Structural Determinants for Phosphatidic Acid Regulation of Phospholipase C-β₁

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Signaling from G protein-coupled receptors to phospholipase C-β (PLC-β) is regulated by coordinate interactions among multiple intracellular signaling molecules. Phosphatidic acid (PA), a signaling phospholipid, binds to and stimulates PLC-β₁ through a mechanism that requires the PLC-β₁ C-terminal domain. PA also modulates Gα₉ stimulation of PLC-β₁. These data suggest that PA may have a key role in the regulation of PLC-β₁ signaling in cells. The present studies addressed the structural requirements and the mechanism for PA regulation of PLC-β₁. We used a combination of enzymatic assays, PA-binding assays, and circular dichroism spectroscopy to evaluate the interaction of PA with wild-type and mutant PLC-β₁ proteins and with fragments of the Gα₉ binding domain. The results identify a region that includes the αA helix and flexible loop of the Gα₉-binding domain as necessary for PA regulation. A mutant PLC-β₁ with multiple alanine/glycine replacements for residues N⁹⁴LIKEHTTKYNIEQ⁹⁵ was markedly impaired in PA regulation. The high affinity and low affinity component of PA stimulation was reduced 70% and PA binding was reduced 45% in this mutant. Relative PLC stimulation by PA increased with PLC-β₁ concentration in a manner suggesting cooperative binding to PA. Similar concentration dependence was observed in the PLC-β₁ mutant. These data are consistent with a model for PA regulation of PLC-β₁ that involves cooperative interactions, probably PLC homodimerization, that require the flexible loop region, as is consistent with the dimeric structure of the Gα₉-binding domain. PA regulation of PLC-β₁ requires unique residues that are not required for Gα₉ stimulation or GTPase-activating protein activity.

Phosphatidic acid (PA) is a novel signaling phospholipid that has been implicated in the regulation of multiple cell functions including cell proliferation, cytoskeletal reorganization and membrane trafficking (1, 2). PA regulates the activity of signaling proteins in vitro, including phospholipases (3–5), kinases (6, 7), G protein regulators (8), and cyclic AMP-phosphodiesterases (9, 10). The importance of PA in the regulation of cellular effectors is shown by in vivo studies that demonstrate loss of cellular regulation in cells overexpressing mutant PA-impaired effectors (6, 7, 9, 10).

Intracellular PA levels are low under resting conditions but increase rapidly upon activation of a number of receptor signaling pathways, including GPCR-PLC-β signaling (11). GPCR-PLC-β signaling is mediated both by the Gα₁₁ family of GTP-binding proteins acting through the Go subunit and by Gβγ subunits supplied primarily by G₁ family members. Rapid hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) by PLC-β generates inositol 1,4,5-trisphosphate and diacylglycerol (DAG). Inositol 1,4,5-trisphosphate increases the levels of regulatory Ca²⁺. DAG activates the protein kinase Cs and other effectors, including DAG kinases, which convert DAG to PA. Increases in intracellular Ca²⁺ levels and protein kinase C activity stimulate phospholipase D (PLD) activity, which generates PA from phosphatidylcholine. PLD is also stimulated in response to GPCR-G₁₂/₁₃ signaling and activation of monomeric GTP-binding proteins, including RhoA. Nine DAG kinase isoforms and two major PLD isoforms have been identified; they differ in subcellular localization and regulation (12, 13). The contribution of the DAG kinase- and PLD-generated pools of PA in the GPCR regulation of cellular function, and the mechanism for PA regulation of effectors remains poorly understood.

In addition to the role of PLC-β₁ in mediating GPCR-dependent increases in PA levels, PLC-β₁ is also regulated by PA in vitro. These data suggest a potential role for PA in the feedback modulation of GPCR-PLC-β₁ signaling (14–16). PLC-β₁ is a member of a large family of PIP₂-specific PLC enzymes that include PLC-β₁₋₄, PLC-γ₁₋₂, PLC-δ₁₋₄, PLC-ε, PLC-ζ, and PLC-η₁₋₂ (17–21). PIP₂-PLCs are grouped by sequence homology and coupling to specific receptor-delineated mechanisms, including those mediated by GPCR and tyrosine kinase receptors. The mechanisms that regulate many of these enzymes are still not well understood, and both specific signaling phospholipids and small molecular weight GTP-binding proteins have recently been implicated in regulation. PLC-β enzymes share phosphatidylinositol 4,5-bisphosphate; PLC, phospholipase C; GPCR, G protein-coupled receptor; GAP, GTPase-activating protein; PC, phosphatidylcholine.
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many of the structural features present in other members of the PIP₂-PLC family, including conserved catalytic X and Y boxes as well as two membrane-phospholipid binding regions, the pleckstrin homology and C2 domains. PLC-β enzymes, however, are distinguished by the presence of an elongated C terminus consisting of ~450 residues, which contains many of the determinants for interaction with Go₅q (22).

GPCR stimulation of PLC-β₁ activity is tightly regulated at the G protein level by cycling of Go₅q between inactive and active states (23). Under basal conditions, Go₅q exists in the relatively inactive GDP-bound state. Agonist binding to GPCR activates Go₅q by promoting exchange of GTP for bound GDP. The active Go₅q-GTP stimulates PLC-β₁ activity. The duration of the Go₅q-GTP state is determined by its intrinsic GTPase activity, which hydrolyzes Go₅q-bound GTP to GDP and thereby terminates activity. GTPase-activating proteins (GAPs), including regulators of G protein signaling and PLC-β, stimulate the intrinsic GTPase activity of Go₅q, accelerating deactivation (24). PLC-β thus functions as both an effector of Go₅q and a GAP specific for the Go₅q family of G proteins (24). The intracellular mechanisms that regulate and integrate these dual functions of PLC-β are not fully understood.

We have shown that PA stimulates PLC-β₁ activity through a mechanism that requires PA binding (14, 16). Unlike other PA-regulated effectors, PA also modulates PLC-β₁ activation in response to known cellular mediators, suggesting that PA targets a mechanism common to both basal and regulated PLC-β₁ activity. PA stimulation of PLC-β₁ activity is synergistic with Go₅q stimulation in vitro (16). The combination of PA and Go₅q-GTPY results in greater stimulation of PLC-β₁ activity than that due to either PA or Go₅q-GTPY alone. PA also potentiates GPCR stimulation of PLC-β₁ activity in membranes, consistent with a role for PA in enhancing receptor-Go₅q stimulation (14, 15). Furthermore, PA antagonizes net inhibition of PLC-β₁ activity by protein kinase C, suggesting a potential role for PA in the regulation of negative feedback inhibition by protein kinase C (16).

Two observations suggest that PA regulation occurs through binding to a site within the PLC-β₁ C terminus and that it is isoform-dependent. First, a calpain-generated 100-kDa PLC-β₁ fragment, which lacks 336 residues C-terminal to His880, does not bind PA and is insensitive to stimulation by 7–15 mol % PA. Stimulation of the 100-kDa fragment requires >15 mol % PA and is not coupled to measurable PA binding (14). Second, PA stimulation of the PLC-β₁ isoform requires >15 mol % PA and is also not associated with PA binding (16).

Sequence diversity within the C-terminal domain of PLC-β isoforms is thought to contribute to isoform differences in sensitivity to stimulation by G protein subunits and regulation by protein kinases (21). The C-terminal domain contains determinants for α₅, stimulation (25, 26), GAP activity (27), electrostatic dependent association with membrane lipids (25, 26), nuclear localization (26), and phosphorylation/regulation by protein kinase C (28, 29). Isolated PLC-β₁ C-terminal tails show complex GAP behavior, which suggests that dimerization/oligomerization has an important role in the regulation of PLC-β₁ function (27). Consistent with this hypothesis, the crystal structure of the turkey PLC-β₂ C-terminal domain shows it to be a homodimer of three-stranded coiled coils in an antiparallel orientation (30). The dimer interface includes the αB and αC helices, and Go₅q is predicted to bind to the dimer interface, further implicating dimerization in Go₅q interaction (31).

To further delineate the role of PA in the modulation of GPCR-Go₅q-PLC-β₁ signaling, the present studies used truncated PLC-β₁ C-terminal fragments and corresponding PLC-β₁ C-terminal mutants (31) to localize the determinants for PA binding and to address the mechanism for PA regulation of PLC-β₁ activity. The data show that the region mapping to the αA helix and flexible αA-αB loop of the Go₅q binding domain is required for both PA binding and stimulation of PLC-β₁ activity. This loop is conserved among mammalian PLC-β₁s but diverges in the other isoforms. In addition, our data suggest a mechanism for PA regulation of PLC-β₁ that involves cooperative interactions, probably homodimerization. Regulated homodimerization is a potential novel mechanism for the integration of signaling from multiple cellular networks to the regulation of GPCR-Go₅q-PLC-β₁ signaling.

**EXPERIMENTAL PROCEDURES**

**Expression and Purification of C-terminal Fragments—**

PLC-β₁ exists as two alternatively spliced variants, 150-kDa PLC-β₁a and 140-kDa PLC-β₁b, that are comparably stimulated by Go₅q. The PLC-β₁b variant replaces the 75 C-terminal residues of PLC-β₁a with 32 residues unique to PLC-β₁b (32, 33). The PLC-β₁ C-terminal sequence common in both variants, Ala878-Lys1141, PLC-β₁b (878–1141) was amplified from rat PLC-β₁ cDNA by PCR using primers 5′-TACCATGGTCC-ACACAGCCAGCCTGTGC-3′ and 5′-CTGGATCTTTCAATT- GTGATGGTGTTGGCCTTCTCATCCAGGATCTGTG-3′. These primers introduce methionine at the N terminus, a His₉ tag at the C terminus, and Ncol and BamH1 sites (underlined). The PCR product was ligated into pET-3d (Novagen). Protein was expressed in BL21 (DE3) grown at 37 °C to an A₆₀₀ of 0.6 in enriched media (30 g of peptone/trypton, 20 g of selectable yeast extract/liter) plus M9 salts. Expression was induced with 400 μM isopropyl 1-thio-β-d-galactopyranoside, and cells were harvested after 3 h. Cells were thawed in 10 mM imidazole, 20 mM phosphate buffer (pH 7.0) containing protease inhibitors (complete EDTA-free protease inhibitor, Roche Applied Science), lysed by sonication on ice, and centrifuged at 15,000 × g for 30 min at 4 °C. Recombinant protein was purified from the supernatant to 80% purity with Talon Metal Affinity Resin (Clontech), as described by the manufacturer. The recombinant protein was identified by its molecular mass, induction of protein expression by isopropyl 1-thio-β-d-galactopyranoside, and reactivity of the His₉ tag with Ni²⁺-nitrilotriacetic acid horseradish peroxidase conjugate (Qiagen) by Western blot. PLC-β₁ (873–1030), PLC-β₁b (878–1010), PLC-β₁ (878–980), and PLC-β₁b (878–950) were produced similarly. PLC-β₁ (1–880) was produced by calpain-1 cleavage of PLC-β₁ as described (14).

**Expression and Purification of Replacement PLC-β₁ Mutants—**

PLC-β₁ mutants were made by PCR mutagenesis using the QuikChange protocol (Stratagene). Wild-type PLC-β₁ and PLC-β₁ mutants were expressed in Escherichia coli SG13009/
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**FIGURE 1.** Binding of PLC-\(\beta\), and PLC-(878–1141) C-terminal fragment to liposomes. Purified PLC-\(\beta\), or PLC-(878–1141) was incubated with liposomes consisting of either 100 mol % PC (lanes 1 and 4), 25 mol % PA/75 mol % PC (lanes 2 and 3), or 25 mol % PS/75 mol % PC (lanes 3 and 6) in the presence of either 0.1 m KCl (lanes 1–3) or 0.4 m KCl (lanes 4–6). Binding was determined as described under “Experimental Procedures.” Gels were stained with Coomassie Blue.

**FIGURE 2.** Full-length PLC-\(\beta\), PLC-(1–880), and PLC C-terminal fragments. The X and Y boxes represent the conserved catalytic domain.

**RESULTS**

**Localization of PA Binding Determinants in PLC-\(\beta\), C-terminal Fragments**

Calpain cleaves PLC-\(\beta\), after His\(^{880}\), to generate a 100-kDa PLC-\(\beta\), fragment, PLC-(1–880), that lacks the 336 C-terminal residues (14, 32). The activity of PLC-(1–880) is not stimulated by low mol % PA, and PLC-(1–880) does not bind PA, indicating that high affinity PA regulation requires the PLC-\(\beta\), C terminus (14). The binding of full-length PLC-\(\beta\), to PA is salt-resistant, consistent with binding mechanisms other than electrostatic (16). To directly address the involvement of the PLC-\(\beta\), C terminus in salt-resistant PA binding, we measured PA binding to a purified recombinant PLC C-terminal fragment corresponding to residues 878–1141 of PLC-\(\beta\), PLC-(878–1141), under highly stringent conditions that included 0.4 m KCl. As shown in Fig. 1, the phospholipid binding profile of PLC-(878–1141) was comparable with that of full-length PLC-\(\beta\), Binding of both PLC-(878–1141) and full-length PLC-\(\beta\), to liposomes that contain PA was resistant to displacement by 0.4 m KCl, indicating that residues 878–1141 contain determinants for salt-resistant PA binding. In contrast, binding of either protein to PS-containing liposomes was inhibited 100% by 0.4 m KCl, showing that PS binds through a predominantly electrostatic mechanism. Less than 5% of either protein bound to PC liposomes.

To further localize the determinants for salt-resistant PA binding, we studied the PA binding of PLC-(1–880) and a series of PLC C-terminal fragments corresponding to residues 878–1030, 878–1010, 878–980, and 878–950 (Fig. 2). Neither PLC-(1–880) nor PLC C-terminal fragments bound appreciably to PC liposomes, less than 5% binding. The binding profile for PLC-(1–880) and PLC C-terminal fragments to PA is shown in Table 1. PLC-(1–880) did not bind to PA in the presence of either 0.1 or 0.3 m KCl. Binding of PLC C-terminal fragments was comparable with PLC-(878–1141) in the presence of 0.1 m KCl. PA increased the binding of all C-terminal fragments to a similar extent. Significant differences in PA binding, however, were apparent in the presence of 0.3 m KCl. PLC-(878–1141), PLC-(878–1030), PLC-(878–1010), and PLC-(878–980) retained 50, 80, 95, and 90% of their total PA binding in the presence of 0.3 m KCl. The increase in KCl-insensitive PA bind-
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TABLE 1
Binding of PLC fragments to PA

| Fragment           | Binding to PA liposomes |
|--------------------|-------------------------|
|                    | 0.1 M KCl               | 0.3 M KCl               |
| PLC-(1–880)        | 8 ± 2                   | 10 ± 4                  |
| PLC-(878–1141)     | 210 ± 42                | 208 ± 42                |
| PLC-(878–1030)     | 216 ± 60                | 180 ± 42                |
| PLC-(878–1010)     | 225 ± 33                | 216 ± 30                |
| PLC-(878–980)      | 210 ± 60                | 195 ± 51                |
| PLC-(878–950)      | 150 ± 30                | 14 ± 3                  |

Purified PLC fragments (300 nM) were incubated with 100 mol % PC or 25 mol % PA/75 mol % PC liposomes the presence of 0.1 or 0.3 m KCl as described under "Experimental Procedures." Binding to 100 mol % PC liposomes was negligible, less than 15 pmol, and is subtracted from binding. Results are the mean ± S.D. of three experiments.

Stimulation of the phospholipase activity of the mutants by PA paralleled their PA binding. PA stimulation of the A-(944–957) and A-(965–975) mutants was reduced 70 and 30% relative to wild-type PLC-β₁, respectively. Again, the decrease in stimulation was statistically significant only for the A-(944–957) mutant. PA stimulation of the other mutants was similar to that of wild-type PLC-β₁ at about 200% of basal level. Interaction with fatty acyl chains appears to contribute appreciably to A-(944–957) binding to PA. When binding was carried out with the short chain PA analog, Di6-PA, binding of A-(944–957) to PA liposomes was 1.9 ± 2 fmol as compared with 17.2 ± 2 fmol for wild-type

FIGURE 3. A, CD spectra of PLC-(878–980) in the absence or presence of DPPA. The CD spectra of purified PLC-(878–980) was determined in the presence of 100 mol % DPPC (●), 25 mol % DPPA/75 mol % DPPC (▼), or 25 mol % DPPS/75 mol % DPPC (▲) as described under "Experimental Procedures." After scanning the spectra, the mixtures were centrifuged, and the protein-lipid pellet was analyzed by SDS-PAGE. The inset shows Coomassie Blue-stained gel lanes for PLC-(878–980) to 100 mol % DPPC (lane 1), 25 mol % DPPA/75 mol % DPPC (lane 2), or 25 mol % DPPA/75 mol % DPPC (lane 3) vesicles. B, concentration dependence for DPPA-induced increase in α helical content of PLC-(878–980). The increase in PLC-(878–980) α helical content was determined by ellipticity at 222 nm. Results are the mean ± S.D. of three experiments.
PLC-\(\beta_1\) (n = 3), a 90% reduction in mutant PA binding. The greater contribution of nonspecific electrostatic binding and fatty acyl interaction in A-(944–957) relative to wild-type PLC-\(\beta_1\) probably underestimates the actual decrease in PA binding. Together, these data identify one mutant with a significant impairment in PA regulation and implicate residues 944–957 in the mechanism for PA binding and stimulation of phospholipase C activity. The A-(944–957) mutation, which replaces LIKEHTTKYNEIQN with AGAGAGAGAG, was characterized further.

**Phospholipase Activation by PA Depends on the Concentration of PLC-\(\beta_1\) **—The relative stimulation of phospholipase activity by PA depends on the concentration of PLC-\(\beta_1\). As shown in Fig. 4, the specific activity of PLC-\(\beta_1\) increased markedly with increasing enzyme concentration up to about 50 pm in either the presence or absence of PA. For wild-type PLC-\(\beta_1\), specific activity increased about 4-fold over the range of enzyme concentrations shown. A qualitatively similar increase was observed for the A-(944–957) mutant. Such behavior suggests that mostly inactive PLC-\(\beta_1\) monomers associate to form a more active dimer or higher oligomer. In the presence of PA, increased specific activity occurred at lower PLC-\(\beta_1\) concentrations than in its absence, which suggests that PA binding promotes dimerization.

The interactive effects of PA and PLC-\(\beta_1\) on self-association are more evident when the PA concentration dependence is examined at low and high PLC-\(\beta_1\) concentrations (Fig. 5). PA stimulated dilute wild-type PLC-\(\beta_1\) more than the concentrated enzyme. Maximal stimulation of 50 pm PLC-\(\beta_1\) was about 8-fold, whereas that of 150 pm PLC-\(\beta_1\) was less than 3-fold. Further, 50 pm PLC-\(\beta_1\) was half-maximally stimulated at about 0.4 mol % PA, but stimulation of 150 pm PLC-\(\beta_1\) did not obviously saturate even at 15 mol %, reflecting a greater contribution of ionic and nonspecific effects. It is therefore likely that at low PLC-\(\beta_1\) concentrations, PA stimulation occurs through a high affinity interaction and that only low affinity interactions occur at higher PLC-\(\beta_1\) concentrations. Stimulation by PA was nearly absent in the A-(944–957) mutant.

Taken together, the data of Figs. 4 and 5 suggest that high affinity PA binding activates PLC-\(\beta_1\) primarily at low enzyme concentrations and that activation by oligomerization supplants the PA effect. Conversely, PA decreases the concentrations of PLC-\(\beta_1\) needed for the display of increased activity. At higher enzyme concentrations, high affinity activation by PA is lost, and the behavior of the A-(944–957) mutant argues that this region of the C-terminal domain is involved in this interaction. The data support the idea that PA stimulates phospholipase activity by promoting its association to form an active oligomer, probably a dimer.

**DISCUSSION**

Previous work from this laboratory has shown that PA regulates PLC-\(\beta_1\) activity through a mechanism that requires PA binding to the C-terminal domain that includes...
the Gαq binding site (14, 16). The observation that PA modulates PLC-β1 regulation by known cellular regulators suggests that PA constitutes part of an important isoform-specific feedback mechanism for regulation of GPCR-PLC-β signaling (16). Further insight into the role and mechanism for PA regulation requires identification of the PA binding region. The present studies used PA binding, CD spectroscopy, and PLC activity measurements and a combination of PLC C-terminal fragments and full-length PLC-β mutants to delineate the region required for interaction with PA. All of the experimental data localized the PA interaction site to the αA helix and the flexible αA-αB loop of the Gαq binding domain. Most notably, mutation of residues 944–957, which includes parts of αA and the loop, caused a significant 45% reduction in PA binding and 70% reduction in PA stimulation. Mutation of residues 965–975, within the loop, also marginally decreased PA binding and PA stimulation but was not statistically significant. Other mutations in the C-terminal domain, A-(928–943), A-(976–990), A-(998–1007), and A-(1018–1028), displayed wild-type or nearly wild-type regulation by PA. Mutants A-(944–957) and A-(965–975) have been characterized previously (31) (Table 3). They show wild-type behavior with respect to Ca2+-stimulated phospholipase activity and wild-type Gαq GAP activity. The A-(944–957) mutant shows only a minor decrease in stimulation by Gαq, and stimulation of A-(965–975) mutant is unaltered. Most of the mutations that decrease the response to Gαq or Gαq GAP activity map to the interface of the C-terminal dimer that occurs between the αB and αC helices or to a proposed Gαq-binding site (30, 31). The present data thus show that interaction with PA is mediated by residues within the Gαq-binding domain that largely do not overlap with those required for Gαq binding or regulation.

The flexible αA-αB loop is highly conserved among mammalian PLC-β enzymes but diverges among the four different PLC-β isoforms. This pattern suggests a possible molecular basis for the isoform specificity in PA regulation. The flexible loop is solvent-exposed and conformationally flexible in full-length PLC-β, and its role in regulation of PLC-β is not understood (30). Solving the crystal structure of the turkey PLC-β C terminus required removal of 76% of the flexible loop (residues 946–978 in turkey PLC-β2) (30). This corresponds to rat PLC-β1 residues 961–993 and lies outside the sequence required for PA regulation as identified in the present study. Removal of this region had little effect on the ability of the turkey PLC-β2 C-terminal fragment to inhibit intrinsic PLC-β2 GAP activity toward Gαq. These residues also do not contribute to PA regulation of PLC-β1, because A-(965–975) and A-(976–993) mutants have wild-type PA regulation. Flexible loops do not appear to have structure-stabilizing roles, but they can accommodate regulatory ligands into binding pockets. In addition, flexible loops allow conformational flexibility, which can govern intramolecular interactions and potentially modulate sensitivity to physiological stimuli (37). The present data show that a portion of the PLC-β1 flexible loop is required for interaction with PA and that these residues lie outside the dimer interface.

The structural nature of the interaction of PA with PLC-β1 remains to be determined. Although PA binding regions have been identified in several proteins (2), there appears to be little overall similarity in the binding sequence or obvious binding determinants except for the presence of positively charged residues that suggest a role for electrostatic binding. The C-terminal domain of the turkey PLC-β shows a large concentration of positive charge at the interface between the αA and αB helices, and this charged region has been proposed to have a role in localizing PLC-β to the membrane (30). The major concentration of charge lies outside the region shown by this study to be required for PA interaction, which suggests that electrostatic interactions are not the

**TABLE 3**
Summary of mutation phenotypes

| Mutation     | Gαq response | Gαq GAP activity | PA stimulation | PA binding |
|--------------|--------------|-----------------|----------------|------------|
| A-(928–943)  | –            | –               | +              | +          |
| A-(944–957)  | m            | +               | m              | –          |
| A-(965–975)  | +            | +               | m              | m          |
| A-(976–990)  | +            | +               | +              | +          |
| A-(998–1007) | +            | +               | +              | +          |
| A-(1018–1028)| +            | +               | +              | +          |
major determinant for PA binding. Additional experimental data support this conclusion. First, PS is also negatively charged but does not produce effects comparable with PA, which is inconsistent with a purely electrostatic mechanism. In addition, PS binding is inhibited 100% by 0.4 M KCl, whereas PA binding is more salt-resistant (Fig. 1). Second, although both DPPA and DPPS promote binding of the PLC-β1 (878–980) fragment to lipid vesicles, only DPPA induces an increase in the secondary structure of the protein (Fig. 3). Third, the most effective PA mutation, A-(944–957) does not change the net charge within PLC-β1. In contrast, both A-(928–943) and A-(965–957), which have no effect or produce a minor decrease in PA regulation, remove 3 and 2 net charges, respectively.

The data presented here suggest that PLC-β1 dimerization, specific (salt-resistant, high affinity) PA binding, and stimulation of phospholipase activity are allosterically coupled. The specific activity of PLC-β1 increases markedly with increasing concentration of the enzyme, and this increase is diminished at high PA concentration, where specific activity is also high. The effect of PA on protein concentration dependence is diminished in the A-(944–957) mutant, which also displays decreased high affinity PA binding. The quantitative relationships among these effects (i.e. the coupling free energies) await more detailed measurements, and it is likely that these interactions will depend in detail on the lipid surface that attaches the PA activator and the PIP2 substrate. It may be that PLC-β1 dimerization is the primary source of activation and that dimerization is driven by PA binding. Alternatively, PA binding might cause activation independently of dimerization. Regardless, the processes of activation, dimerization, and PA binding are clearly thermodynamically linked. Ultimately, the PLC-β1 dimer appears to offer the optimal surface to which Gαq binds to cause full activation (30, 31).

At least two structural models for PA-induced dimerization are suggested by these data. Two binding sites for PA may exist, a high affinity and a low affinity binding site near the αA helix and adjacent loop. Cooperative interactions or dimerization might then modulate the contribution of each binding region to overall binding. Alternatively, only one PA binding site may exist, with its affinity for PA regulated by intramolecular interactions that occur in the αA helix/loop region. Nonspecific ionic PA binding may also be structurally delocalized and mechanistically unrelated to the interactions described here. Although the binding data suggest the existence of only one high affinity, salt-resistant PA binding site (supplemental Fig. 1), more detailed characterization of binding at different protein concentrations is necessary to address this question.

These studies suggest a novel role for PA in the regulation of GPCR signal transduction that is mediated through PLC-β1 homodimerization. Activation of Gαq-PLC-β1 signaling increases PA levels through activation of both DAG kinases and phospholipase Ds. The PA species thus generated would be chemically distinct because of their different precursors, and they may each exert unique regulation of PLC-β1. PA increases PLC-β1 dimerization and promotes PLC-β1 localization to membrane-associated signaling proteins. PA regulation is mediated by residues that include the flexible αA-B loop and an αA helix of the Gαq binding domain but are not required for Gαq stimulation or GAP activity. PLC-β1 binding to Gαq, stimulation of phospholipase activity by Gαq, and regulation by protein kinase C are all altered as a consequence of PA binding. PA thus coordinately modulates spatial and temporal GPCR signaling through PLC-β1. Cross-talk with PA generated by activation of other receptors may confer additional levels of regulation on GPCR-PLC-β1 signaling.

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