were removed by centrifugation. The supernatant, containing GST-Rap1A, GST-Rap2A, and GST-Rap2B proteins, was incubated with glutathione-Sepharose beads for 30 min at 4 °C. Thereafter, the beads were washed three times with buffer A to remove unbound proteins. Rap proteins were released from the parent GST fusion proteins bound to the beads by incubation with thrombin (10 units) overnight at 4 °C in a buffer containing 150 mM NaCl, 5 mM MgCl2, 2.5 mM CaCl2, 1 mM dithiothreitol, and 50 mM Tris-HCl, pH 8. Thereafter, the beads were removed by centrifugation, and the excess of thrombin was removed by the addition of p-amino-benzamidine beads. The homogeneity of the recombinant Rap proteins was analyzed by Coomassie Blue staining of SDS-PAGE gels. The C-terminally truncated Rap1A used for the experiment shown in Fig. 8A was a generous gift of Dr. N. van den Berghe.

Similarly, hRac1 and the C-terminally truncated sRalA (amino acids 1–177) and hRalB (amino acids 1–178) were produced as GST fusion proteins in the protease-poor strain AD202 (53) and purified over E. coli beads. The homogeneity of the recombinant Rap proteins was analyzed by Coomassie Blue staining of SDS-PAGE gels. The C-terminally truncated Rap1A used for the experiment shown in Fig. 8A was a generous gift of Dr. N. van den Berghe.

**Immunoblot Analysis**—For immunoblot analysis of PKC isozymes, cells were cultured in T500-cm² culture flasks to near confluence, before the addition of TcdB-1470 (300 pg/ml) for 24 h. Thereafter, the cells were detached from the culture flasks, pelleted by centrifugation, resuspended in PBS, and homogenized in a glass Teflon homogenizer. An aliquot of the homogenates and/or membranes (corresponding to about 50 μg of protein) was subjected to SDS-PAGE on 10% (mass/volume) acrylamide gels for the separation of PKC isozymes. Proteins were transferred to nitrocellulose membranes and blotted with anti-c-PCPK and anti-a-PCPK antibodies (1:400 dilution, 1-h incubation). For detection of Ral and Ras proteins in HEK-293 cell membranes, 100 μg of membrane proteins were subjected to SDS-PAGE on 15% (mass/volume) acrylamide gels, transferred to nitrocellulose membranes, and blotted with anti-RalA (1:5000 dilution, 1-h incubation), anti-RalB (1:250 dilution, 1-h incubation), and anti-Ras (1:500 dilution, 1-h incubation) antibodies. For detection of hPLD1α and hPLD1β, we used an anti-hPLD1-specific peptide antibody (1:2000 dilution, 1-h incubation) (54).

**Data Presentation**—Data are shown as mean ± S.D. from one experiment performed in triplicate, repeated as indicated in the figure legends.

**Fig. 1. Influence of C. difficile TcdB-1470 on PMA- and mACHR-stimulated PLD activities in HEK-293 cells**.

In previous studies, we have demonstrated that PLD stimulation in HEK-293 cells by the G protein-coupled m3 mAChR and phorbol ester-activated PKC are apparently independent and that m3 mAChR- but not PKC-mediated PLD stimulation involves ARF and Rho proteins (17, 27, 42, 43). To further characterize the two signaling pathways leading to PLD activation, we made use of two large clostridial cytotoxins, namely C. difficile TcdB-1470 and C. sordellii TeS1, which monoglucosylate a specific subset of small GTPases (see Refs. 45–48, and see below). Pretreatment of m3 mAChR-expressing HEK-293 cells with C. difficile TcdB-1470 caused a time- and concentration-dependent inhibition of PMA-stimulated PLD activity. [3H]PtdEtOH formation induced by PMA (0.1 μM) was inhibited by up to 85% in [3H]oleic acid-prelabeled cells pretreated for increasing periods of time with 300 pg/ml TcdB-1470 (Fig. 1, left panel). Half-maximal inhibition was observed after approximately 9 h of treatment with this toxin concentration. In contrast, PLD stimulation by the receptor agonist carbachol (1 mM) was virtually unaffected by TcdB-1470 (Fig. 1, right panel). Only very prolonged treatment of HEK-293 cells (>48 h) with TcdB-1470 (300 pg/ml) caused a slight reduction in carbachol-stimulated PLD activity. When pretreated for 24 h, PMA-stimulated PLD activity was half-maximally inhibited with 3–10 pg/ml TcdB-1470 and reduced by 70% upon treatment with 100–300 pg/ml TcdB-1470 (Fig. 2, left panel). In contrast, 24-h treatment of HEK-293 cells with any of these TcdB-1470 concentrations (0.3–300 pg/ml) did not alter carbachol-stimulated PLD activity (Fig. 2, right panel). At 3 ng/ml TcdB-1470, carbachol-stimulated PLD activity was reduced by 10–15% (data not shown). Basal [3H]PtdEtOH accumulation was not affected by TcdB-1470 treatment of HEK-293 cells (Figs. 1 and 2), even when treated with a high TcdB-1470 concentration (3 ng/ml, 24 h) (data not shown). These data suggest that TcdB-1470 potently and selectively interferes with PKC signaling to PLD.

TcdB-1470 and C. sordellii TeS1 have been reported to induce similar changes of cell morphology (tube-like cell shape with membrane blebbing), distinct from those induced by TcdB (44, 45). Similar distinct cell morphology changes induced by TcdB at one hand and TcdB-1470 and TeS1 on the other hand were observed in HEK-293 cells (data not shown). Therefore, we studied whether TeS1, known to inactivate Rac, Ras, Rap, and Ral proteins (46–48), may induce similar differential inhibitory effects on mAChR- and phorbol ester-stimulated PLD activities in HEK-293 cells as observed with TcdB-1470. Indeed, pretreatment of HEK-293 cells with TeS1 had no effect on PLD stimulation by carbachol (data not shown) but caused a time- and concentration-dependent in-
Inhibition of PMA-stimulated PLD activity. [3H]PtdEtOH accumulation induced by PMA (0.1 μM) was reduced by up to 70% in cells pretreated for increasing periods of time with 100 ng/ml TcsL (Fig. 3, left panel). When pretreated for 24 h, PMA-stimulated PLD activity was half-maximally reduced with 50 ng/ml TcsL, and 100 ng/ml TcsL inhibited PMA stimulation by 70% (Fig. 3, right panel). TcsL treatment had no effect on basal PLD activity.

To exclude the possibility that inhibition of PMA-induced PLD stimulation by TcdB-1470 and TcsL, causing drastic changes in cell morphology (44, 45), was due to their effects 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 G proteins (55, 56). Treatment of HEK-293 cells with cytochalasin B and C. botulinum C2 toxin were used as controls. Both agents, by distinct mechanisms, cause depolymerization of actin but notably without involving G proteins (55, 56). Treatment of HEK-293 cells with cytochalasin B and C. botulinum C2 toxin were used as controls. Both agents, by distinct mechanisms, cause depolymerization of actin but notably without involving G proteins (55, 56). Treatment of HEK-293 cells with cytochalasin B (5 μg/ml, 15 min) and C2 toxin (20 ng/ml component I plus 40 ng/ml component II, 24 h) did not reduce PMA-stimulated [3H]PtdEtOH accumulation, which amounted to 2.55 ± 0.03% and 2.89 ± 0.12% of total phospholipids, respectively, compared with 2.71 ± 0.23% in control cells.

To further dissect m3 mAChR and PKC signaling to PLD, we studied the effects of TcdB-1470 and TcsL on PLD activation by direct G protein stimulation. In control cells, direct activation of heterotrimeric G proteins by AlF4−S (100 μM) and PMA (0.1 μM) by 3–4-fold. Pretreatment of intact cells with TcdB-1470 (300 pg/ml, 24 h) did not reduce the response to PMA by about 60%. In contrast, basal PLD activity and PLD stimulation by GTPγS were not affected by pretreatment with TcdB-1470.

To study whether TcdB-1470 treatment may alter PLD per se, we measured PLD activities in membranes of control and TcdB-1470-treated HEK-293 cells with exogenous PtdCho as enzyme substrate, using two distinct assay conditions. TcdB-1470 treatment (300 pg/ml, 24 h) had no effect on basal PLD activity, measured with [3H]PtdCho/sodium oleate/sodium cholate vesicles, either in the absence or in the presence of the stimulating PLD cofactor, PtdIns(4,5)P2 (Fig. 5, left). Furthermore, basal and GTPγS-stimulated PLD activities, measured with [3H]PtdCho/PtdIns(4,5)P2 vesicles, were not affected by prior treatment of HEK-293 cells with TcdB-1470 (Fig. 5, right panel). In contrast, stimulation of PLD activity by PMA (0.1 μM) was markedly reduced, by about 60%, in membranes of HEK-293 cells pretreated with TcdB-1470.

TcsL mimicked the effects of TcdB-1470 on PMA-stimulated PLD activity, measured in HEK-293 cell membranes with [3H]PtdCho/PtdIns(4,5)P2 vesicles. Basal and GTPγS-stimulated PLD activities were not altered by prior treatment of HEK-293 cells with TcsL (100 ng/ml, 24 h), whereas PLD stimulation by PMA (0.1 μM) was reduced by about 60% (Fig. 6, left panel). As reported before (40), treatment of HEK-293 cells with TcdB (100 pg/ml, 24 h) also had no effect on basal and GTPγS-stimulated PLD activities measured in membranes with [3H]PtdCho/PtdIns(4,5)P2 vesicles as enzyme substrate (Fig. 6, right panel). However, in contrast to TcdB-1470 and TcsL, stimulation of PLD activity by PMA (0.1 μM) was not reduced, and was even slightly enhanced, in membranes of TcdB-treated cells. In previous studies, we demonstrated that TcdB blocks receptor-induced PLD stimulation in HEK-293
cells, apparently by reducing the cellular level of the PLD cofactor, PtdIns(4,5)P2 (27, 40, 41). In contrast, treatment of HEK-293 cells with TcdB-1470 (up to 3 ng/ml, 24 h, left) or TcsL (100 ng/ml, 24 h, right), followed by measurement of [3H]PtdEtoH formation in the absence (Basal) and presence of carbamol (1 μM), PMA (0.1 μM), or NaF (10 mM NaF plus 10 μM AlCl3), B, [3H]Oleic acid-prelabeled HEK-293 cells were treated for 24 h without (Control) and with TcdB-1470 (300 pg/ml). Thereafter, formation of [3H]PtdEtoH was determined in digitonin-permeabilized cells without (Basal) and with 100 μM GTPyS or 0.1 μM PMA as described under “Experimental Procedures.” Data are representative of three similar experiments.

To examine whether the inhibition of PMA-stimulated PLD activity was caused by potential degradation of PKC isozymes, the levels of c-PKC (α, β, γ) and a-PKC (ζ, η) isoforms were measured in membranes of control and TcdB-1470-treated HEK-293 cells with anti-PKC antibodies. A typical blot of three similar experiments performed in HEK-293 cell membranes is illustrated in Fig. 7, which indicates that the levels of the PKC isoforms were not different between control and TcdB-1470-treated cells. Similar data were obtained in cell homogenates (data not shown).

Since TcsL and TcdB-1470 caused similar alterations in PLD stimulation, quite distinct from those induced by TcdB, it was tempting to speculate that TcsL and TcdB-1470 modify and inactivate a similar set of GTPases. To study this, we determined 14C glucosylation of recombinant GTPases and GTPases in lysates of HEK-293 cells by TcdB, TcsL, and TcdB-1470. As shown in Fig. 8A, recombinant Rac1 served as glucosylation substrate for TcdB, TcsL, and TcdB-1470. In contrast, recombinant Rap and Ral proteins acted as substrates for TcsL and TcdB-1470 only, and Ras was exclusively glucosylated by TcsL. RhoA, which is a glucosylation substrate for TcdB (57), was glucosylated neither by TcsL (46, 47) nor by TcdB-1470 (data not shown). In lysates of untreated HEK-293 cells, TcdB, TcsL, and TcdB-1470 induced incorporation of 14C glucose into several proteins in the 20–30-kDa range (Fig. 8B). While each of the three toxins caused 14C glucosylation of proteins migrating in the 20–22-kDa range, only TcsL and TcdB-1470 induced incorporation of 14C glucose into a protein migrating at ~25 kDa. From comigration studies with recombinant proteins, the ~25 kDa is tentatively assigned to Ral proteins, while RhoA, Rac, Ras, and Rap proteins migrated within the 20–22-kDa range.
range. Pretreatment of intact HEK-293 cells with TcsL (100 ng/ml, 24 h) or TcdB-1470 (300 pg/ml, 24 h) reduced the subsequent $^{14}$C glucosylation catalyzed by either toxin by at least 50%. Pretreatment of HEK-293 cells with TcsL and TcdB-1470 did not reduce the levels of immunologically detectable Ral and Ras proteins (data not shown).

Considering the GTPase specificities of TcdB, TcsL, and TcdB-1470 (see Refs. 46–48 and Fig. 8A) on one hand and the distinct inhibitory patterns of these toxins on mAChR- and PMA-induced PLD stimulation on the other hand, it was tempting to speculate that a specific subset of GTPases participates in the PMA-induced PLD activation. Therefore, we studied the effects of various purified recombinant GTPases on PLD activity in membranes of toxin-pretreated HEK-293 cells. Rac proteins are common substrates of TcdB, TcsL, and TcdB-1470, suggesting that Rac proteins may not be essential for PKC signaling to PLD in HEK-293 cells. Indeed, as shown in Fig. 9, the addition of purified recombinant Rac1 had no effect on basal and PMA-stimulated PLD activities in control membranes and did not restore the TcdB-1470-inhibited PLD stimulation by PMA.

Since Ras proteins are substrates for TcsL, but not TcdB-1470, it was likely that the addition of Ras proteins might restore PLD stimulation by PMA in membranes of cells pretreated with TcsL, while being ineffective in TcdB-1470-treated membranes. Indeed, the addition of recombinant RasG12V, a constitutively active Ras mutant, to membranes of HEK-293 cells pretreated with TcdB-1470 (300 pg/ml, 24 h) did not restore PLD stimulation by PMA (Fig. 10, left panel). However, the addition of recombinant RasG12V also had no effect on PMA-induced PLD stimulation in membranes of cells pretreated with TcsL (100 ng/ml, 24 h) (Fig. 10, right panel). Similar to Rac1, the addition of RasG12V did not alter basal and PMA-stimulated PLD activities in membranes of control untreated cells. Thus, Rac and Ras proteins seem not to be crucial for stimulation of PLD by PKC in HEK-293 cells.

Since Rap and Ral proteins are common targets of TcsL and TcdB-1470, but not TcdB, their participation in the PMA-induced PLD activation was rather likely. However, as shown in Fig. 11, the addition of different Rap proteins (Rap1A, Rap2A, and Rap2B) did not restore the TcdB-1470-inhibited PLD stimulation by PMA. The addition of Ral proteins, which were detected by immunoblotting in the membrane preparations (data not shown), had no effects on basal and GTP$_\gamma$S-stimulated PLD activities in control membranes (data not shown). Furthermore, as illustrated in Fig. 12A, the addition of RalA (up to 30 mM) did not affect basal and GTP$_\gamma$S-stimulated PLD activities in membranes of TcdB-1470-treated HEK-293 cells. However, stimulation of membrane PLD activity by PMA, which was reduced by prior TcdB-1470 treatment of intact HEK-293 cells, could be fully restored by the addition of RalA in a concentration-dependent manner. RalB had similar ef-
fects, whereas heat-inactivated Ral proteins were inactive (data not shown).

As recently reported (43), PLD stimulation by PMA in membranes of HEK-293 cells is absolutely dependent on the presence of MgATP (Fig. 12A). Half-maximal and maximal stimulation of PLD activity by PMA (0.1 μM) was observed at about 50 μM and 1 mM MgATP, respectively. In membranes of TcdB-1470-treated HEK-293 cells, the remaining PMA-stimulated PLD activity (about 30%) also exhibited a strict MgATP dependence, with half-maximal and maximal stimulation by PMA (0.1 μM) occurring at about 10 μM and 100 μM MgATP, respectively. The addition of recombinant RalA fully restored the TcdB-1470-inhibited PLD stimulation by PMA, exhibiting an identical MgATP dependence as in control untreated membranes (Fig. 12B).

Finally, since TesL inactivates Ras and Ral proteins and evidence has been provided that Ras/Ral signaling cascades can be involved in PLD stimulation (30, 58, 59), we examined whether Ral proteins alone can restore PLD stimulation by PMA in membranes of TesL-treated cells or whether a combination of Ras and Ral proteins is required. As shown above (see Fig. 10), the addition of RasG12V alone to membranes of TesL-treated HEK-293 cells had no effect on the TesL-inhibited PMA-stimulated PLD activity (Fig. 13). In contrast, the addition of RalA fully restored PLD stimulation by PMA. The co-addition of RasG12V did not affect basal PLD activity; nor was the PMA-induced PLD stimulation restored by RalA further enhanced. An additive or synergistic effect of RasG12V addition also was not observed when RalA was added at a half-maximally effective concentration (data not shown).

**DISCUSSION**

In the present study, we have investigated the involvement of Ras-like GTPases in signal transduction pathways leading to PLD stimulation in HEK-293 cells stably expressing the m3 mAChR. Previous studies indicated that at least two distinct signaling pathways evoke PLD activation in HEK-293 cells (42). PLD stimulation by the agonist-activated m3 mAChR is dependent on G proteins of the ARF and Rho protein family (17, 27). While ARF proteins may directly stimulate PLD activity as recently reported for purified PLD1, by interacting with specific PLD domains (8, 9), Rho proteins appear to participate indirectly in receptor-mediated PLD activation in HEK-293 cells, i.e. by controlling the supply of the stimulatory PLD cofactor, PtdIns(4,5)P2 (17, 27, 40, 41). In line with this notion, specific binding of partially purified rat brain PLD to PtdIns(4,5)P2 has recently been described (60) as well as direct activation of purified recombinant PLD isozymes (hPLD1, mPLD2, and rPLD2) by PtdIns(4,5)P2 (8, 10, 11). In contrast to mAChR activation, PLD stimulation in HEK-293 cells by phorbol ester-activated PKC is apparently independent of ARF and Rho proteins (17, 27, 43). This may reflect the existence of multiple signaling pathways, activating the lipase through dif-
different signal transduction components, and/or the existence of different PLD isozymes, being under control of distinct cellular components. Indeed, the existence of different PLD isozymes in one cell type has recently been reported (61, 62).

The role of different GTPases, specifically members of the Rho and Ras protein family, in receptor and PKC signaling to PLD in HEK-293 cells was studied with the use of different large clostridial cytoxins, belonging to a family of glucosyltransferases that monoglucosylate a specific subset of small G proteins, among which TRC proteins in intact or permeabilized HEK-293 cells as well as in membrane preparations where an exogenous PLD substrate was added. It should be noted that TcdB-1470 and TcsL did not cause a complete (100%) loss of PMA-induced PLD stimulation (see e.g. Figs. 1–3), suggesting that a small part of PMA-stimulated PLD activity is independent of the G proteins being substrates for TcdB-1470 and TcsL. As recently reported with membrane-bound, partially purified, and purified PLD enzymes, PLD can be directly stimulated by purified phorbol ester-activated PKC-α, which is apparently independent of the enzymatic kinase activity (8, 9, 36, 39). In membranes of HEK-293 cells, PMA-induced PLD stimulation required the presence of MgATP (see Ref. 43 and Fig. 12), suggesting an enzymatic mechanism of action as also reported for PLD stimulation by PKC isozymes (PKC-α, PKC-β1, and PKC-γ) in membranes of human neutrophils (35). Thus, the small part of PMA-stimulated PLD activity that was resistant to treatment of HEK-293 cells with TcdB-1470 and TcsL may be due to a direct interaction of a PKC isozyme with PLD, although it must be noted that the remaining PMA-stimulated PLD activity observed in membranes of TcdB-1470-treated cells was also MgATP-dependent. In contrast to PLD stimulation by PMA, stimulation of PLD activity by the m3 mAChR and direct activation of G proteins in intact or permeabilized HEK-293 cells as well as membrane preparations was not or was only marginally reduced by either TcdB-1470 or TcsL, and basal activity was not affected at all. On the other hand, mAChR-mediated PLD stimulation was potently inhibited by TcdB (27), which did not affect PMA-stimulated PLD activity. Second, as studied with TcdB-1470 and/or TcsL, the reduction in PMA-induced PLD stimulation cannot be attributed to a loss or altered
activity of individual components potentially involved in PLD stimulation. Specifically, TcdB-1470 treatment did not reduce levels of c- and a-PKC isoforms. Furthermore, the MgATP dependence of PMA-induced PLD stimulation in membranes of TcdB-1470-treated HEK-293 cells reconstituted with RaLA was identical to that of control membranes, indicating that the enzymatic mechanism of action of PKC isozyme(s) involved in PMA-stimulated PLD activity in HEK-293 cells was not altered by TcdB-1470. Third, in contrast to TcdB, which reduced cellular PtdIns(4,5)P_2 levels and basal PLD activity measured in the absence of PtdIns(4,5)P_2 (40, 41), treatment of HEK-293 cells with TcdB-1470 and TcsL had only a slight effect on cellular PtdIns(4,5)P_2 level and did not reduce basal PLD activity measured in the absence of PtdIns(4,5)P_2. Fourth, direct measurement of TcdB-1470- and TcsL-catalyzed glucosylation of recombinant GTPases and GTpases endogenously expressed in HEK-293 cells suggested that RaL proteins are glucosylation substrates of either toxin. Finally, and most importantly, the addition of purified recombinant RaL proteins (RaLA and RaLB) fully restored PMA-induced PLD stimulation in membranes of HEK-293 cells pretreated with either TcSL or TcdB-1470. On the other hand, purified recombinant Rap proteins, which are also substrates of TcsL and TcdB-1470, were inactive in this respect. Furthermore, recombinant Rac1, serving as glucosylation substrate for TcSL, but not TcdB-1470, did not restore TcdB-1470-inhibited PMA-induced PLD stimulation. Interestingly, recombinant Ras (RasG12V), serving as glucosylation substrate for TcSL, but not TcdB-1470, did not rescue PMA-induced PLD stimulation, either in membranes of HEK-293 cells treated with TcdB-1470 or in those treated with TcSL. The small GTPases used in the reconstitution rescued PMA-induced PLD stimulation, either in membranes of HEK-293 cells pretreated with TcSL or TcdB-1470. The small GTPases used in the reconstitution rescues PMA-induced PLD stimulation, either in membranes of HEK-293 cells treated with TcdB-1470 or TcsL, and TcdB-1470, which glucosylate and thereby inactivate Ras-like GTPases, most likely RaL proteins. As mentioned above, m3 mACHr-mediated PLD stimulation in HEK-293 cells is independent of phospholipase C activation in HEK-293 cells (41, 42), it is likely that the PKC isozyme(s) responsible for PMA-stimulated PLD activity is different from the PKC isozyme(s) activated by the m3 mACHr, subsequent to the phospholipase C stimulation, or is present in a distinct cellular compartment.

Recently, a participation of RaL proteins in PLD stimulation in Balb/c and NIH 3T3 fibroblasts has been reported (30). However, in contrast to the data presented in this report in HEK-293 cells, the RaL-mediated PLD stimulation in those cells was caused by v-Src and was apparently not PKC-mediated. The v-Src-induced activation of PLD in Balb/c and NIH 3T3 fibroblasts required, in addition to RaL proteins, activated Ras and the RaL guanine nucleotide dissociation-stimulating factor, RaL-GDS. Furthermore, the RaL-mediated PLD activation was apparently independent of the GTP/GDP-binding state and the effector domain of RaLa but was attributed to the unique NH_2-terminus of this G protein (30). Similar to that observed in Balb/c and NIH 3T3 fibroblasts (30), PMA-stimulated PLD activity in HEK-293 cells seems to be largely independent of the GTP/GDP binding state of RaL proteins but may not involve active Ras. First, full restoration of PMA-stimulated PLD activity in membranes of HEK-293 cells treated with TcSL or TcdB-1470 by RaL proteins was observed without adding GTP or GDPyS. Second, PLD stimulation by PMA and GDPyS in HEK-293 cells was simply additive (42), and the latter one was not affected by inactivation of Ras-like GTPases. Third, the addition of recombinant RasG12V, a constitutively active Ras mutant, to membranes of HEK-293 cells treated or not treated with TcdB-1470 or TcsL did not alter basal and GDPyS- or PMA-stimulated PLD activities. Finally, the simultaneous addition of RasG12V and RaLA had no additive or synergistic effect on TcsL-inhibited PMA-induced PLD stimulation. However, although the reconstitution experiments suggest that Ras is not essential for PKC-induced PLD stimulation mediated by RaL proteins, the possibility of a signaling cascade involving Ras, RaL-GDS, and RaL proteins in PKC-induced PLD stimulation in intact HEK-293 cells cannot be excluded and is presently under investigation. Recently, direct interaction of RaL with the ARF-responsive PtdIns(4,5)P_2-dependent recombinant PLD1 isozyme has been demonstrated (65). HEK-293 cells apparently express hPLD1, as shown by immunoblot studies with anti-hPLD1-specific peptide antibodies (54) in membrane preparations (data not shown). Thus, it remains to be studied whether RaL proteins interact directly with hPLD1 in HEK-293 cells as well and whether this process is under the control of phosphor ester-activated PKC.

In conclusion, we demonstrate that C. sordellii TcSL and C. difficile TcdB-1470, which inactivate Ras-like GTPases, potentially and specifically inhibit PLD stimulation by phosphor ester-activated PKC in HEK-293 cells. By comparing the effects of different large clostridial cytotoxins, namely TcdB, TcSL, and TcdB-1470, which glucosylate and thereby inactivate Ras-like GTPases, we provide evidence that RaL proteins are essentially required for PKC signaling to PLD in HEK-293 cells. In contrast, receptor stimulation of PLD activity, which was highly sensitive to inactivation of Rho proteins by TcdB, was resistant to inactivation of Ras-like GTPases by TcdB-1470 and TcSL, further strengthening the possibility that distinct signal transduction pathways leading to stimulation of PLD activity exist in HEK-293 cells.

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