Cardiac Phospholipase D2 Localizes to Sarcolemmal Membranes and Is Inhibited by α-Actinin in an ADP-ribosylation Factor-reversible Manner*

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Myocardial phospholipase D (PLD) has been implicated in the regulation of Ca2+ mobilization and contractile performance in the heart. However, the molecular identity of this myocardial PLD and the mechanisms that regulate it are not well understood. Using subcellular fractionation and Western blot analysis, we found that PLD2 is the major myocardial PLD and that it localizes primarily to sarcolemmal membranes. A 100-kDa PLD2-interacting cardiac protein was detected using a protein overlay assay employing purified PLD2 and then identified as α-actinin using peptide-mass fingerprinting with matrix-assisted laser desorption/ionization mass spectroscopy. The direct association between PLD2 and α-actinin was confirmed using an in vitro binding assay and localized to PLD2's N-terminal 185 amino acids. Purified α-actinin potently inhibits PLD2 activity (IC50 = 80 nM) in an interaction-dependent and ADP-ribosylation factor-reversible manner. Finally, α-actinin co-localizes with actin and with PLD2 in the detergent-insoluble fraction from sarcolemmal membranes. These results suggest that PLD2 is reciprocally regulated in sarcolemmal membranes by α-actinin and ARF1 and accordingly that a major role for PLD2 in cardiac function may involve reorganization of the actin cytoskeleton.

Mammalian phospholipase D (PLD)1 hydrolyzes phosphatidylcholine (PC) to generate the signaling lipid, phosphatidic acid (PA) (1), and has been implicated as a regulator in multiple settings in the heart (2, 3). These settings include stimulating inositol 1,4,5-triphosphate production in cardiacocytes (4), inducing phosphorylation of cardiac proteins (5), and increasing intracellular free Ca2+ and contractile force (6). Prolonged activation of cardiac PLD has also been linked to cardiac hypertrophy, potentially through the conversion of PA to diacylglycerol, the endogenous activator of protein kinase C (PKC) (1). Many hormones, neurotransmitters and growth factors exert their cellular effects through PA. Catecholamine (7), angiotensin II (8), and endothelin-1 (9) are all able to stimulate the production of PA in cardiomyocytes, presumably through some combination of Ca2+ mobilization and the actions of small GTP-binding proteins (ARF and Rho family members), PKC, and tyrosine phosphorylation (1). However, the molecular identity of myocardial PLD and the specific mechanisms that regulate it remain unknown.

Mammalian PLD has been implicated in a wide range of physiological processes and diseases including inflammation, secretion, mitogenesis, cytoskeletal rearrangement and respiratory bursts in neutrophils (10). Two types of mammalian PLD, PLD1 and PLD2, have been cloned (1). PLD1 has low basal activity, requires PIP2 for its activation, and is activated by PKC and ARF and Rho small G-protein family members via direct association (11–14). PLD2 also requires PIP2 for its enzymatic activity (15), but unlike PLD1, it can be activated by unsaturated fatty acids such as oleate (16). PLD2 is constitutively active in vitro, and its activity in that setting is relatively insensitive to the PLD1-activating factors PKCα, ARF, and Rho (17). The finding that purified PLD2 is constitutively active in vitro led to the proposal that PLD2 may be regulated in vivo by negative regulatory agents that are released upon agonist stimulation.

In this study, we report that the major myocardial PLD isozyme is PLD2 and that it localizes to the sarcolemmal (SL) membrane. Furthermore, we suggest that PLD2 is negatively regulated by α-actinin through direct interaction and that this inhibition can be overcome by ARF. These results provide the basis for a more detailed model for PLD2 regulation in vivo.

EXPERIMENTAL PROCEDURES

Materials—The enhanced chemiluminescence kit and dipalmitoylphosphatidyl [methyl-3H]choline were from Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Dipalmitoylphosphatidylcholine (dipalmitoyl-PC), phosphatidylinositol 4,5-bisphosphate (PIP2), dioleoylphosphatidylethanolamine, and GTPγS were obtained from Roche Molecular Biochemicals (Mannheim, Germany). Anti-α-actinin antibody and sodium oleate were purchased from Sigma. A polyclonal antibody that recognized both PLD1 and PLD2 was produced as described previously (16). The anti-ARF and the PLD2-specific polyclonal antibodies were a generous gift from Dr. J. David Lambeth (Emory University). Horseradish peroxidase-conjugated goat anti-rab-

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1The abbreviations used are: PLD, phospholipase D; ARF, ADP-ribosylation factor; GTPγS, guanosine 5′-O-(3-thiotriphosphate); PA, phosphatidic acid; PC, phosphatidylcholine; PIP2, phosphatidyl 4,5-bisphosphate; PKC, protein kinase C; small G proteins, low molecular weight GTP-binding proteins; MOPS, morpholinopropansulfonic acid; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[3-cholamidopropyl(dimethylammonio)-1-propanesulfonic acid; SL, sarcolemmal; PX, phox.
Inhibition of PLD2 by α-Actinin

Purification of α-Actinin and ARF1—α-Actinin was purified from rat heart as described previously (23) and judged to be >95% pure by SDS-PAGE. Myristoylated recombinant ARF1 was expressed in E. coli and purified as described (24).

In Vitro Binding Analysis—Wild-type murine PLD2, N-terminal 185-amino acid-deleted mutant PLD2 (PLD2Δ1–185), and the N-terminal 308-amino acid-deleted mutant PLD2 (PLD2Δ1–308) (25) were transfected as described (26). Transfected cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 150 mM NaCl, 1% Triton X-100, and 1% sodium cholate) containing protease inhibitors. After centrifugation (12,000 × g for 15 min), aliquots of the soluble extract were incubated with anti-PLD antibody immobilized on protein A-Sepharose. The PLD2, PLD2Δ1–185, and PLD2Δ1–308 immune complexes were then incubated with purified α-actinin in 0.5 ml of buffer C (20 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 1 mM CaCl₂, 150 mM NaCl) for 1 h at 4 °C. The resulting immune complexes were washed three times with 1 ml of buffer C containing 0.25% CHAPS and subjected to SDS-PAGE, followed by immunoblot analysis with anti-α-actinin or anti-PLD antibody.

Preparation of COS-7 Cell Membranes—Cells transfected with the wild-type murine PLD2, N-terminal 185-amino acid-deleted mutant PLD2 (PLD2Δ1–185), and the N-terminal 308-amino acid-deleted mutant PLD2 (PLD2Δ1–308) were disrupted by sonication in 1 ml of ice-cold buffer D (50 mM HEPES, pH 7.3, 3 mM EGTA, 2 mM CaCl₂, 3 mM MgCl₂, 80 mM KC1, and 1 mM dithiothreitol). The lysates were centrifuged at 100,000 × g for 1 h at 4 °C, and the pellet resuspended in buffer A and referred to as membranes.

Measurement of PLD Activity—PIP₂-dependent PLD activity was assessed by measuring choline release from phosphatidylcholine (PC) essentially as described previously (27). Aliquots of the purified PLD preparation were added to a standard assay mixture (150 μl) containing 125 μl of buffer D (50 mM HEPES, pH 7.3, 3 mM EGTA, 2 mM CaCl₂, 3 mM MgCl₂, 80 mM KC1, and 1 mM dithiothreitol) and 25 μl of phospholipid vesicles composed of dioleoylphosphatidylethanolamine, PIP₂, and dipalmityl-PC in a molar ratio of 16:1.4:1 and dipalmitylphosphatidyl-[(methyl-3H)choline (total 200,000 dpm/assay). The final concentration of PC was 3.3 μM. The assay mixtures were incubated at 37 °C for 15 min. Oleate-dependent activity was assayed as described earlier (16). In brief, PC vesicle (25 μl) containing 5 nmol of dipalmitoyl-PC and 200,000 dpm of dipalmitylphosphatidyl-[(methyl-3H)choline were added to a reaction mixture (175 μl) containing 50 mM HEPES-NaOH, pH 7.0, 2 mM EGTA, 1.7 mM CaCl₂, 20 μM sodium olate, and 100 mM KC1. The final concentration of PC was 25 μM in the reaction mixtures. The assay mixtures were incubated at 30 °C for 1 h and the reactions terminated by the addition of 0.5 ml of 1 N HCl in 5 mM EGTA and 1 ml of chloroform:methanol:HCI (50:50:0.3). After a brief centrifugation, the [methyl-3H]choline in 0.5 ml of the aqueous phase was quantified by liquid scintillation counting.

Co-immunoprecipitation of α-Actinin and ARF1 with PLD2—Purified recombinant human PLD2 (0.1 μg) was precipitated using immobilized anti-PLD antibody. The immune complexes were incubated with various concentrations of ARF1 and 100 nm α-actinin in 150 μl of buffer A at 37 °C for 15 min. The resulting immune complexes were washed twice with buffer A containing 0.25% CHAPS and analyzed by immunoblot with anti-ARF or anti-α-actinin antibody.

Immunoblotting—Immunoblotting was performed as previously reported (12). In brief, proteins were transferred to nitrocellulose membranes by SDS-PAGE. The membranes were blocked with TTBS buffer (50 mM Tris-HCl, pH 7.4, 0.05% Tween 20, 150 mM NaCl) containing 5% skim milk. Subsequently, the blots were incubated with the primary antibody for 3 h. The membrane was washed four times for 10 min with TTBS before being incubated with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h. Visualization of the immune complexes was performed with horseradish peroxidase-dependent enhanced chemiluminescence (ECL).

RESULTS

PLD2 Is the Major Cardiac PLD Isoenzyme and Localizes to the Sarcolemmal Membrane—We used polyclonal antisera that recognize both PLD1 and PLD2 (anti-PLD antibody) to determine which isoforms are expressed in heart. A band corresponding to PLD2 (105 kDa, as shown in the control lane) was weakly detected in a homogenate of rat heart (Fig. 1A) and was highly enriched in a fraction consisting of sarcolemmal (plasma) membranes (SL). A band at the position that would correspond to PLD1 (120 kDa, as shown in the control lane) was not detected.
visualized in any fraction but this band was slightly detected in sarcolemma after long time-exposure (data not shown). To confirm that the band strongly recognized by the pan-PLD-specific antiserum was PLD2, immunoblot analysis was performed utilizing an anti-PLD2-specific antibody (Fig. 1B). This antibody detected a band at the same position on the Western blot (105 kDa) and in the same fractionation samples as the more generic anti-PLD antibody. These results suggest that the major PLD isozyme in the rat heart is PLD2 and that it is present primarily in the sarcolemmal membrane, consistent with its localization primarily in the plasma membrane in other cell types (17, 28, 37).

Partial Purification of a Protein Interacting with PLD2—To search for proteins that interact with PLD2 in the sarcolemmal membrane, we carried out a protein overlay assay. Nitrocellulose blots of proteins from rat heart membrane extracts were incubated with purified PLD2, and the PLD2 bound was then detected using the anti-PLD antibody. A band corresponding to a protein 100 kDa (p100) in size was visualized and partially purified by sequential column chromatography as described under “Experimental Procedures.” On an anionic Q-Sepharose exchange column, p100 eluted with a relatively broad profile (fractions 36–60, Fig. 2A). These fractions were pooled and further purified on phenyl-Sepharose, from which p100 eluted as a sharper peak (fractions 35–45). Analysis by SDS-PAGE revealed that these fractions contained a protein band with a molecular mass of 100 kDa, which matched the molecular weight of the protein reactive in the overlay assay (Fig. 2B).

Identification of the 100-kDa PLD2-binding Protein as α-Actinin—To pursue identification of p100, the partially purified preparation was resolved using SDS-PAGE. The p100 candidate band was excised after Coomassie Brilliant Blue staining and “in-gel” digested with trypsin. The resultant peptides were eluted and analyzed by matrix-assisted laser desorption/ionization mass spectrometer (Fig. 3A). A search for these masses in a comprehensive sequence data base showed that 14 masses matched to the calculated tryptic peptide masses of α-actinin with an accuracy of >50 ppm. These peptides covered 20% of the sequence of α-actinin (Fig. 3B). Finally, the band at 100 kDa that was enriched in fractions 35–45 in the phenyl-Sepharose elute and detected using the PLD2 overlay assay was also detected by specific antibody to α-actinin (Fig. 3C).

PLD2 Directly Interacts with α-Actinin and Co-localizes with It in the Sarcolemmal Membrane—To further confirm the direct interaction between PLD2 and α-actinin, we incubated a purified recombinant human PLD2-anti-PLD-antibody complex with α-actinin purified from rat heart. Immunoblot analysis of the immune complex with anti-α-actinin antibody demonstrated that α-actinin was co-immunoprecipitated only when PLD2 was present (Fig. 4A, lane 3) and that the α-actinin-reactive band did not derive from the Sf9 cells from which the PLD2 was prepared (lane 2). Next, we explored whether PLD2 and α-actinin co-localize to the sarcolemmal membrane. Immunoblot analysis (Fig. 4B) revealed that α-actinin and PLD2 are both present in sarcolemmal membranes and are enriched in the detergent-insoluble membrane fraction, consistent with the several reports that PLD2 can be found in this fraction when it is prepared from plasma membranes of several cell lines (15, 55). Actin was weakly detected in the SL membranes but highly enriched in the detergent-insoluble membrane fraction, suggesting that not only do PLD2 and α-actinin associate, but they do so in the context of the actin cytoskeleton.

The N-terminal region (1–185) of PLD2 Is Responsible for the
Fig. 3. Identification of p100 as α-actinin. A, the map was produced using the peptide supernatant obtained after in-gel digestion of the excised band with trypsin as described under “Experimental Procedures.” A database search for the measured tryptic peptide masses uniquely identified p100 as α-actinin. The peaks labeled with an *A* matched the calculated tryptic peptide masses from α-actinin within 50 ppm. Trypsin autolysis products are marked by a T. B, coverage map for α-actinin. The matching peptides are underlined. C, the fractions (25–55) of phenyl-Sepharose (Fig. 2B) were immunoblot-analyzed with anti-α-actinin antibody.

**Direct Interaction between PLD2 and α-Actinin—**PLD2 has four conserved catalytic regions (I–IV), a PIP2-binding site, a pleckstrin homology domain, and an N-terminal phox (PX) domain that has been proposed to mediate a wide variety of protein–protein interactions (1, 45) (Fig. 5A). Using COS-7 cells to overexpress wild-type and N-terminally deleted mutants of PLD2 (PLD2Δ1–185 and PLD2Δ1–308), we found that truncation of the N-terminal 185 amino acids from PLD2 (which removes the PX domains but still yields a catalytically active enzyme) resulted in the complete loss of α-actinin binding to PLD2 (Fig. 5B). It appears, therefore, that the N-terminal 185 amino acids contain a site critical for the interaction of PLD2 with α-actinin.

**α-Actinin Inhibits PLD2 Activity via Direct Interaction—**We next examined whether α-actinin plays a role in the regulation of PLD2 by determining whether it affected PLD2 activity in the in vitro setting. As shown in Fig. 6A, purified α-actinin inhibited PIP2-dependent PLD2 activity in a concentration-dependent manner. The IC50 of the α-actinin-mediated inhibition was 80 nM, and inhibition was complete at 300 nM. α-Actinin does not have a pleckstrin homology domain but has been reported to bind PIP2 (29, 30), and PIP2 is a required co-factor for PLD2 activity in the standard in vitro assay (15, 16, 25). To exclude the possibility that the mechanism of the α-actinin inhibition might involve PIP2 sequestration, we examined the effect of α-actinin on oleate-dependent activation of PLD2, which is an alternate method for activation of PLD2 that does not require PIP2 (16). α-Actinin similarly inhibited the oleate-dependent activation in a concentration-dependent manner. To further delineate the interaction and inhibition of α-actinin on PLD2 activity, we examined the effect of α-actinin on the deletion mutants of PLD2. We overexpressed wild-type and N-terminally deleted mutants of PLD2 (PLD2Δ1–185) and PLD2Δ1–308), and used then COS-7 cell membranes as a source of PLD2. As in the above binding assay, α-actinin did not inhibit either of the N-terminally deleted mutants (PLD2Δ1–185 and PLD2Δ1–308), whereas it inhibited whole PLD2 in a concentration-dependent manner (Fig. 6B). These results led us to the conclusion that the observed inhibition occurred through direct interaction between α-actinin and the N-terminal region (1–185) of PLD2.

The Inhibition of PLD2 by α-Actinin Is Overcome by ARF1—ARF1 has been implicated as a potent activator of PLD1 in a variety of settings (1). Recent evidence has suggested the possibility that PLD2 may also be activated by ARF (25, 28, 37), and it was proposed that this might be physiologically relevant in settings where the PLD2 high basal activity had been suppressed (25). We accordingly examined the possibility that inhibition of PLD2 by α-actinin might be countered by ARF1 stimulation. Upon addition of ARF1, PLD2 basal activity increased modestly, whereas the 60% inhibition mediated by addition of 100 nM α-actinin was almost completely eliminated. In the presence of added ARF1, α-actinin inhibition was modest, but the IC50 was increased 7-fold (Fig. 6C).
The interaction of independent preparations of PLD2 with anti-PLD or anti-α-actinin antibody. The results shown are those of a single experiment representative of two experiments performed with independent preparations.

FIG. 5. The N-terminal region (1–185) of PLD2 is required for the interaction of α-actinin with PLD2. A, schematic outline of the structural domains of PLD2. Boxes, location of regions of highly conserved sequence in PLD: PX, pleckstrin homology; CR, conserved region. B, COS-7 cells were transfected with the empty vector (MOCK), N-terminal 185-amino acid-truncated PLD2 (PLD2Δ(1–185)), N-terminal 308-amino acid-truncated PLD2 (PLD2Δ(1–308)), or wild-type PLD2 (PLD2). Immune complexes were prepared as described under “Experimental Procedures.” Purified α-actinin and the immune complexes were incubated and then subjected to immunoblot analysis with anti-PLD or anti-α-actinin antibody. The assay was performed to measure PIP2-dependent (open rectangles) or oleate-dependent (closed rectangles) PLD2 activity as described under “Experimental Procedures.” The PLD2 activity was determined as a function of the α-actinin concentration. The data represent means ± S.E. of three experiments performed in duplicate. B, N-terminal 185 amino acid region is responsible for the inhibition of PLD2 by α-actinin. COS-7 cell membranes overexpressing N-terminal 185-amino acid-truncated PLD2 (PLD2Δ(1–185)) (closed rectangles), N-terminal 308-amino acid-truncated PLD2 (PLD2Δ(1–308)) (open rectangles), or wild-type PLD2 (open circles) were prepared as described under “Experimental Procedures” and used as a source of PLD2. PLD activity was determined with 0.5 μg of membranes and various concentrations of purified α-actinin. The data represent the means ± S.E. of three experiments performed in duplicate.

FIG. 6. Inhibition of PLD2 activity by α-actinin. A, effect of α-actinin on the PIP2, or oleate-dependent activity of PLD2 is plotted. The assay was performed to measure PIP2-dependent (open rectangles) or oleate-dependent (closed rectangles) PLD2 activity as described under “Experimental Procedures.” The PLD2 activity was determined as a function of the α-actinin concentration. The data represent means ± S.E. of three experiments performed in duplicate. B, N-terminal 185 amino acid region is responsible for the inhibition of PLD2 by α-actinin. COS-7 cell membranes overexpressing N-terminal 185-amino acid-truncated PLD2 (PLD2Δ(1–185)) (closed rectangles), N-terminal 308-amino acid-truncated PLD2 (PLD2Δ(1–308)) (open rectangles), or wild-type PLD2 (open circles) were prepared as described under “Experimental Procedures” and used as a source of PLD2. PLD activity was determined with 0.5 μg of membranes and various concentrations of purified α-actinin. The data represent the means ± S.E. of three experiments performed in duplicate.

DISCUSSION

PLD is thought to play an important role in several different aspects of cardiac physiology (2, 3). PLD activity is enriched in sarcomemal membranes (31), and its functional significance has been linked to Ca2+ mobilization and changes in the force of contraction of the heart (6). Although oleate (32) and several agonists that activate PI-PLC activity (7–9) are known to enhance myocardial PLD activity, the molecular identity and the regulatory mechanism of myocardial PLD have not previously been described. We report here that PLD2 is the major myocardial PLD, that it is enriched in sarcomemal membranes, and that α-actinin inhibits the high basal activity of PLD2 in an ARF-reversible manner.

Both PLD1 and PLD2 mRNA have been reported to be expressed in the heart (33), and evidence has been presented that there are two types of PLD activity in the heart: one in the sarcomemal and the other in the sarcoplasmic reticulum. We found that PLD2 is major myocardial PLD and the majority of it was present in the sarcomemal. Supporting our molecular data that the sarcomemal isoform is PLD2, it was previously reported that this activity can also be stimulated by unsaturated fatty acids such as oleate (32), which is characteristic of PLD2 (16).

It has been reported that PLD may be functionally associated with the actin-based cytoskeleton. Stimulation of PLD in fibroblasts and endothelial cells leads to the formation of actin stress fibers, and this effect is mimicked by the addition of purified PLD or PA and blocked by inhibitors of PLD-dependent generation of PA (34, 35). Recently, Iyer et al. (36) reported association of PLD1 activity with the actin-based membrane skeleton in U937 cells. PLD2 also has been implicated in the regulation of the cytoskeleton. Overexpression of PLD2 promoted cytoskeletal reorganization in serum-stimulated fibroblasts (17), and it co-localized with membrane ruffles induced by EGF (37). In this report, we identified α-actinin as a PLD2-binding protein, which potently inhibits the basal activity of PLD2. α-Actinin is known as a key regulator of actin-based structures such as stress fibers and focal adhesions (38). Binding of α-actinin to phosphatidylinositol 3-kinase, protein kinase N (39), and vinculin (40) is consistent with functional roles of α-actinin in signal transduction and cytoskeletal reorganiza-
PLD2 interacts with the actin cytoskeleton. The insoluble fraction of sarcolemma membranes provides strong evidence that PLD2 is part of the actin cytoskeleton. However, the combination of mild detergents and the use of an immunoprecipitation approach, because PLD2 and α-actinin are tightly associated with membrane structures, proved difficult. LD2 coupled immune complexes were incubated with 100 nM α-actinin, and the indicated concentrations of ARF1 were added to the assay mixtures. The resultants pellets were subjected to immunoblot analysis with anti-α-actinin and anti-ARF antibody. The results shown are those of a single experiment, representative of three experiments performed with independent preparations. Therefore, our results suggest that regulation of PLD2 by α-actinin might be involved in cytoskeletal reorganization.

It proved technically difficult to demonstrate in vivo association of α-actinin with PLD2 in the sarcolemmal membrane using an immunoprecipitation approach, because PLD2 and α-actinin are tightly associated with membrane structures and we found that we could not extract them using the mild detergent conditions required to observe the interaction in vitro (1% cholic acid was required to extract them, whereas in vitro interaction was disrupted at cholic acid concentrations greater than 0.5%; data not shown). However, the combination of in vitro association and of co-localization in vivo to the detergent-insoluble fraction of sarcolemma membranes provides strong support for their interaction and further supports the idea that PLD2 interacts with the actin cytoskeleton. α-Actinin shares sequence homology to fodrin, which was previously reported to have sequence homology to fodrin (41). Our findings indicate that the mechanism of α-actinin inhibition of PLD2 does not involve blockade of PIP2 but rather direct interaction, suggesting that these related cytoskeletal proteins use different mechanisms to achieve the same end: suppression of PLD activity in cells. It has also been reported that PLD2 can be inhibited by synuclein (42), which regulates synaptic transmission, and by proteins involved in endocytosis (43), suggesting that different cells may suppress PLD2 activity using different mechanisms, each one appropriate for the primary role played by PLD2 in that particular cell type.

Purified PLD2 exhibits a high basal activity in the standard in vitro assay and after transfection into COS-7 cells, and it can be minimally stimulated further by the well-known activators of PLD1 (17). This seemed paradoxical, because, although PLD2 mRNA is expressed in many tissues and mammalian cell lines (44), they nonetheless do not exhibit elevated PLD basal activities. Recently, Sung et al. (25) reported that an N-terminal truncated PLD2 exhibited low basal activity in vitro but elevated activity in vivo. The authors hypothesized that unknown negative regulatory factors might inhibit the basal activity of PLD2 through interaction with its N terminus. This hypothesis is consistent with our finding that α-actinin directly interacts with PLD2 through its N-terminal 185 amino acids as inhibits it.

PLD2 carries a PX motif in the N-terminal region (Fig. 5A) (45). PX motifs have been reported to mediate a wide variety of protein-protein interactions including kinase or SH3-domain binding (46). Although significantly conserved PX domain is present in animal and yeast PLD (46), its function is unknown. In this study, we have shown that α-actinin inhibits PLD2 activity through direct interaction with N-terminal 185 amino acids which contain the PX motif. This result raised the possibility that PX domain might play a regulatory role through direct interaction with regulatory proteins such as α-actinin.

Although ARF has long been known to be a potent activator of PLD1, several recent findings raised the possibility that ARF could also stimulate PLD2 activity. It has been reported that ARF stimulated the activity of hPLD2 1.6-fold in an in vitro reconstitution assay (25) and that it mediated insulin-dependent PLD2 activation in HIREc cells (28). Moreover, a PLD2 mutant form that lacked the N-terminal 308 amino acids and exhibited reduced basal activity was stimulated more than 5-fold by ARF (25). Our observation that ARF1 reversed the α-actinin inhibition of PLD2 activity strongly supports the hypothesis that ARF1 can potently activate PLD2 activity in the in vivo setting where it is suppressed by inhibitory proteins.

Of interest is the mechanism through which ARF blocks the interaction of α-actinin with PLD2 and accordingly its inhibitory effect on PLD2 activity. It seems unlikely that the mechanism involves a simple competition for an identical or overlapping binding site, since α-actinin interacts with the N terminus (Fig. 5B) and ARF does not (25). However, several lines of evidence suggest that there are interactions between the N terminus and C terminus for PLD. First, there is synergism between factors that stimulate PLD1 through the N terminus such as PKC and factors that stimulate PLD1 through the C terminus, such as ARF and Rho and this synergism has been suggested to involve conformational changes in the PLD structure (47). Second, PLD1 and PLD2 encode a “split” catalytic site, one part of which is in the N-terminal half of the protein (but not in the first 308 amino acids), and the other in the C-terminal half. It has been reported that both catalytic sites are needed to be functional (48), that they are likely to associate in the three-dimensional structure (49), and that the N-terminal and C-terminal halves by themselves show affinity for each other (50). Taken together, these findings suggest two possibilities for the ARF-mediated reversal of α-actinin inhibition of PLD. First, the sites of interactions for α-actinin and ARF1 with PLD2 may lie far apart in the primary structure but close together in the tertiary structure of PLD2. Accordingly, ARF might conceivably physically interfere with the α-actinin-PLD2 interaction. Alternatively, a conformational change induced by ARF might alter the structure of the N terminus and accordingly eliminate the binding site for α-actinin.

PLD activation and ARF translocation is tightly coupled. FMLP (formyl-methionyl-leucyl-phenylalanine) or phorbol 12-myristate 13-acetate leads to the translocation of ARF proteins to the particulate membrane as well as activation of PLD in neutrophils and mast cells (51, 52). Translocation of ARF to the actin cytoskeleton has also been reported and suggested to be involved in actin reorganization (53, 54). Recently, Iyer et al.
nists can stimulate PLD2 activity in the actin cytoskeleton
incubated with GTP (36) demonstrated that PLD activity can be stimulated in de-
et al. (1997) J. Biol. Chem. 272, 424–2259
13. Yamazaki, M., Zhang, Y., Watanabe, H., Yokozeki, T., Ohno, S., Kaibuchi, K.,
12. Kim, J. H., Lee, S. D., Han, J. M., Lee, T. G., Kim, Y., Park, J. B., Lambeth,
14. Hammond, S. M., Jenco, J. M., Nakashima, S., Cadwallader, K., Gu, Q., Cook,
11. Xu, Y. J., Botsford, M. W., Panagia, V., and Dhalla, N. S. (1996) J. Biol. Chem.
10. Exton, J. H. (1997) J. Biol. Chem. 272, 2717–2724
9. Ye, H., Wolf, R. A., Kurz, T., and Wolf, R. A. (1995) J. Biol. Chem. 270, 1347–1358
8. Sadoshima, J., and Izumo, S. (1993) Circ. Res. 73, 424–438
7. Nowacka, O., Drakova, J., Kubista, V., and Novak, F. (1994) Physiol. Res. 43, 151–156
6. Xu, Y. J., Panagia, V., Shao, Q., Wang, X., and Dhalla, N. S. (1996) Am. J. Physiol.
5. Cross, M. J., Roberts, S., Ridley, A. J., Hodgkin, M. N., Stewart, A., Claesson-
4. Henry, R. A., Boyce, S. Y., Kurz, T., and Wolf, R. A. (1995) Am. J. Physiol. 269, 349–358
3. Xu, Y. J., Botsford, M. W., Panagia, V., and Dhalla, N. S. (1996) Can. J. Cardi-
2. Frohman, M. A., and Morris, A. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6210–6213
1. Frohman, M. A., and Morris, A. J. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 6210–6213
REFERENCES

(36) demonstrated that PLD activity can be stimulated in de-
tergent-insoluble fractions derived from U937 cells membranes
incubated with GTPyS and that the level of ARF was increased
moreover, Honda et al. (37) have reported that ARF6 co-localizes with PLD2 in ruffling membranes after treatment with EGF. our finding that PLD2 is regulated by ARF1 and
-actinin further extends the hypothesis that extracellular ago-
nists can stimulate PLD2 activity in the actin cytoskeleton through the action of ARF proteins.
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