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The Activation of Phospholipase D by Endothelin-1, Angiotensin II, and Platelet-Derived Growth Factor in Vascular Smooth Muscle A10 Cells Is Mediated by Small G Proteins of the ADP-Ribosylation Factor Family*

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ABSTRACT

We show here that A10 cells express the phospholipase D (PLD) isoforms PLD1b and PLD2. The activation of PLD in these cells by angiotensin II (AngII), endothelin-1 (ET-1), and platelet-derived growth factor (PDGF) was found to be sensitive to inhibitors of the activation of ADP-ribosylation factor (ARF) but not to blockers of Rho protein function. PDGF, AngII, and ET-1 induced the binding of ARF proteins to cell membranes in a permeabilized cell assay. Cells permeabilized and depleted of ARF were no longer sensitive to stimulation with AngII, ET-1, or PDGF, but the addition of recombinant myristoylated human ARF1 restored agonist-dependent PLD activity. Expression of dominant negative ARF mutants blocked receptor-dependent activation of PLD. PLD activity was also potently stimulated by treatment with phorbol esters, but this activity was only partially inhibited by brefeldin A or by the overexpression of ARF dominant negative mutants. Transient expression of catalytically inactive mutants of PLD2, but not PLD1, inhibited significantly PDGF- and AngII-dependent PLD activity. We conclude: 1) the activation of PLD by cell surface receptors occurs primarily by an ARF-dependent mechanism in A10 cells, whereas the activation of PLD by protein kinase C-dependent pathways is only partially dependent on the regulation of ARF proteins; and 2) cell surface receptors, such as AngII and PDGF, signal primarily via PLD2 in A10 cells. (*Endocrinology* **141:** 2200–2208, 2000)

PHOSPHATIDIC acid (PA) is an important lipid second messenger that has been implicated in the regulation of cell differentiation and proliferation in many cell types (1–4). The main source of PA in most cells and tissues is the hydrolysis of phosphatidylcholine by the action of specific phospholipases D (PLD) (5). Many of the external agents that promote the proliferation of vascular smooth muscle cells (VSMC) activate PLD to various extents (6–10). However, the mechanisms by which these agents regulate PLD activity remain to be elucidated.

The recent cloning and study of specific isoforms of PLD (11–13) have led to the suggestion of three main mechanisms for the regulation of PLD activity by cell surface receptors. These mechanisms are mediated by: 1) G proteins of the ADP-ribosylation factor (ARF) family; 2) G proteins of the Rac/Rho/Cdc42 family; and 3) protein kinase C (PKC) (11–16). All of these are likely to act simultaneously in some cell systems to produce complete activation of the PLD pathway. Furthermore, the evidence suggests that these three mechanisms are not independent and that substantial cross-talk among these pathways exists (17).

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Recent work has suggested that PLD plays a very important role in the proliferation of VSMC. Wilkie *et al.* (6) suggested in 1996 that PLD activity and mitogen activated protein kinase (MAPK) activation are both required for agonist-induced proliferation of VSMC. Furthermore, comparative analysis suggests that the VSMC hyperplasia observed in a spontaneous hypertensive rat model is associated with a significant increase in the activity of PLD in response to external agents, when compared with the levels observed in the normotensive rat (WKY) (18). These observations are significant due to the important contribution of VSMC proliferation in the development of atherosclerotic plaques and the thickening of the blood vessel wall in hypertension. Therefore, the analysis of the mechanisms by which external stimuli regulate the activity of PLD in VSMC is of significant interest. However, these mechanisms remain largely undefined. In this paper, we show that the regulation of PLD activity by angiotensin II (AngII), endothelin-1 (ET-1), platelet-derived growth factor (PDGF), and phorbol 12-myristate 13-acetate (PMA) in A10 cells, a cell model widely used to study the basic biology of VSMC, is mediated, at least in part, by proteins of the ARF family. We also show that Rho plays a minor role in the regulation of PLD activity in these cells. A general model for the activation of PLD and its functional role in VSMC biology is proposed.

Materials and Methods

Cell culture

A10 smooth muscle cells were cultured in 60-mm dishes using DMEM supplemented with 10% FBS. Cells were used within 3 days of subcul-

ture and were serum-starved for 20 h in medium containing 0.1% BSA before treatment.

ARF proteins

The following ARF proteins were used: wild-type (wt)-ARF1, wt-ARF6, Q71L-ARF1, Q67L-ARF6, T31N-ARF1, and T27N-ARF6. The latter are putative dominant negative mutants with reduced affinity for GTP. These mutants were generated by site-directed mutagenesis, cloned in the multiple cloning site of the vector pGEFP-N1 (CLONTECH Laboratories, Inc., Palo Alto, CA), and fused to the green fluorescent protein (GFP) of *A. victoria*, as described (19). Cells were transfected with these constructs as described elsewhere (20). The efficiency of transfection was estimated by counting cells using DIC and fluorescence microscopy of the same fields. Based exclusively on the ratio of green fluorescent cells to cells detected by DIC, the transfection efficiency was better that 50%. When the efficiency of transfection was measured in terms of the number of viable cells (as determined by propidium iodide exclusion), the transfection efficiency was very near 90%. The PLD activity of ARF-GFP-transfected cells was determined approximately 48 h after transfection.

Detection of PLD isoforms

PLD1a, PLD1b, and PLD2 expression was quantitated using a semi-quantitative PCR procedure. Two 100-mm plates were used to isolate total RNA using commercial kits (QIAGEN, Valencia, CA). Two micrograms of total RNA were used for 1st-strand complementary DNA (cDNA) synthesis (using a CLONTECH Laboratories, Inc. kit). Once the cDNA was obtained, the following primers were used to amplify PLD 1a (expected 527 bp) and 1b (expected 413 bp): 5′-CGTGAACCACAGAACCAATG-3′ and 5′-TCTCACGGCAGCATCAGTAG-3′. PLD 2 was amplified (expected 467 bp) with 5′-CAGGAGGCGGTTGAGGTAAT-3′ and 5′-AGTTGCACATGGAGCCAGAT-3′. Additionally, the glyceraldehyde-3-phosphate dehydrogenase primers, 5′-TACTCCTTGGAGGCCATGTA-3′ and 5′-CGTGGAGTCTACTGGCGTCT-3′, were used for quantitation purposes. The amplification cycles were designed so that all the reactions would occur simultaneously (annealing at 60 C for 45 sec and elongation at 72 C for 60 sec). This cycle was repeated for a total of 30 times. Fifteen-microliter aliquots of the resulting PCR products were loaded in each lane of a 3% agarose gel.

PLD mutants

Wild-type and catalytically inactive variants of PLD1 and PLD2 (K898R-PLD1 and K758R-PLD2) were made as previously described (21), and fused to *A. victoria* green fluorescent protein by subcloning into pEFGP-C1 (CLONTECH Laboratories, Inc.). The enzymatic activity of the wild-type enzyme-GFP chimeras expressed in *Baculovirus* was determined *in vitro* to confirm that the GFP tag did not generate an inactive phenotype. Cells were transfected with Lipofectamine, as described (21). Transfection efficiencies were determined as described above.

Immunoblotting

ARF was detected with 2 μg/ml affinity-purified 1D9 (a monoclonal antibody that recognizes most members of the ARF family (22); a gift from R. Kahn). Bound antibodies were detected by chemiluminescence.

PLD assays

Cells (60-mm plates; 70–80% confluence) were serum starved and labeled overnight with ^{3}H-palmitate (5 μCi/ml) in DMEM/F12 containing 0.1% BSA. Cells were stimulated with PDGF (30 ng/ml), ET-1 (100 nM), AngII (1 μM), or PMA (500 nM) in the presence of 0.3% ethanol for 20 min. The reaction was stopped by addition of chloroform:methanol (1:1). The lipid phase was extracted and developed by TLC on silica gel 60 plates using ethyl acetate:trimethylpentane:acetic acid (9:5:2) as the solvent. The position of major phospholipids was determined using true standards (Avanti Biochemicals) and autoradiography. The TLC plates were scraped, and the total amount of radioactivity associated with each lipid species was determined by liquid scintillation counting. The data were expressed as the number of counts associated with the phosphatidylethanol spot normalized by the total number of counts of lipid.

Digitonin treatment

A10 cells were serum starved, scraped gently, resuspended in PBS, and treated with 8 μM digitonin in the presence or absence of PDGF (30 ng/ml), ET-1 (100 nM), AngII (1 μM), or PMA (500 nM), with or without the addition of GTPγS (100 μM) at 37 C for 15–20 min. ATP (1 mM) and magnesium (2 mM) were included in all the experiments. To release intracellular proteins, digitonin-treated cells were centrifuged in a microfuge for 20 min. Supernatants and pellets were collected separately and resolved by SDS-PAGE. ARF proteins were detected by immunoblotting, as mentioned above. PLD activity of digitonin-treated cells was determined using cells that had been labeled overnight with ^{3}H-palmitate (see above). Recombinant ARF was isolated from bacteria that coexpress ARF1 and yeast *N*-myristoyltransferase, as described (23, 24). Recombinant ARF protein (final concentration, 8 μg/ml) was added to cytosol-depleted cells where indicated. Human recombinant PKCα (Calbiochem; 5 μg/ml, final concentration) was included in all assays of the effects of PMA. The choice of PKCα was arbitrary, and was included with the purpose of maximizing the effects of PMA, because endogenous PKC is likely to leak out of the cell during the permeabilization and centrifugation procedures.

Treatment with ARF and Rho inhibitors

Cells were treated with brefeldin A (BFA) (25 μg/ml, final) 10 min before stimulation with the agonists in serum-free medium. C3 exotoxin treatment was done by the scrape loading method of Malcolm *et al.* (25). Briefly, cells were grown in 10-cm plates and scraped gently in 500 μl buffer [10 mM Tris (pH 7.2), 114 mM KCl, 25 mM NaCl, 5.5 mM $MgCl_2$] with or without 5 μg/ml C3 transferase. The cells were then distributed onto 6-cm plates and allowed to recover overnight. Cells were then serum-starved and labeled with 5 μCi/ml ^{3}H-palmitate for 17 h, as described previously (23). The effectiveness of the treatment with C3 was determined by *in vitro* ADP-ribosylation of cell extracts after treatment with the toxin, as described by Shome *et al.* (24). According to these controls, ADP-ribosylation of endogenous Rho proteins was at least 95% complete under the conditions of the scrape-loading assay described.

MAP kinase activity

A10 cells were treated as described above or as described in the figure legends. Cells were washed with cold PBS and scraped into microcentrifuge tubes in Buffer A [10 mM HEPES (pH 7.4), 2 mM EDTA, 1 mM Na_3VO_4, and 1 mM phenylmethylsulfonylflouride] and then pelleted via centrifugation. Cells were resuspended in a 0.5 ml detergent lysis buffer [50 mM HEPES (pH 7.4), 0.1 M NaCl, 1.5% sodium cholate, 1 mM EDTA, 1 mM EGTA, 5 μg/ml leupeptin, 1 mM phenylmethylsulfonylflouride, and 1 mg/ml soybean trypsin inhibitor] and lysed for 30 min at 4 C. Cell lysates were analyzed by SDS-PAGE, followed by immunoblotting with a phosphospecific anti-MAPK antibody (New England Biolabs, Inc.). Immunocomplexes were detected by ECL.

Confocal microscopy

A10 cells were transfected with ARF-GFP chimeras using Lipofectamine. The cells were examined by confocal microscopy 24 h after transfection using a 2002 instrument (Molecular Dynamics, Inc.), equipped with an argon laser. Data were obtained using the 488-nm band of the laser and a 530 ± 35-nm bandpass filter. This filter was used to reduce errors caused by the background autofluorescence associated with the use of Lipofectamine.

Results

Effects of BFA and C. botulinum C3 transferase on the activation of PLD by external agonists in A10 cells.

Previous work from our laboratory has shown that the activation of PLD by cell surface receptors is sensitive to the fungal metabolite BFA in rat fibroblasts (23, 24). BFA is a

potent inhibitor of the activation of ARF proteins *in vitro* (19, 26) and *in vivo* (20), and our previous work has shown that the inhibition of PLD activation by BFA reflects a role for ARF proteins in the mechanism of activation of PLD by external stimuli (23, 24). Therefore, our initial studies concentrated on the effects of BFA on receptor-mediated PLD activation in A10 cells. The data shown in Fig. 1 summarize these studies. As shown, AngII, ET-1, PDGF, and PMA considerably stimulated PLD activity (2- to 5-fold). The addition of 25 μg/ml BFA, 10 min before stimulation of the cells with these agents, consistently inhibited the activation of PLD. AngII, ET-1, and PDGF-dependent PLD activation was completely blocked by BFA treatment, whereas the activation of PLD by PMA was reduced by approximately 50% (Fig. 1).

To assess the role of proteins of the Rho family on the activation of PLD, the cells were pretreated with *C. botulinum* exotoxin C3, an ADP-ribosyl transferase that blocks the activity of small G proteins of the Rho family (27). The cells were treated with C3 transferase using a scrape-loading procedure, as described previously (24). Its effects on the activation of PLD by external stimuli were not significant (Fig. 2). These experiments suggest that the activation of PLD by AngII, ET-1, and PDGF is mediated by ARF proteins and that Rho proteins do not play an essential role in receptor-mediated activation of PLD in A10 cells.

External stimuli promote the activation of ARF proteins in A10 cells

We next studied the effects of external stimuli in the activation of ARF proteins. This was done by determining the translocation of ARF proteins to cell membranes, as described (23, 24).

Figure 3 shows the results of a representative experiment using AngII and PDGF as agonists. As shown, ARF leaked out of permeabilized cells during treatment (digitonin lane), and the inclusion of GTPγS, AngII, or PDGF alone during the permeabilization procedure did not induce membrane association of ARF proteins. Incubation with agonist and GTPγS during permeabilization resulted in a significant increase translocation of ARF to cell membranes (Fig. 3, 5th and 7th lanes). Identical results were obtained with ET-1 and PMA (not shown). These data show unequivocally that the activation of ARF proteins can be regulated by the agonists AngII, ET-1, and PDGF and by the activation of PKC by PMA.

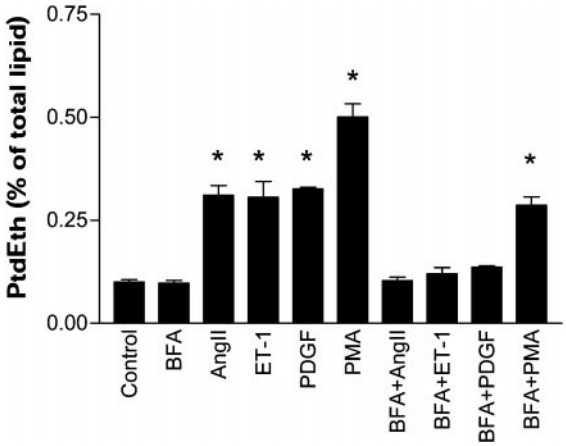

FIG. 1. Acute pretreatment with BFA inhibits agonist-induced activation of PLD in A10 cells. Cells were pretreated with 25 μg/ml BFA for 10 min, before treatment with one of the following: AngII (1 μM), ET-1 (100 nM), PDGF (30 ng/ml), or PMA (500 nM). PLD activation was determined as described in the text. The data shown represent the average of five independent experiments. *Asterisks*, Samples that had statistically significant greater activity that that shown in the control (nonstimulated) cells ($P < 0.01$). *Bars not labeled* were indistinguishable from the controls.

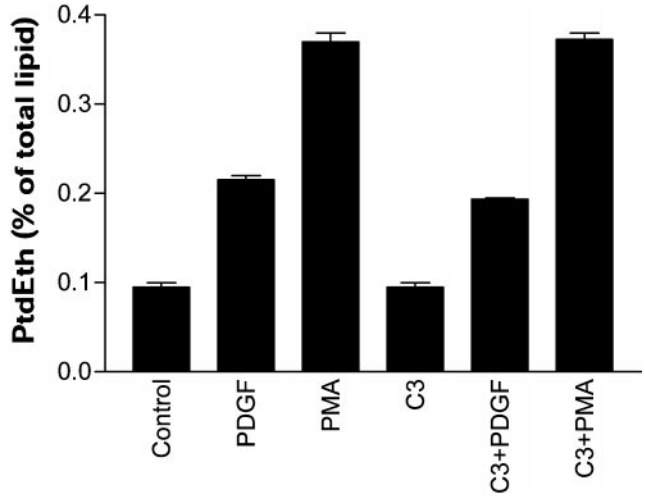

FIG. 2. The effects of *C. botulinum* C3 transferase on the activation of PLD by PDGF and PMA. Cells were scrape-loaded with the transferase (as described in the text), replated, serum-starved, and labeled with ^{3}H-palmitate. The activity of PLD was determined as described in the text. The data show the average of three independent experiments. Statistical analyses, to determine whether C3 affected agonist responses, were made by Student's *t* tests, comparing the C3-treated group to its respective control. There were no statistically significant effects of the treatment with C3.

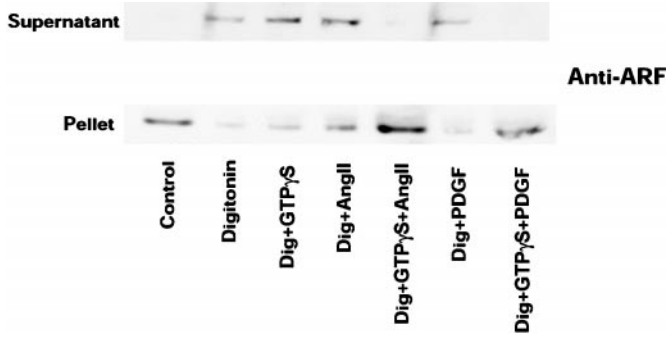

FIG. 3. Agonist- and GTPγS-induced translocation of ARF to cell membranes. Cells were permeabilized with digitonin (Dig), as described, and fractionated by centrifugation. Pellets and supernatants were analyzed by SDS-PAGE and immunoblotting by separate. The incubation mixture contained ATP (1 mM) and $MgCl_2$ (2 mM). The lane labeled *Control* indicates results obtained with cells incubated in the absence of digitonin.

Reconstitution of agonist-dependent PLD activation in permeabilized cells

To examine the effects of ARF depletion on agonist-dependent activation of PLD, A10 cells were permeabilized in the absence or presence of agonists and GTPγS, following the protocol described above; and the activation of PLD was

measured by the production of phosphatidylethanol. Cells that had been permeabilized in the presence of agonist and GTPγS showed high levels of PLD activity that were comparable to those observed with intact cells after agonist stimulation (Figs. 1, 4A, and 4C). In contrast, the PLD activity of cells permeabilized in the presence of agonist alone or GTPγS alone was indistinguishable from that of control cells.

We next examined cells that had been permeabilized with digitonin in the absence of agonist and GTPγS. These cells contained very little ARF, because of the depletion of their intracellular contents during the permeabilization procedure (Fig 3). Figure 4, B and D, shows that the addition of GTPγS, agonist, or a solution containing both GTPγS and the corresponding agonist failed to restore PLD activity. However, when recombinant, myristoylated human ARF1 was included in this assay, the activity of PLD was fully recovered to levels comparable with those obtained by the stimulation of intact cells with the corresponding agonist.

Inhibition of receptor-mediated PLD activation by dominant negative mutants of ARF

A10 cells were transfected with ARF proteins that had been subcloned in pEGFP-C1 as fusion proteins containing an *Aequorea victoria* GFP tag in their C-terminus. The following chimeras were tested: wild-type ARF1-GFP, wt-ARF6-GFP, and the dominant negative mutants T31N-ARF1-GFP and T27N-ARF6-GFP. The GFP fusion constructs were selected to facilitate the estimation of the transfection efficiency, using phase contrast and fluorescent microscopy.

The data obtained are shown in Fig. 5. Additional controls were run using the empty vector, and the PLD activity of these cells was indistinguishable from that of untransfected cells. The overexpression of wt-ARF1-GFP and wt-ARF6-GFP produced a small (but significant) increase in agonist-dependent PLD activity. More important, the expression of the dominant negative mutants T31N-ARF1-GFP and T27N-

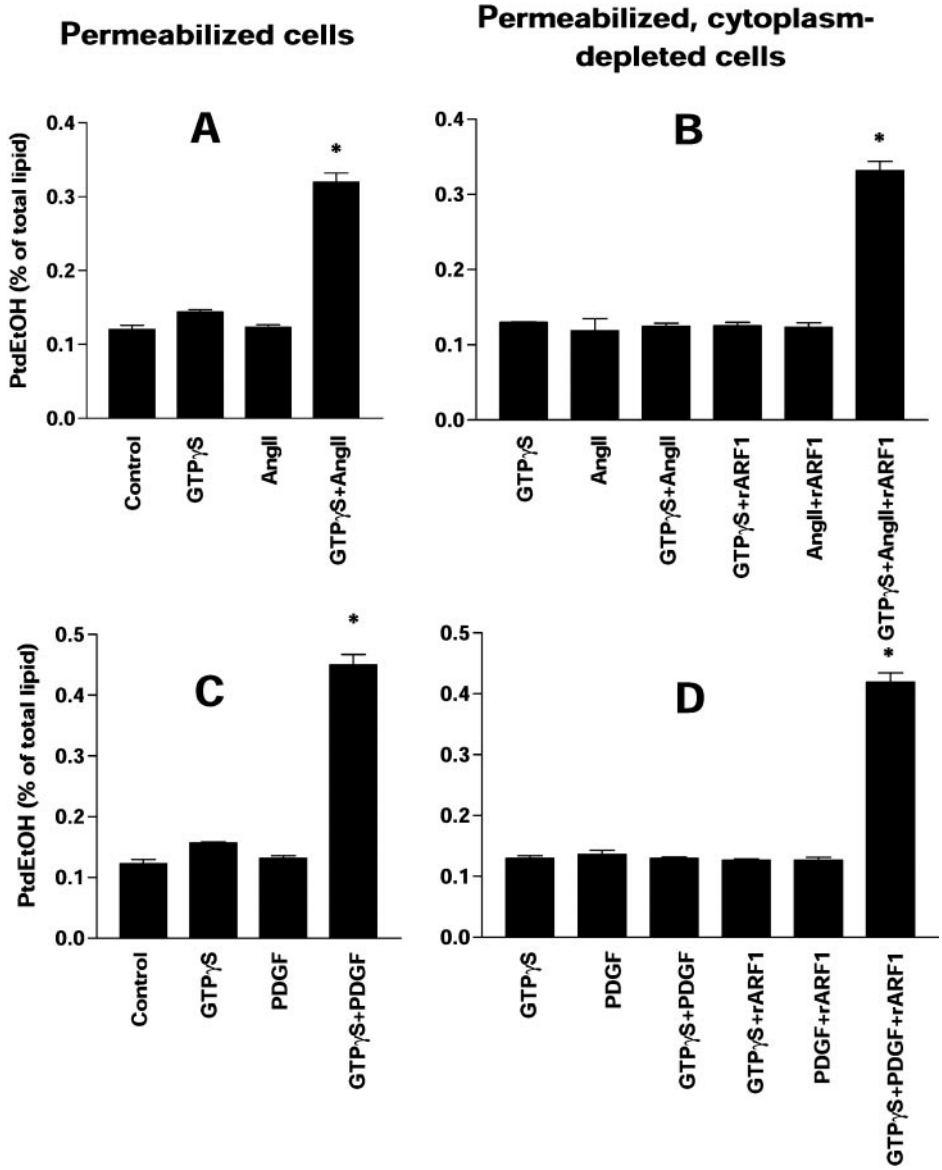

FIG. 4. Reconstitution of AngII- and PDGF-induced PLD activity in permeabilized cells. A, Cells were prelabeled with ^{3}H-palmitate and treated with digitonin with no additions (*Control*) or in the presence of AngII and/or GTPγS. PLD was assayed as described. B, Cells were treated with digitonin in the absence of agonist and GTPγS. Where indicated, AngII, GTPγS, and myristoylated recombinant human ARF1 were added to these permeabilized cell preparations, and the activity of PLD was assayed as described. C, Cells were prelabeled with ^{3}H-palmitate and treated with digitonin with no additions (*Control*) or in the presence of PDGF and/or GTPγS. PLD was assayed as described. D, Cells were treated with digitonin in the absence of agonist and GTPγS. Where indicated, PDGF, GTPγS, and myristoylated recombinant human ARF1 were added to these permeabilized cell preparations, and the activity of PLD was assayed as described. All incubations contained ATP (1 mM) and $MgCl_2$ (2 mM). The data shown represent the average of three independent data sets. *Asterisks*, Statistically significant differences among specific groups and the respective controls, as determined by ANOVA and post-ANOVA Bonferroni statistics.

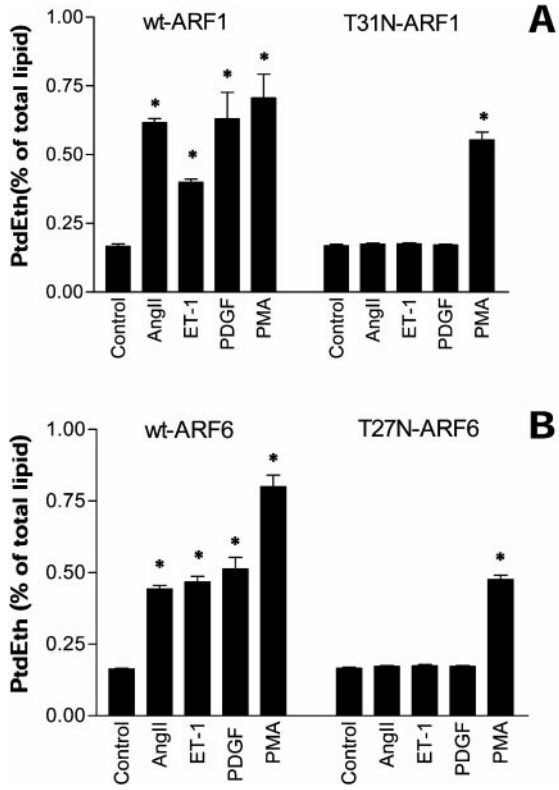

FIG. 5. The effects of transient overexpression of ARF proteins on agonist-dependent PLD activation. Cells were transiently transfected with the following ARF-GFP chimeras: wt-ARF1-GFP, wt-ARF6-GFP, T31N-ARF1-GFP, and T27N-ARF6-GFP. After 24 h, the cells were labeled with ^{3}H-palmitate and were serum-starved as described. PLD activity was determined as described in the text. The data shown represent the mean ± SEM of three independent experiments. *Asterisks*, Statistically significant differences among specific data sets and the Control data, as determined by ANOVA followed by Bonferroni post-test analysis.

ARF6-GFP abolished the activation of PLD by AngII, ET-1, and PDGF. In contrast, the dominant negative ARF1 mutant had no significant effects on PMA-dependent PLD activity, whereas the dominant negative ARF6 mutants reduced the effects of PMA by about 40%. These results show that AngII, ET-1, and PDGF activate PLD by a mechanism mediated primarily by the activation of ARF proteins. In contrast, PMA activates PLD by a mechanism that is only partially dependent on the activation of ARF.

Both ARF dominant negative mutants inhibited agonist-mediated PLD activation in A10 cells. However, the intracellular distribution of ARF6 has been reported to be very different from that of other members of the ARF family (28, 29). Because PLD1 and PLD2 have been found confined to cell membranes (11, 12), we studied the distribution of wt-ARF1-GFP, wt-ARF6-GFP, T31N-ARF-GFP, and T27N-ARF6-GFP by confocal microscopy. Additional controls included the mutants Q71L-ARF1-GFP and Q67L-ARF6-GFP, two mutants with reduced GTPase activity, that have been described to act as dominant activated mutants in other systems (22, 28). The results are shown in Fig. 6. Wild-type-ARF1 and the dominant activated mutant Q71L-ARF1 were both distributed between the Golgi and the cytosol. Q71L-ARF1-GFP also localized to some punctate intracellular structures or on the plasma membrane, although the fraction of the chimera in these regions was small. In contrast, T31N-ARF1-GFP was mainly cytosolic. All ARF6 chimeras were found associated with small intracellular vesicles. However, a significant fraction of the wild-type and GTPase-deficient chimeras were also found on the plasma membrane, in regions that seem to be membrane ruffles (see Fig. 6).

The production of PA acid is necessary for the activation of the MAP kinase cascade

We have, so far, shown that PLD activation is ARF-dependent in A10 cells. We next examined the relevance of this pathway in the activation of downstream effects. We have recently shown that ARF-dependent PLD activation is essential for the regulation of the MAP kinase cascade by insulin (21). We thus examined the effects of the inhibition of the generation of PA on the activation of the MAP kinase pathway. In these experiments, A10 cells were treated with BFA (25 μg/ml), for 10 min, before addition of the agonists, and the state of phosphorylation of MAP kinase was determined by SDS-PAGE and immunoblotting with a specific anti-phospho-MAPK antibody. Both PDGF and AngII were potent stimulators of MAPK phosphorylation, whereas ET-1 gave weak and barely detectable responses. A representative experiment with AngII and PDGF is shown in Fig. 7. A short acute preexposure to BFA blocked the activation of MAP kinase by either PDGF or AngII. However, addition of PA reversed the inhibitory effects of BFA, suggesting a role for PLD-dependent generation of phosphatidate in the modulation of the MAP kinase cascade by extracellular signals in A10 cells.

PDGF and AngII activate primarily PLD2

The discovery of several isoforms of PLD has originated a significant amount of interest in the identification of the PLD species responsible for receptor-mediated generation of PA. To address this question, we first examined the expression of PLD in A10 cells using semiquantitative PCR. The primers chosen were based on the published sequences for rat PLD1 and PLD2. The data are shown in Fig. 8A. A10 cells expressed primarily PLD1b and PLD2, whereas the expression of PLD1a was barely detectable. Also, as shown, the predominant PLD expressed in A10 cells is PLD2.

To determine which PLD was the primary target for receptor-mediated PLD activation, we transiently transfected A10 cells with GFP-tagged catalytically inactive forms of PLD1b and PLD2. These experiments are based on the hypothesis that overexpression of catalitycally inactive forms of the specific target enzymes will scavenge the regulatory proteins that modulate PLD activity. Figure 8b shows clearly that the overexpression of the catalytically inactive K758R-PLD2 completely abolished PDGF- and AngII-dependent PLD activity, whereas the overexpression of the corresponding PLD1 mutant had no apparent effects. These data demonstrate that the activation of PLD by PDGF and AngII in A10 cells is primarily mediated by PLD2.

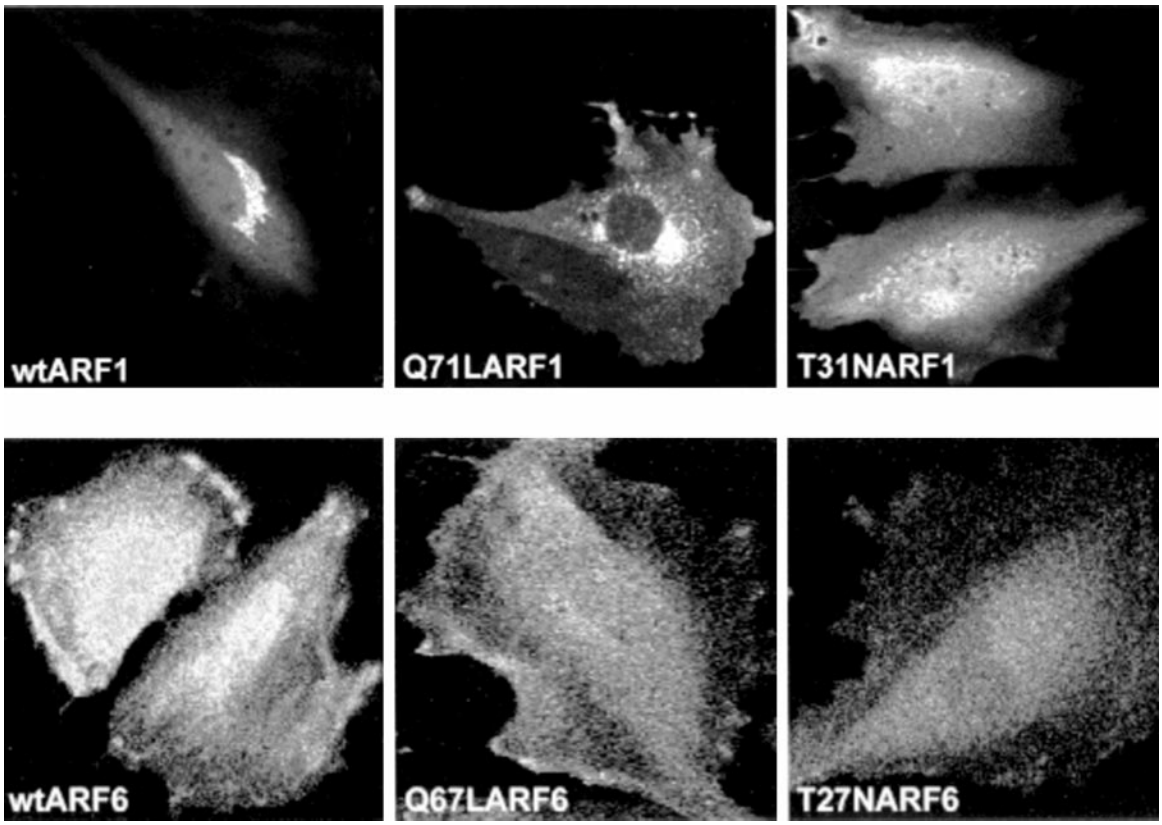

FIG. 6. The intracellular localization of ARF1-GFP and ARF6-GFP in A10 cells. A10 cells were transfected with the following chimeras: wt-ARF1-GFP, wt-ARF6-GFP, T31N-ARF1-GFP, T27N-ARF6-GFP, Q71L-ARF1-GFP, and Q67L-ARF6-GFP. Cells were examined by confocal microscopy, 24 h after transfection, using a Molecular Dynamics, Inc. 2002 instrument.

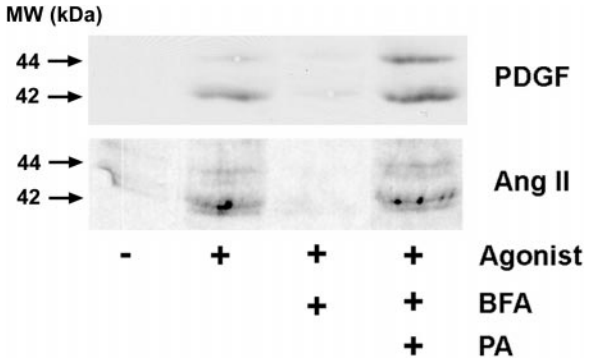

FIG. 7. BFA treatment blocks the activation of the MAPK cascade by PDGF and AngII. Cells were treated with BFA (25 μg/ml), for 10 min, before the addition of agonist. Where indicated, 200 μM phosphatidate was added simultaneously with the agonist. These data are representative of three separate experiments.

Discussion

Recent data suggest that the generation of PA by the hydrolytic action of PLD is an essential component of the signaling pathways that lead to the activation of MAP kinase (21). Many of the mitogenic stimuli that regulate VSMC proliferation have been shown to activate PLD. These include AngII (6, 18), ET-1 (8), PDGF (7), and high glucose concentration (9). However, little is known about the mechanisms by which cell surface receptors regulate PLD activity in VSMC. In this report, we have examined the role of proteins of the ARF family of small GTPases in the regulation of PLD by agents that modulate VSMC proliferation. Our data suggest a predominant role for an ARF-mediated pathway in the regulation of PLD activation by ET-1, AngII, and PDGF.

Two different PLD genes have been identified in mammals (11, 12). One of these, PLD1, codes for two different gene products generated by alternative splicing (30). PLD1a and PLD1b are activated *in vitro* by small GTPases of the ARF and Rho families and by direct interaction with PKC (31). Although the sensitivity of PLD2 to regulatory stimuli is lost during purification (12), the PLD activity of crude extracts prepared from cells that overexpress this protein are sensitive to ARF stimulation (32). The linkage between receptor-mediated activation of PLD and the activation of PLD1 and PLD2 has not been examined in detail. In particular, the study of the relative roles of ARF, Rho, and PKC requires a significant amount of attention.

We have recently developed a two-pronged approach to study the role of small GTPases in the regulation of PLD activity in HIRcB fibroblasts (23, 24). This approach is based on a combination of permeabilization/reconstitution assays and on the use of dominant negative mutants of these GTPases. Using this approach, we have recently shown that ARF plays a major role in the modulation of PLD activity by insulin and PDGF in fibroblasts (23, 24), thus proving that the regulation of PLD by receptor tyrosine kinases is primarily mediated by ARF. Some evidence linking ARF to the activation of PLD by G protein-linked receptors has also been

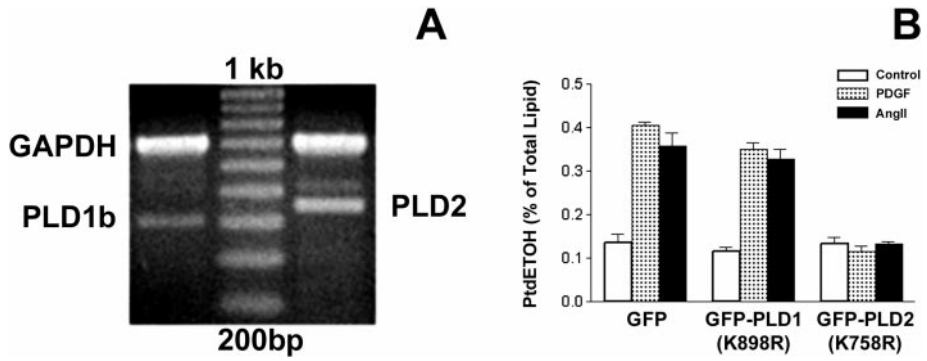

FIG. 8. Expression of PLD isoforms in A10 cells. A, Quantitative PCR demonstrates that A10 cells express primarily PLD1b and PLD2. Specific PLD1 and PLD2 primers were used to amplify cDNA obtained from A10 cells. The location of the PLD1b and PLD2 amplified fragments is indicated. The cDNA of glyceraldehyde-3-phosphate dehydrogenase was simultaneously amplified for quantitative purposes. B, A10 cells were transfected with GFP or with the catalytically inactive mutants GFP-K898R-PLD1 and GFP-K758R-PLD2, as indicated, and the effects of PDGF and AngII on the stimulation of PLD activity was determined 48 h after transfection. As shown, overexpression of the mutant PLD1 had no effects on receptor-mediated activation of PLD, whereas the catalytically inactive PLD2 completely abolished receptor-mediated activation.

reported (33, 34). Here, we extend these studies to mitogenic agents that regulate the proliferation of VSMC.

We also investigated the effects of ARF1 and ARF6 dominant negative mutants on the activation of PLD. ARF6 was chosen because it is the only member of the ARF family abundantly found on the plasma membrane (28, 29). ARF1 was selected because: 1) it is the most abundant of the ARF proteins in mammalian cells; and 2) it is representative of the remaining members of the family, in that it exists in a steady-state distribution between cytosolic and membrane-bound forms. Our data show that the overexpression of both ARF1 and ARF6 dominant negative mutants inhibits agonist-dependent PLD activation. This is consistent with previous data from our laboratory (24). Therefore, the question of which ARF mediates this signaling pathway remains undecided. Examination of cells that express ARF-GFP chimeras failed to provide conclusive results. ARF6 was found on the plasma membrane or on intracellular vesicles, a distribution that corresponds closely to that of mammalian PLDs (11–13). However, although most of the ARF1-GFP chimeras were found localized to the Golgi and cytosol (Fig. 6), a small fraction was visible in the plasma membrane and small intracellular vesicles. Furthermore, agonist treatment failed to produce a noticeable displacement of either ARF1-GFP or ARF6-GFP to the plasma membrane. This result seems to be at odds with the observed complete displacement of endogenous ARF proteins to the membrane in the permeabilization experiments shown in Fig. 3. Our interpretation of this apparent discrepancy is that, when ARF1 and ARF6 are overexpressed, then the concentrations of these proteins are so high that only a very small fraction of the protein is activated and displaced to the membrane. Because the cytosolic protein is also intensely fluorescent, the displacement of a small fraction of the ARF-GFP chimeras is very difficult to examine using conventional live-cell confocal microscopy techniques.

Significantly, both ARF dominant negatives were found to be equally effective in the inhibition of PLD activation by ET-1, AngII, and PDGF. The mechanism by which these dominant negative mutants inhibit PLD activity is likely to result from the competition with endogenous ARF proteins for a limited number of ARF activators (guanine nucleotide exchange factors, or GEFs). The fact that both ARF dominant negatives are effective in the blockade of the activation of PLD suggests that these GEFs cannot distinguish between ARF1 and ARF6. Several ARF-GEFs have been described in recent years (35–39). They are characterized by their homology to the yeast protein Sec7, which is itself an ARF-GEF (35). Many of these ARF-GEFs are not sensitive to BFA *in vitro* (36, 37). However, our data show clearly that BFA inhibits the activation of PLD in A10 cells. The ARF-GEFs ARNO and GRP-1 have been shown to be recruited to the plasma membrane by a mechanism that seems to involve the activation of PI3 kinase (40, 41). However, the activation of ARF by these GEFs *in vitro* does not seem to be BFA sensitive (37). There are two possible explanations for these discrepancies: either the GEFs that mediate the activation of ARF by cell surface receptors in A10 cells are BFA sensitive, or the activation of these GEFs by cell surface receptors is BFA sensitive. Our laboratory is presently working on the resolution of this issue. Finally, the dominant negative mutants of ARF1 and ARF6 had very small effects on the activation of PLD by phorbol esters. This suggests that ARF proteins do not mediate the effects of PKC on PLD. Because PKC has been shown to activate directly PLD1 (16, 17), PKC-dependent pathways for the activation of PLD may bypass completely the ARF signaling pathway.

Our data show that PDGF and AngII activate PLD2, rather than PLD1. This observation is particularly interesting, considering that the activity of PLD2 *in vitro* is independent of ARF (12); and thus PLD2 was thought to be an unlikely candidate for receptor-sensitive PLD activity. Contrary to this expectation, we find that the catalytically inactive variant of PLD2, but not PLD1, functions as a dominant negative and blocks PDGF- and AngII-dependent activation of PLD, suggesting that PLD2 mediates receptor-responsive PLD activity in A10 cells. Interestingly, this further suggests that PLD2 is regulated by ARF *in vivo*, as follows from the fact that the PDGF- and AngII-dependent PLD activity in this model requires ARF activation. Recent evidence from Sung *et al.* (32) supports this model. Although immunopurified PLD2 was found to be unresponsive to ARF, PLD2 overexpressed in COS-7 cells was activated by ARF preloaded with GTPγS,

suggesting that PLD2 may be regulated by ARF *in vivo*. Furthermore, a PLD2 mutant lacking the N-terminal 308 amino acids displays both a reduced *in vitro* activity and ARF-sensitive activity. These results are consistent with a model for ARF-mediated PLD2 activation in response to cell surface receptors.

Finally, it is important to consider the role of PLD activation in the biology of VSMC cells. Several pieces of work have strongly suggested that PLD activation is essential for the proliferative responses of VSMC cells to a variety of stimuli (6–10). However, the mechanism by which PLD modulates proliferation is not clearly understood. Recent work by Rizzo *et al.* (21) has shown that the activation of the MAP kinase pathway by cell surface receptors is BFA sensitive and requires the generation of PA, but that PA alone is insufficient to activate the MAPK cascade. This seems to be a direct consequence of the effects of PA on the catalysis of the recruitment of Raf to membrane compartments (21). This function of PA was originally described in fibroblasts. Our data demonstrate a similar phenomenon in A10 cells. Furthermore, in agreement with our previous data, PLD activation alone is insufficient to activate the MAPK cascade in A10 cells (21). This is evidenced by the fact that ET-1 was a very poor activator of MAPK phosphorylation, although the activation of PLD by ET-1 was comparable with that observed upon AngII and PDGF treatment. This suggests that the different effects of ET-1, PDGF, and AngII in the phosphorylation of MAPK probably reflect differences in the mechanisms of activation of Ras or Raf-1 and that this mechanisms are either not present or not fully active in A10 cells.

Taken altogether, our observations suggest a working model for the mechanism of activation and function of PLD in A10 cells. Agonists stimulate the activation of ARF proteins by a mechanism that is likely mediated by ARF-GEFs related to ARNO. Activated, GTP-bound ARF interacts with PLD and promotes its activation. Once activated, PLD hydrolyzes phosphatidylcholine to choline and phosphatidate. The latter, in conjunction with Ras, catalyzes the activation of Raf-1, thus initiating the events that lead to the activation of MAP kinase and cell proliferation. The general aspects of this model are strongly supported by our data, but additional studies are required, to validate its details.

References

1. **Zhang H, Desai NN, Murphey JM, Spiegel S** 1990 Increases in phosphatidic acid levels accompany sphingosine-stimulated proliferation of quiescent Swiss 3T3 cells. J Biol Chem 265:21309–21316
2. **Knauss TC, Jaffer FE, Abboud HE** 1990 Phosphatidic acid modulates DNA synthesis, phospholipase C, and platelet-derived growth factor mRNAs in cultured mesangial cells. Role of protein kinase C. J Biol Chem 265:14457–14463
3. **Suzuki A, Kozawa O, Shinoda J, Watanabe Y, Saito H, Oiso Y** 1996 Thrombin induces proliferation of osteoblast-like cells through phosphatidylcholine hydrolysis. J Cell Physiol 168:209–216
4. **Flores I, Casaseca T, Martinez AC, Kanoh H, Merida I** 1996 Phosphatidic acid generation through interleukin 2 (IL-2)-induced α-diacylglycerol kinase activation is an essential step in IL-2-mediated lymphocyte proliferation. J Biol Chem 271:10334–10340
5. **Exton JH** 1994 Messenger molecules derived from membrane lipids. Curr Opin Cell Biol 6:226–229
6. **Wilkie N, Morton L, Ng LL, Boarder ML** 1996 Stimulated mitogen-activated protein kinase is necessary but not sufficient for the mitogenic response to angiotensin II. A role for phospholipase D. J Biol Chem 271:32447–32453
7. **Inui H, Kitami Y, Tani M, Kondo T, Inagami T** 1994 Differences in signal transduction between platelet-derived growth fact (PDGF) α and β receptors in vascular smooth muscle cells. J Biol Chem 269:30546–30552
8. **Jones LG, Ella KM, Bradshaw CD, Gause KC, Dey M, Wisehart-Johnson AE, Spivey EC, Meier KE** 1994 Activation of mitogen-activated protein kinases and phospholipase D in A7r5 vascular smooth muscle cells. J Biol Chem 269:23790–23799
9. **Yasunari K, Kohno M, Kano H, Yokokawa K, Horio T, Yoshikawa J** 1996 Possible involvement of phospholipase D and protein kinase C in vascular growth induced by elevated glucose concentration. Hypertension 28:159–168
10. **Jones AW, Magliola L, Waters CB, Rubin LJ** 1998 Endothelin-1 activates phospholipases and channels at similar concentrations in porcine coronary arteries. Am J Physiol 274:C1583–C1591
11. **Hammond SM, Altshuller YM, Sung TC, Rudge SA, Rose K, Engebrecht J, Morris AJ, Frohman MA** 1995 Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new, and highly conserved gene family. J Biol Chem 270:29640–29643
12. **Colley WC, Sung TC, Roll R, Jenco J, Hammond SM, Altshuller Y, Bar-Sagi D, Morris AJ, Frohman MA** 1997 Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. Curr Biol 7:191–201
13. **Park SK, Provost JJ, Bae CD, Ho WT, Exton JH** 1997 Cloning and characterization of phospholipase D from rat brain. J Biol Chem 272:29263–29271
14. **Brown HA, Gutowski S, Moomaw CR, Slaughter C, Sternweis PC** 1993 ADP-ribosylation factor, a small GTP-dependent regulatory protein, stimulates phospholipase D activity. Cell 75:1137–1144
15. **Hess JA, Ross AH, Qiu RG, Symons M, Exton JH** 1997 Role of Rho family proteins in phospholipase D activation by growth factors. J Biol Chem 272:1615–1620
16. **Singer WD, Brown HA, Jiang X, Sternweis PC** 1996 Regulation of phospholipase D by protein kinase C is synergistic with ADP-ribosylation factor and independent of protein kinase activity. J Biol Chem 271:4504–4510
17. **Rumenapp U, Schmidt M, Wahn F, Tapp E, Grannass A, Jakobs KH** 1997 Characteristics of protein-kinase-C- and ADP-ribosylation-factor-stimulated phospholipase D activities in human embryonic kidney cells. Eur J Biochem 248:407–414
18. **Morton C, Baines R, Masood I, Ng L, Boarder ML** 1995 Stimulation of two vascular smooth muscle cell lines by angiotensin II: differential second messenger responses leading to mitogenesis. Br J Pharmacol 115:361–367
19. **Donaldson** JG, Finazzi D, Klausner RD 1992 Brefeldin A inhibits Golgi membrane-catalysed exchange of guanine nucleotide onto ARF protein. Nature 360:350–352
20. **Vasudevan C, Han W, Tan Y, Nie Y, Li D, Shome K, Watkins SC, Levitan ES, Romero G** 1998 The distribution and translocation of the G protein ADP-ribosylation factor 1 in live cells is determined by its GTPase activity. J Cell Sci 111:1277–1285
21. **Rizzo MA, Shome K, Vasudevan C, Sotltz DB, Sung TC, Frohman MA, Watkins SC, Romero G** 1999 Phospholipase D, and its product, phosphatidic acid, mediate agonist-dependent Raf-1 translocation to the plasma membrane and the activation of the mitogen-activated protein kinase pathway. J Biol Chem 274:1131–1139
22. **Zhang CJ, Rosenwald AG, Willingham MC, Skuntz S, Clark J, Kahn RA** 1994 Expression of a dominant allele of human ARF1 inhibits membrane traffic *in vivo*. J Cell Biol 124:289–300
23. **Shome K, Vasudevan C, Romero G** 1997 ARF proteins mediate insulin-dependent activation of phospholipase D. Curr Biol 7:387–396
24. **Shome K, Nie Y, Romero G** 1998 ADP-ribosylation factor proteins mediate agonist-induced activation of phospholipase D. J Biol Chem 273:30836–30841
25. **Malcolm KC, Elliott CM, Exton JH** 1996 Evidence for Rho-mediated agonist stimulation of phospholipase D in rat1 fibroblasts. Effects of clostridium botulinum C3 exoenzyme. J Biol Chem 271:13135–13139
26. **Helms JB, Rothman JE** 1992 Inhibition by brefeldin A of a Golgi membrane enzyme that catalyses exchange of guanine nucleotide bound to ARF. Nature 360:352–354
27. **Aktories K** 1997 Bacterial toxins that target Rho proteins. J Clin Invest 99:827–829
28. **D'Souza-Schorey C, Li G, Colombo MI, Stahl PD** 1995 A regulatory role for ARF6 in receptor-mediated endocytosis. Science 267:1175–1178
29. **Cavenagh MM, Whitney JA, Carroll K, Zhang CJ, Boman AL, Rosenwald AG, Mellman I, Kahn RA** 1996 Intracellular distribution of Arf proteins in mammalian cells. Arf6 is uniquely localized to the plasma membrane. J Biol Chem 271:21767–21774
30. **Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q, Cook S, Nozawa Y, Prestwich GD, Frohman MA, Morris AJ** 1997 Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4:5-bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP-binding proteins and protein kinase C-α. J Biol Chem 272:3860–3868
31. **Park SK, Min DS, Exton JH** 1998 Definition of the protein kinase C interaction site of phospholipase D. Biochem Biophys Res Commun 244:364–367
32. **Sung TC, Altshuller YM, Morris AJ, Frohman MA** 1999 Molecular analysis of mammalian phospholipase D2. J Biol Chem 274:494–502
33. **Fensome F, Whatmore J, Morgan C, Jones D, Cockcroft S** 1998 ADP-ribosylation factor, and Rho proteins mediate fMLP-dependent activation of phospholipase D in human neutrophils. J Biol Chem 273:13157–13164

34. **Rumenapp U, Geiszt M, Wahn F, Schmidt M, Jakobs KH** 1995 Evidence for ADP-ribosylation-factor-mediated activation of phospholipase D by m3 muscarinic acetylcholine receptor. Eur J Biochem 234:240–244
35. **Sata M, Donaldson JG, Moss J, Vaughan M** 1998 Brefeldin A-inhibited guanine nucleotide-exchange activity of Sec7 domain from yeast Sec7 with yeast and mammalian ADP ribosylation factors. Proc Natl Acad Sci USA 95:4204–4208
36. **Chardin P, Paris S, Antonny B, Robineau S, Beraud-Dufour S, Jackson CL, Chabre M** 1996 A human exchange factor for ARF contains Sec7- and pleckstrin-homology domains. Nature 384:481–484
37. **Meacci E, Tsai SC, Adamik R, Moss J, Vaughan M** 1997 Cytohesin-1, a cytosolic guanine nucleotide-exchange protein for ADP-ribosylation factor. Proc Natl Acad Sci USA 94:1745–1748
38. **Klarlund JK, Guilherme A, Holik JJ, Virbasius JV, Chawla A, Czech MP** 1997 Signaling by phosphoinositide-3,4,5-trisphosphate through proteins containing pleckstrin and Sec7 homology domains. Science 275:1927–1930
39. **Peyroche A, Paris S, Jackson CL** 1996 Nucleotide exchange on ARF mediated by yeast Gea1 protein. Nature 384:479–481
40. **Venkateswarlu K, Oatey PB, Tavare JM, Cullen PJ** 1998 Insulin-dependent translocation of ARNO to the plasma membrane of adipocytes requires phosphatidylinositol 3-kinase. Curr Biol 8:463–466
41. **Venkateswarlu K, Gunnmoore F, Oatey PB, Tavare JM, Cullen PJ** 1998 Nerve growth factor- and epidermal growth factor-stimulated translocation of the ADP ribosylation factor exchange factor GRP-1 to the plasma membrane of PC12 cells requires activation of phosphatidylinositol 3-kinase and the GRP1 pleckstrin homology domain. Biochem J 335:139–146