Evidence for Rho-mediated Agonist Stimulation of Phospholipase D in Rat1 Fibroblasts

KENNETH C. MALCOLM, CASSONDRA M. ELLIOTT, AND JOHN H. EXTON†

From the Howard Hughes Medical Institute and the Departments of Molecular Physiology and Biophysics and Pharmacology, Vanderbilt University School of Medicine, Nashville, Tennessee 37232-0295

Small GTP-binding proteins of the Rho family are implicated in the in vitro regulation of phosphatidylcholine-hydrolyzing phospholipase D (PLD). However, their role in agonist-stimulated PLD activity in whole cells is not clear. The ribosyltransferase C3 from Clostridium botulinum modifies Rho proteins and inhibits their function. When introduced into rat1 fibroblasts by scrape-loading, C3 inhibited PLD activity stimulated by lysophosphatidic acid (LPA), endothelin-1, or phorbol ester. Neither the time course nor agonist dose response for LPA-stimulated PLD activity was altered in C3-treated cells. In contrast to the effects of C3 on PLD activity, agonist-stimulated phosphatidylinositol-phospholipase C activity was not altered in C3-treated cells. Surprisingly, C3 treatment led to a decrease in the amount of RhoA protein, indicating that the loss of PLD activity in response to agonist was partly due to the loss of Rho proteins. As described previously, C3 treatment led to the inhibition of LPA-stimulated actin filament formation. However, disruption of actin filaments with cytochalasin D caused only a minor inhibition of LPA-stimulated PLD activity. Interestingly, stimulation of cells with LPA caused a rapid enrichment of RhoA in the particulate fraction of cell lysates. These data support an in vivo role for RhoA in agonist-stimulated PLD activity that is separate from its role in actin fiber formation.

Phosphatidylcholine-hydrolyzing phospholipase D (PLD) activity has been identified in a wide variety of cell types in response to extracellular signals (1, 2). However, the mechanisms by which receptor activation leads to stimulation of PLD activity are poorly understood and appear to be multiple (1, 2). Early studies using fractionated or permeabilized cells indicated the involvement of G proteins in this response (3–6); GTPγS, a nonhydrolyzable analog of GTP, alone or in combination with agonists for seven transmembrane-spanning receptors was shown to stimulate PLD activity as measured by production of phosphatidylalcohols, the unique product of PLD in the presence of primary alcohols. In some cases, the stimulation of PLD activity in response to GTPγS or agonist was insensitive to inhibition by pertussis toxin (7, 8), an agent that prevents receptor coupling to G1 or Gα, thereby discounting these heterotrimeric G proteins in the activation of PLD.

Recent studies have concluded that GTPγS-stimulated PLD activity is mediated by Ras-related low molecular weight (small) G proteins of the Rho and ARF families (9–16). The Rho family includes RhoA, -B, and -C, Rac1 and -2, Cdc42, and TC10, which are involved in membrane movement, cytoskeletal rearrangements, and cell growth (17–22). In broken or permeabilized cell systems, purified or recombinant Rho proteins stimulate PLD activity in the presence of GTPγS but not GDP (10, 11, 13). RhoA displays the greatest activity in these assays, whereas Rac1 and Cdc42 have variable activity. RhoGDI inhibits GTPγS-stimulated PLD activity in membranes (9–11, 13) and is able to bind tightly to and extract membrane-bound Rho, Rac, and Cdc42 (10, 13, 23). Stimulation of PLD activity by Rho-family proteins often requires prior removal of endogenous Rho from membrane preparations by RhoGDI (10, 11). Although many experiments have indicated the involvement of Rho family proteins in GTPγS-stimulated PLD activity in vitro, their involvement in receptor-mediated activation of PLD in vivo has not been well documented.

Fibroblasts provide a useful model system in which to explore mechanisms of signal transduction from the plasma membrane. Manipulation of the activity of cellular Rho has been demonstrated using the C3 exoenzyme of Clostridium botulinum which catalyzes an ADP-ribosyltransferase reaction. Modification of Rho by this enzyme interferes with its biological effects including actin stress fiber formation (18, 24, 25). In the present study, we have used the method of scrape-loading to introduce the C3 transferase directly into cells. In addition to causing ADP-ribosylation of Rho, the treatment led to a loss of RhoA from cells. More importantly, an inhibition of agonist- and phorbol ester-stimulated PLD activity was observed. These observations support the idea that Rho proteins are mediators of agonist-stimulated PLD activity and that protein kinase C-mediated activation of PLD may also involve Rho proteins.

EXPERIMENTAL PROCEDURES

Materials—Lysophosphatidic acid (LPA) was from Avanti Polar Lipids, endothelin-1 (ET-1) from Calbiochem, and phorbol 12-myristate 13-acetate (PMA) from Sigma. BODIPY 558/568 phalloidin was from Molecular Probes and SDS-polyacrylamide gels from NOVEX. Anti-RhoA antibodies were from Santa Cruz Biotechnology and C3 exoenzyme from List Biologicals. [3H]Myristic acid and [3H]inositol were from DuPont NEN.

Cell Culture Conditions—Rat1 fibroblasts or rat1 fibroblasts transfected with a vector conferring puromycin resistance (pBabe purom) were grown in a 5% CO2 incubator in Dulbecco’s modified Eagle’s medium...
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Scrape Loading—Rat1 fibroblasts growing on 100-mm dishes in serum-containing medium were washed in 5 ml of phosphate-buffered saline (PBS) and 2 ml of scraping buffer (114 mM KCl, 15 mM NaCl, 5.5 mM MgCl2, 10 mM Tris-HCl). Scraping buffer (0.5 ml) was then added in the presence or absence of C3 transferase (5 μM/ml). Cells were gently scraped (26, 27) and resuspended in DMEM/10% fetal bovine serum, split into six-well dishes, and grown overnight. Confluent dishes were routinely used for scraping and gave the best viability. An estimated >90% of viable cells incorporated large molecules as determined by scrape-loading fluorescent M, 70,000 dextran.

PLD Assay—Scrape-loaded cells were serum-starved and labeled with [3H]myristic acid (1 μCi/ml) overnight (28). Unincorporated [3H]myristic acid was removed by washing with DMEM, and cells were incubated in DMEM for 50 min at 37 °C followed by incubation in 0.3% 1-butanol for 10 min. Cells were stimulated with the indicated concentration of agonist for 20 min at 37 °C, or as indicated. The reaction was terminated by washing in PBS and addition of 1.2 ml of ice-cold methanol. Cells were scraped into 1.2 ml of CHCl3 and 1.2 ml of H2O and extracted overnight. Phosphatidylbutanol, the transphosphatidylation reaction product of PLD in the presence of butanol, was separated as described previously (10), localized with a BioScan 200 Imaging Scanner, scraped from the plate, and quantified by scintillation counting.

Phosphoinositide Phospholipase C Assay—Cells were scrape-loaded, in the absence or presence of C3 transferase as described, into six-well plates, serum-deprived in inositol-free DMEM, and labeled with 1 μCi/ml myo-[3H]inositol for 18 h. Unincorporated myo-[3H]inositol was removed by washing in DMEM followed by incubation for 10 min in DMEM containing 20 μM LiCl. Cells were stimulated with LPA (100 μM) or ET-1 (100 nM) for 20 min at 37 °C. Reactions were stopped with 750 μl of 20 mM formic acid, incubated on ice for 30 min, and neutralized with 100 μl of 0.7 M NaOH. Cells were scraped into Eppendorf tubes and centrifuged at 14,000 g for 10 min, and the supernatant was applied to Dowex AG1–8x columns in a modification of Berridge et al. (29). myo-[3H]inositol-labeled metabolites were eluted consecutively with 4 ml of H2O, 4 ml of 40 mM ammonium formate, 0.1 M formic acid, and 5 ml of 1 M ammonium formate, 0.1 M formic acid to isolate inositol phosphates ([3H]IP fraction). PLC activity was calculated from the ratio ([3H]IP/([3H]inositol + [3H]PI) and converted to fold-stimulation of activity in control-scraped cells in the absence of agonists.

Actin Polymerization—Scrape-loaded cells were grown on glass coverslips, serum-deprived for 18 h, washed in DMEM, and incubated for 50 min before addition of 100 μM LPA for 5 min. After washing in PBS, cells were fixed in 3.7% formaldehyde for 20 min, permeabilized in 0.2% Triton X-100 for 5 min, and incubated with BODIPY 558/568 phallolidin for 30 min as described by Molecular Probes, Inc. Coverslips were washed, air-dried, and mounted on slides with clear nail polish. Fluorescence was observed with a 100 × objective using a Leica DMRB microscope.

Localization of RhoA—Cells growing in 100-mm dishes were serum-starved in DMEM for 18 h and stimulated in the absence or presence of LPA (100 μM) for 2 min, washed in PBS, and scraped into 1.0 ml of PBS that included protease inhibitors (10 μM leupeptin, 10 μM antipain, 0.2 mM phenylmethylsulfonyl fluoride). Cells were lysed by 6 passes through a 25-gauge needle (30). Examination of lysates indicated >95% disruption of the plasma membrane by trypan blue staining. Suspensions were centrifuged at 125,000 × g for 45 min. The resulting cytosolic and particulate fractions were suspended in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and examined for RhoA content by Western blotting.

Western Analysis—Cells were washed in PBS and solubilized in SDS-PAGE sample buffer, and protein concentrations were determined by BCA (Pierce). Equal amounts of cellular or cell fraction proteins (0.3–0.5 μg) were separated by SDS-PAGE on a 14% gel, transferred to Immobilon-P, blocked in 10% non-fat dry milk, and detected using the specified antibody, as described previously (10).

RESULTS

To determine the role of Rho proteins in agonist activation of PLD, rat1 fibroblasts were stimulated with LPA, an agonist that acts via a G protein-coupled receptor (31, 32). LPA has been shown to stimulate the activity of Rho in Swiss 3T3 fibroblasts, but not that of other members of the Rho family (17, 18). LPA stimulated a dose-dependent increase in PLD activity in serum-starved rat1 cells, as measured by production of PtdBut, with an EC50 of approximately 1 μM (Fig. 1A). LPA activation by LPA was rapid, but transient, with maximal PtdBut formation being attained at 2 min (Fig. 1B). In agreement with findings in Swiss 3T3 cells (17, 18), LPA stimulated the formation of actin stress fibers (Fig. 2, A and B).

We investigated the involvement of Rho in agonist-stimulated PLD activation using C3 transferase. Cells scrape-loaded with C3 transferase showed striking morphological differences to control-scraped cells, characterized by spindle formation and rounding of the cell body (Fig. 2, C and D). Control-scraped cells responded normally to agonist stimulation. PLD activity was enhanced 18-, 22-, and 42-fold by LPA, ET-1, and PMA, respectively (Fig. 3). In contrast, in C3-treated cells, PLD activation by these agents was inhibited by 63–65% (Fig. 3). Treatment of cells with C3 transferase did not significantly alter the time course of PtdBut formation by LPA (Fig. 1B), i.e. PtdBut formation was inhibited at all time points measured. Therefore, C3 treatment did not act to slow the PLD response to LPA or alter the metabolism of PtdBut.

Analysis of the LPA dose-response curves on PLD activity indicated that C3 transferase did not act at the level of the LPA receptor. Whereas the extent of PLD activity was inhibited by C3 transferase, the efficacy of LPA changed very little since the EC50 values were 1.0 and 0.5 μM in control- and C3-treated
cells, respectively (Fig. 1A). In addition, the activation of PI-PLC by LPA or ET-1 in scraped cells was not significantly altered by C3 treatment (Fig. 4). The formation of membrane ruffles by platelet-derived growth factor was also unaffected in C3-treated cells (data not shown).

The effects of C3 transferase on levels of RhoA protein were studied by Western blot analysis. Surprisingly, treatment of cells with C3 resulted in a greater than 80% loss of immuno-detectable RhoA (Fig. 5). This was not due to an inability of the RhoA antibody to detect modified RhoA since other studies have shown that C3-induced ADP-ribosylation does not alter the detection of recombinant RhoA by the antibodies used in

Fig. 2. Actin polymerization and cell morphology are altered by C3 transferase. Cells were scraped in the absence (A and B) or presence (C and D) of 5 μg/ml C3 transferase. Serum-starved cells were stimulated for 5 min with 100 μM LPA (B and D) or a bovine serum albumin solution (A and C).

Fig. 3. Receptor-dependent and -independent stimulation of PLD is inhibited by C3 transferase. PLD activity was measured as described in Fig. 1 in response to LPA (100 μM), ET-1 (100 nM), or PMA (100 nM) for 20 min in control or C3-treated cells. PtdInsOEt production in the absence of agonist was 252 ± 58 cpm in control cells and 464 ± 68 cpm in C3-treated cells.

Fig. 4. Receptor-stimulated PI-PLC activity is unaffected by C3 transferase. PI-PLC activity was measured as described under “Experimental Procedures” in response to stimulation with LPA (100 μM) or ET-1 (100 nM) for 20 min in control or C3-treated cells.
our study (33).

The known role of Rho in actin polymerization prompted us to investigate the role of the cytoskeleton in agonist-mediated PLD activity (17, 18). Treatment of cells with cytochalasin D leads to a breakdown of cytoskeletal structure and disruption of actin filament formation. Treatment of rat 1 cells with cytochalasin D (2 μM) had only a small (23%) inhibitory effect on LPA-stimulated PLD activity (Fig. 6). As expected, the cellular network of polymerized actin was obscured by this treatment (data not shown).

The site of action of Rho on PLD is likely to be at a cellular membrane(s). However, much of the cellular Rho is contained in the cytosol in association with RhoGDI (34). Therefore, we studied the effects of LPA on the cellular localization of RhoA. Treatment of cells with LPA for 2 min caused a redistribution of RhoA from the cytosolic to the particulate fraction (Fig. 7). The effect was transient, however, since no accumulation of RhoA in the particulate fraction was observable at 5 min (data not shown).

DISCUSSION

The present data using the C3 transferase of C. botulinum indicate that Rho proteins play a role in agonist-stimulated PLD activity in rat 1 fibroblasts (Figs. 1 and 3). The C3 transferase has been useful in elucidating Rho-mediated events, but the inability to readily incorporate it into a population of cells has limited studies of the cellular function of Rho proteins. The technique of scrape-loading successfully incorporates molecules into adherent cells (26) and has been used in studies of oncogenic Ras (27, 35). The present findings indicate its feasibility to investigate the role of Rho proteins in the regulation of PLD and other activities in cell populations.

The incorporation and function of C3 in scrape-loaded cells was confirmed by (i) morphological changes in C3-treated cells (Fig. 2) and (ii) decreased incorporation of [32P]NAD in extracts of cells previously treated with C3 transferase (data not shown). Interestingly, the loss of labeling by [32P]NAD was due not only to the incorporation of endogenous NAD but mainly to a decrease in the amount of immunoreactive RhoA. Since recombinant RhoA and RhoA modified by ADP-ribosylation are recognized equally by the antibody (33), the decrease represents a loss of protein. Furthermore, ADP-ribosylation of recombinant RhoA and RhoA in C3-treated cells is often accompanied by an upward gel shift (10, 36), and this was seen in some cases in the present study. It is not known by what mechanism ADP-ribosylation of RhoA leads to its net degradation. Recently, Dillon et al. (36) have demonstrated a similar degradation of RhoA in C. difficile toxin B-treated fibroblasts. Therefore, modification of RhoA by these agents may be a general destabilizing signal.

FIG. 5. Incorporation of C3 transferase by scrape-loading decreases RhoA protein. Cells scraped in the presence or absence of C3 transferase were treated as for PLD assays but serum-starved in the absence of [3H]myristic acid. Cellular proteins were solubilized in sample buffer, and proteins (0.5 μg) were separated by SDS-PAGE on a 14% gel. RhoA was identified by Western blotting.

FIG. 6. Effect of cytochalasin D on LPA-stimulated PLD activity. Cells labeled with [3H]myristic acid were treated with 0.1% dimethyl sulfoxide or 2 μM cytochalasin D (cyto D) for 1 h before stimulation with 100 μM LPA.

Loss of RhoA in C3-treated cells corresponded to the inhibition of agonist- and PMA-stimulated PLD activity. Based on the lack of inhibition of both agonist-stimulated P1-PLC activity (Fig. 4) and platelet-derived growth factor-induced membrane ruffling, the inhibition of PLD activity appears selective. In addition, LPA dose-response curves on PLD activity showed little change in EC50 values (Fig. 1A). These observations indicate that receptor function is not impaired. Together, our data suggest a role for Rho proteins in agonist-stimulated PLD activation in vivo. Very recently, Schmidt et al. (37) used a similar approach using Clostridium difficile toxin B to conclude also that Rho proteins are involved in the PLD response to agonists.

Tyrosine phosphorylation of proteins occurs in response to LPA (38, 39) and has been implicated in the regulation of PLD activity (40–42). Furthermore, C3-treated Swiss 3T3 cells have an impaired ability to phosphorylate proteins on tyrosine in response to LPA (39). Therefore, inhibition of Rho-dependent PLD-activated tyrosine phosphorylation in C3-treated cells may explain our results. However, efforts to inhibit PLD activity to the same extent as in C3-treated cells were unsuccessful using tyrosine kinase inhibitors (data not shown) or cytochalasin D, which also inhibits agonist-stimulated tyrosine phosphorylation (data not shown). Cytochalasin B, a compound of similar specificity to that of cytochalasin D, had no effect also on receptor-stimulated PLD activity in HEK cells (37). Recently, Rho-dependent serine/threonine kinases have been identified, and these could be involved in PLD activation in vivo (43–46).

PMA-stimulated PLD activity was also inhibited in C3-treated cells (Fig. 3) indicating that Rho proteins act in a pathway involving PKC in rat 1 fibroblasts. Recent studies indicate a functional interaction between RhoA and PKC (47). However, a significant component of the PMA effect was not...
affected by C3 transference, and little or no inhibition of PMA-stimulated PLD activity was observed in C. difficile toxin B-treated HEK cells (37). These results highlight the complexity of regulation of PLD activity in various tissues and cell types, perhaps reflecting differences in the distribution of different PLD and PKC isoforms and of accessory proteins that modulate the effects of Rho and ARF on PLD (48, 49). The mechanism of action of PKC on PLD is still undefined and may partly involve a nonphosphorylation mechanism (50).

We and others (9, 10) have reported that C3 treatment of either recombinant RhoA or membranes does not inhibit membrane-associated PLD activity assayed in the presence of GTPγS. Thus, it is possible that C3 modification of Rho may not interfere with the interaction of Rho with PLD at the membrane but may block activation of Rho by agonists or alter the access of Rho to the site(s) at which it activates PLD.

RhoA localization is changed by treatment of cells with LPA, the G protein translocates from the cytosolic to the particulate fraction (Fig. 7). Furthermore, activation of PLD and movement of RhoA to the particulate fraction both occur rapidly. Maximal PLD activation occurs within 2 min of LPA treatment and does not increase thereafter (Fig. 1B), and the shift in RhoA from the cytosol to the particulate fraction is equally rapid (Fig. 7), although its membrane association declines more rapidly (data not shown).2 Agonist-stimulated translocation of ARF from a detailed study of the translocation of Rho-family proteins in response to various agonists is being conducted and will be reported elsewhere (I. Fleming, and J. H. Exton, unpublished observations).

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Kenneth C. Malcolm, Cassondra M. Elliott and John H. Exton

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