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PI(3,4,5)P₃ Potentiates Phospholipase C-β Activity

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Abstract

Phospholipase C-β (PLC-β) isozymes are key effectors in G protein-coupled signaling pathways. Previously, we had shown that PLC-β1 and PLC-β3 bound immobilized PIP₃. In this study, PIP₃ was found to potentiate Ca²⁺-stimulated PLC-β activities using an *in vitro* reconstitution assay. LY294002, a specific PI 3-kinase inhibitor, significantly inhibited 10 minutes agonist-stimulated total IP accumulation. Both LY294002 and wortmannin inhibited 90 seconds agonist-stimulated IP₃ accumulation in intact cells. Moreover, transfected p110CAAX, a constitutively activated PI 3-kinase catalytic subunit, increased 90 seconds oxytocin-stimulated IP₃ accumulation. Receptor-ligand binding assays indicated that LY294002 did not affect G protein-coupled receptors directly, suggesting a physiological role for PIP₃ in directly potentiating PLC-β activity. When co-expressed with p110CAAX, fluorescence-tagged PLC-β3 was increasingly localized to the plasma membrane. Additional observations suggest that the PH domain of PLC-β is not important for p110CAAX-induced membrane association.

Keywords

phospholipase C-β; phosphatidylinositol 3; 4; 5-trisphosphate; G protein-coupled receptors; phosphatidylinositol 3-kinase

Introduction

Phosphoinositide-specific phospholipase C (PLC) isozymes are key effector enzymes in multiple cellular signaling pathways, hydrolyzing phosphatidylinositol 4,5-bisphosphate (PI (4,5)P₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol (DAG), second messengers which subsequently regulate Ca²⁺ release from intracellular stores and protein kinase C activation. To date, thirteen identified mammalian PLC isozymes have been classified by sequence homology into six families, PLC-β, -γ, -δ, -ε, -ζ, and -η, each having a unique mechanism of activation and regulation [1]. The activation of PLC-β isoenzymes is regulated by GTP-bound Gα subunit of Gα_q family, Gβγ subunit and the small GTPase Rac [2]. G protein-coupled receptors (GPCR) that activate PLC-β include angiotensin receptors, histamine receptors, oxytocin receptors, muscarinic receptors, α₁-adrenergic receptors, lysophosphatidic acid receptors and endothelin receptors among many others, indicating the crucial roles of PLC-β in cell signaling cascades [1].

The PLC-β family exists as four identified isoforms, β1 – β4. Among them, PLC-β1 and β3 are ubiquitously expressed in a variety of tissues, whereas β2 and β4 have limited tissue

distribution. Identified by sequence homology, PLC- β isoforms contain at least five distinct structural domains, including a PH (pleckstrin homology), four EF-hand domains, catalytic X and Y domains, a C2 domain, and a unique C-tail (carboxy-terminal) domain [1]. The amino-terminal PH domain of PLC- β is known as a protein interaction domain that binds to G $\beta\gamma$ [3] or Rac1 [4] in certain contexts. The central XY domain is highly conserved among PLC isoforms and structurally forms a catalytic TIM (Triosephosphate isomerase) barrel to create the catalytic pocket for PI(4,5)P₂ binding [4]. PLC- β isoforms are distinguished by a structurally unique C-tail domain (approximately 400 amino acids) that is involved in regulation by G α_q subunits [1,5] and dimerization [6-8].

There are at least eight mammalian phosphatidylinositol 3-kinases (PI 3-kinases) which are divided into three classes according to their structure and substrate specificity. Of particular interest are the class 1 PI 3-kinases, the only class that produces phosphatidylinositol 3,4,5-trisphosphate (PIP₃) and comprises a p110 catalytic subunit and a separate regulatory subunit. Class 1 PI 3-kinases are further subdivided into class 1A (PI 3-kinase- α , - β , - δ), which are activated by receptor tyrosine kinases, and class 1B (PI 3-kinase- γ), which is uniquely activated by heterotrimeric G proteins [9]. Stimulation of a range of receptors, such as growth factor receptors and G protein-coupled receptors, results in the activation of PI 3-kinases, which generate PIP₃ by phosphorylation of PI(4,5)P₂ at the 3-position of the inositol ring. PIP₃ has been proposed as a second messenger that affects numerous cellular responses, including exocytosis, cytoskeleton remodeling, chemotaxis, and regulation of ion channels [10]. PIP₃ mediates its effects by directly regulating functional activity and/or acting as a docking site for a number of proteins containing PH domains such as Akt/PKB (protein kinase B), Btk (Bruton's tyrosine kinase) and GRP1 (general receptor for phosphoinositides-1), and recruiting them to the plasma membrane [11].

Previously, we have reported that several phosphatidylinositides, including PIP₃, bound PLC- β 1 and PLC- β 3 *in vitro* using a phospholipid protein overlay assay [12]. To further understand the interaction between PIP₃ and PLC- β , we sought to determine the effect of PIP₃ on PLC- β activity *in vitro* and in intact cells. Since PIP₃ is a poor substrate of PLC- β [13], our data suggest that PIP₃ could be an important allosteric regulator of PLC- β activity.

Materials and methods

Materials

Carbamylcholine chloride (carbachol) and oxytocin were obtained from Calbiochem (San Diego, California). LY294002 and phosphor-Akt (Ser473) antibody were from Cell Signaling Technology (Danvers, MA) and wortmannin was from LC Laboratories (Woburn, MA). PI (4,5)P₂, PI(3,5)P₂, PE (phosphatidylethanolamine) and PIP₃ were purchased from Avanti Polar Lipids (Alabaster, AL). [³H]oxytocin and [³H]PI(4,5)P₂ were from PerkinElmer (Waltham, MA). [³H]myo-inositol and [³H]QNB were from American Radiolabeled Chemicals (St. Louis, MO). Atropine sulfate was from Acros Organics (Geel, Belgium). β -actin antibody was from Abcam (Cambridge, MA).

Plasmids

pCDNA3-hOTR (human oxytocin receptor) was purchased from UMR cDNA Resource Center (Rolla, MO) and p110CAAX was a gift from Dr. Andrew Henderson [14]. GFP-Akt-PH and GFP-Akt-PH (R25C) were gifts from Dr. Craig Montell [15]. PH domain of PLC- β 3 (PH- β 3, residues 1-138) was amplified by PCR (polymerase chain reaction) using the following sets of primers: 5'-ATGAATTCATGGCGGGCGCCAGC-3' and 5'-CTCGAGTCACAGCTTGAATAGCTCCTCAGAC-3' and PH domain of PLC- β 1 (PH- β 1, residues 1-166) was amplified with 5'-GATGTCGACCATGGCCGGGGCTCAAC-3' and 5'-

GATGGTACCTCATTCTGGAGTGACTTGCAGCTT-3'. PCR-amplified PH- β 3 was ligated into EcoRI and SalI sites of pEGFP-C2 vector (Clontech, Mountain View, CA) and PH- β 1 was ligated into SalI and KpnI sites in pEGFP-C4 vector. Full length PLC- β 3 was subcloned into EcoRI and XhoI sites of pEGFP-C4 vector. All constructs were confirmed by sequencing.

Cell culture and generation of HEK 293 Cells Stably Expressing OTR

1321N1, H9c2 and HEK 293 cells were cultured in 90% DMEM, 10% fetal bovine serum, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C under 5% CO₂ in humidified air [6]. One day prior to transfection, HEK 293 cells were plated at a density of 5×10^5 cells/10 cm culture dish. pCDNA3-OTR plasmid (4 μ g/10 cm plate), which includes a neomycin resistance gene, was transfected into cells using JetPEI kit (Polyplus-transfection, New York, NY) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were selected by treatment with 0.7 mg/ml G418 for at least 4 weeks following transfection. Drug-resistant clones were isolated, expanded and evaluated for OTR receptor expression by binding assay as described below.

Protein expression and purification

PLC- β 1, PLC- β 2, PLC- β 3, G α_{11} and G $\beta_1\gamma_2$ were expressed in Sf9 cells following baculovirus infection and purified as described previously [6,16].

Reconstitution assay

The catalytic activity of PLC- β was quantitated using [³H]PIP₂ substrate as described previously [12]. Briefly, 35 ng of purified PLC- β 3 was added in 20 μ l Buffer 1 (50mM HEPES pH 7.2, 3 mM EGTA, 80 mM KCl, 1mM DTT). 15 μ M PI(4,5)P₂, 135 μ M PE, and 9000 DPM [³H]PI(4,5)P₂ with or without 5 μ M PIP₃ or 5 μ M PI(3,5)P₂ were dried under nitrogen and resuspended in 20 μ l Buffer 1 by 3×10 sec bursts of sonication. For assay of G-protein-stimulated activity of PLC- β , 10 μ l of Buffer 2 (50 mM HEPES pH 7.2, 1mM EDTA, 3 mM EGTA, 5mM MgCl₂, 1 mM DTT, 100 mM NaCl, 1% cholate) containing 60 ng G $\beta\gamma$ or 50 ng G α_{11} with or without prior activation by 0.15 μ M GTP γ S was added to each reaction. For assays of Ca²⁺-stimulated (non-G protein activated) PLC- β activity, 12 ng PLC- β 1, 40 ng PLC- β 2 or 35 ng PLC- β 3 was added in Buffer 2 without G-protein along with 10 μ l Buffer 1 with 9 mM CaCl₂ to yield a final assay volume of 60 μ l. Reactions were incubated at 30°C for 12 min and terminated by the addition of 375 μ l of chloroform, methanol and hydrochloric acid in a ratio of 80:40:1, followed by the addition of 125 μ l chloroform and 125 μ l 0.1 N HCl with vigorous mixing. Aqueous and organic phases were separated by centrifugation for 5 min at 3000 $\times$ g. [³H]IP₃ product was quantitated by scintillation counting of 320 μ l of the upper phase. Triplicate samples were tested in two separate experiments.

Inositol phosphate assay

Whole cell inositol phosphate (IP) assays were performed in 1321N1 cells or HEK 293 cells expressing OTR as previously described [12]. 1×10^5 1321N1 cells or HEK 293 cells were subcultured in 24-well plates overnight and then labeled overnight with 2 mCi/ml [³H]myo-inositol in bicarbonate-buffered inositol-free DMEM without additives. For assay, cells were pre-treated with 0.1% DMSO or 50 μ M LY294002 for 30 min in 10 mM LiCl, 20mM Hepes-buffered DMEM (pH 7.4) at 37°C in room air. 1321N1 cells were stimulated with 1 mM carbachol and HEK 293 were stimulated with 100 nM oxytocin for 10 min. Cells were lysed and total inositol phosphates (IP) were extracted and quantitated.

Inositol trisphosphate (IP₃) accumulation assay

1321N1 cells or HEK 293 cells stably expressing hOTR were seeded at 2×10^5 cells/well in 12-well plates, allowed to attach overnight, and labeled for 18 h with 2 mCi/ml [³H]myo-inositol

in inositol-free, bicarbonate-buffered DMEM. Following radiolabeling, cells were pre-treated with 0.1% DMSO or 50 μ M LY294002 or 0.5 μ M wortmanin for 30 min in 0.5 ml 10 mM LiCl, 20mM Hepes-buffered DMEM (pH 7.4) at 37°C in room air. 1321N1 cells were then stimulated with 1 mM carbachol and HEK 293 cells was stimulated with 100 nM oxytocin for 90 second. The reaction was stopped by adding 1 ml 15% trichloroacetic acid solution directly to the wells. The cell lysates were extracted, and IP₃ was separated by anion exchange chromatography and quantitated as described previously [12].

Membrane preparation

1321N1 cells or HEK 293 cells were washed thrice with ice-cold PBS, collected by scraping, and pelleted at 500 $\times$ g for 5 min. Ice-cold hypotonic lysis buffer (25 mM Hepes, pH 7.4, 2 mM EDTA, 0.1 mM PMSF, 1 μ M pepstatin A, 10 μ M leupeptin) was added to each sample and incubated on ice for 15 min. Cells were lysed with 15 strokes of a Dounce homogenizer and the lysate centrifuged at 500 $\times$ g, 4°C for 5 min to pellet nuclei and intact cells. The resulting supernatants were centrifuged at 200,000 $\times$ g, 4°C for 30 min. The resulting crude membrane pellets were suspended in binding buffer (25 mM Tris, pH 7.4, 10 mM MgCl₂, 0.1 mM PMSF, 1 μ M pepstatin A, 10 μ M leupeptin). Protein concentration was determined using Bradford reagent (Bio-Rad, Hercules, CA) with BSA as standards [17].

Receptor binding assays

Cell membranes were diluted to 0.5-1 μ g/ μ l in binding buffer (25 mM Tris, pH 7.4, 10 mM MgCl₂, 0.2% BSA). Total binding assays (200 μ l final volume) containing 50-100 μ g cell membranes, varying concentrations of [³H]OT (for transfected HEK 293 membranes) or [³H]QNB (for 1321N1 cell membranes) and 50 μ M LY294002 or 0.1% DMSO vehicle were incubated at 30°C for 30 min. Nonspecific binding was assessed by including 2 μ M unlabeled OT for [³H]OT binding or by including 0.1 mM atropine sulfate for [³H]QNB binding. Following incubation, assays were filtered through No. 32 glass-fiber filters (Whatman, Sanford, ME) on a Brandel Cell Harvester (Gaithersburg, MD), washed three times with ice-cold binding buffer and radioactivity retained by filters quantitated using a Beckman LS 6500 Scintillation counter [17].

Fluorescence microscopy

HEK 293 cells grown on poly-lysine coated glass cover slips were transiently transfected by JetPEI kit with 100 ng of plasmid DNA. Cells were washed twice with PBS after 48 h transfection, fixed in 3% formaldehyde (Tousimis, Rockville, MD) for 20 minutes at room temperature, and mounted on slides with a ProLong antifade kit (Molecular Probes, Eugene, OR). Confocal images were obtained using a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY) with a 63 $\times$ oil immersion objective as previously described [6].

Western blot

Cells were lysed in ice-cold hypotonic buffer (25 mM Hepes, pH 7.4, 2 mM EDTA, 0.1 mM PMSF, 1 μ M pepstatin A, 10 μ M leupeptin) and homogenized by 15 strokes using a Dounce homogenizer. After removal of nuclei and intact cells by low speed centrifugation (500 $\times$ g for 5 min), the resulting supernatant was precipitated with chloroform and methanol before separation by SDS-PAGE and visualization by western blot. For phosphor-Akt assay, cells were serum-starving overnight before pretreatment with vehicle or inhibitor in Kreg's solution (118 mM NaCl, 4.69 mM KCl, 1.18 mM MgSO₄, 1.29 mM CaCl₂, 1.18 mM KH₂PO₄, 11.67 mM glucose, and 25mM Hepes, pH 7.4) for 30 min and stimulation with agonists. All primary antibodies were used at a concentration of 0.1 μ g/ml, and secondary antibodies at 0.04 μ g/ml. Immunoreactive bands were detected using SuperSignal West Pico chemiluminescent substrates (Pierce, Rockford, IL).

Curve fitting and data analysis

Curve fitting and data analysis were performed using GraphPad Prism 4 (GraphPad software, San Diego, CA). All saturation isotherm specific binding data were best fit to a one-site binding parabola. Data are presented as mean $\pm$ S.D with $p < 0.05$ considered significant as determined by Student's unpaired two-tailed t-test unless otherwise indicated. All experiments were reproduced at least twice.

Results

PIP₃ increases Ca²⁺-stimulated PLC- β activity *in vitro*

Previously, we had shown that several phospholipids, including PIP₃, could bind PLC- β 1 and PLC- β 3 using a phospholipid blot (PipStrip®) protein overlay with antibody detection [12]. Because PIP₃ is critical in signaling transduction, we sought to determine whether PIP₃ affected PLC- β activity *in vitro*, despite a lack of known PLC- β hydrolytic activity towards PIP₃. A reconstitution assay was performed using purified PLC- β protein, purified G protein subunits and purified lipids. As illustrated in Figure 1A, PIP₃ increased basal (in the presence of inactivated G α_{11}) PLC- β 3 activity by 39% while having no effect on PLC- β 3 activity stimulated by GTP γ S-activated G α_{11} (G α_{11} + GTP γ S). PIP₃ also increased Ca²⁺-stimulated PLC- β 3 activity by 54% but not G $\beta\gamma$ -stimulated PLC- β 3 activity (Fig. 1B). Importantly, PI (3,5)P₂, another negatively charged non-substrate phospholipid, did not change either Ca²⁺-stimulated or G $\beta\gamma$ -stimulated PLC- β 3 activity (Fig. 1C), suggesting a unique effect of PIP₃ compared to other phosphoinositol phospholipids. Moreover, PIP₃ potentiated the Ca²⁺-stimulated activities of PLC- β 3 as well as PLC- β 1 and PLC- β 2 by 61% and 30%, respectively (Fig. 1D), suggesting a general effect of PIP₃ on Ca²⁺-stimulated PLC- β isozyme activities.

PI 3-Kinase inhibitor inhibits PLC activity in whole cells

To ascertain the potential PIP₃ effect on PLC β activity in a more physiological context, we treated 1321N1 cells with LY294002 (LY), a specific PI 3-kinase inhibitor, and assayed for inositol phosphates (IP) accumulation. 1321N1 cells, human astrocytoma cells, express muscarinic receptors which are coupled to PLC- β , presumably PLC- β 1 [18]. In the unstimulated basal state, LY294002 did not affect IP accumulation in 1321N1 cells. However, when 1321N1 cells were stimulated for 10 minutes with 1 mM carbachol, a muscarinic receptor agonist, LY294002 significantly inhibited carbachol-stimulated IP accumulation. LY294002 inhibition of carbachol-stimulated IP accumulation was dose dependent with 10 μ M and 50 μ M LY294002 significantly inhibiting IP hydrolysis by 21% and 44%, respectively (Fig. 2A).

To determine whether the inhibition of PLC activity by LY294002 was cell line specific or receptor specific, similar experiments were conducted in another cell model, transfected HEK 293 cells stably expressing human oxytocin receptor (hOTR). HEK 293 cells express PLC- β 1 and PLC- β 3 predominantly and hOTR receptors presumably couple to PLC- β 3 [19]. In this system, 50 μ M LY294002 had no significant effect on basal IP accumulation but markedly inhibited 100 nM oxytocin (OT)-stimulated IP accumulation by 45% (Fig. 2B). Similar data were obtained in HEK 293 cells expressing ovine OTR (data not shown).

To verify the activity of LY294002 in whole cells to reduce PIP₃ levels by inhibition of PI 3-kinase, we monitored Akt phosphorylation which is responsive to changing PIP₃ levels [20]. In unstimulated cells, weak Akt phosphorylation was detected in 1321N1 cells (Fig. 2C, lane 1) and 50 μ M LY294002 was without effect as expected (Fig. 2C, lane 2) by monitoring with phospho-specific anti-Akt antibodies. However, when 1321N1 cells were treated with 1 mM carbachol for 10 minutes to stimulate PLC- β activity, phospho-specific Akt bands were noticeably increased in intensity (Fig. 2C, lanes 3 and 4); carbachol-induced Akt phosphorylation was abolished by treatment with 50 μ M LY294002 (Fig. 2C, lanes 5 and 6).

Similar effects of LY294002 on oxytocin (OT)-induced Akt phosphorylation were observed in OTR-expressing HEK 293 cells (data not shown).

PI 3-Kinase activity is related to PLC- β activity in whole cells

To rule out the involvement of other PLC isozymes besides PLC- β in IP₃ accumulation stimulated by 10 minutes of agonist treatment (e.g. transactivation of PLC- δ or PLC- ϵ), we repeated the above experiments restricting agonist treatment to 90 seconds and selectively recovering IP₃ for quantitation. Previous experiments demonstrated that the majority of IP₃ production following G protein-coupled receptor activation was attributable to PLC- β activation when cells were stimulated by agonists up to 90 seconds [21]. 50 μ M LY294002 had no effect on basal IP₃ accumulation but inhibited 90 seconds carbachol-stimulated IP₃ accumulation by 47% in 1321N1 cells or oxytocin-stimulated IP₃ accumulation by 60% in hOTR expressing HEK 293 cells (Fig. 3A and 3B). Furthermore, LY294002 inhibited IP₃ accumulation by 59% in H9c2 cells, rat myoblast cells which exclusively express PLC- β 3 but not other PLC- β isozymes (supplemental data, Fig. 1 and data not shown).

To substantiate that LY294002 was blocking PI 3-kinase to inhibit agonist-stimulated IP₃ accumulation, we reproduced the effects of LY294002 to inhibit 90 seconds OT-induced IP₃ accumulation in hOTR expressing HEK 293 cells with a structurally unrelated PI 3-kinase inhibitor, wortmannin, as shown in Fig. 3C. Wortmannin (500 nM) inhibited oxytocin-stimulated IP₃ accumulation by 29 % while it had no effect on basal IP₃ accumulation. The difference in effectiveness of wortmannin versus LY294002 in this system (29 % versus 60%) may be attributable to the sub-maximal dose of wortmannin chosen to avoid non-selective inhibition of myosin light chain kinase at μ M concentrations [22].

To attempt to address the effects of increased PIP₃ levels on PLC- β activity, we measured agonist-stimulated IP₃ accumulation in cells transfected with p110CAAX, a constitutively activated PI 3-Kinase catalytic subunit widely used to increase cellular PIP₃ concentration [14,23]. In hOTR expressing HEK 293 cells, p110CAAX transfection increased oxytocin-stimulated IP₃ accumulation by 26% compared to non-transfected cells (Fig. 4A). Phospho-Akt Western blot assays suggested that over-expression of p110CAAX induced Akt phosphorylation (Fig. 4B, lanes 3 and 4) as compared to mock transfected HEK 293 cells (Fig. 4B, lanes 1 and 2) in the absence of agonist stimulation. Thus, increasing PI 3-kinase activity constitutively does not affect basal IP₃ levels, but potentiates agonist-stimulated IP₃ accumulation.

PI 3-Kinase inhibitor does not affect G protein-coupled receptors

To address whether LY294002 may reduce PLC- β -mediated signal transduction by directly inhibiting G protein-coupled receptors, receptor-ligand binding assays were conducted. 1321N1 cells exhibited saturable and specific muscarinic receptor binding sites for [³H]QNB. 50 μ M LY294002 had no effect on [³H]QNB specific binding in terms of total binding sites or affinity for [³H]QNB. No significant differences in the apparent B_{max} values (41.0 ± 2.0 vs. 39.5 ± 1.74 fmol/mg protein for control and LY294002 treated groups, respectively) or the apparent K_d values (3.12 ± 0.37 vs. 3.02 ± 0.32 nM for control and LY294002 treated groups, respectively) were detected between DMSO vehicle treatment and LY294002 treatment (supplemental data, Fig. 2A).

In addition, [³H]OT binding assay was performed in hOTR expressing HEK 293 cells (Fig. 5B). When membrane preparations from HEK 293 cells were treated with 0.1% DMSO or 50 μ M LY294002, the specific [³H]OT binding curves almost overlapped. As above, no significant differences in the apparent B_{max} values (601.0 ± 9.3 vs. 592.7 ± 7.3 fmol/mg protein for control and LY294002 treatment groups, respectively) or the apparent K_d values ($4.38 \pm$

0.19 vs. 4.13 ± 0.14 nM for control and LY294002 treated groups, respectively) were detected (supplemental data, Fig. 2B). These data suggest LY294002 had no direct effect on agonist binding to G protein-coupled receptors to inhibit IP accumulation.

Membrane association of PLC- β 3 is related to PIP₃

PIP₃ has been well documented to act as a docking site for a range of proteins containing PH domains, such as Akt, by recruitment to the plasma membrane [11]. To further dissect the mechanism by which PIP₃ potentiates PLC- β activity, we attempted to assess PLC- β 3 localization under varying cellular PIP₃ concentrations. We sought to detect the intracellular localization of PLC- β 3 under conditions of increased PIP₃ levels by co-transfecting constitutively activated p110CAAX with GFP (green fluorescent protein)-tagged PLC- β constructs. As control experiments for visualizing membrane and cytoplasmic protein localization, GFP-tagged Akt-PH domain, a PIP₃ binding probe, or a non-PIP₃ binding mutant, GFP-Akt-PH (R25C) was transfected into HEK 293 cells [15]. As expected, GFP-Akt-PH was exclusively localized at membrane (Fig. 5A, left panel), whereas its non-PIP₃ binding mutant, GFP-Akt-PH (R25C), was expressed in the cytosol and nuclei (Fig. 5A, right panel). When GFP-PLC- β 3 was co-transfected with vector (pSG5) into HEK 293 cells, PLC- β 3 mainly localized to the cytosol (Fig. 5B, left panel). This majority cytosolic localization of transfected GFP-PLC- β 3 is a pattern that we have observed previously [6]. Interestingly, GFP-PLC- β 3 was increasingly localized to the plasma membrane when co-transfected with p110CAAX into HEK 293 cells (Fig. 5B, right panel), suggesting that increased PIP₃ levels in the p110CAAX-transfected cells may influence PLC- β 3 subcellular localization.

The PH domain of PLC- β is not important for p110CAAX-induced membrane association of the full length protein

PH domains have been described in multiple protein contexts as phosphoinositide binding domains [24] and PLC- β has a putative PH domain homology sequence at its amino terminus [4]. To further delineate the domain of PLC- β involved in membrane localization and potential PIP₃ interaction, GFP-tagged PH domains from PLC- β 1 (Fig. 6, left panel) or PLC- β 3 (Fig. 6, right panel) were constructed and transfected into HEK 293 cells. Both GFP-tagged PH domains were detected predominantly in the cytosol of transfected HEK 293 cells. Co-expression of either PH construct with p110CAAX did not affect the localization pattern (data not shown). These data suggest that other domains of PLC- β besides the PH domain are important for PIP₃-induced PLC- β membrane association.

Discussion

Previously, we observed that PLC- β bound PIP₃ along with several other anionic phospholipids on nitrocellulose membranes, implying that PIP₃ might have an effect on PLC- β [12]. In the present study, we quantitated PIP₃'s effect on PLC- β activity *in vitro*, defined the effect of blocking PIP₃ increases with PI 3-kinase inhibitors on inositol phosphate accumulation in whole cells, and investigated the role of PIP₃ in PLC- β 3 localization. Cumulatively, these data support a role for PIP₃ in potentiating Ca²⁺-stimulated PLC- β activity.

Our *in vitro* phospholipase activity data suggest that PIP₃ potentiates Ca²⁺-stimulated, but not G protein-stimulated, PLC- β activity (Fig. 1). Previous studies have determined that PIP₃ is a very poor substrate of PLC- β [13], which excludes the possibility that the increased IP₃ accumulation was a result of hydrolysis of PIP₃ at the concentrations tested. Thus PIP₃ potentially allosterically increases Ca²⁺-stimulated PLC- β activity.

In addition, PI(3,5)P₂, another negatively charged phosphatidylinositol phospholipid, did not affect PLC- β 3 activity, which is consistent with other studies showing that PI(3,4)P₂ has no

effect on PLC- β activity [25]. These data illustrate that the effect of PIP₃ on PLC- β is specific and not due to non-specific binding of anionic phospholipids. In our *in vitro* assay system, free Ca²⁺ concentration is 0.15 μ M, which is in the range of normal physiological Ca²⁺ concentrations (0.05-0.5 μ M). PLC- β catalytic activity is very sensitive to changes in Ca²⁺ in the physiological range [26,27]. Moreover, by pre-incubating whole cells with EGTA and thapsigargin to block the increase of cytosolic Ca²⁺, IP₃ production stimulated by carbachol in SH-SY5Y cells was reduced by $47 \pm 4\%$ [28]. Thus 50% of the activation of PLC- β in whole cells following GPCR stimulation may be attributable to the feed forward rise in cytosolic Ca²⁺ to further stimulate PLC- β in a very short time frame. Therefore, although PIP₃ appears to mostly affect Ca²⁺-stimulated PLC- β activity *in vitro*, it may significantly affect GPCR-stimulated IP accumulation in whole cells.

To test this hypothesis, we used LY294002 and wortmannin, structurally unrelated and widely studied PI 3-kinase inhibitors. LY294002 (50 μ M) completely and specifically abolishes PI 3-kinase activity, but not other protein kinases such as PKA, PKC, MAP kinase, Src or diacylglycerol kinase [29]. Furthermore, multiple reports have demonstrated that LY294002 does not affect the cellular level of PI(4,5)P₂, a substrate of PLC- β [29-32]. Here, we observed that LY294002 inhibited carbachol and OT-stimulated IP accumulation in 1321N1 cells and HEK 293 cells expressing OTR, respectively (Fig. 2). LY294002 inhibition of OT effects was reproduced with wortmannin. However, wortmannin has a lower selectivity for PI 3-kinase than LY294002, so we limited dose to 0.5 μ M resulting in consistent but reduced effectiveness compared to LY294002.

Our findings of PI 3-kinase inhibition leading to reduced agonist-induced IP₃ accumulation are reminiscent of previous reports. LY294002 diminished serotonin -induced inositol phosphate formation in primary cultures of rat smooth muscle cells [32]. Wortmannin (0.1 μ M) or LY294002 inhibited lysophosphatidic acid-induced IP₃ formation by 50% in Cos-7 cells [33]. However, a mechanism for these observations was not previously determined.

Although carbachol and oxytocin are agonists for G α_q -coupled receptors and can activate PLC- β , other PLC isoforms, such as PLC- γ , PLC- δ or PLC- ϵ , might be trans-activated when cells are stimulated for 10 minutes. A recent study demonstrates that PLC- β 3 is predominantly involved in acute IP accumulation (up to 90 seconds) while PLC- ϵ is involved in sustained IP accumulation when Rat-1 fibroblast cells are stimulated by different agonists for G protein-coupled receptors [21]. To rule out the involvement of PLC isozymes besides PLC- β in LY294002 inhibition of IP accumulation, 1321N1 cells, HEK 293 cells expressing OTR, and H9c2 cells were stimulated with agonists for only 90 seconds and assayed for IP₃ accumulation. LY294002 inhibits IP₃ accumulation at the early time point (Fig. 3 and data not shown).

LY294002 does not affect agonist binding to G protein-coupled receptors in our system. From our [³H]QNB binding data in 1321N1 cells and [³H]OT binding data in HEK 293 cells expressing OTR, no change of apparent B_{max} or K_d were observed when cell membrane fractions were treated with LY294002 (supplementary Fig. 2). Nevertheless, LY294002 may affect G protein-coupled activation of phosphatidylinositol hydrolysis through targets in the signaling cascade between agonist activation of the receptor and stimulation of PLC- β . RGS (Regulator of G Protein Signaling) proteins are important signaling factors involved in regulation of IP₃ accumulation. Previous reports suggest that PIP₃ binds to RGS4 and inhibits its GTPase-accelerating activity [34, 35]. Suppression of RGS inhibition activity may play a role in potentiation of PLC- β activity by PIP₃ in whole cell assays. While we do not rule out an effect of PIP₃ to stimulate PLC- β by attenuation of RGS inhibition, our *in vitro* assay data demonstrate that PIP₃ can directly affect PLC- β activity. Defining the relative overall contribution of RGS suppression and direct potentiation of PLC- β activity by PIP₃ to increase agonist-induced inositol phosphate accumulation awaits further study.

To define a possible mechanism by which PIP₃ potentiates PLC-β activity in whole cells, we investigated the effect of increased PIP₃ levels on the localization of PLC-β3 by co-transfecting p110CAAX, a constitutively active PI 3-kinase [14,23]. Interestingly, more PLC-β3 localized at the membrane in cells co-transfected with p110CAAX (Fig. 5).

PIP₃ recruits several PH domain-containing proteins to the plasma membrane [11]. Although the PH domain of PLC-δ1 binds PI(4,5)P₂ with high affinity and specificity [36], the PH domain of PLC-β was found predominantly in the cytosol of transfected HEK 293 cells (Fig. 6), suggesting other domains of PLC-β are important for membrane association. Previous work on the structure of PLC-β2 [4], as well as homology models of PLC-β isozymes [37], indicate that the PLC-β PH domains lack electrostatic potentials to bind phosphoinositide phospholipids with any significant affinity.

To identify a potential PIP₃ binding domain in PLC-β3, we scanned the amino acid sequence for polybasic clusters that may bind negatively charged lipids for membrane targeting [38]. Two polybasic amino acid clusters were identified within the C-tail of PLC-β; these clusters were previously identified in PLC-β1 as involved in Gα_q-dependent activation [5]. In addition, the crystal structure of the C-tail of turkey PLC-β is highly electrostatically polarized with a large basic surface [7], suggesting a role the C-tail domain in membrane and anionic phospholipid binding. A role for the C2 domain of protein kinase Cα in binding PIP₃ has recently been proposed and represents another potential PIP₃ binding site in PLC-β isoenzymes [39].

Lipids are known to be important in PLC regulation. PLC-γ can be activated by PIP₃. The PH domain and Src homology 2 (SH2) domains of PLC-γ have been proposed to be important for PIP₃ binding [25,40]. PI 3-kinase inhibitor (LY294002) blocks the stimulation of PLC-γ activity by calcium in human keratinocytes [41], which is similar to our *in vitro* data on LY294002 inhibition of Ca²⁺-stimulated PLC-β activity. Phosphatidic acid has been demonstrated to bind PLC-β1 and increases Ca²⁺-stimulated activity *in vitro* [42] through a region that includes the αA helix and flexible loop of the Gα_q-binding domain of the C-tail [43]. Moreover, one group has reported that the PLC-β1 PH domain translocates rapidly but transiently to the plasma membrane upon stimulation of cells with serum or lysophosphatidic acid, and the PLC-β1 PH domain appears to bind PI-3P *in vitro* [33].

It is interesting that modulation of PI 3-kinase has been reported to affect agonist-stimulated smooth muscle contraction [44,45], a physiological effect of PLC-β activation [46,47]. In aortic strips from normal Wistar-Kyoto rats, LY294002 inhibits noradrenaline-induced contraction mediated by α₁-adrenergic receptors coupled to Gα_q [44]. Similarly, using intact medial strips of the swine carotid artery, histamine-stimulated contraction was significantly decreased in the presence of LY294002 [45]. A PI 3-kinase-γ knockout study indicated that PI 3-kinase-γ makes a small but significant contribution to thrombin-stimulated calcium response in platelets [48], suggesting that PI 3-kinase-γ, which is uniquely activated by heterotrimeric G proteins [9], plays a role in the PLC-β-mediated release of calcium. However, LY294002, an inhibitor affecting all known PI 3-kinases, impairs the thrombin-induced calcium rise by a larger extent [48], implicating that more than one PI 3-kinase isoforms, rather than PI 3-kinase-γ alone, are involved in PLC-β activation, presumably through elevation of PIP₃. These data support our findings for a physiological relevance of PIP₃ effects on PLC-β at the organ level. Although these phenomena are intriguing, the related molecular mechanisms for LY294002 inhibition of PLC-β-dependent processes were not clearly defined.

In conclusion, our study shows that PIP₃ potentiates Ca²⁺-stimulated PLC-β activities using an *in vitro* reconstitution assay. Both LY294002 and wortmannin, inhibitors of PI 3-kinase, inhibit PLC-β activity in whole cells. In addition, LY294002 does not affect agonist binding

to G protein-coupled receptors. Increasing cellular PIP₃ levels by co-transfecting p110CAAX, a constitutively active PI 3-kinase, promotes more PLC-β₃ localized at the membrane in cells. Based on our data, we propose a model whereby agonists of GPCR induce PIP₃ production, presumably via PI 3-kinase coupled to Gβγ, and potentiate PLC-β activity, possibly by membrane recruitment, repression of RGS activity or both.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. PIP₃ increases Ca^{2+} stimulated, but not $G\alpha_{11}$ or $G\beta\gamma$ stimulated, PLC-β activity *in vitro*
 Reconstitution PIP₂ hydrolysis assay was performed as described in materials and methods.
A. Stimulation of PLC-β3 (35 ng) activity by 50 ng $G\alpha_{11}$ or $GTP\gamma S$ -activated $G\alpha_{11}$ ($G\alpha_{11}$ + $GTP\gamma S$) was quantitated in the presence of 15 μM PI(4,5)P₂ (white bar) or 15 μM PI(4,5)P₂ plus 5 μM PIP₃ (gray bar). **B.** Stimulation of PLC-β3 (35 ng) activity by 0.15 μM free Ca^{2+} (Ca^{2+} buffer) or 60 ng $G\beta_1\gamma_2$ ($G\beta\gamma$) was quantitated in the presence of 15 μM PI(4,5)P₂ (white bar) or 15 μM PI(4,5)P₂ plus 5 μM PIP₃ (gray bar). **C.** Stimulation of PLC-β3 (35 ng) activity by 0.15 μM free Ca^{2+} (Ca^{2+} buffer) or 60 ng $G\beta_1\gamma_2$ ($G\beta\gamma$) was quantitated in the presence of 15 μM PI(4,5)P₂ (white bar) or 15 μM PI(4,5)P₂ plus 5 μM PI(3,5)P₂ (gray bar). **D.** Ca^{2+} -stimulated PLC-β1 (12 ng), PLC-β2 (40 ng) or PLC-β3 (35 ng) activity was quantitated in the

presence of 15 μM PI(4,5) P_2 (white bar) or 15 μM PI(4,5) P_2 plus 5 μM PIP $_3$ (gray bar). Results are mean $\pm$ S.D for triplicate determination and are representative of two experiments. ** indicates $p < 0.01$ and *** indicates $p < 0.001$ compared with controls.

Figure 2. LY294002 inhibits agonist-stimulated IPn accumulation in whole cells

A, 1321N1 cells were pre-treated with 0.1% DMSO vehicle (white bars) or two concentrations of LY294002, 10 μ M (grey bars) or 50 μ M (black bars), for 30 minutes followed by stimulation with 1 mM Carbachol (Carbachol) or DMEM vehicle (Basal) for 10 minutes. Cells were lysed and total inositol phosphates (IP) were extracted and quantitated as described in materials and methods. **B**, Transfected HEK 293 cells stably expressing human oxytocin receptor were pre-treated with 0.1% DMSO vehicle (white bars) or 50 μ M LY294002 (grey bars) for 30 minutes followed by stimulation with 100 nM oxytocin (OT) or DMEM vehicle (Basal) for 10 minutes. Cells were lysed and total inositol phosphates (IP) were quantitated. Results are mean $\pm$ S.D for triplicate determination and *** indicates $p < 0.001$ compared with DMSO controls. **C**,

1321N1 cells were pretreated with 0.1% DMSO vehicle (DM, lanes 1, 3 and 4) or 50 μ M LY294002 (LY, lanes 2, 5 and 6) in Kreb's solution for 30 min followed by stimulation with 1mM carbachol for 10 minutes (Carb, lanes 3-6). Cell lysates were separated by SDS-PAGE and phospho-Akt (top panel) or β -actin (bottom panel) was detected by Western blot with selective antibodies.

Fig. 3

Figure 3. PI 3-kinase inhibitors inhibit agonist-stimulated IP₃ accumulation in whole cells

A, 1321N1 cells were pre-treated with 0.1% DMSO vehicle (white bars) or 50 μ M LY294002 (grey bars) for 30 minutes followed by stimulation with 1 mM Carbachol or DMEM vehicle (Basal) for 90 seconds. Cells were lysed and total inositol trisphosphate (IP₃) was extracted and quantitated as described materials and methods. **B**, Transfected HEK 293 cells stably expressing human oxytocin receptor were pre-treated with 0.1% DMSO vehicle (white bars) or 50 μ M LY294002 (grey bars) for 30 minutes followed by stimulation with 100 nM oxytocin (OT) or DMEM vehicle (Basal) for 90 seconds. Cells were lysed and total inositol trisphosphate (IP₃) was quantitated. **C**, Transfected HEK 293 cells stably expressing human oxytocin receptor were pre-treated with 0.05% DMSO vehicle (white bars) or 0.5 μ M wortmannin (grey

bars) for 30 minutes followed by stimulation with 100 nM oxytocin (OT) or DMEM vehicle (Basal) for 90 seconds. Cells were lysed and total inositol trisphosphate (IP₃) was quantitated. Results are mean $\pm$ S.D for triplicates. *** indicates $p < 0.001$ and ** indicates $p < 0.01$ compared with DMSO controls.

Figure 4. p110CAAX, constitutively activated PI 3-kinase, potentiates agonist-stimulated IP₃ accumulation in whole cells

A, HEK 293 cells stably expressing human oxytocin receptor were transfected with vehicle control (Mock) or p110CAAX (PI3K*) for 48 hours followed by stimulation with 100 nM oxytocin (OT) or DMEM vehicle (Basal) for 90 seconds. Cells were lysed and total inositol trisphosphate (IP₃) was quantitated. Results are mean $\pm$ S.D for triplicate determination and * indicates $p < 0.05$ compared with DMSO controls. **B**, HEK 293 cells were transfected with vehicle control (Mock, lanes 1 and 2) or p110CAAX (PI3K*, lanes 3 and 4) for 48 hours and cell lysates were separated by SDS-PAGE. Phospho-Akt (top panel) or β -actin (bottom panel) was detected by Western blot with selective antibodies.

Fig. 5

Figure 5. p110CAAX, constitutively activated PI 3-kinase, increases PLC-β3 membrane association
A, HEK 293 cells were transiently transfected with GFP tagged Akt-PH domain (Akt-PH) or GFP tagged Akt-PH R25C mutant (Akt-PH (R25C)). **B**, HEK 293 cells were co-transfected with GFP tagged PLC-β3 plus mock vector (β3+Mock) or GFP tagged PLC-β3 plus p110CAAX (β3+PI3K*). After transfected 48 hours, cells were fixed and GFP fluorescence was detected at 488 nm by confocal microscopy. Scale bar is 5 μm.

Figure 6. PLC-β PH domain localization to cytosol is not affected by p110CAAX-over-expression
HEK 293 cells were transiently transfected with GFP tagged PLC-β1 PH domain (PLC-β1 PH) or GFP tagged PLC-β3 PH domain (PLC-β3 PH). After transfected 48 hours, cells were fixed and GFP fluorescence was detected at 488 nm by confocal microscopy. Scale bar is 2 μm.