ADP-ribosylation Factor Proteins Mediate Agonist-induced Activation of Phospholipase D*

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The role of small G proteins of the ADP-ribosylation factor (ARF) and Rho families on the activation of phospholipase D (PLD) by platelet-derived growth factor (PDGF) and phorbol esters (PMA) has been investigated. The activation of PLD by PDGF and PMA was blocked by brefeldin A (BFA), an inhibitor of ARF activation, but not by Clostridium botulinum C3 exotoxin, an inhibitor of the activity of Rho. PDGF and PMA, in the presence of GTPyS, promoted the association of ARF and RhoA with cell membranes. Cells depleted of ARF and Rho by digitonin permeabilization showed a significant reduction of the activity of phospholipase D. Recombinant ARF was sufficient to restore agonist-induced PLD activity to digitonin-permeabiled, cytoplasm-depleted cells. In contrast, isoprenylated recombinant RhoA had no effects in this reconstitution assay. HIREB cells were transiently transfected with wild-type and dominant-negative mutants of ARF1 and ARF6. Neither wt-ARF1 nor wt-ARF6 had any effects on agonist-dependent PLD activity. However, dominant-negative ARF1 and ARF6 mutants blocked the stimulation of PLD by PDGF but only partially inhibited the effects of PMA. These results demonstrate that ARF rather than Rho proteins mediate the activation of PLD by PDGF and phorbol esters in HIREB fibroblasts.

Platelet-derived growth factor (PDGF)* stimulates a number of cellular responses following the binding to its specific cell surface receptor and activation of the intrinsic tyrosine kinase (1, 2). Some of the actions of PDGF are mediated by lipid second messengers, including phosphatidylinositol derivatives, diacylglycerol and phosphatidic acid (PA) (3–5). PA is a product of the hydrolysis of phosphatidylincholine by phospholipase D (PLD). PA has been proposed to play an important role in intracellular signaling. On one hand, PA is a precursor of other second messengers, such as diacylglycerol (by removal of the phosphate from the head group) or lyso-PA (by removal of the fatty acid from the sn-2 position by phospholipase A₂). On the other, PA has been shown to activate tyrosine phosphorylation, to inhibit Ras-GAP, and to stimulate the recruitment of Raf-1 to membranes (6–8).

The mechanism by which PDGF stimulates PLD activity has not been well established. Several lines of evidence suggest that the effects of PDGF on the activation of PLD require the activation of the phospholipase C-diacylglycerol/PKC signaling cascade (5, 9–11). In vitro data suggest that PKC may activate PLD by a direct interaction mechanism (12). Furthermore, recent data support the idea that PLD may be tyrosine-phosphorylated (13), thus suggesting a potential mechanism by which PDGF might regulate PLD activity.

However, the most potent physiological activators of PLD are small G proteins of the Ras superfamily. Several members of the family appear to regulate PLD activity, including most members of the ARF and Rho families (14–19). Of these, ARF proteins appear to be the most active in vitro. Reports by Hammond et al. (20) and Frohman and Morris (21) have confirmed that Rho and ARF proteins act as direct stimulators of PLD.

The relationship between the activation of small G proteins and receptor-mediated PLD activation has been explored in a few recent studies. Karnam et al. (22) have shown that insulin induces the translocation of ARF and Rho to the plasma membrane of rat adipocytes and that the activation of PLD by insulin correlates with the activation of Rho proteins. We showed that ARF mediates insulin-dependent activation of PLD in a rat fibroblast cell line (23). Studies using Rat1 fibroblasts strongly suggest that Rac proteins mediate the stimulation of PLD by epidermal growth factor, another member of the Rho family (11). Morgan et al. (24) have reported that ARF-regulated PLD activity is translocated to the plasma membrane from secretory vesicles upon stimulation of neutrophils with formyl-methionyl-leucyl-phenylalanine. Thus, small G proteins of the ARF and Rho families are likely to play an important role in the regulation of PLD activity by cell surface receptors although the details of these roles have not been fully established.

Here we provide evidence that links the activation of ARF by PDGF and phorbol esters to the effects of these agonists on the activation of PLD. First, we show that the activation of PLD by PDGF and PMA is inhibited by brefeldin A (BFA), an inhibitor of the activation of ARF proteins, (25–27) but not by exotoxin C3, an inhibitor of the function of Rho proteins. Second, using a permeabilized cell assay, we show that PDGF in the presence of ATP and GTPyS stimulates the recruitment of ARF and Rho proteins to the membrane. PMA had similar effects but only in the presence of exogenous PKCα. Third, we show that permeabilized, cytoplasm-depleted cells lose their ability to stimulate PLD in response to agonist and GTPyS, and that the observed responses are reconstituted, at least partially, by the addition of recombinant ARF but not by RhoA. Finally, we show that...
overexpression of putative dominant-negative mutants of ARF1 and ARF6 block the activation of PLD by PDGF and significantly reduce the effects of PMA. These results indicate that the activation of PLD by PDGF and PMA in HIRcB cells is mediated by ARF proteins and that the role of Rho in this process is not essential.

EXPERIMENTAL PROCEDURES

Cell Culture—HIRcB cells were cultured in 100-mm dishes using Dulbecco’s modified Eagle’s medium/F-12 supplemented with 10% fetal bovine serum as described (23, 28). Cells were used within 3 days of subculture and were serum-starved for 20 h in medium containing 0.1% bovine serum albumin before treatment.

ARF Proteins—The following ARF proteins were used: wt-ARF1, wt-ARF6, T31N-ARF1, and T27N-ARF6. The latter are putative dominant-negative mutants with reduced affinity for GTP. These mutants were cloned in the multiple cloning site of the vector pGEFP-N1 (CLONTECH) and fused to the green fluorescent protein of Aequorea victoria as described (29). Cells were transfected with these constructs as described elsewhere (29). The efficiency of transfection was estimated by counting cells using phase contrast and fluorescence microscopy of the same fields. The transfection efficiency was 60–80% for all the experiments reported. The PLD activity of ARF-GFP-transfected cells was determined approximately 48 h after transfection.

Immunoblotting—Protein bands resolved by SDS-PAGE were transferred to nitrocellulose filters. The filters were blocked for 2 h at room temperature in blocking buffer (phosphate-buffered saline containing 5% non-fat milk and 0.1% Tween 20). ARF was detected with 2 μg/ml affinity purified 1D9 (a monoclonal antibody that recognizes most members of the ARF family (30); a gift from R. Kahn) or a commercial anti-RhoA antibody obtained from Santa Cruz Biotech. Bound antibodies were detected by chemiluminescence.

PLD Assays—Cells (100-mm plates; 70–80% confluency) were serum-starved and labeled overnight with [3H]palmitate (5 μCi/ml) in Dulbecco’s modified Eagle’s medium/F-12 containing 0.1% bovine serum albumin. Cells were stimulated with PDGF (30 ng/ml) or PMA (500 nM) in the presence of 0.3% butanol for 20 min. PDGFB-mediated PLD activity was significantly reduced. The activity of PLD was significantly reduced. The activity of PLD was significantly reduced. The activity of PLD was significantly reduced. The activity of PLD was significantly reduced.

Supernatants and pellets were collected separately and resolved by SDS-PAGE. The bands were transferred to nitrocellulose and immunoblotted with the appropriate antibodies. Western blot analysis revealed a single band corresponding to the 55-kDa PtdBu band. The specific activity of PLD was determined by liquid scintillation counting. The data are expressed as the number of counts of lipid.

RESULTS

Activation of Phospholipase D by PDGF and PMA Is Brefeldin A-sensitive—Stimulation of HIRcB cells with PDGF resulted in a large increase in the levels of PLD activity (480 ± 45%) (Fig. 1A). Treatment with BFA resulted in an even greater increase (955 ± 78%) (Fig. 1B). When BFA (50 μg/ml) was added 10 min before stimulation of the cells with either agonist, the activity of PLD was significantly reduced. The effects of PDGF were completely abolished by BFA treatment (Fig. 1A) whereas the effects of PMA were reduced by 62% (Fig. 1B). PMA-stimulated PLD activity was significantly reduced but never completely abolished by the inhibitor. Since BFA is a potent inhibitor of the activation of ARF proteins (25–27) this finding suggests that the activation of ARF is a key step in the signaling pathway by which PDGF regulates PLD activity. In contrast, the activation of PLD by PMA could only be partially blocked by BFA, suggesting that the activation of ARF is one of two or more parallel pathways by which the activation of PKC.
modulates PLD activity. Since the exposure to BFA was acute (10 min), the effects of the drug cannot be related to the long-term cytotoxic effects of BFA.

Fig. 2 shows the dose response of PDGF-induced PLD activity to increasing concentrations of BFA. As shown, the half-maximal dose of BFA was less than 5 µg/ml, and doses of 25–50 µg/ml completely ablated the stimulatory effects of PDGF. In contrast, the inactive BFA analog B17 (25 µg/ml) had no effects on the stimulation of PLD activity by PDGF. These experiments confirmed that the effects of BFA were specific.

The Activation of PLD by PDGF and PMA Is Not Sensitive to Pretreatment with Exotoxin C3—The effects of Clostridium botulinum C3 exotoxin were also examined. This protein catalyzes the ADP-ribosylation of proteins of the Rho family and has been reported to inhibit agonist-induced PLD activation in some cellular systems (10, 11, 22). Cells were treated with C3 using the scrape loading procedure reported by Malcolm et al. (10). Cells loaded with C3 and controls were incubated overnight. The action of the toxin was evident, since treated cells were rounded and were not tightly attached to the culture dish. Furthermore, the level of ADP-ribosylation of Rho was determined by incubating cell extracts with additional C3 in the presence of 32P-labeled NAD. After incubation for 90 min, aliquots of the reaction mixture, normalized according to their protein contents, were electrophoresed, and the level of incorporation of 32P to specific bands was determined by autoradiography. As shown in Fig. 3A, control untreated cells were extensively labeled during the in vitro ADP-ribosylation reaction, whereas cells pretreated with C3 incorporated very little [32P]ADP-ribose. This confirms that the scrape-loading procedure was effective in the modification of endogenous Rho proteins. Fig. 3B shows the activity of PLD in HIRcB cells scrape-loaded with C3 transerase and mock-scraped cells. Both agonist and PMA induced a significant activation of PLD although to a smaller level than that observed with cells that had not been previously subjected to scraping (compare Fig. 1 and Fig. 3B). However, C3 exotoxin had no significant effects on the activation of PLD by either agent. These results clearly support the hypothesis that ARF rather than Rho activation is necessary for the stimulation of PLD by PDGF and PKC.

**PDGF and PMA Induced the Translocation of ARF and Rho Proteins to a Membrane Fraction**—We have recently used a simple cell permeabilization and reconstitution assay to analyze the role of cytosolic factors in the stimulation of membrane-bound PLD (23). This assay is based on the use of low concentrations of digitonin to permeabilize the cells. Since digitonin induces the formation of membrane pores large enough to allow the passage of macromolecules, this permeabilization procedure results in the depletion of cytoplasmic proteins (23, 33). Fig. 4 shows that, after digitonin permeabilization, both ARF and Rho proteins leak out of the cell in substantial quantities. This suggests that both ARF and Rho proteins are mainly cytosolic in non-stimulated cells. However, when the cells were permeabilized in the presence of GTPγS (100 µM), PDGF (30 ng/ml), ATP (1 mM), and Mg2+ (2 mM), most of the G protein was found associated to a particulate fraction. Similar effects were obtained when PDGF was replaced by PMA (500 nM), but the effects of PMA required the addition of PKCα (5 µg/ml). This demonstrates the recruitment of both ARF and Rho to membranes by the combined effect of the agonist and the GTP analogue. Neither PDGF nor PMA in the absence of GTPγS had any effects on the translocation of ARF and RhoA. Likewise, the guanine nucleotide alone was ineffective in promoting the translocation of ARF and RhoA in the absence of agonist.

**Reconstitution of Agonist- and GTPγS-dependent PLD Activity with Recombinant ARF1 and RhoA**—Fig. 4 also shows that digitonin-treated cells can be ARF- and Rho-depleted when permeabilization is done in the absence of agonists and GTP analogues. We took advantage of this phenomenon to study the involvement of these G proteins in the process of activation of PLD by PDGF and PMA. During permeabilization, GTPγS and PDGF added separately did not alter PLD activity whereas the inclusion of both agents together activated PLD significantly (Fig. 5). Cells that were permeabilized in the absence of agonist and GTPγS (conditions in which they were depleted of ARF and Rho proteins, as shown in Fig. 4) failed to respond to PDGF and GTPγS added after permeabilization. This shows that some cytosolic factor lost during the permeabilization treatment is necessary to mediate the effects of PDGF on PLD activity. As shown in Fig. 5, the addition of myristoylated recombinant ARF1 was sufficient to restore the sensitivity of PLD to PDGF- and GTPγS. In contrast, addition of either recombinant RhoA that had been geranyl-geranylated in vitro or recombinant RhoA-GDI complexes produced in yeast failed to reconstitute agonist-dependent PLD activation. These results demonstrate
that ARF proteins mediate the effects of PDGF on PLD and that Rho proteins are not necessary for the effects of PDGF on the activity of PLD. This result confirms the data obtained with exotoxin C3 reported above.

The role of ARF proteins in the PKC-dependent activation of PLD was studied using a similar experimental design. In these studies, however, all permeabilization studies (including controls) were carried out in the presence of ATP and additional PKCa (5 μg/ml). Fig. 6 shows that the PLD activity of cells permeabilized in the absence of agonist and GTPγS was no longer sensitive to the addition of PMA and GTP analogues. Addition of myristoylated recombinant ARF1 restored PMA and GTPγS sensitivity to PLD, but recombinant RhoA had no effects. Therefore, PKC-mediated activation of PLD is also mediated, at least in part, by ARF proteins. The evidence presented here shows that the activation of Rho is not necessary for PKC- or PDGF-dependent PLD activation.

**FIG. 4.** Agonist- and GTPγS-induced translocation of ARF and RhoA to cell membranes. Cells were permeabilized with digitonin as described and fractionated by centrifugation. Pellets and supernatants were analyzed by SDS-PAGE and immunoblotting by separate. A, the translocation of ARF to membranes is induced by PDGF and PMA in the presence of GTPγS. The incubation mixture also contained ATP (1 mM), MgCl₂ (2 mM), and, in the case of the incubations with PMA, human recombinant PKCa (5 μg/ml). The lane labeled Control represents the results obtained with cells incubated in culture medium in the absence of digitonin. B, the translocation of RhoA to membranes under conditions identical to those shown in A.

**FIG. 5.** Reconstitution of PDGF-induced PLD activity in permeabilized cells. A, cells were prelabeled with [³H]palmitate and treated with digitonin with no additions (Control) or in the presence of PDGF and/or GTPγS. PLD was assayed as described. B, cells were treated with digitonin in the absence of agonist and GTPγS and then were treated with these agents and, where indicated, myristoylated recombinant human ARF1, geranyl-geranylated recombinant human RhoA, or recombinant human RhoA produced in yeast. All incubations contained ATP (1 mM) and MgCl₂ (2 mM). The data show the average of 4 independent experiments.

**Effects of the Overexpression of ARF on PLD Activity**—To confirm the conclusion that ARF proteins mediate PDGF and PMA-dependent activation of PLD, HIRcB cells were transiently transfected with ARF-GFP chimeras created using wild-type and mutated ARF1 and ARF6. We have previously shown that ARF1-GFP chimeras behave very similarly to ARF1 (29). Therefore, these ARF-GFP constructs behave like the endogenous ARF proteins with the additional advantage that, because of the expression of the fluorescent chimera, the efficiency of the transfection procedure can be determined for each plate of cells by epifluorescence and phase contrast microscopy. The effects of the overexpression of wt-ARF1, wt-ARF6, T31N-ARF1, and T27N-ARF6 on the activation of PLD by PDGF and PMA are shown in Fig. 7. The overexpression of either wild-type protein had no effect on the stimulation of PLD by either PDGF or PMA. In contrast, the overexpression of the putative dominant-negative mutants T31N-ARF1 and T27N-ARF6 blocked completely the effects of PDGF and significantly reduced the response to phorbol esters.

**DISCUSSION**

We have previously shown that ARF proteins mediate the activation of PLD by insulin in HIRcB cells. The data reported here suggest that ARF proteins also mediate the activation of PLD by PDGF and PMA. This conclusion is based on the following observations: 1) BFA, an inhibitor of ARF activation, inhibits PDGF- and PMA-induced PLD activation; 2) addition of recombinant ARF is sufficient to restore agonist-dependent PLD activation to permeabilized, cytoplasm-depleted cells; and 3) dominant-negative mutants of ARF1 and ARF6 inhibit the response of cellular PLD to stimulation by PDGF or phorbol esters.

Since a significant amount of evidence suggestive of a direct role of Rho proteins in receptor-mediated PLD activation has been published in the past few years, we also examined the role of Rho in PDGF- and PMA-dependent PLD activation in HIRcB cells. This was done by delivering C3 transferase to cells by the scrape-loading procedure of Malcolm et al. (10). Preloading of cells with exotoxin C3 was apparently toxic to cells and significantly reduced the levels of incorporation of labeled fatty acid. Because of this reduced uptake (20–30% of that of control cells), the amount of PtdBu generated after addition of the agonist was significantly smaller than that observed with control cells. However, when the amount of PtdBu was normalized to the total levels of radiolabeled lipids, we observed that the...
experiments. The data shown represent the average of 3 independent experiments.

The effects of transient overexpression of ARF proteins in PDGF- and PMA-induced PLD activity. Cells were transiently transfected with the following ARF-GFP chimeras: wt-ARF1-GFP, wt-ARF6-GFP, T31N-ARF1-GFP, and T27N-ARF6-GFP. After 24 h, the cells were labeled with [3H]palmitate and serum-starved as described. PLD activity was determined as described above. Transfection efficiency was determined for each set of plates by examination of the plates using an inverted fluorescence microscope optimized for GFP visualization and equipped with phase contrast optics. Only plates in which the transfection efficiency was better that 60% were used in these assays. The data shown represent the average ± S.E. of 3 independent experiments.

fraction of the lipid converted to PtdBu was the same in C3-treated and untreated cells. Thus, we conclude that the net activity of PLD remained unchanged after C3 treatment. These results appear to contradict the reports of Malcolm et al. (10). Since these morphological changes could have been attributed to a small but measurable fraction of Rho being ADP-ribosylated, we also confirmed the extent of the modification of Rho by a standard ADP-ribosylation assay. This assay is based on the incorporation of [32P]ADP-ribose to Rho extracted from cells after pretreatment with C3 transferase. Our data, shown in Fig. 3A, clearly confirm that greater than 95% of the intracellular Rho had been modified during the treatment with the transferase in vivo. On close inspection, the discrepancies between our conclusions and those of Malcolm et al. (10) are likely consequences of the normalization of our data. Malcolm et al. reported total amounts of PtdBu formation (10), whereas our data are expressed in terms of the fraction of lipid converted to PtdBu. Since the incorporation of radiolabeled fatty acid was much smaller in the C3-treated cells, normalization of the data to the total amount of available substrate eliminates the apparent effect of C3 on PLD activity.

We also observed a few additional features of the scraped cells. For instance, agonist- and PMA-induced PLD activities of cells that had been previously scraped and replated were always significantly smaller than those of cells that had not been scraped, independently of whether or not the cells were loaded with C3 exotoxin. An explanation of this is that during the scraping procedure some cell components that participate in the physiological activation of PLD may be lost. In agreement with this idea, the levels of activation of PLD in scraped cells were comparable with those observed in digitonin-permeabilized cells.

These observations do not exclude a role for Rho in the mediation of the activation of PLD in these cells. The data show that PDGF and PMA induce Rho translocation, suggesting that these agents promote the activation of the G protein. However, ARF translocation occurs as well (Fig. 4). Thus, a simple correlation between the activation of these G proteins and PLD activity is insufficient to prove a role in agonist-mediated activation of PLD. A more detailed analysis is shown in Figs. 5 and 6. In these experiments, untreated cells were permeabilized with digitonin and then treated with PDGF and PMA. To validate our observations, several independent analyses were performed to confirm the effectiveness of the C3 exotoxin scrape-loading procedures. First, we examined the morphology of the scrape-loaded cells. Our observations confirmed that at least 80% of the C3-treated cells had a round morphology and were loosely attached to the plate, as reported by Malcolm et al. (10). Since these morphological changes could have been attributed to a small but measurable fraction of Rho being ADP-ribosylated, we also confirmed the extent of the modification of Rho by a standard ADP-ribosylation assay. This assay is based on the incorporation of [32P]ADP-ribose to Rho extracted from cells after pretreatment with C3 transferase. Our data, shown in Fig. 3A, clearly confirm that greater than 95% of the intracellular Rho had been modified during the treatment with the transferase in vivo. On close inspection, the discrepancies between our conclusions and those of Malcolm et al. (10) are likely consequences of the normalization of our data. Malcolm et al. reported total amounts of PtdBu formation (10), whereas our data are expressed in terms of the fraction of lipid converted to PtdBu. Since the incorporation of radiolabeled fatty acid was much smaller in the C3-treated cells, normalization of the data to the total amount of available substrate eliminates the apparent effect of C3 on PLD activity.

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thermore, experiments done with cells transiently overexpressing ARF mutants further strengthen this conclusion: putative dominant-negative mutants of ARF1 and ARF6 inhibit PDGFinduced PMA-dependent PLD activation (although to different degrees) while the overexpression of the wild-type proteins had no effects. Therefore, we conclude that the main pathway by which growth factors and PMA modulate PLD activity in these cells involves the activation of ARF proteins.

EGF-mediated modulation of PLD activity appears to be mediated by Rac1 proteins in Rat1 fibroblasts (11). PKC activates PLD directly by a mechanism that does not appear to involve protein phosphorylation (12). The involvement of PKC in growth factor-dependent PLD activation has been shown in 3T3 cells (5), but the role of PKC in PLD activation has been questioned in other systems (34). A role for RhoA has been suggested in several systems (5, 10, 11, 22, 35), and there is unquestionable evidence that RhoA activates PLD in vitro (14, 20) and in cell culture systems (23, 33). Therefore, many signaling pathways appear to converge on the activation of PLD.

Finally, the ARF proteins have been shown to activate PLD in vitro (14, 20) and in cell culture systems (23, 33). Therefore, many signaling pathways appear to converge on the activation of PLD. This level of redundancy in the pathways that lead to PLD activation is strongly suggestive of an essential role for these enzymes in cellular signaling. Much further work is needed to determine the relative roles of these diverse pathways in receptor-mediated PLD regulation and to understand the precise functional role of PLD in cell physiology.

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