Regulation of Phospholipase C-β Activity by Phosphatidic Acid: Isoform Dependence, Role of Protein Kinase C, and G Protein Subunits[†]

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ABSTRACT: Phosphatidic acid (PA) stimulates phospholipase C-β_1 (PLC-β_1) activity and promotes G protein stimulation of PLC-β_1 activity. The isoform dependence for PA regulation of PLC-β activity as well as the role of PA in modulating regulation of PLC-β activity by protein kinase C (PKC) and G protein subunits was determined. As compared to PLC-β_1, the phospholipase C-β_3 (PLC-β_3) isoform was less sensitive to PA, requiring greater than 15 mol % PA for stimulation. PLC-β_3 bound weakly to PA. PKC had little effect on PA stimulation of PLC-β_3 activity. PKC, however, inhibited PA stimulation of PLC-β_1 activity through a mechanism dependent on the mol % PA. Stimulation by 7.5 mol % PA was completely inhibited by PKC. Increasing the PA and Ca^{2+} concentration attenuated PKC inhibition. The binding of PLC-β_1 to PA containing phospholipid vesicles was also reduced by PKC, in a manner dependent on the mol % PA. PA increased the stimulation of PLC-β_1 activity by Gαq but had little effect on the stimulation by $\beta\gamma$ subunits. These results demonstrate that PA stimulation of PLC-β activity is tightly regulated, suggesting the existence of a distinct PA binding region in PLC-β_1. PA may be an important component of a receptor mediated signaling mechanism that determines PLC-β_1 activation.

The phospholipase C-β_1 isoform (PLC-β_1) is a member of a large family of PLC-β enzymes, regulated by cell surface receptors and linked to the production of intracellular messengers. Activation of G protein coupled receptors (GPCR) at the plasma membrane stimulates PLC-β_1 activity through the $G_{q/11}$ family of G proteins (*1*−*3*). Mitogenic agents stimulate the activity of a nuclear pool of PLC-β_1 through a separate signaling pathway that involves the mitogen activated protein kinase cascade (*4*). PLC-β isoforms exhibit marked differences in sensitivity to regulation by individual G protein subunits and phosphorylation by protein kinases (*1*−*4*) suggesting that different isoforms constitute part of unique signaling cascades.

Receptor stimulation of PLC-β_1 activity results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate, elevation of intracellular Ca^{2+} levels, and formation of diacylglycerol (DAG). Calcium and DAG activate a number of signaling pathways including protein kinase C (PKC), a family of serine and threonine kinases with multiple cellular targets (*5*). DAG is also a substrate for the diacylglycerol kinase (DGK)[1] family of enzymes (*6*). DGKs mediate the conversion of DAG to phosphatidic acid (PA), thereby terminating DAG signaling but generating a new signaling molecule. PA stimulates the activity of a number of enzymes and has been proposed to function as a novel intracellular mediator of hormone action (*7*−*13*). How PA signaling integrates with other receptor mediated signaling pathways that regulate protein function is not well-understood.

PA stimulates PLC-β_1 activity (*14*) and promotes G protein stimulation of PLC-β_1 activity in membranes (*14*, *15*). These data suggest a potential role for PA in a positive feedback mechanism that regulates agonist stimulation of PLC-β_1 activity. Consistent with this proposed role of PA in regulating PLC-β_1 stimulation, DGKϵ knockout mice demonstrate a reduced stimulation of PLC-β_1 activity (*16*).

The isoform dependence for PA regulation of PLC-β activity and the role of PA in determining PLC-β_1 activity, in the context of other receptor initiated intracellular signaling pathways, is therefore of interest to determine. PKC phosphorylates PLC-β_1 (*17*) and confers negative modulation of PLC-β_1 activity in vitro (*18*, *19*) and in vivo (*20*). PKC inhibition of the phospholipase C-β_3 isoform (PLC-β_3) has been demonstrated in vivo (*21*).

The present studies determined the PLC-β isoform dependence for PA regulation by comparing regulation of PLC-β_3 with PLC-β_1. Furthermore, the role of PA in modulating PKC and Gq regulation of PLC-β_1 activity was determined. These data demonstrate that PA regulation of PLC-β is isoform dependent. PA and PKC regulate PLC-β_1 activity in a reciprocal manner. PA and Gαq, however, function in concert to stimulate PLC-β_1 activity. These results are consistent with an important role for PA in modulating agonist dependent stimulation of the PLC-β_1 isoform.

EXPERIMENTAL PROCEDURES

Purification of PLC-β and G Protein Subunits. Native PLC-β_1 and PLC-β_3 were purified from bovine brain and

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[1] Abbreviations: DGK, diacylglycerol kinase; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis (β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; pCa, negative log calcium concentration; PA, phosphatidic acid; PC, phosphatidylcholine; PIP_2, phosphatidylinositol-4,5-bisphosphate; PLD, phospholipase D; PS, phosphatidylserine; SLUV, sucrose loaded large unilamellar vesicles.

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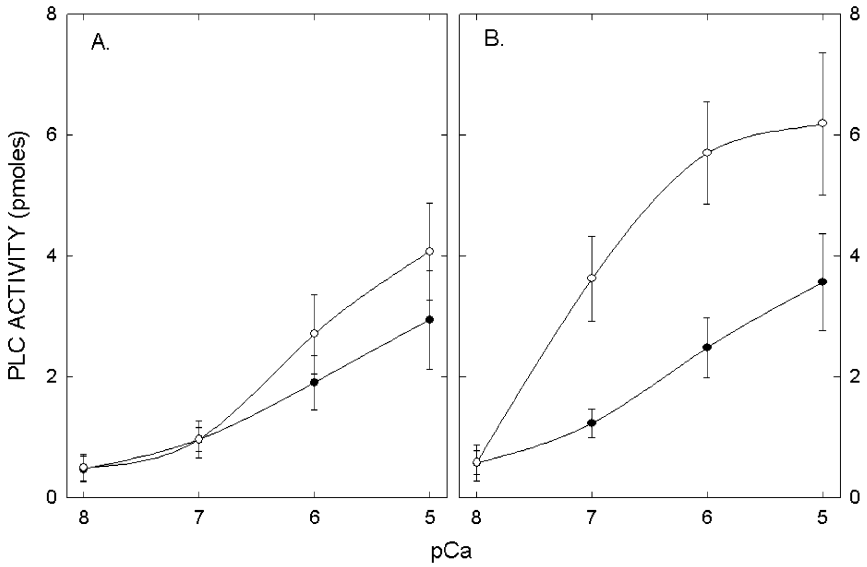

FIGURE 1: Regulation of PLC-β_3 and PLC-β_1 activity by PA. PLC-β_3 (panel A) or PLC-β_1 (panel B) was incubated in the absence (●) or presence (○) of 15 mol % PA over the indicated range of Ca^{2+} concentrations. Results shown are the mean ± SE of four experiments.

bovine heart, respectively, as described (*19*). G protein $\beta\gamma$ subunits were purified from bovine brain, as described (*19*). Recombinant PLC-β_1 $(His)_6$ in pET-15b vector (Novagen) was expressed in BL21 (DE3) pLysS (Novagen). Purification of rPLC-β_1 was essentially as described (*22*). The purified rPLC-β_1 $(His)_6$ was approximately 90% pure, as judged by a Coomassie stained 4−20% Tris Glycine gel (ICN). Purified Gαq subunit, expressed in Sf9 cells, was a generous gift from Dr. Elliott M. Ross (University of Texas, Southwestern Medical Center, Dallas, Texas).

Phospholipase C Assay. PLC-β (generally 2.5−10 ng) was added to 50 μL of buffer consisting of the indicated free Ca^{2+} concentration, 0.5 mM $MgCl_2$, 100 mM KCl, 25 mM HEPES (pH 7.0), and 14 μM [^{3}H]-phosphatidylinositol-4,5-bisphosphate (labeled plus unlabeled) in combination with phosphatidylcholine (PC) and PA to achieve the appropriate mol % PA. The free Ca^{2+} concentration was set with a Ca−EGTA buffer (*14*). Total lipid concentration was 150 μM. In experiments that compared different PLC-β enzymes, the amount of each enzyme added to the assay was adjusted to attain comparable basal activities in the absence of PA. G protein subunit regulation of PLC-β_1 activity was determined by the addition of purified $\beta\gamma$ subunits or activated αq subunits to the assay mixture, at pCa 5 (*19*). Prior to use, the αq subunit was activated by a 2 h incubation at 30 °C in 50 mM HEPES (pH 8.0), 1 mM EDTA, 3 mM EGTA, 5 mM $MgCl_2$, 2 mM DTT, 100 mM NaCl, 1% cholate, and 1 mM GTP-γ-S.

PKC Phosphorylation. PLC-β_1 was incubated in the presence of 5 nM PKCα (or PKCβ for PLC-β_3) plus PKC cofactors (0.4 mM $CaCl_2$, 200 μM phosphatidylserine, 100 nM phorbol-12-myristate 13-acetate, and 5 mM ATP) for 2 h at 30 °C. Control incubations were done in the absence of PKCα but in the presence of PKC cofactors and PKCα dilution buffer (20 mM Tris/HCl (pH 7.5), 0.5 mM EGTA, 0.5 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, 0.5% Triton X-100, and 400 mM NaCl) (*19*). The control and PKC phosphorylated PLC-β was subsequently assayed for phospholipase C activity or binding to phospholipid vesicles.

Phospholipid Binding Assay. Phospholipid binding was determined with sucrose loaded large unilamellar vesicles (SLUV) (*14*). Briefly, PLC-β_1 or PLC-β_3 was added to a 100 μL assay mixture consisting of SLUV, PC (100%), or PA/PC (30:70, molar ratio) in 10 mM HEPES (pH 7.0) and 0.25 μg of fatty acid free bovine serum albumin plus 100 mM NaCl. Total phospholipid concentration was 400 μM. After 10 min on ice, the lipid bound PLC-β was separated from unbound PLC-β by centrifugation. The lipid bound PLC-β was determined as described (*14*). Recovery of the SLUV, as determined by the inclusion of a tracer amount of radioactive ^{3}H-PIP_2 to the lipid mixture, was approximately 90%. There was little significant difference in the recovery of PC or PA/PC vesicles following centrifugation.

Other. PKCα or PKCβ, human recombinant form, was from Panvera. [^{3}H]-phosphatidylinositol-4,5-bisphosphate was from NEN/Dupont. Phospholipids were from Avanti Polar Lipids. Other reagents, unless indicated, were from Sigma.

RESULTS

PA Regulation Is Isoform Dependent. The effect of PA on PLC-β_3 activity was determined over a range of Ca^{2+} concentrations. The results, shown in Figure 1A, demonstrate that 15 mol % PA had little effect on PLC-β_3 activity. PA produced less than a 40% increase in PLC-β_3 activity over the concentration range of 10 nM to 10 μM Ca^{2+}. PA stimulation of PLC-β_3 activity was markedly less than that previously observed with PLC-β_1 (*14*). As a control, PA stimulation of PLC-β_1 activity was determined, in paired experiments, using the same phospholipid preparation. The amount of PLC-β_1 added to the assay was adjusted to attain a basal enzymatic activity comparable to that of PLC-β_3. The data in Figure 1B demonstrate that the basal Ca^{2+} sensitivity of PLC-β_1 and PLC-β_3 was comparable. However, PA stimulated a marked increase in PLC-β_1 activity. PA, at 15 mol %, increased PLC-β_1 activity by 200% at 0.1 μM Ca^{2+}.

PA stimulation of PLC-β_3 and PLC-β_1 activity, as a function of PA mol %, is shown in Figure 2A. Stimulation

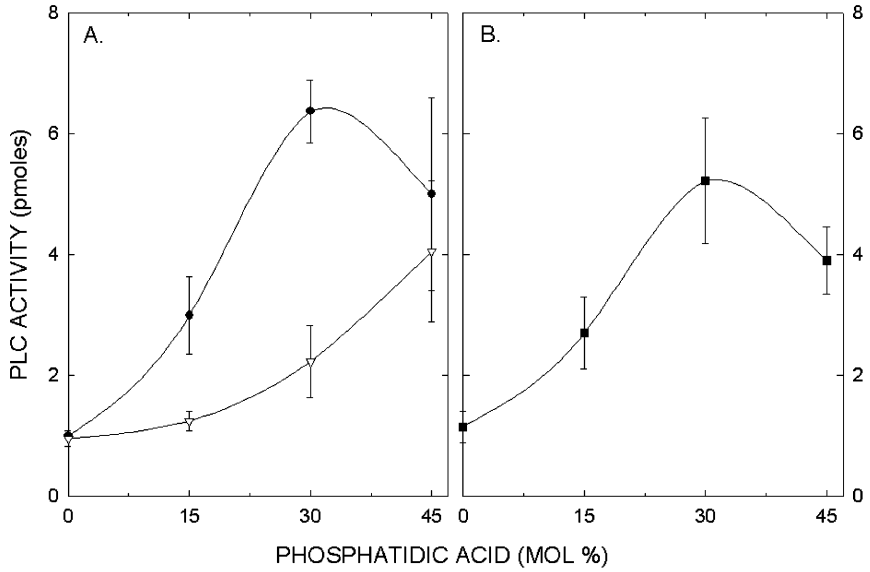

FIGURE 2: Effect of PA mol % on PLC-β_3 and PLC-β_1 activity. In panel A, PLC-β_3 (▽) or PLC-β_1 (●) was incubated with the indicated mol % PA at pCa 7.0. In panel B, recombinant PLC-β_1 was incubated with the indicated PA mol %. The results shown are the mean ± SE of four experiments.

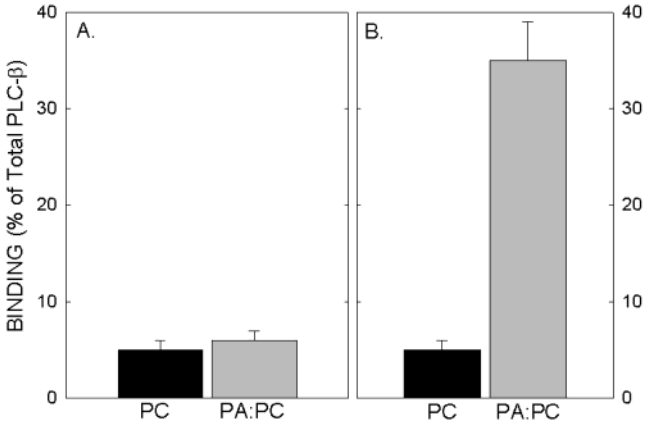

FIGURE 3: PLC-β_3 and PLC-β_1 binding to phospholipid vesicles. PLC-β_3 (panel A) or PLC-β_1 (panel B) was incubated with sucrose loaded large unilamellar vesicles (SLUV) containing either PC (100) or PA/PC (30:70) in the presence of 300 mM NaCl. After incubation, the samples were centrifuged, and lipid bound PLC-β was determined by SDS−PAGE analysis as described (*14*). The percent of total PLC-β protein bound to SLUV is shown. PLC-β_3 was recovered in the supernatant fraction (data not shown). Results shown are the mean ± SE of three experiments.

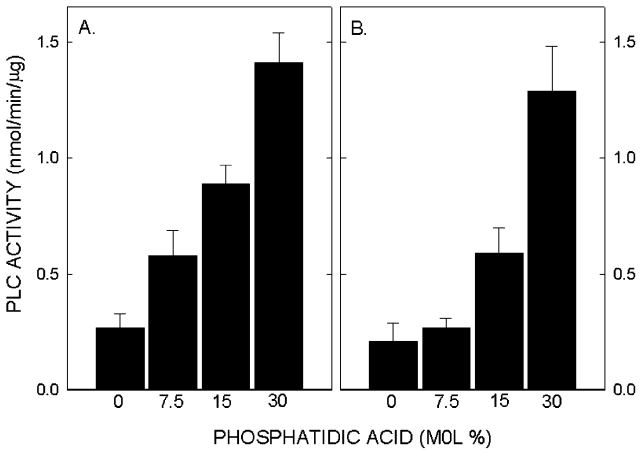

FIGURE 4: PKC and PA regulation of PLC-β_1 activity. The enzymatic activity of PLC-β_1, incubated without (panel A) or with (panel B) PKCα was determined in the presence of the indicated mol % PA at pCa 7.0. Results shown are the mean ± SE of four experiments.

of PLC-β_3 activity was evident at greater than 15 mol % PA. PA stimulation of PLC-β_3 activity occurred up to 45 mol % PA, the highest PA mol % used. In contrast, stimulation of PLC-β_1 activity was appreciable with 15 mol % PA. Previous studies had demonstrated a significant stimulation of PLC-β_1 activity with 7.5 mol % PA (*14*). Maximal stimulation of PLC-β_1 activity was attained at 30 mol % PA, followed by a decline from maximal activity with increasing mol % PA. The sensitivity of the recombinant PLC-β_1 to PA stimulation was comparable to the native PLC-β_1 enzyme (Figure 2B).

PA binding to PLC-β_1 is required for stimulation of enzyme activity (*14*). The binding of PLC-β_3 to PA containing SLUV is shown in Figure 3A. PLC-β_3 bound weakly to PC containing SLUV. Binding of PLC-β_3 to SLUV was not increased by the inclusion of PA. In contrast, as reported previously, PA enhanced the binding of PLC-β_1 to phosphatidylcholine (PC) containing SLUV (Figure 3B). Thus, PLC-β_3 does not bind to PA under conditions that promote PLC-β_1 binding. These data suggest that the lack of PA binding may be responsible for the insensitivity of PLC-β_3 to stimulation by low mol % PA.

PA Regulation Is Modulated by PKC. The interaction between PA and PKC in the regulation of PLC-β activity was determined. PKCα does not phosphorylate PLC-β_3 in vitro (*19*). However, PKCβ phosphorylates PLC-β_3, producing substoichiometric phosphorylation in vitro (*21*). PKCβ had little effect on the stimulation of PLC-β_3 activity by PA. PA, at 30 mol %, stimulated a 210 and 190% increase in the activity of the control and PKC phosphorylated PLC-β_3, respectively (data not shown).

PKCα inhibited basal PLC-β_1 activity and the stimulation of PLC-β_1 activity by 7.5−15 mol % PA (Figure 4). At 30 mol % PA, however, the enzymatic activities of the control and PKC inhibited PLC-β_1 were comparable. These results demonstrate that PKC inhibits stimulation of PLC-β_1 activity

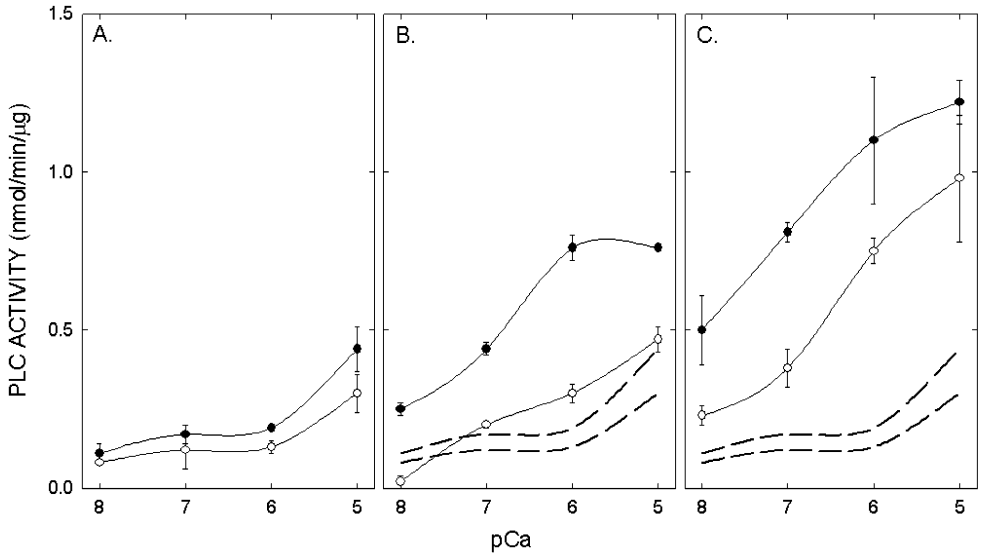

FIGURE 5: Effect of Ca^{2+} concentration on the regulation of PLC-β_1 activity by PKC and PA. PLC-β_1 was incubated without (●) or with (○) PKCα for 2 h in the presence of PKC cofactors. Phospholipase C activity was determined in the presence of no added PA (panel A), 7.5 mol % PA (panel B), or 15 mol % PA (panel C) and the indicated pCa. In panels B and C, the basal activity of the control and PKC treated PLC-β_1 (from panel A) is depicted with dashes. Results shown are the mean ± SE of three experiments.

by low mol % PA. Furthermore, PKC inhibition of PLC-β_1 activity is attenuated at levels of PA that produce maximum stimulation of PLC-β_1 activity (i.e., 30 mol % PA).

The magnitude of PLC-β_1 inhibition by PKC was also dependent on the Ca^{2+} concentration (Figure 5). PKC inhibited the stimulation of PLC-β_1 activity by 7.5 mol % PA by approximately 50% within the Ca^{2+} concentration range of 10 nM to 1 μM Ca^{2+}. Inhibition by PKC was slightly less at 10 μM Ca^{2+}. PKC also inhibited the stimulation of PLC-β_1 activity by 15 mol % PA, by approximately 50%, at 10 and 100 nM Ca^{2+}. Inhibition by PKC was reduced to 25% at 1 and 10 μM Ca^{2+} in the presence of 15 mol % PA. Antagonism of PKC inhibition required both Ca^{2+} and PA since a comparable reversal of inhibition was not observed upon increasing only the Ca^{2+} concentration.

The effect of PKC on PLC-β_1 binding to PA is shown in Figure 6. Phosphorylation reduced PLC-β_1 binding to SLUV containing 15 mol % PA. Binding of the phosphorylated PLC-β_1 to SLUV containing 30 mol % PA, however, approached control values. These data demonstrate that PKC inhibits PA stimulation and PA binding to PLC-β_1 through a mechanism dependent on the mol % PA.

PA Modulates Gαq Stimulation of PLC-β_1. The regulation of G protein stimulated PLC-β_1 activity by PA was determined. As shown in Figure 7, 1 nM αq-GTP-γ-S stimulated a 67% increase in PLC-β_1 activity. Basal activity was increased by 35% in the presence of 7.5 mol % PA. The combination of PA plus αq-GTP-γ-S produced a 150% increase in PLC-β_1 activity. Thus, stimulation of PLC-β_1 activity by PA plus αq-GTP-γ-S was greater than additive.

Under comparable assay conditions, 500 nM $\beta\gamma$ had little effect on PLC-β_1 activity, producing a 12 and 10% increase in activity in the absence or presence of PA, respectively.

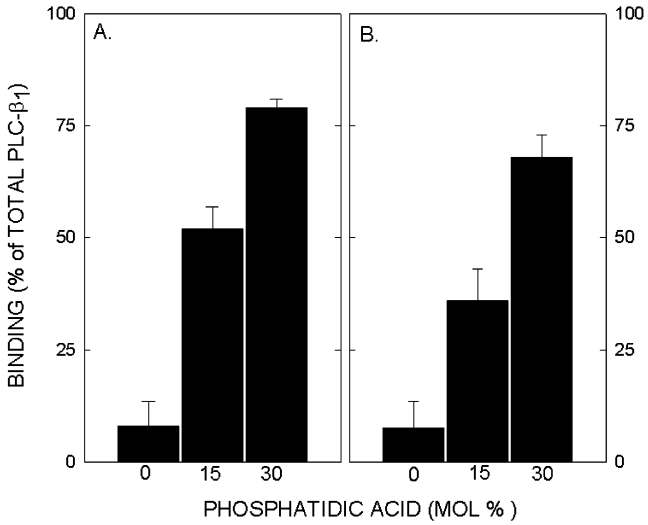

FIGURE 6: Effect of PKC phosphorylation on PLC-β_1 binding to PA. Control (panel A) or PKC phosphorylated PLC-β_1 (panel B) was added to SLUV containing the indicated mol % PA. After 10 min, the SLUV-bound PLC-β_1 was separated from the unbound PLC-β_1 by centrifugation. The enzymatic activity of the supernatant, corresponding to unbound activity, was determined. Bound PLC-β_1 was calculated by subtracting the unbound activity from the total PLC-β_1 activity added to the binding assay. Binding is expressed as percent of total PLC-β_1. Results are the mean ± SE of five experiments.

DISCUSSION

The present studies demonstrate that PA regulation of PLC-β activity is isoform dependent. Major differences in the regulation of PLC-β_3 and PLC-β_1 enzyme activity by PA were evident. PLC-β_3 was considerably less sensitive to PA stimulation than PLC-β_1. The EC_{50} for PA stimulation of PLC-β_1 was 15 mol % (ref *14*, Figure 2). In contrast, 15 mol % PA had little effect on PLC-β_3 activity. The EC_{50} for PA stimulation of PLC-β_3 activity could not be determined from the present studies because saturation of stimulation was not attained at 45 mol % PA, the highest mol % used in these studies. However, it can be estimated to be greater than 30 mol %. Finally, PLC-β_3 did not bind PA under conditions where PLC-β_1 binding was evident. These data suggest that the insensitivity of PLC-β_3 to stimulation by low mol % PA may be due to the weak binding of PLC-β_3 to PA.

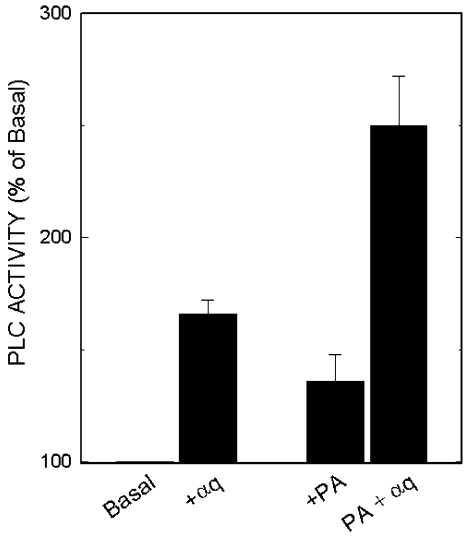

FIGURE 7: PA regulation of Gαq stimulated PLC-β_1 activity. PLC-β_1 was incubated in the presence or absence of 1 nM αq-GTP-γ-S and with or without 7.5 mol % PA for 2 min. Results shown are the mean ± SE of three experiments.

These data are consistent with previous studies demonstrating that PA binding is required for stimulation of PLC-β_1 activity by low mol % PA (*14*). While full length PLC-β_1 is stimulated by low mol % PA, a 100 kDa PLC-β_1 catalytic fragment, generated by calpain proteolysis of PLC-β_1, requires greater than 15 mol % PA for stimulation. The 100 kDa PLC-β_1 catalytic fragment lacks the C-terminal region and does not bind PA (*14*). Thus, the absence of a PA binding C-terminal region is associated with a diminished sensitivity to PA stimulation.

PKC regulation of PLC-β_3 and PLC-β_1 also differs. PKC has been implicated in the negative feedback regulation of PLC-β_3 activity in vivo (*21*). PKCβ had little effect on PA stimulation of PLC-β_3 activity suggesting that PKC does not modulate PA stimulation of PLC-β_3 activity. However, the lack of PKC regulation may be related to the inefficient phosphorylation of PLC-β_3 that occurs in vitro. PKC phosphorylation of PLC-β_3 is isoform dependent. PKCα does not phosphorylate PLC-β_3 in vitro (*19*). PKCβ phosphorylates PLC-β_3 activity in vitro, but phosphorylation is substoichiometric, achieving 0.4 nmol Pi/nmol protein (*21*). Comparable substoichiometric phosphorylation has been observed in this laboratory (data not shown). The reason for the inefficient PKC phosphorylation of PLC-β_3 in vitro remains to be determined.

In contrast, PKC phosphorylation of PLC-β_1 is stoichiometric (*17*, *18*). PKC inhibited PA stimulation and PA binding to PLC-β_1 at low mol PA %. The magnitude of PKC inhibition was reduced at higher mol % PA and Ca^{2+}. These findings suggest a possible interaction between PA and PKC signaling in the regulation of PLC-β_1 activity in vivo.

In addition to modulating PKC regulation, PA also affected the stimulation of PLC-β_1 by Gq. PA promotes G protein stimulation of PLC-β_1 in membranes (*14*, *15*), but the effect of PA on the stimulation by the individual G protein subunits had not been addressed. As shown in Figure 7, PA enhanced the stimulation of PLC-β_1 activity by Gαq. G protein $\beta\gamma$ subunits produced little stimulation of PLC-β_1 activity under these conditions. PA had little effect on the stimulation by $\beta\gamma$ subunits.

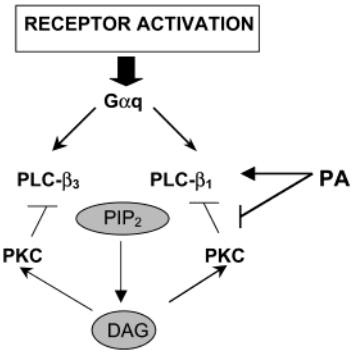

FIGURE 8: Model for phosphatidic acid regulation of PLC-β_1 activity. Stimulation of PLC-β_1 and PLC-β_3 activity by activated G protein coupled receptors (GPCR) results in the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) and production of diacylglycerol (DAG), which activates protein kinase C (PKC). PKC phosphorylates PLC-β_1 and PLC-β_3, initiating negative feedback regulation of these enzymes. GPCR stimulation also increases intracellular phosphatidic acid (PA) levels through activation of diacylglycerol kinase and phospholipase D activity. PA promotes PLC-β_1 stimulation through two mechanisms. PA binds to PLC-β_1, possibly at an allosteric regulatory site, and increases PLC-β_1 activation in response to GPCR stimulation. PA also antagonizes inhibition of PLC-β_1 activity by PKC. In contrast, PLC-β_3 is less sensitive to PA stimulation. As a result, inhibition of PLC-β_3 activity by PKC may be more pronounced in vivo.

These results suggest a model for PA regulation of PLC-β activity in vivo. As depicted in Figure 8, GPCR stimulation of PLC-β_1 and PLC-β_3 activity results in the production of DAG from PIP_2 hydrolysis and activation of PKC. PA levels increase because of DGK (*6*) and phospholipase D (PLD) activation (*23*). This increase in intracellular PA levels has two actions. First, PA stimulates PLC-β_1 activity, thereby increasing PLC-β_1 activity in response to GPCR stimulation. Second, PA antagonizes the inhibition of PLC-β_1 activity by PKC. The data in Figures 4 and 5 suggest that stimulation of PLC-β_1 activity by low levels of PA would be inhibited by PKC. However, as intracellular PA and Ca^{2+} levels increase, the extent of PKC inhibition would diminish. The control and PKC phosphorylated PLC-β_1 activity would be comparable when the local concentration of PA begins to approximate 30 mol %. In this manner, PA and PKC may confer temporal regulation of PLC-β_1 signaling. Termination of PA signaling would occur upon the degradation of PA by phospholipase A_2 or phosphatidate hydrolase. On the otherhand, PKC inhibition of PLC-β_3 activity may be more effective in vivo since PLC-β_3 is less sensitive to PA.

The role of PA in the regulation of GPCR signaling may thus depend on the specific PLC-β isoform expressed in cells and the level of PA attained in response to agonist stimulation. Both DGK (*24*, *25*) and PLD (*26*, *27*) are compartmentalized. Thus, agonist stimulated increases in PA may be localized. Localization of DGK to receptor generated pools of DAG and PA has been reported (*28*). While the concentration of PA attained near PLC-β is not known, total PA levels can reach 100 μM in stimulated neutrophils (*28*, *29*). In addition, pathological conditions are associated with large increases in PA levels. Ischemia results in an increase in PA levels (*30*). Malignant transformed cells have elevated PA levels (*31*, *32*). It will be of interest to determine whether these changes in PA levels impact PLC-β_1 signaling.

Isoform differences in PA regulation have been demonstrated for other enzymes. Cyclic AMP phosphodiesterase

exhibits isoform dependence for PA regulation (*11*). PA activates some but not other isoforms of the type 4 cyclic AMP phosphodiesterases (PDE4) in vitro. The PDE4D1 isoform binds weakly to PA and is not stimulated by PA, while the PDE4D3 isoform binds PA and is activated by PA. SHP-1 phosphatase has a high affinity binding site for PA, which is absent from a related protein tyrosine phosphatase SHP-2 (*10*). These studies suggest the existence of specific determinants that define a functional interaction between PA and its target protein. A PA binding region has been identified in raf 1 kinase (*7*), SH-1 phosphatase (*10*), and PDE4D1 (*11*).

PLC-β_1 is stimulated by GPCR linked to the $G\alpha_{q/11}$ family of G proteins. The activation of PLC-β_1 signaling is tightly regulated. Several negative feedback mechanisms contribute to regulate signaling at the level of the GPCR and G protein. These include the G protein coupled receptor kinases (*33*) as well as GTPase activating proteins such as the regulators of G protein signaling (*34*) and PLC-β_1 (*35*). In addition, PKC phosphorylates and inhibits PLC-β activity (*19*, *21*). Despite the existence of these negative feedback regulatory mechanisms, GPCR stimulated PLC-β_1 activity can remain sustained for a long time in some cells (*36*−*40*). Thus, additional control mechanisms, possibly mediated by local increases in PA levels, may modulate inhibitory feedback regulation of GPCR signaling.

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