A Role for Rho-kinase in Rho-controlled Phospholipase D Stimulation by the m3 Muscarinic Acetylcholine Receptor*

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Stimulation of phospholipase D (PLD) by membrane receptors is now recognized as a major signal transduction pathway involved in diverse cellular functions. Rho proteins control receptor signaling to PLD, and these GTPases have been shown to directly stimulate purified recombinant PLD1 enzymes in vitro. Here we report that stimulation of PLD activity, measured in the presence of phosphatidylinositol 4,5-bisphosphate, by RhoA in membranes of HEK-293 cells expressing the m3 muscarinic acetylcholine receptor (mAChR) is phosphorylation-dependent. Therefore, the possible involvement of the RhoA-stimulated serine/threonine kinase, Rho-kinase, was investigated. Overexpression of Rho-kinase and constitutively active Rho-kinase (Rho-kinase-CAT) but not of kinase-deficient Rho-kinase-CAT markedly increased m3 mAChR-mediated but not protein kinase C-mediated PLD stimulation, similar to overexpression of RhoA. Expression of the Rho-inactivating C3 transferase abrogated the stimulatory effect of wild-type Rho-kinase, but not of Rho-kinase-CAT. Recombinant Rho-kinase-CAT mimicked the phosphorylation-dependent PLD stimulation by RhoA in HEK-293 cell membranes. Finally, the Rho-kinase inhibitor HA-1077 largely inhibited RhoA-induced PLD stimulation in membranes as well as PLD stimulation by the m3 mAChR but not by protein kinase C in intact HEK-293 cells. We conclude that Rho-kinase is involved in Rho-dependent PLD stimulation by the G protein-coupled m3 mAChR in HEK-293 cells. Thus, our findings identify Rho-kinase as a novel player in the receptor-controlled PLD signaling pathway.

Phospholipase D (PLD) enzymes belong to a newly identified enzyme family known to exist in plant, bacteria, yeast, and mammalian sources. Stimulation of PLD has been described in many cellular systems in response to a large variety of agonist-activated tyrosine kinase receptors and receptors coupled to heterotrimeric G proteins and is apparently involved in various signaling processes (1–3). Specifically, PLD and its immediate reaction product, phosphatidic acid, have been reported to regulate diverse cellular events, such as vesicular trafficking, actin stress fiber formation, activation of Raf-1 kinase, and phosphatidylinositol 4-phosphate (PtdIns4P)-5-kinase isofoms, to name but a few (4–9).

The two mammalian PLD isoforms identified thus far, PLD1 (with the two splice variants PLD1a and PLD1b) and PLD2, differ greatly in their regulatory properties. PLD2 is thought to be solely stimulated by the phosphoinositide phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P2; Refs. 10 and 11), although recent reports on cloned PLD2 enzymes suggest that this PLD isoform may also be activated, but very modestly, by ADP ribosylation factor (ARF), a member of the low molecular weight GTPase superfamily (12, 13). On the other hand, PLD1 enzymes are strongly stimulated by PtdIns(4,5)P2 and ARF and, in addition, by some protein kinase C (PKC) isoforms and by Rho GTPases (14, 15). Specifically, the Rho family GTPases RhoA, Rac1, and Cdc42, which are activated by the stable GTP analog guanosine 5′-O-(3-thio)triphosphate (GTPγS), have been shown to stimulate purified recombinant PLD1 enzymes, apparently by a direct interaction of PLD1 with these GTPases (16–19). Furthermore, studies performed with toxins inactivating Rho GTPases indicated that these GTPases are also involved in PLD stimulation by G protein-coupled and growth factor receptors in intact cells (20–23). However, stimulation of endogenous PLD by Rho GTPases seems to be rather complex. Whereas RhoA stimulation of ARF-sensitive PLD has been reported in some cell-free systems (24–26), it was without effect in others or could even be resolved from the ARF-stimulated PLD (15, 26, 27). Rho GTPases may also indirectly stimulate PLD enzymes by increasing the synthesis of PtdIns(4,5)P2 by PtdIns4P 5-kinases (28), which has been demonstrated in various cellular systems to be of crucial importance for signaling to PLD (2, 3, 7, 29, 30).

We have recently reported that in HEK-293 cells stably expressing the G protein-coupled m3 muscarinic acetylcholine receptor (mAChR), PLD activity depends on PtdIns(4,5)P2 and that PLD stimulation by phorbol ester-activated PKC involves the Ras-related Ral proteins, whereas m3 mAChR signaling to PLD is mediated by members of the ARF and Rho GTPase families (20, 30–34). The aim of the present study was to identify the mechanism of PLD stimulation by Rho proteins in HEK-293 cells. We demonstrate here that the PLD stimulatory effect of recombinant RhoA in HEK-293 cell membranes is phosphorylation-dependent. In the search for the putative kinase, we studied the effect of the RhoA-stimulated serine/threonine kinase Rho-kinase (35), which has also been termed ROCKα (36) and ROCK-II (37), on PLD regulation. We found...
that overexpression of Rho-kinase greatly increases m3 AchR-mediated but not PKC-mediated PLD stimulation in intact cells. Furthermore, we show that HA-1077, a Rho-kinase inhibitor, specifically suppresses receptor-mediated PLD stimulation and that recombinant Rho-kinase mimics the stimulatory effect of RhoA on PLD activity in HEK-293 cell membranes. These findings strongly suggest that Rho-kinase is involved in Rho-controlled PLD stimulation by the G protein-coupled m3 AchR in HEK-293 cells.

EXPERIMENTAL PROCEDURES

Materials—[3H]Oleic acid (10 Ci/mmole) and 1-palmitoyl-2-[3H]palmitoyl-glycerophosphocholine ([3H]PtdCho; 37.5 Ci/mmole) were from New England Nuclear. HA-1077 was from Calbiochem, and glutathione-Sepharose was from Amersham Pharmacia Biotech. Unlabeled PtdCho, phorbol 12-myristate 13-acetate (PMA), and TNM-FI insect medium were from Sigma, and PtdIns(4,5)P2 and GTP-S were from Roche Molecular Biochemicals. Antibodies against RhoA and Rho-kinase were purchased from Santa Cruz Biotechnology.

Plasmids—DNA encoding human RhoA was subcloned into pRK5 expression vector. DNA encoding myc-tagged C3 transferase subcloned in pEF (38) was a kind gift of Dr. A. Hall. DNAs encoding myc-tagged wild-type Rho-kinase, the catalytic domain of Rho-kinase, Rho-kinase-CAT (amino acids 6–553), and the kinase-deficient mutant of Rho-kinase-CAT, Rho-kinase-CAT-KD (Rho-kinase-CAT K121G), were subcloned into pEF (39). For expression in Sf9 cells, DNA encoding RhoA was subcloned into a pAcGHLL baculovirus transfer vector (PharMinigen) and DNA encoding Rho-kinase-CAT was subcloned into a pAcGLT transfer vector (39).

Cell Culture and Transfection—Culture conditions of HEK-293 cells stably expressing the m3 AchR were as reported previously (31). For experiments, cells subcultured in Dulbecco's modified Eagle's medium/F-12 medium were grown to near confluence (145-mm culture dishes) and transfected with either the indicated concentrations of DNA encoding RhoA, myc-tagged C3 transferase, Rho-kinase, Rho-kinase-CAT or RhoA-KD, or the corresponding vectors using the calcium phosphate method (40). Transfection efficiency of HEK-293 cells, which ranged from 50% to 80%, was determined by in situ staining for β-galactosidase activity of the cells cotransfected with the constitutively active pSVβ-gal (Promega). All assays were performed 48 h after transfection. Transient overexpression of the proteins was verified by immunoblotting of cell lysates using specific antibodies. Transient overexpression of C3 transferase was detected by the mobility shift of the calcium-ribosylated endogenously expressed RhoA (41). Morphological changes induced by overexpression of RhoA and Rho-kinase were visualized by phase-contrast microscopy (Nikon TMS).

Assay of PLD Activity in Intact Cells—For measurement of PLD activity in intact HEK-293 cells, the cells were replated 24 h after transfection on 145-mm culture dishes. Cellular phospholipids were labeled by incubating monolayers for 20–24 h with [3H]oleic acid (2 μCi/ml) in growth medium. Thereafter, cells were detached from the dishes, washed 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 to pH 7.4 with 15 mM HEPES, and resuspended at a cell concentration of 1 × 106 cells/ml. PLD activity was measured for 60 min at 37 °C in a total volume of 200 μl containing 100 μl of cell suspension (1 × 106 cells), 400 μl methanol, and the indicated stimulatory agents. The reaction was stopped, and labeled phospholipids, including the specific PLD product [3H]phosphatidylethanol ([3H]PtdEtoH), were isolated as described previously (31). The formation of [3H]PtdEtoH is expressed as a percentage of the total amount of labeled phospholipids. Data shown are the mean ± S.D. from one experiment performed in triplicate and repeated as indicated in the figure legends.

Assay of PLD Activity in Membranes—To measure PLD activity in HEK-293 cell membranes prepared as described previously (30), [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 EGTA, 80 mM KCl, and 1 mM dithiothreitol, followed by sonication on ice. PLD activity was determined as described previously (30) with [3H]PtdCho/PtdIns(4,5)P2 (200 μM/25 μM) as substrate vesicles and 200 μg of membrane protein for 60 min at 37 °C or for 15 min at 30 °C.

FIG. 1. Phosphorylation-dependent PLD stimulation by RhoA. PLD activity was measured in HEK-293 cell membranes as described under "Experimental Procedures" with [3H]PtdCho/PtdIns(4,5)P2 substrate vesicles in the absence (Basal) and presence of 100 μM HA-1077 or 10 μM Rho plus 100 μM GTP·S, added alone (No MgATP) or with 1 mM MgATP, for 60 min at 37 °C. Data are representative of three experiments.

for 48 h at 25 °C. Thereafter, the cells were centrifuged, resuspended in Buffer A (50 mM NaCl, 10 mM MgCl2, 1 mM dithiothreitol, 10 μM phenylmethylsulfonyl fluoride, and 10 mM Tris-HCl, pH 7.5), and homogenized by sonication on ice. The lysates were centrifuged for 1 h at 20,000 × g. The supernatant, which contained GST-RhoA or GST-Rho-kinase-CAT, 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. RhoA and Rho-kinase-CAT were released from the parent GST-fusion proteins bound to the beads by incubation with thrombin (PharMinigen; 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 8.0. The beads were removed by centrifugation, and the excess thrombin was removed by the addition of p-aminobenzamidine beads. The homogeneity of the recombinant RhoA and Rho-kinase-CAT proteins was analyzed by SDS-polyacrylamide gel electrophoresis.

Immunoblot Analysis—For immunoblot analysis, an aliquot of the supernatant was subjected to SDS-polyacrylamide gel electrophoresis on 10% acrylamide gels to separate the proteins. After a transfer to nitrocellulose membranes and a 1-h incubation with anti-RhoA (1:500 dilution) or anti-Rho-kinase (1:200 dilution) antibodies, the proteins were visualized by enhanced chemiluminescence.

RESULTS

PLD Stimulation by RhoA in HEK-293 Cell Membranes Is Phosphorylation-dependent—We have reported previously that PLD stimulation by the m3 AchR, which activates endogenous RhoA in HEK-293 cells (42), is potently inhibited by the inactivation of Rho family GTPases with Clostridium difficile toxin B (20). Furthermore, it has been shown that toxin B and the Rho-specific C3 transferase (41) decrease the cellular level of PtdIns(4,5)P2 and that PtdIns(4,5)P2 regulates PLD activity in HEK-293 cell membranes (30, 32, 43). Thus, to study PLD stimulation by Rho proteins in HEK-293 cells, we measured PLD activities in the membranes of HEK-293 cells in the presence of PtdIns(4,5)P2. Several previous studies have demonstrated that activated RhoA stimulates purified recombinant PLD1 enzymes under this condition, apparently by a direct Rho-A-PLD1 interaction (16–19). In HEK-293 cell membranes, the addition of GTP·S (100 μM) alone caused about a 2-fold increase in PLD activity, which is probably due to the activation of endogenous membrane-associated ARF proteins (30, 32). Surprisingly, however, the addition of purified recombinant RhoA (10 μM) in the presence of GTP·S (100 μM) had no effect on PLD activity (Fig. 1). Similar data were obtained in the membranes of HEK-293 cells pretreated with toxin B, causing the inactivation of endogenous Rho proteins (data not shown). In contrast, under the same assay conditions, GTP·S-
activated recombinant RhoA stimulated PLD activity in the membranes of human PLD1a-expressing Sf9 cells about 20-fold, from 50 ± 10 to 1070 ± 80 pmol h⁻¹ mg protein⁻¹ (mean ± SD; n = 5 experiments).

Besides PtdIns4P 5-kinases, Rho GTPases can stimulate various protein kinases (for reviews, see Refs. 28 and 44). Therefore, we studied whether a phosphorylation reaction is involved in RhoA stimulation of HEK-293 cell PLD activity. For this study, PLD activity was measured in the presence of 1 mM MgATP. Under this condition, the addition of RhoA in the presence of GTPγS markedly increased PLD activity in HEK-293 cell membranes (Fig. 1). Although a permissive effect of MgATP on GTPγS binding by RhoA cannot be excluded, we first considered the possibility that a RhoA-dependent lipid or protein kinase is involved in PLD stimulation by RhoA. The involvement of a RhoA-stimulated PtdIns(4,5)P₂ 5-kinase was unlikely for the following reasons: (a) PtdIns(4,5)P₂ was degraded at the end of the incubation period in the absence of MgATP to 10 μmol, this PtdIns(4,5)P₂ concentration was virtually identical to that (12 μmol) used by many others to demonstrate RhoA stimulation of PLD in vitro (16–19, 45); and (c) in the presence of MgATP, which by itself prevented (by ~50%) the degradation of added PtdIns(4,5)P₂, the addition of RhoA had a very modest protective effect on the PtdIns(4,5)P₂ level (data not shown), which was quite distinct from the marked PLD stimulation by RhoA under this condition. Thus, the phosphorylation-dependent PLD stimulation by RhoA in HEK-293 cell membranes may involve a protein kinase rather than PtdIns4P 5-kinase.

Rho-Kinase Stimulates PLD in HEK-293 Cells—Recently, several direct RhoA target proteins have been identified, including the two Rho-stimulated serine/threonine kinases: (a) Rho-kinase, also termed ROCKs or ROCK-II, and (b) p160ROCK, also termed ROCK-I (35–37, 46). To test the hypothesis that Rho-induced PLD stimulation involves Rho-kinase, we studied the effects of HEK-293 cell transfection with RhoA and different Rho-kinase constructs on cell morphology and PLD activity. Expression of RhoA and the myc-tagged Rho-kinases was verified by immunofluorescence and immunoblotting (data not shown). Overexpression of RhoA caused drastic changes in HEK-293 cell morphology, as demonstrated by the occurrence of a high number of rounded cells (Fig. 2). As reported previously by others (36, 44, 47), similar morphology changes were observed in HEK-293 cells overexpressing Rho-kinase-CAT, which lacks the regulatory Rho-binding and PH domains and is constitutively active, and although less pronounced, in wild-type Rho-kinase-overexpressing cells. In contrast, overexpression of a kinase-deficient mutant of Rho-kinase-CAT, Rho-kinase-CAT-KD, did not cause rounding of HEK-293 cells.

We then determined the effects of the same proteins on PLD activity in HEK-293 cells. As illustrated in Fig. 3, overexpression of RhoA and either wild-type Rho-kinase or Rho-kinase-CAT markedly increased m3 mAChR-mediated PLD stimulation without significantly altering basal PLD activity. The PLD stimulatory effects were most pronounced in cells overexpressing RhoA and Rho-kinase-CAT. For example, the transfection of HEK-293 cells with 100 μg of RhoA DNA and Rho-kinase-CAT DNA increased PLD stimulation by carbachol (1 mM) by about 150% and 250%, respectively (Fig. 3, A and C). A significant but less pronounced increase in m3 mAChR-mediated PLD stimulation was observed in HEK-293 cells overexpressing wild-type Rho-kinase (Fig. 3B). In contrast, the transfection of HEK-293 cells with up to 150 μg of kinase-deficient Rho-kinase-CAT-KD DNA did not change PLD stimulation by carbachol (Fig. 3D). The potentiating effect of Rho-kinase and Rho-kinase-CAT overexpression on m3 mAChR-mediated PLD stimulation was even more evident at low agonist concentrations. For example, at 3 μM, carbachol had only a very small effect on PLD activity in control cells, but it increased the PLD activity in Rho-kinase-CAT-expressing cells to the same extent as 1 mM carbachol in control cells (Fig. 4). Finally, the effects of wild-type Rho-kinase and Rho-kinase-CAT overexpression on PLD stimulation by phorbol ester-activated PKC were studied. In contrast to m3 mAChR-mediated PLD stimulation, stimulation of PLD by PMA (100 nM) was not altered by the transfection of HEK-293 cells with either wild-type Rho-kinase or Rho-kinase-CAT (Fig. 5).

To study the involvement of Rho in the potentiating effect of Rho-kinase on m3 mAChR-mediated PLD stimulation, cotransfection experiments with C3 transferase were performed. Overexpressed C3 transferase reduced the carbachol (1 mM)-stimulated PLD activity in control cells and fully prevented the stimulatory effect of coexpressed wild-type Rho-kinase (Fig. 6A). In contrast, cotransfection with C3 transferase did not inhibit the potentiating effect of Rho-kinase-CAT on m3 mAChR-mediated PLD stimulation (Fig. 6B).

Rho-Kinase Stimulates PLD in Vitro—To study whether Rho-kinase activates PLD in vitro, the effects of purified recombinant Rho-kinase-CAT were measured on PLD activity in HEK-293 cell membranes. In the absence of MgATP, Rho-kinase-CAT had no effect on PLD activity in either the absence or presence of GTPγS (data not shown). However, in the presence of MgATP (1 mM), the addition of Rho-kinase-CAT (2 μM) markedly increased PLD activity (Fig. 7). The net increase in
activity was similar in both the absence and presence of GTPγS (100 μM), which enhanced PLD activity by itself about 2-fold. In contrast, in the presence of RhoA (10 μM) and GTPγS (100 μM), further addition of Rho-kinase-CAT had no effect on enzyme activity. These results indicate that Rho-kinase can stimulate HEK-293 cell PLD activity in vitro almost as efficiently as GTPγS-activated RhoA.

DISCUSSION
In the present study, we provide evidence that the Rho-stimulated serine/threonine kinase Rho-kinase participates in...
PLD stimulation by the G protein-coupled m3 mAChR in HEK-293 cells. The evidence is based on the following findings: (a) stimulation of PLD activity in HEK-293 cell membranes measured in the presence of PtdIns(4,5)P₂ by purified GTPγS-activated RhoA was fully MgATP-dependent; (b) overexpression of wild-type Rho-kinase and particularly of Rho-kinase-CAT but not of Rho-kinase-CAT-KD potentiated and sensitized m3 mAChR-mediated PLD stimulation in intact HEK-293 cells, similar to overexpression of RhoA; (c) inactivation of Rho by coexpressed C3 transferase blocked the stimulatory effect of wild-type Rho-kinase but not of Rho-kinase-CAT; (d) recombinant Rho-kinase-CAT mimicked the phosphorylation-dependent stimulatory effect of GTPγS-activated RhoA on PLD stimulation in HEK-293 cell membranes; and (e) the Rho-kinase inhibitor HA-1077 largely suppressed PLD stimulation by RhoA in HEK-293 cell membranes as well as by the m3 mAChR in intact cells.

We reported previously that the inactivation of Rho family GTPases with C. difficile toxin B potently inhibits m3 mAChR-mediated PLD stimulation in HEK-293 cells (20). Furthermore, we showed that toxin B and Rho-specific C3 transferase reduce the cellular level of PtdIns(4,5)P₂ and that PtdIns(4,5)P₂ stimulates PLD activity in HEK-293 cell membranes (30, 43). Therefore, we examined the effect of activated RhoA on PLD activity in HEK-293 cell membranes in the presence of PtdIns(4,5)P₂. Previous studies on various crude cell-free preparations (24–26) and with purified recombinant PLD1 enzymes (16–19) using similar in vitro PLD assay conditions reported that activated RhoA stimulates PLD activity. Furthermore, the data obtained with purified PLD1 enzymes strongly suggested that activated RhoA directly interacts with and stimulates PLD1 activity. Using membranes of human PLD1a-expressing SF9 cells, we also observed that activated RhoA potently stimulates PLD activity. However, there are also reports on the failing effects of RhoA on PLD activity. For example, Vinggaard et al. (27) reported that GTPγS-activated RhoA stimulates PLD activity in crude membranes of the human placenta, but that this stimulation is lost after partial PLD purification, in contrast to the stimulatory effect of ARF3 that was maintained with the purified enzyme preparation. Furthermore, Park et al. (15) showed that activated ARF3 but not activated RhoA stimulates PLD activity in native COS-7 cell membranes, whereas in the membranes of COS-7 cells, overexpressing rat PLD1 enzyme RhoA stimulated PLD activity. Thus, stimulation of PLD by RhoA can apparently occur by both direct and indirect mechanisms.

In contrast to human PLD1a-expressing SF9 cells, activated RhoA did not stimulate PLD activity in the membranes of HEK-293 cells. The ineffectiveness of RhoA was not due to saturation of the membranes with endogenous Rho, because RhoA was similarly ineffective in the membranes of HEK-293 cells in which the endogenous Rho proteins had been inactivated with toxin B. However, when MgATP was added, RhoA strongly stimulated PLD activity, thus suggesting that a phosphorylation reaction is involved. Because the PLD assay was performed in the presence of a saturating concentration of PtdIns(4,5)P₂ (30) and because the addition of RhoA in the presence of MgATP had only a very small effect on the PLD assay conditions reported that activated RhoA stimulates PLD activity. Furthermore, the data obtained with purified PLD1 enzymes strongly suggested that activated RhoA directly interacts with and stimulates PLD1 activity. Using membranes of human PLD1a-expressing SF9 cells, we also observed that activated RhoA potently stimulates PLD activity. However, there are also reports on the failing effects of RhoA on PLD activity. For example, Vinggaard et al. (27) reported that GTPγS-activated RhoA stimulates PLD activity in crude membranes of the human placenta, but that this stimulation is lost after partial PLD purification, in contrast to the stimulatory effect of ARF3 that was maintained with the purified enzyme preparation. Furthermore, Park et al. (15) showed that activated ARF3 but not activated RhoA stimulates PLD activity in native COS-7 cell membranes, whereas in the membranes of COS-7 cells, overexpressing rat PLD1 enzyme RhoA stimulated PLD activity. Thus, stimulation of PLD by RhoA can apparently occur by both direct and indirect mechanisms.

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PLD stimulation by PMA-activated PKC, which is less sensitive to the inactivation of Rho GTPases by toxin B than the receptor response (20, 32, 33), was not affected. C3 transferase fully blocked the stimulatory effect of wild-type Rho-kinase, indicating that even the overexpressed Rho-kinase is under tight control by endogenous Rho proteins. Finally, purified Rho-kinase-CAT increased PLD activity in HEK-293 cell membranes, similar to activated RhoA and in a non-additive manner, suggesting a common pathway for PLD stimulation by these two proteins.

Overexpressed RhoA and wild-type Rho-kinase increased the receptor-induced PLD stimulation but had no effect on the basal PLD activity. Strikingly, the constitutively active Rho-kinase-CAT, which stimulated PLD activity in HEK-293 cell membranes, also had no effect on basal PLD activity in intact cells, but it required the input by the activated receptor, and this effect was C3-insensitive. These findings suggest that for stimulation of PLD by Rho and Rho-kinase in intact HEK-293 cells, an additional component or signal is necessary, which is apparently provided by the receptor and may sensitize PLD for stimulation by Rho-kinase. The second signal required for PLD stimulation could be ARF activated by the receptor (34). A similar bifurcating pathway involving both Rho and ARF proteins has been described for PLD stimulation by the formyl peptide receptor in human neutrophils (23). Alternatively, the receptor may inactivate or cause the redistribution of PLD inhibitory proteins, which are mainly identified in and purified from brain cytosol (50–53), which may prevent PLD stimulation by Rho-kinase-CAT in intact cells in the absence of receptor stimulation. In contrast, in membrane preparations, Rho-kinase-CAT may have unrestricted access to stimulate PLD. Among the PLD inhibitory proteins, synucleins have recently been identified as selective inhibitors of PLD2 enzymes (53). Furthermore, evidence has recently been provided suggesting that PLD2 enzymes can be activated by epidermal growth factor and insulin receptors (54, 55). However, which of the PLD isoforms endogenously expressed in HEK-293 cells represents the enzyme stimulated by the m3 mAChR is presently unknown. Furthermore, although very attractive, in preliminary experiments with membranes of HEK-293 cells overexpressing human PLD1α or mPLD2 and purified Rho-kinase-CAT, no clear evidence for phosphorylation of the expressed PLD proteins has been obtained thus far (data not shown).

In conclusion, this study identifies Rho-kinase as a novel and essential component of the Rho-dependent signaling pathway leading to PLD stimulation by a G protein-coupled receptor.

**FIG. 8.** Inhibition of m3 mAChR-induced PLD stimulation by a Rho-kinase inhibitor. m3 mAChR-expressing HEK-293 cells prelabeled with [3H]oleic acid were treated with HA-1077 at the indicated concentrations for 15 min, followed by PLD activity measurements (A) in the absence (Basal, ◊) or presence of 1 mM carbachol (●) or (B) in the absence (Basal, □) and presence of 100 μM PMA (■). Data are representative of three to five similar experiments.

**FIG. 9.** Inhibition of Rho-induced PLD stimulation by a Rho-kinase inhibitor. PLD activities were measured in HEK-293 cell membranes pretreated or not pretreated for 15 min at 4 °C with 10 μM HA-1077 in the absence (Basal) and presence of 1 mM MgATP plus 100 μM GTPγS, 10 μM recombinant RhoA, or the indicated combinations for 60 min at 37 °C. Data are representative of four similar experiments.
Thus, for the first time, a role for Rho-kinase in phospholipid signaling is demonstrated.

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