Regulation of Phospholipase C-β_1 Activity by Phosphatidic Acid[†]

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ABSTRACT: The role of phosphatidic acid (PA) in regulating phospholipase C-β_1 (PLC-β_1) activity was determined. PA promoted the binding of PLC-β_1 to sucrose-loaded unilamellar vesicles (SLUV) containing phosphatidylcholine. PA increased enzymatic activity over a range of Ca^{2+} concentrations and reduced the Ca^{2+} concentration required for half-maximal stimulation of activity. PA did not affect the apparent K_m for phosphatidylinositol 4,5-bisphosphate. Lysophosphatidic acid also enhanced the binding of PLC-β_1 to SLUV but was less effective in stimulating enzymatic activity. Diacylglycerol, phosphatidylserine, and oleic acid had little effect on activity. Anionic and neutral detergents did not stimulate activity. PA stimulation was relatively independent of acyl chain length. Dipalmitoyl-PA (16:0) was comparable to PA from egg lecithin and dimyristoyl-PA (C14:0) in stimulating activity, while dilauroyl-PA (C12:0) was slightly less effective. A 100 kDa catalytic fragment of PLC-β_1 lacking amino acid residues C-terminal to His^{880} did not bind to PA and was insensitive to stimulation by 7−15 mol % PA. Stimulation of 100 kDa enzymatic activity required 30 mol % PA. PA increased receptor−G protein stimulation of PLC-β_1 activity in membranes. These results demonstrate that PA stimulates basal and receptor−G protein-regulated PLC-β_1 activity. PA stimulation occurs through both a C-terminal-dependent and an independent mechanism. The C-terminal-mediated mechanism for stimulation may constitute an important pathway for conferring specific regulation of PLC-β_1 in response to increases in cellular PA levels.

Phospholipase C-β (PLC-β)[1] has a key role in promoting the increase in intracellular Ca^{2+} levels and protein kinase C activity in response to hormone stimulation (1). PLC-β_1 hydrolyzes a minor membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2), resulting in the formation of diacylglycerol and inositol 1,4,5-trisphosphate. PLC-β_1 activity is regulated by receptor−G protein-coupled signaling pathways. The $G_{q/11}$ family of GTP binding proteins, via $\alpha_{q/11}$ and $\beta\gamma$ subunits, mediate receptor stimulation of enzymatic activity (*2*).

While the mechanisms by which G proteins activate PLC-β_1 are well understood, other mechanisms of regulation, particularly those mediated by protein−lipid interactions, are less well understood. Regulation of protein function by specific lipid interactions has been shown for several important enzymes (*3*−*5*). Phosphatidic acid (PA), whose levels increase markedly and transiently during hormone stimulation (*6*−*10*), has been the focus of numerous studies. PA stimulates the activity of several enzymes, including cyclic AMP phosphodiesterase (*11*, *12*), protein kinases (*13*, *14*), NADPH oxidase (*15*, *16*), phospholipase C-γ (*17*, *18*), phospholipase C-δ_3 (*19*), and phospholipase D (*20*). These findings suggest that PA may function as an intracellular second messenger to affect cell function.

The present studies were initiated to determine the role of PA in the regulation of PLC-β_1 activity. The results demonstrate that PA enhances the binding of PLC-β_1 to sucrose-loaded large unilamellar vesicles, stimulates basal PLC-β_1 activity, and increases net stimulation of activity in response to receptor−G protein activation. PLC-β_1 binding to PA and stimulation of enzymatic activity by low concentrations of PA require the PLC-β_1 C-terminal region. High concentrations of PA can stimulate activity through a mechanism that does not require the C-terminal region or binding to PA. Thus, regulation of PLC-β_1 activity by PA occurs through a mechanism which differs from that of PLC-δ and PLC-γ, which lack the C-terminal region characteristic of all PLC-β isoforms. This may be important in conferring selective regulation of the G protein-coupled PLC-β_1 signaling pathway in response to increases in intracellular PA levels.

EXPERIMENTAL PROCEDURES

(1) Purification of PLC-β_1. PLC-β_1 was purified from bovine brain membranes as previously described (*21*).

(2) Lipid Binding Assay. Lipid binding was performed with sucrose-loaded large unilamellar vesicles (SLUV) as described (*22*, *23*). SLUV consisting of phosphatidylcholine (PC) plus phosphatidic acid (PA), phosphatidylethanolamine (PE), phosphatidylserine (PS), or lysophosphatidic acid (LPA) were resuspended in buffer containing 0.1 M KCl in 10 mM HEPES (pH 7.0) and used for the binding assays. Final total lipid concentration in the assay was 400 μM.

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[1] Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; GTP-γ-S, guanosine 5′-*O*-(3-thiotrisphosphate); LPA, lysophosphatidic acid; pCa, negative logarithm of calcium concentration; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLC-β_1, G protein-regulated phosphatidylinositol 4,5-bisphosphate-specific-phospholipase C; PS, phosphatidylserine; SLUV, sucrose-loaded large unilamellar vesicles.

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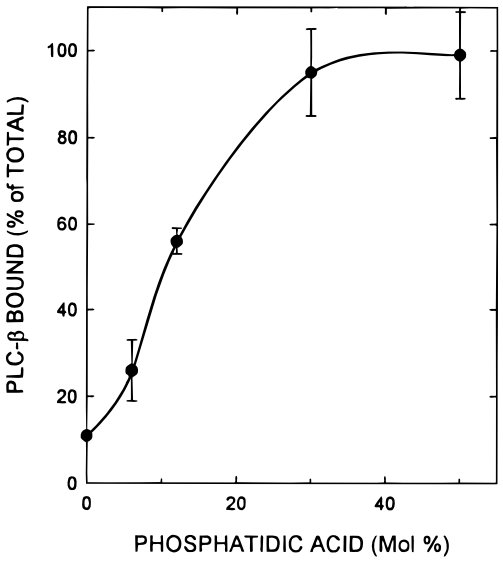

FIGURE 1: Effect of phosphatidic acid on the binding of PLC-β_1 to sucrose-loaded large unilamellar vesicles. PLC-β_1 was incubated with SLUV containing phosphatidylcholine plus the indicated mole percent phosphatidic acid. The total lipid concentration was maintained at 0.4 mM. The amount of lipid-bound PLC-β_1 was determined as described under Materials and Methods. Results are the mean ± SE of three experiments.

Binding was carried out in siliconized, BSA-treated tubes containing 100 μL of SLUV solution, 700 ng of PLC-β_1 and 1 μg of bovine serum albumin. Incubation was for 10 min at room temperature. After incubation, lipid vesicles containing bound protein were separated from free protein by centrifugation in a Beckman Airfuge (100000*g*, 60 min). The supernatant was removed and the enzymatic activity in the supernatant, corresponding to free enzymatic activity, was determined. Bound PLC-β_1 was calculated by subtracting free enzymatic activity from the total PLC-β_1 activity. Total PLC-β_1 activity corresponded to the enzymatic activity of PLC-β_1 in the absence of lipid vesicles. The results obtained from the enzymatic assay were comparable to those obtained from a direct measurement of lipid bound protein (data not shown). For this, the pellet from the 100000*g* centrifugation was resuspended in SDS sample buffer and proteins separated by electrophoresis on SDS−7.5% PAGE. Proteins were electrophoretically transferred to nitrocellulose. PLC-β_1 was identified by Western blot with anti-PLC-β_1 antibody. Detection was by enhanced chemiluminesence (Amersham). Quantitation of protein levels was by the NIH Image program from the scanned ECL film.

(3) PLC Assay. PLC activity was determined essentially as previously described (*21*). PLC-β_1 (3 ng) was added to 50 μL of buffer containing the indicated Ca^{2+} concentration as set by a Ca-EGTA buffer, 0.5 mM $MgCl_2$, 150 mM KCl, and 25 mM HEPES (pH 7.0). [^{3}H]phosphatidylinositol 4,5-bisphosphate (labeled plus unlabeled to a final concentration of 14 μM) was added in combination with phosphatidylcholine (PC) plus PA, phosphatidylserine (PS), lysophosphatidic acid (LPA), or other additions as indicated in the text to achieve the desired mole percent of each phospholipid. Total lipid concentration in the assay was 200 μM. Membranes were assayed with substrate prepared as described (*24*) except that PIP_2 was added at 25 μM in combination with phosphatidylethanolamine (PE) to a total lipid concentration of 140 μM.

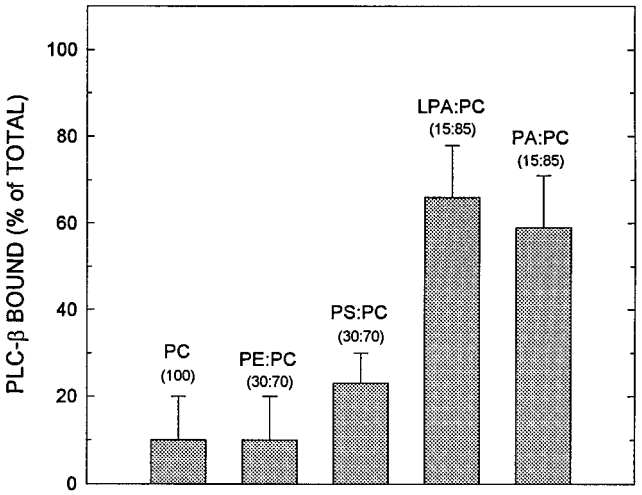

FIGURE 2: Binding of PLC-β_1 to sucrose-loaded large unilamellar vesicles containing different phospholipids. PLC-β_1 was incubated with SLUV containing 100 mol % phosphatidylcholine (PC) or PC plus 30 mol % phosphatidylethanolamine (PE:PC), 30 mol % phosphatidylserine (PS:PC), or 15 mol % lysophosphatidic acid (LPA:PC). Binding to PC SLUV containing 15 mol % PA (PA:PC) is shown for comparison. The total lipid concentration was maintained at 0.4 mM. Results are the mean ± SE of three experiments.

(4) Generation of the 100 kDa Catalytic Fragment Lacking the C-Terminal Region. PLC-β_1 (1 μg) was incubated in 30 μL of reaction mixture containing 12.5 milliunits/μL calpain in buffer containing 50 mM HEPES (pH 7.5), 1 mM DTT, 8 mM EGTA, and 12 mM $CaCl_2$. Fifteen microliters was removed and added to a new vial containing EGTA and leupeptin to a final concentration of 4.5 mM and 1 μg/mL, respectively. This mixture, corresponding to unhydrolyzed PLC-β_1, was maintained on ice. The remaining mixture was incubated at 30 °C for up to 2 h, as needed. Following incubation, the incubated mixture was transferred to ice for the remainder of the incubation. EGTA plus leupeptin were added as above to terminate the proteolysis. An aliquot was taken for analysis by SDS−PAGE to verify the extent of proteolysis.

(5) Isolation of Membranes. Rat cerebral cortical membranes were isolated and assayed for PLC activity as described previously (*24*, *25*).

(6) Other. Anti-PLC-β_1 was from Santa Cruz. [^{3}H]Phosphatidylinositol 4,5-bisphosphate was from NEN/Dupont. Calpain was from Calbiochem. Lipids were from Avanti Polar Lipids. PA (egg lecithin) was used in the majority of studies unless otherwise indicated. Other reagents, unless indicated, were from Sigma.

RESULTS

(1) PA Promotes Binding of PLC-β_1 to SLUV. The binding of PLC-β_1 to SLUV containing PC and increasing mol % PA is shown in Figure 1. PLC-β_1 bound weakly to SLUV containing 100 mol % PC. Increasing mole percent PA increased the binding of PLC-β_1 to SLUV. Half-maximal binding required 14 mol % PA, with near-maximal binding at 30 mol % PA. The inclusion of 0.5 M NaCl, in the binding assay, inhibited the binding of PLC-β_1 to SLUV containing PA by 90% (data not shown).

The binding of PLC-β_1 to PC SLUV containing other phospholipids is shown in Figure 2. The binding of PLC-β_1

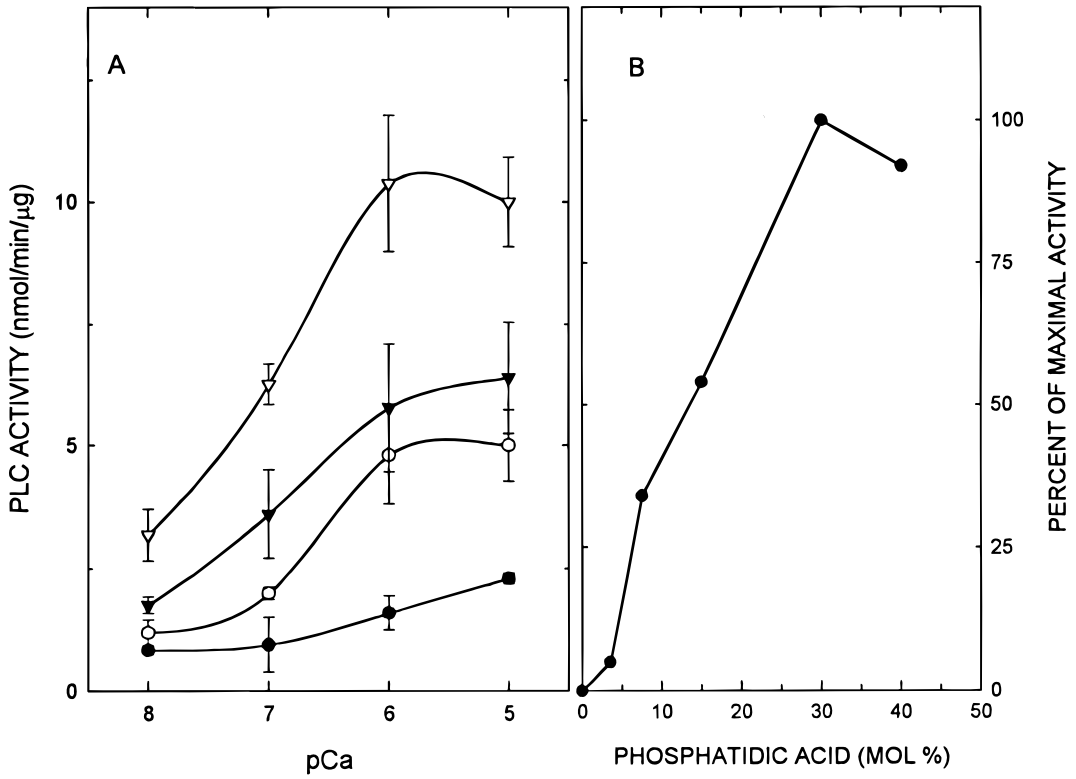

FIGURE 3: Effect of phosphatidic acid on the Ca^{2+}-dependent stimulation of PLC-β_1 activity. (A) PLC-β_1 activity was assayed over a range of pCa in the absence of PA (●) or in the presence of 7.5 mol % PA (○), 15 mol % PA (▼), or 30 mol % PA (▽). Results shown are the mean ± SE of 3−4 experiments. In panel B, the data are plotted as percent maximal activity versus mole percent PA.

to SLUV was little affected by the inclusion of 30 mol % phosphatidylethanolamine (PE). Binding was enhanced by including negatively charged phosphatidylserine (PS) at 30 mol %. The binding of PLC-β_1 to SLUV containing 30 mol % PS, however, was less then that to SLUV containing 15 mol % lysophosphatidic acid (LPA), which carries two negative charges. LPA promoted the binding of PLC-β_1 to SLUV to an extent comparable to that of PA.

(2) PA Stimulates PLC-β_1 Enzymatic Activity. The effect of PA on PLC-β_1 activity, as determined over a range of Ca^{2+} concentrations, is shown in Figure 3A. Basal enzymatic activity was stimulated as the Ca^{2+} concentration was increased from 10 nM to 10 μM. PA, at 7.5 mol %, had little effect on basal activity at 10 nM Ca^{2+} but stimulated enzymatic activity at 100 nM Ca^{2+} or greater. Increasing the PA to 15 and 30 mol % resulted in a further stimulation of activity with maximal stimulation of activity occurring at 1−10 μM Ca^{2+}. Enzymatic activity at 1 μM Ca^{2+} was increased by 200%, 260%, and 550% in the presence of 7.5, 15, and 30 mol % PA, respectively.

PA also reduced the Ca^{2+} concentration required for half-maximal stimulation of activity. Maximal stimulation of basal activity by Ca^{2+} was not attained in these experiments. However, half-maximal stimulation can be estimated to occur near 1 μM (pCa 6.0). In the presence of 7.5 and 15 mol % PA, the Ca^{2+} concentration required for half-maximal stimulation of activity was shifted to 250 nM (pCa 6.6) and 150 nM (pCa 6.8), respectively. There was little additional change in Ca^{2+} sensitivity upon increasing the PA from 15 to 30 mol %.

The dependence of enzymatic activity on the mole percent PA is also shown in Figure 3B. Half-maximal stimulation occurred at 15 mol % PA in the presence of 1 μM Ca^{2+}. These results demonstrate that binding and stimulation of enzymatic activity occur at a similar mole percent PA.

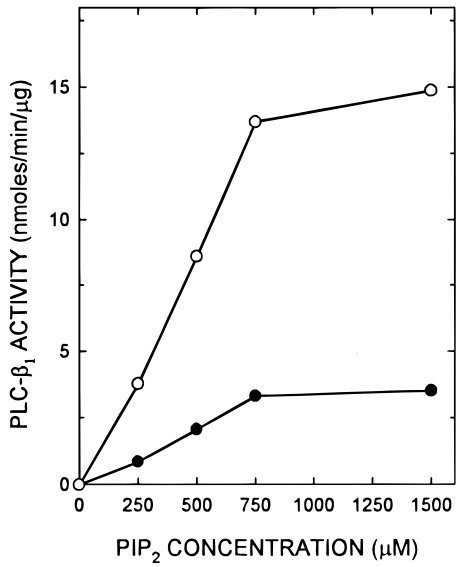

FIGURE 4: Effect of phosphatidic acid on the kinetic properties of PLC-β_1. PLC-β_1 activity was assayed in the presence of increasing bulk concentrations of PIP_2 at a fixed 7 mol % PIP_2 and in the absence (●) or presence (○) of 15 mol % PA. Results are from one of two experiments which gave identical results.

The effect of PA on the kinetic properties of PLC-β_1 is shown in Figure 4. Enzymatic activity was determined at a fixed 7 mol % PIP_2 and 1−1.5 mM PIP_2. The apparent K_m was 450 μM PIP_2. PA increased total activity but did not affect the concentration dependence for stimulation of activity.

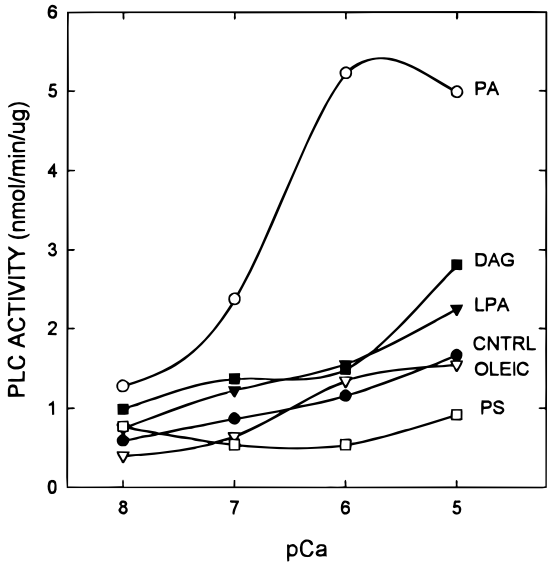

FIGURE 5: Effect of lipids on the Ca^{2+}-dependent stimulation of PLC-β_1. PLC-β_1 activity was assayed over a range of pCa in the absence of PA (●) or in the presence of 15 mol % PA (○), diacylglycerol (■), lysophosphatidic acid (▼), or oleic acid (▽). The effect of 30 mol % PS (□) is also shown. Results shown are from one of two experiments that gave identical results.

The effect of other lipids on PLC-β_1 activity is shown in Figure 5. Diacylglycerol (DAG) had little effect on enzyme activity. PA is a negatively charged phospholipid. Oleic acid, which is negatively charged, had little effect on activity. Lysophosphatidic acid (LPA) produced a slight increase in activity that was considerably less than the increase due to PA. Thirty mole percent PS, a concentration that promotes binding of PLC-β_1 to SLUV, did not increase activity and actually slightly reduced PLC-β_1 enzymatic activity.

To determine whether the stimulatory effects of PA could be due to detergentlike properties, the effect of anionic detergents on enzyme activity was determined. The anionic detergent deoxycholate inhibited basal and PA-stimulated enzyme activity at a concentration of 0.1 mM or greater (Figure 6). Similar results were obtained with cholic acid (data not shown). The neutral detergent Triton X-100 also inhibited activity but to a slightly lesser extent than that observed with deoxycholate. These results indicate that PA stimulation of PLC-β_1 activity is not due to detergentlike properties.

The effect of acyl chain length on the PA stimulation of PLC-β_1 activity is shown in Figure 7. Dipalmitoyl-PA (16:0) was comparable to PA derived from egg lecithin, which was comparable to dimyristoyl-PA (C14:0) in stimulating enzymatic activity over the range of pCa examined. Dilauroyl-PA (C12:0) was the least effective but nonetheless produced stimulation of activity. Thus stimulation of activity is relatively independent of the acyl chain length.

Competition experiments were done to determine whether PA stimulation of activity required the presence of both PA and PIP_2 in the lipid vesicles. The effect of adding vesicles containing PC plus PA or LPA on the activity of PLC-β_1 toward labeled substrate vesicles containing PC plus PA was determined. If stimulation was dependent on the binding of PLC-β_1 to substrate vesicles containing PA plus PIP_2, then the activity toward lipid vesicles containing substrate should be reduced by the addition of PA vesicles that lack substrate. These vesicles should bind PLC-β_1 and thus compete for PLC-β_1 binding to substrate containing vesicles. As shown in Figure 8, the addition of PC vesicles containing PA or LPA to the PLC assay mixture reduced the total enzymatic activity toward the labeled substrate vesicles containing 7.5 mol % PA (15 μM). Enzymatic activity was reduced by the addition of 7 μM PA or LPA vesicles. In contrast, the addition of vesicles containing PE, which does not bind PLC-β_1, had little effect on measured activity. These results demonstrate that PA stimulation requires the binding of enzyme to vesicles containing both PA and PIP_2. Increasing the total amount of PA in the assay is not sufficient to increase activity and reduces activity, most likely by binding the enzyme and thus reducing the amount available for interaction with vesicles containing substrate.

(3) C-Terminal Region is Required for PA Regulation. Calpain cleaves PLC-β_1 between His^{880} and Ser^{881}, producing

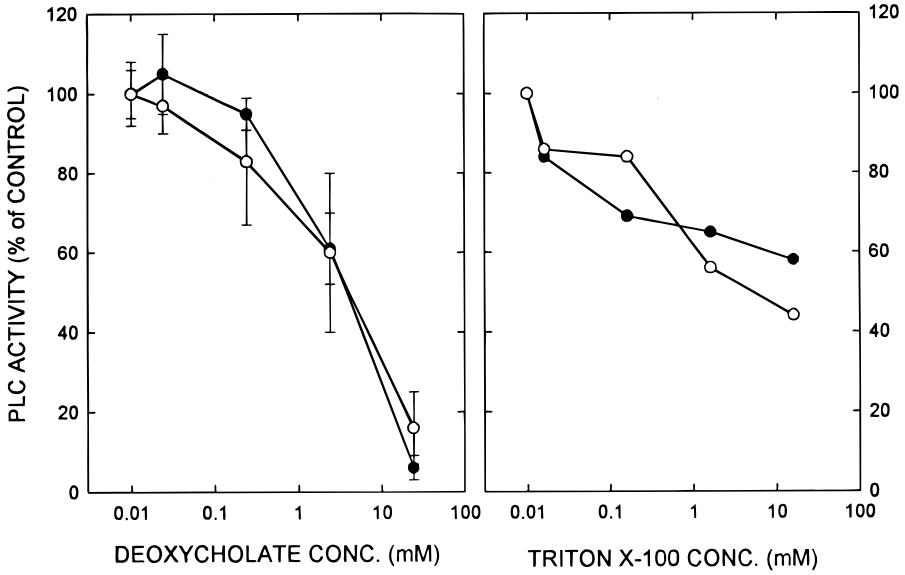

FIGURE 6: Effect of detergents on PLC-β_1 activity. PLC-β_1 activity was assayed without PA (●) or with 15 mol % PA (○) in the presence of the indicated concentration of deoxycholate or Triton X-100. Results shown are the mean ± SE of three experiments for deoxycholate and two experiments for Triton X-100. Data are expressed as the percent activity measured in the absence of detergent.

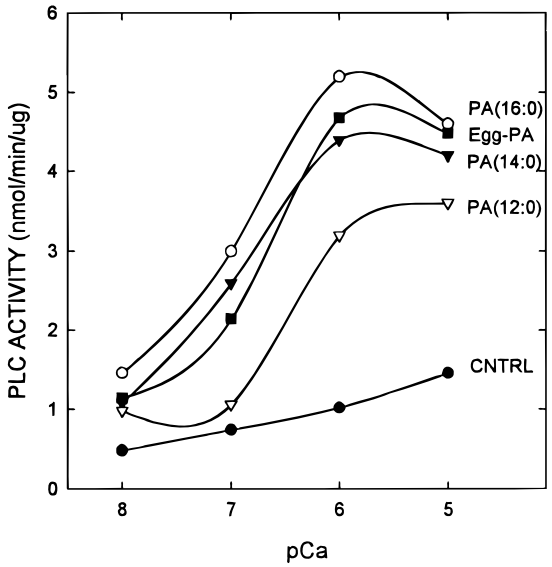

FIGURE 7: Effect of acyl chain derivatives of phosphatidic acid on the Ca^{2+} stimulation of PLC-β_1 activity. PLC-β_1 activity was assayed over a range of pCa in the absence of PA (●) or presence of 15 mol % egg lecithin PA (■), dipalmitoyl-PA ($C_{16:0}$) (○), dimyristoyl-PA ($C_{14:0}$) (▼), or dilauroyl PA ($C_{12:0}$) (▽). Results are from one of two experiments that gave identical results.

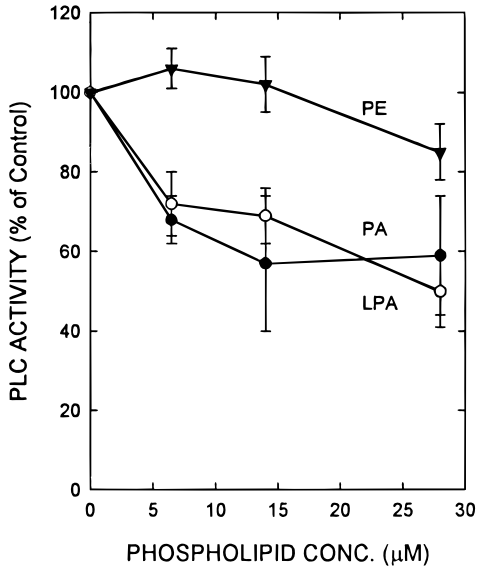

FIGURE 8: Effect of addition of unlabeled lipid vesicles on PLC-β_1 activity. PLC-β_1 was incubated with substrate vesicles containing phosphatidylinositol 4,5-bisphosphate (labeled plus unlabeled), PC, and 7.5 mol % PA at pCa 6.0 for 6 min as described under Materials and Methods. Unlabeled PC lipid vesicles containing 7, 14, or 28 μM PA (●), LPA (○), or PE (▼) were added to the PLC assay mixture as indicated. Activity in the presence of the added lipid vesicles is shown as a percent of control activity (without vesicle addition).

a 100 kDa fragment with catalytic activity and a 45 kDa fragment corresponding to the C-terminal region of PLC-β_1 (*26*). The effect of Ca^{2+} and PA on the enzymatic activity of PLC-β_1 and the 100 kDa catalytic fragment is shown in Figure 9. Ca^{2+}-dependent stimulation of PLC-β_1 and the 100 kDa fragment enzymatic activity was comparable (Figure 9A). PA at 7.5−15 mol %, which produced a marked stimulation of PLC-β_1 activity, had little effect on the enzymatic activity of the 100 kDa fragment. However, increasing the PA to 30 mol % resulted in stimulation of the 100 kDa enzymatic activity to an extent almost comparable to that of PLC-β_1. SDS−PAGE analysis of the calpain-treated PLC-β_1 indicated that greater than 90% of the PLC-β_1 had been hydrolyzed to 100 kDa fragment (Figure 9B).

Removal of the C-terminal region by calpain proteolysis also resulted in a loss of binding to SLUV containing 30 mol % PA. There was little binding of the 100 kDa fragment to SLUV containing 30 mol % PA (data not shown). These results demonstrate that the PLC-β_1 C-terminal region is required for PA stimulation of enzymatic activity and for binding to PA containing SLUV. Stimulation of activity by high concentrations of PA occurs through a mechanism that does not require binding to the C-terminal region.

(4) PA Increases Receptor−G Protein-Stimulated PLC-β_1 activity. Rat cerebral cortical membranes exhibit receptor−$G_{q/11}$-dependent regulation of PLC-β_1 activity (*24*, *25*). The effect of PA on receptor−$G_{q/11}$ stimulation of PLC-β_1 activity in membranes is shown in Table 1. Carbachol, an M_1 receptor agonist, in the presence of GTP-γ-S stimulated a 176% increase in basal activity. PA increased basal activity by 55%. Carbachol and GTP-γ-S further increased the PA-stimulated activity, resulting in a greater net increase over basal activity, i.e., 357% increase over basal activity. The ability of the activated G protein to increase basal and PA-stimulated activity was comparable, i.e., 176% and 194% in the absence and presence of PA, respectively. LPA was less effective then PA in stimulating activity in either the absence or presence of carbachol plus GTP-γ-S. These results demonstrate that PA increases net simulation of PLC-β_1 in response to receptor−G protein activation.

DISCUSSION

The present studies demonstrate that PA regulates two major properties of PLC-β_1. PA promotes the binding of PLC-β_1 to SLUV (Figure 1) and stimulates enzymatic activity (Figure 3). The mol % PA required to promote half-maximal binding of PLC-β_1 to SLUV is comparable to that required to stimulate Raf-1 kinase binding to lipid vesicles (*13*). The PA-mediated recruitment of Raf-1 kinase from the cytosol to the membrane is important for the activation of Raf-1 kinase (*13*). Association of PLC-β_1 with the plasma membrane is also thought to be important for rapid and efficient regulation of this enzyme by localizing PLC-β_1 near its membrane substrate and membrane-associated regulatory G proteins. However, the mechanisms involved in localizing PLC-β_1 to membranes are not well understood. A major fraction of cellular PLC-β_1 can be present in the nucleus as well as in the cytoplasm (*27*). The C-terminal region of PLC-β_1 is required for particulate association (*28*). Neither $\alpha_{q/11}$ nor $\beta\gamma$ subunits nor PIP_2 promotes the binding of PLC-β_1 to lipid vesicles (*23*). PS, the major acidic phospholipid present in membranes (*29*, *30*), does promote PLC-β_1 association with SLUV (Figure 2) but does not appear to regulate activity (Figure 5). PS may thus help anchor PLC-β_1 to membranes as has been shown for other proteins including myristoylated alanine rich C kinase substrate (*31*) and synaptotagmin (*32*).

Binding of PLC-β_1 to PA was inhibited by 0.5 M NaCl, suggesting an electrostatic mechanism for binding. LPA also increased the binding of PLC-β_1 to SLUV to an extent comparable to that of PA. However, LPA was considerably

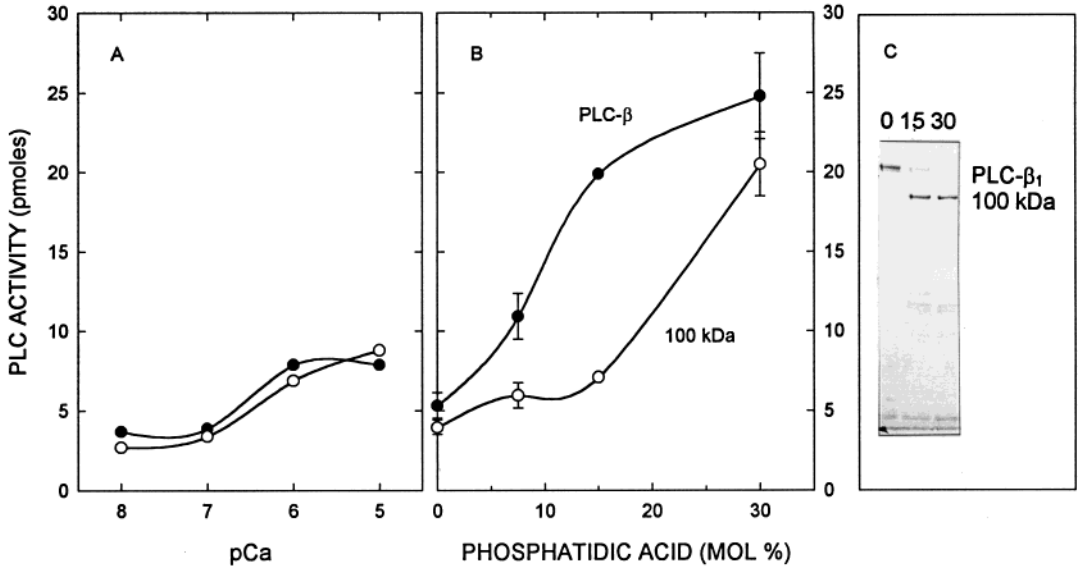

FIGURE 9: Effect of phosphatidic acid on the enzymatic activity of the 100 kDa catalytic fragment of PLC-β_1. (A) PLC-β_1 was incubated with calpain as described under Materials and Methods. The Ca^{2+}-dependent stimulation of PLC-β_1 (●) or the catalytic 100 kDa fragment (○) is shown in panel A. Stimulation of activity due to increasing mol percent of PA as determined at pCa 6.0 is shown in panel B. Results are the mean ± SE of three experiments. (C) Coomassie blue-stained SDS−11% PAGE of PLC-β_1 (without calpain incubation) and 100 kDa fragment generated after 15 and 30 min incubations with calpain.

Table 1: Effect of Phosphatidic Acid on Receptor−G Protein Activation of PLC-β_1[a]

addition	PLC Activity (pmol min^{-1} μg^{-1})
basal	0.69 ± 0.06
+ carbachol + GTP-γ-S	1.91 ± 0.01
PA	1.07 ± 0.18
PA + carbachol + GTP-γ-S	3.15 ± 0.46
LPA	0.79 ± 0.08
LPA + carbachol + GTP-γ-S	1.78 ± 0.20

[a] Cerebral cortical membranes (1.5 μg) were incubated in the absence of any addition, with 15 μM PA or LPA, and with or without 300 μM carbachol plus 330 nM GTP-γ-S for 6 min at 30 °C. Activity in the presence of 330 nM GTP-γ-S was 0.92 ± 0.03 pmol min^{-1} μg^{-1}. Assay conditions were as described under Materials and Methods.

less effective then PA in stimulating enzymatic activity. Diacylglycerol, the lipid precursor for PA, had little effect on basal activity. Oleic acid, which is negatively charged, had little effect on activity (Figure 4). PS did not stimulate activity at concentrations that promoted the binding of PLC-β_1 to SLUV. The presence of PS actually reduced enzymatic activity. These results indicate that while net lipid charge has a role in promoting the association of PLC-β_1 with SLUV, lipid charge is not sufficient to affect a change in enzymatic activity. Furthermore, PA stimulation of activity is not due to its detergentlike properties since anionic detergents did not stimulate activity (Figure 6). Rather, inhibition of activity was observed with the addition of detergent. This could reflect detergent-mediated disruption of the substrate micelle or inhibition of protein−lipid interactions.

Binding to PA and stimulation of PLC-β_1 enzymatic activity by 7.5−15 mol % PA required the C-terminal region. A 100 kDa catalytic fragment lacking residues C-terminal to His^{880} was insensitive to stimulation by 15 mol % PA and did not bind to PA vesicles. The 100 kDa fragment was activated, however, in the presence of 30 mol % PA. Deletion of the C-terminal region had little effect on the Ca^{2+}-dependent stimulation of activity as has been reported (*26*), indicating that the basic catalytic function was unaffected by the proteolysis. Thus, the C-terminal region is required for stimulation of activity by low concentrations of PA as well as for binding to PA. The stimulation observed in the presence of 30 mol % PA suggests that there is a second site for PA regulation. Alternatively, activation at this level of PA could be due to an effect at the level of substrate.

Other PIP_2−PLC enzymes are also stimulated by PA but the mechanisms involved appear to differ. PA stimulates PLC-γ activity and increases its affinity for substrate (*17*, *18*). PA has little effect on the binding of PLC-γ to lipid vesicles (*33*), suggesting that PA binding does not constitute part of the activation mechanism. Half-maximal stimulation of PLC-γ activity requires near 20 mol % PA with little effect on activity at less than 10 mol % PA (*17*). PA stimulates PLC-δ_1 and PLC-δ_3 activity as well as enhancing their binding to lipid vesicles (*19*, *33*). PA stimulation of PLC-δ_3 requires at least 20 mol % PA and is associated with an increase in affinity for PIP_2. Binding to PA is mediated through the N-terminal pleckstrin homology domain (PH domain) of PLC-δ_3 (*19*). The PLC-δ PH domain confers high affinity binding of this enzyme to PIP_2 (*23*, *34*). It has been proposed that PA may serve as a mechanism for promoting the association of PLC-δ with membranes and regulation of activity (*33*).

With PLC-β_1, there was little effect of PA on the apparent K_m for PIP_2. PA binding requires the C-terminal region of PLC-β_1. The N-terminal PH domain of PLC-β_1, which differs in sequence from the PLC-δ PH domain, does not bind PA, as evidenced by the lack of PA binding by the 100 kDa catalytic fragment of PLC-β_1 containing the PH domain. PLC-β isoforms differ from the other PIP_2−PLC enzymes, PLC-δ and PLC-γ, in having a long sequence of amino acids C-terminal to the catalytic Y domain. The PLC-β_1 C-terminal region confers several additional levels of regulation that are absent in PLC-δ and PLC-γ. The C-terminal region promotes particulate association and uptake of PLC-β_1 into the nucleus

(*28*). The C-terminal region is required for $\alpha_{q/11}$ stimulation of enzymatic activity (*26*, *35*) and has GTPase activating activity on $\alpha_{q/11}$ (*36*). Protein kinase C phosphorylates at Ser^{887} within the C-terminal region (*37*), resulting in inhibition of activity (*38*). The present studies indicate that the C-terminal region also has a role in mediating PA regulation of activity.

PA is present at less than 2 mol % in most cells (*39*−*41*). However, agonist stimulation can produce a 150−300% increase in total PA mass levels, depending on the cell type (*40*−*43*). PA concentrations can reach 50−100 μM in stimulated neutrophils (*16*, *44*, *45*). However, the actual hormone stimulated increase in PA levels near PLC-β_1 may be underestimated. Cellular diacylglycerol kinase activity is restricted to receptor-generated pools and may be associated with PIP_2−PLC (*46*). Hormone-sensitive pools of PIP_2, a source of diacylglycerol and phosphatidic acid, are compartmentalized to low-density caveolae-enriched fractions (*47*) or low-density detergent-insoluble domains (*48*). Thus, hormone-stimulated increases in PA levels may be localized to the vicinity of the enzyme, achieving concentrations much greater than that inferred from changes in whole cell PA. The concentrations of PA used in this study are likely to be physiologically relevant.

PA stimulation of PLC-β_1 activity has physiological implications. PA, generated in response to hormone stimulation, may function as a positive feedback regulator of PLC-β_1. PLC-β_1 is a Ca^{2+}-regulated enzyme and Ca^{2+} is required for maximal receptor activation of enzyme activity (*49*, *50*). PA increases enzyme activity over a range of Ca^{2+} concentrations and increases the sensitivity to Ca^{2+} (Figure 3). Thus changes in intracellular Ca^{2+} concentration that occur in response to hormone stimulation can have a much greater effect on PLC-β_1 activity in the presence of PA.

PA also increases the attained activity in response to receptor stimulation. As shown in Table 1, receptor−G protein stimulation increased both basal and PA-stimulated activities to a comparable extent. The total attained activity, however, was higher with the PA-stimulated enzyme. LPA produced only a slight increase in activity and had little effect on the stimulation by carbachol plus GTP-γ-S. Thus, PA stimulation was not due to LPA acting on an LPA receptor. These results are consistent with a regulatory mechanism by which PA stimulates PLC-β_1 catalytic activity, which is then further increased with receptor stimulation. PA action would be terminated by PLA_2, resulting in the formation of LPA, which has little effect on PLC-β_1 activity (Figure 5) or the response to G protein activation. PA levels would also decrease due to its recruitment for lipid resynthesis.

PA stimulation in membranes was less than that observed with purified enzyme. The reason for this remains to be determined. It is possible that this reflects a difference between membrane- and enzyme-based assays. Both the substrate and PA are presented in vesicles, which may be more accessible to the isolated enzyme then to the membrane associated enzyme.

PA can also be formed as a consequence of phosphatidylcholine hydrolysis via phospholipase D (PLD). Many agonists activate both PIP_2−PLC and PLD (*51*). PLD activation results in the generation of saturated/monosaturated lipid species while PIP_2−PLC activation results in primarily polyunsaturated species (*52*). It remains to be determined whether the PA generated by PLD or PIP_2−PLC has the major role in regulating PLC-β_1 activity.

PA stimulates inositol phosphate production in astrocytes (*53*), fibroblasts (*54*), cardiac myocytes (*55*), keratinocytes (*56*), and other cells. Stimulation of inositol phosphate production is not due to the presence of trace amounts of LPA in the PA (*56*). Stimulation of inositol phosphate production is dependent on the fatty acyl chain length, with dimyristoyl-PA and dilauroyl-PA being totally ineffective (*53*, *54*). This dependency on fatty acyl chain length for PA stimulation in cells differs from the results obtained with purified PLC-β_1, where stimulation was observed with all species (Figure 4). Membranes also demonstrate a lack of dependence on fatty acyl chain length for PA stimulation (Litosch, manuscript in preparation). One possible explanation for this difference in acyl chain dependence is that the acyl chain length affects the membrane-partitioning properties of PA and thus the ability of PA to activate intracellular enzymes in cells. The mechanism by which PA stimulates inositol production in cells remains to be determined.

In summary, the present studies demonstrate that PA promotes the binding of PLC-β_1 to SLUV, stimulates PLC-β_1 activity, and increases the activity attained in response to receptor−G protein stimulation. Binding to PA requires the C-terminal region of PLC-β_1. Stimulation of enzymatic activity by a low mole percent PA requires the C-terminal region, while stimulation by a high mole percent PA can occur in the absence of the C-terminal region. The C-terminal region is unique to the PLC-β family of enzymes. Thus regulation through the C-terminal region may allow for specificity in the regulation of this class of enzymes in response to hormone-mediated increases in PA levels.

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