Calmodulin Is a Phospholipase C-β Interacting Protein

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Phospholipase C-β (PLCβ) is an important effector enzyme in G protein-coupled signaling pathways. Activation of PLCβ by Go and Gβγ subunits has been fairly well characterized, but little is known about other protein interactions that may also regulate PLCβ function. A yeast two-hybrid screen of a mouse brain cDNA library with the amino terminus of PLCβ3 has yielded potential PLCβ3 interacting proteins including calmodulin (CaM). Physical interaction between CaM and PLCβ3 is supported by a positive secondary screen in yeast and the identification of a CaM binding site in the amino terminus of PLCβ3. Co-precipitation of in vitro translated and transcribed amino- and carboxyl-terminal PLCβ3 revealed CaM binding at a putative amino-terminal binding site. Direct physical interaction of PLCβ3 and PLCβ isoforms with CaM is supported by pull-down of both isoenzymes with CaM-Sepharose beads from 1321N1 cell lysates. CaM inhibitors reduced M1-muscarinic receptor stimulation of inositol phospholipid hydrolysis in 1321N1 astrocytoma cells consistent with a physiologic role for CaM in modulation of PLCβ activity. There was no effect of CaM kinase II inhibitors, KN-93 and KN-62, on M1-muscarinic receptor stimulation of inositol phosphate hydrolysis, consistent with a direct interaction between PLCβ isoforms and CaM.

Phospholipase C-β (PLCβ) enzymes are key effectors in G protein-linked receptor-mediated signaling cascades. In response to external cellular stimuli and subsequent activation of G protein-coupled receptors, PLCβ cleaves phosphatidylinositol bisphosphate into inositol triphosphate (IP3) and diacylglycerol. IP3 mediates an increase in cytosolic Ca2+ by releasing intracellular stores while diacylglycerol activates protein kinase C (1). Four isoforms of PLCβ, 1–4, have been identified in mammals. PLCβ2 and PLCβ4 have limited tissue distributions, whereas PLCβ1 and PLCβ3 are nearly ubiquitous in human tissues; PLCβ1 is dominant in brain and PLCβ3 is dominant in rat heart and smooth muscle (2–10). All four mammalian PLCβ isoenzymes are activated by Goα-type G-protein subunits to various degrees, but only PLCβ1 and PLCβ3 are activated by Gβγ dimers. PLCβs, with some isozyme differences, may be purified from both cytosolic and particulate fractions of cells (11, 12), suggesting that in addition to activation, translocation to substrate at the membrane or maintenance of the enzyme at the plasma membrane may be an additional means of regulation.

PLCβ proteins, as a family, contain at least five distinct structural domains, identified by homology to the crystal structure of PLCγ (13). The amino terminus contains a pleckstrin homology (PH) domain, a known protein interaction and membrane-binding domain that may also be a region for Gβγ subunits interaction with PLCβ1 and -β3 isoforms (14, 15). In all PLCβ isoenzymes, the PH domain is followed sequentially by four EF-hand domains, which bind Ca2+ in other protein contexts but whose function in PLCβs has yet to be characterized. The middle third of the PLCβ proteins contains catalytic X and Y domains, the activity of which requires a Ca2+ co-factor. The COOH-terminal portion of PLCβ isoforms has recently been crystallized as a dimer from the turkey PLCβ homologue and hypothesized to be the domain responsible for the dimerization of the full-length enzyme (16). This region also contains a site for activation by Goα subunits (17, 18) in all PLCβ isoenzymes and a 4-amino acid PDZ-binding domain in PLCβ1, 2, and 3 (19, 20). Whereas there is significant homology among mammalian PLCβ isoforms (35–55% in full-length protein and 80–90% in catalytic domains), the differences in G-protein selectivity for activation and subcellular localization among isoforms suggest that each has unique mechanisms of regulation.

Given the ubiquitous distribution of PLCβ1 and PLCβ3 and their differential regulation by Gβγ G-protein subunits, isozyme-specific modifiers of PLCβ activity may be postulated. A yeast two-hybrid method was used to screen a mouse cDNA library and identify proteins that interact with the PH and/or the EF-hand domain of PLCβ. Several candidates have been identified, most intriguing being calmodulin (CaM). PLCβ3 is a Ca2+-sensitive protein whose activation leads to increases in cytosolic Ca2+ levels. CaM acts as an intracellular Ca2+ sensor and is a known regulator of other membrane-associated proteins and thus is a good candidate for interaction with and regulation of PLCβ3. We have detected PLCβ3 and PLCβ3 expression in 1321N1 human astrocytomas, and using this cell line as a model system, determined that CaM is a regulator of G protein-coupled receptor-stimulated PLCβ activity.
PLCβ Interaction with Calmodulin

EXPERIMENTAL PROCEDURES

Materials—KN-62, KN-93, KN-92, W-13, and fluphenazine were obtained from Calbiochem (San Diego, CA). Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech Cellgro (Herdon, VA). Fetal bovine serum was purchased from Hyclone (Logan, UT). myo-[3H]inositol and protein A-Sepharose were purchased from Amersham Biosciences. PLCβ-selective polyclonal rabbit antisera and alkaline phosphatase-conjugated goat anti-rabbit IgG secondary antibody were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Immunoblots were subsequently stripped and purchased from Bio-Rad. CaM-Sepharose 4B was purchased from Amersham Biosciences. pBTM116 and pBL1 vectors, and L40 and PL3α Saccharomyces cerevisiae yeast strains were kindly provided by Dr. Mark Leid (Oregon State University, Corvallis, OR).

Yeast Two-hybrid Screening Assay—Yeast two-hybrid screening was performed as described previously (21, 22) with the following modifications. cDNA coding for the amino-terminal PH and EF-hand domain regions of PLCβ2 (amino acids 2–315; PH-EF-β2) was PCR amplified to incorporate 5′ EcoRI and 3′ XhoI restriction sites with the following primers: 5′-ATATATCTGGCCGGGCGC and 3′-TATATACTGGATCAGATCCGGTTTCAGG. The PCR-amplified fragment was subcloned into phIP116 (a tryptophan-selectable yeast expression plasmid) in-frame with the lexA DNA-binding domain as the “bait” vector. PCR amplification and in-frame insertion was confirmed by dideoxy sequencing using pBTM116-specific forward and reverse sequencing primers. A random-primer mouse brain cDNA library subcloned in-frame with the GAL4 activation domain into the pACT2 vector (a leucine-selectable yeast expression plasmid; Clontech) was used as bait. S. cerevisiae L40 reporter yeast strain containing the PH-EF-PLCβ135/315; PH-EF-β2 bait plasmid was co-transformed with pLexA reporter activity under the control of estrogen response elements. Transformants were selected for growth on Ura−Leu−Trp− selective plates in the presence of 50 mM aminotriazole to suppress LexA−α-galactosidase activity in the absence of interaction. 150 growth-positive clones were isolated and screened for β-galactosidase activity in liquid assay as described previously (21). Positive clones with β-galactosidase activities of at least twice background (defined by β-galactosidase activity from L40 yeast transformed with bait plasmid only) were selected for further characterization.

For amplification and sequencing of positive clones, growth selection, and β-galactosidase-positive yeast were grown for 3 days on His−Leu−medium to allow for loss of bait plasmid. Plasmid DNA was isolated, amplified using pACT2 forward and reverse primers (Clontech), sequenced with the same primers, and analyzed by BLAST search (National Institutes of Health, National Library of Medicine) for similarity to known sequences. Previously identified sequences were assessed in a secondary hybridization bindscreen interaction with PH-EF-β2 bait plasmid in a different vector context. For secondary screening, positive clones in the pACT2 vector were co-transformed into the S. cerevisiae strain L315αalong with pBL1 (a histidine-selectable yeast expression plasmid) containing the PH-EF-PLCβ fragment subcloned in-frame with the estrogen receptor DNA-binding domain. PL3α yeast contain a URA3 reporter activity under the control of estrogen response elements. Transformants were selected for growth on Ura−His−Leu−medium.

Inositol Phosphate Assay—Inositol phosphate (IP) assays were performed with 1321N1 cells as previously reported (23). 1321N1 cells were routinely subcultured in DMEM, 10% fetal bovine serum and standard antibiotics (penicillin and streptomycin) in humidified 5% CO2 at 37 °C. For in vivo experiments, 1321N1 cells were subcultured in 24-well plates at a density of 1 × 105 cells/well and allowed to attach overnight. Cells were then labeled overnight with myo-[3H]inositol, 1 µCi/0.5 ml well, prepared in sterile inositol-free, bicarbonate-buffered DMEM without additives. Following radiolabeling, cells were pre-treated for 30 min (KN-62, KN-92, and KN-93) or 10 min (W-13 and fluphenazine) in 20 mM Hepes-buffered DMEM, pH 7.4 (HDMEM), at 37 °C in room air. Following pre-treatment, 10 mM LiCl was added for 10 min followed by stimulation of muscarinic receptors with 1 mM carbobachol for 20 min. Following stimulation, cells were lysed with 0.5% cold trichloroacetic acid and the soluble lysate was ether extracted three times. Inositol phosphates (IP1, IP2, and IP3) were purified by anion exchange chromatography with ammonium formate. Lipids were solubilized with 1 N NaOH and collected to quantitate [3H]inositol phospholipids. Samples were harvested into scintillation vials and radioactivity was quantitated in a liquid scintillation counter. Percent (×) conversion was calculated as [(IP3 radioactivity (dpm) − [3H]inositol phospholipids (dpm)) / [3H]inositol phospholipids (dpm)]. All assays were performed in triplicate and values reported reflect an average of at least three experiments ± S.E. Student's t tests were performed to assess statistical significance where indicated.

Cell Fractionation and Immunoprecipitation—1321N1 cells were plated at 3 × 104 cells/cm2 plate and grown for 3 days under standard conditions. On day 3, cells were washed twice with HDMEM and collected by scraping and pelleting at 500 × g for 5 min. Cold lysis buffer (0.6 M 10 mM Tris, pH 7.4, 5 mM MgCl2, and protease inhibitors, 200 µM benzamidine, 200 µM PMSF, 2 µg pepstatin A, and 2 µg leupeptin) was added to each sample and incubated on ice for 10 min. Cells were lysed with 15 strokes of a Dounce homogenizer and a 50-µl fraction was saved as crude lysate. The remainder of the lysate was centrifuged at 500 × g, 4 °C, for 5 min to pellet nuclei and intact cells. The low speed supernatant was centrifuged at 500,000 × g, 4 °C, for 25 min to pellet soluble and particulate fractions. The particulate (membrane) fraction was resuspended in 0.6 ml of extraction buffer (50 mM HEPES, 2.5 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM dithiothreitol, and protease inhibitors) and incubated at 4 °C with inversion. After extraction, the membrane fraction was centrifuged at 16,000 × g, 4 °C, for 30 min to pellet detergent-insoluble particulates. Supernatant was saved as membrane extract.

For immunoprecipitation, anti-PLCβ isozyme-selective polyclonal antibodies were added to soluble (cytosol) and membrane extract fractions at a 1:200 dilution and incubated at 4 °C overnight with continuous inversion. Immune complexes were precipitated with 50 µl of Protein A-Sepharose that had been washed and equilibrated in extraction buffer (v/v) according to the manufacturer’s specifications. The resulting immunoprecipitates were separated by 7.5% SDS-PAGE. The separated proteins were transferred electrophoretically to nitrocellulose paper, incubated with PLCβ-selective polyclonal antibodies at a 1:1000 dilution followed by alkaline phosphatase-conjugated goat anti-rabbit antibodies at 1:5000 dilution, and visualized with alkaline phosphatase chemiluminescent substrate (ImmuneStar™) according to the manufacturer’s specifications.

For preparation of cell lysates for CaM-Sepharose 4B binding assay, 1321N1 cells were plated at 3 × 104 cells/cm2 plate and grown for 3 days at 37 °C with 5% CO2 in DMEM + 10% fetal bovine serum and antibiotics. Plated cells were washed twice with HDMEM. Cells were scraped into tubes and pelleted at 500 × g for 5 min. Cold lysis buffer (0.6 M 10 mM Tris, pH 7.4, 5 mM MgCl2, 2 mM CaCl2, and protease inhibitors) was added to each sample and incubated on ice for 10 min. Cells were lysed with 15 strokes of a Dounce homogenizer. The lysate was centrifuged at 500 × g, 4 °C, for 5 min to pellet nuclei and intact cells prior to incubation with CaM-Sepharose 4B beads as described below.

Transcription and Translation of Radiolabeled Proteins—[35S]Methionine-labeled amino-terminal and carboxyl-terminal PLCβ2 were prepared according to the manufacturer’s instructions using the in vitro transcription/translation system (Promega, Madison, WI). Briefly, 1 µg of pCDNA3 plasmid containing cDNA sequence for the amino- (amino acids 1–315, N-PLCβ2) or carboxyl- (amino acids 670–1173, C-PLCβ2) terminal sequences of PLCβ2, were incubated with TNT™ reticulocyte lysate and 25 µCi of [35S]methionine in a total volume of 50 µl for 90 min at 30 °C. Labeling efficiency and level of protein expression were evaluated by SDS-PAGE and autoradiography (described below).

CaM-Sepharose 4B Binding Assay—The CaM-Sepharose binding assay with [35S]methionine-labeled proteins was performed according to the protocol of Chuang et al. (24) with the following modifications. CaM-Sepharose 4B resin was washed twice in 500 µl of D2O, 4 °C, and equilibrated for 1 h in binding buffers (50 mM MOPS, pH 7.2, 100 mM NaCl, 2 mM CaCl2, and 1 mM dithiothreitol) or (in the absence of Ca2+) made with calcium calibration buffers (K2-EGTA and/or CaEGTA in varying amounts) from Molecular Probes (Eugene, OR). Free Ca2+ concentrations ranged from 0 to 39 µM. Equivalent radioactive quantities of radiolabeled [35S]-N-PLCβ2 or [35S]-C-PLCβ2 were incubated with 50 µl of pre-equilibrated calmodulin-Sepharose 4B resin in a total volume of 200 µl overnight at 4 °C with incubation of the resin for 3 h with CaCl2. Ca2+-bound material was removed by washing three times with 200 µl of binding buffer. SDS sample buffer was added to the final pellet and the calmodulin-Sepharose precipitates were resolved by 12.5% SDS-PAGE. For autoradiography the resultant gel was soaked in glacial acetic acid for 20 min, impregnated with 10% 2,4-dihydroxyxazole scintillant for 15 min, washed in ddH2O for 10 min before being dried. Curved gel material was cut out with a filter paper. The gels were exposed overnight to x-ray film and the [35S]radioactive bands were visualized.

For cell lysates (prepared as described in the cell fractionation and immunoprecipitation section) 70 µl of a 50% slurry of CaM-Sepharose...
Table 1
Specific interaction of PLCβ, with CaM

Table of β-galactosidase activity of representative growth-positve clones from the yeast two-hybrid assay. β-Galactosidase-positive clones were identified by activities at least 2-fold higher than background. Mitochondrial proteins are often considered to be false positives in yeast two-hybrid screens and were not pursued further.

| Selected positive clones | Fold above background |
|--------------------------|----------------------|
| Calmodulin | 3.5 |
| 14–3-3δ | 7.5 |
| γ-Actin | 4.0 |
| Cytochrome C oxidase | 3.5 |

Identification of CaM/PLCβ interaction—To identify novel protein interactions with PLCβ, we utilized a yeast two-hybrid screening system. A mouse brain cDNA library was screened with the NH2-terminal (N-PLCβ) as bait. Of the positive clones identified in this screen, full-length mouse calmodulin was isolated at least three times and interaction was confirmed by a β-galactosidase assay (Table 1) and a secondary growth screen as described under “Experimental Procedures” (Fig. 1).

The Calmodulin Binding Site—Binding site search and analysis site The Calmodulin Target Database was used for computer analysis of the PLCβ protein sequence. Three putative calmodulin-binding sites were identified in the full-length PLCβ protein sequence by hydropathy, α-helical propensity, residue weight, residue charge, hydrophobic residue content, helical class, and occurrence of key residues. Two of these sites are located in the COOH-terminal region and one in the NH2-terminal region of PLCβ. The NH2-terminal CaM-binding site is shared by PLCβ and has 90% identity to the NH2-terminal-binding site in PLCβ (Fig. 2A). When compared with other CaM-binding domains, conserved hydrophobic and positively charged amino acids were identified in the PLCβ and PLCβ sequences consistent with putative calmodulin-binding domains (Fig. 2B).

PLCβ and PLCβ Are Expressed in 1321N1 Cells—1321N1 cells express Gα-coupled muscarinic receptors that are activated by carbachol to hydrolyze phospholipids. To establish which PLCβ isoforms are expressed in 1321N1 cells to mediate G protein-activated inositol phospholipid hydrolysis, we performed immunoprecipitation and Western blot analysis using PLCβ isoenzyme selective antibodies. We determined that PLCβ and PLCβ isoenzymes are expressed in 1321N1 cells, whereas PLCβ and PLCβ are not expressed at detectable levels (Fig. 3). Having confirmed the presence of PLCβ in 1321N1 cells, we investigated the possible interaction of the enzyme with calmodulin in vitro.

N-PLCβ Physically Interacts with CaM—To verify that the CaM-binding domains of PLCβ physically interact with CaM, a co-precipitation assay was performed using 35S-radiolabeled NH2-terminal (N-PLCβ) or C-terminal (C-PLCβ) regions of PLCβ (see “Experimental Procedures”) and CaM-Sepharose beads. In vitro transcription and translation and 35S radiolabeling of N-PLCβ and C-PLCβ were verified by SDS-PAGE and autoradiography (not shown). Co-precipitation assays were performed with 35S-N-PLCβ (Fig. 4A) in the presence of binding buffers containing the indicated concentrations of free Ca2+ (Fig. 4A). N-PLCβ protein bound most strongly in the buffer containing no free Ca2+ and bound less effectively with increased Ca2+. Co-precipitation of C-PLCβ (Fig. 4B) with CaM-Sepharose in the presence of binding buffers containing the indicated concentrations of free Ca2+ revealed that C-PLCβ did not bind CaM-Sepharose under any of the above conditions (Fig. 4B). Binding of N-PLCβ to CaM occurred in the absence of free Ca2+ in the presence of varying NaCl concentrations up to 200 mM (data not shown).

Full-length PLCβ and PLCβ Are Isolated from Cell Lysates by CaM-Sepharose Beads—To determine whether the physical interaction between CaM and N-PLCβ identified by the in vitro co-precipitation assay occurred with the full-length protein, we used CaM-Sepharose beads to precipitate PLCβ or

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*a* calcium.uhnres.utoronto.ca/ctdb/pub_pages/general/index.htm.
PLCβ₁ from 1321N1 whole cell lysates. Cell lysates (supernatant from a low speed centrifugation) were incubated overnight with CaM-Sepharose beads in 1% Triton X-100. The beads were pelleted, washed, and the CaM-binding proteins were eluted with SDS-PAGE loading buffer. The eluted proteins were separated by SDS-PAGE and Western blotting was performed with anti-PLCβ-specific antibodies: PLCβ₁ (A), PLCβ₂ (B), PLCβ₃ (C), and PLCβ₁ (D). Migration of molecular weight markers (kDa) are indicated on the right. Arrows indicate migration of purified protein standards for PLCβ₁ and PLCβ₂, or expected migration by size of PLCβ₁ and PLCβ₂.

**Calmodulin Antagonists Inhibit Activity of PLCβ in Vivo**—To ascertain the potential physiological consequences of the interaction between CaM and PLCβ, we treated 1321N1 cells with CaM inhibitors (W-13 and fluphenazine) and calmodulin-dependent kinase II (CaMKII) inhibitors (KN-93 and KN-62), and assayed for effects on PLCβ activity by measuring subsequent carbachol-stimulated IP hydrolysis. CaM antagonists, W-13 and fluphenazine, reduced carbachol-stimulated IP hydrolysis in intact 1321N1 cells by 52 and 60%, respectively, compared with carbachol-stimulated IP hydrolysis in pre-treated cells (Fig. 5A), suggesting that CaM is supportive of PLCβ activity in 1321N1 cells.

To determine whether the inhibition of PLCβ activity by W-13 was sensitive to stimulation of IP hydrolysis or changes in intracellular Ca²⁺ concentration, we investigated effects of calmodulin inhibitor W-13 on IP hydrolysis either concurrent with carbachol activation or 10 min post-activation. When W-13 was added concurrently with carbachol, IP hydrolysis was inhibited by 50%, whereas only 25% inhibition occurred when W-13 was added 10 min post-activation (Fig. 5B).

To address whether CaM kinase may mediate the effects of CaM on PLCβ activity, 1321N1 cells were treated with CaMKII inhibitors, 30 µM KN-93 or 2 µM KN-62 (26, 27). In 1321N1 cells pre-treated with CaMKII inhibitors for 30 min, there was no effect on basal or carbachol-stimulated IP hydrolysis (Fig. 6). CaMKII inhibitors had no effect on basal or carbachol-stimulated IP hydrolysis in 1321N1 cells at concentrations ranging from 1 nM to 100 µM (data not shown). The lack of effect of CaMKII antagonists in this system supports a physiological role for direct interaction between PLCβ enzymes and CaM without requiring activation of CaMKII.

**DISCUSSION**

The PLCβ class of enzymes is a key component in G-protein-linked receptor-mediated signaling cascades. In response to external cellular stimuli and subsequent activation of G protein-coupled receptors, PLCβ is responsible for the production of IP₃ and DAG, two important second messengers in a variety of cellular functions. Examples of a few of the receptors that act through the G-protein/PLCβ pathway include thromboxane A₂, bradykinin, angiotensin, histamine, vasopressin, M₁-type muscarinic cholinergic, and thyroid stimulating hormone. Efforts are being made to elucidate differences in signaling through PLCβ pathways to identify tissue and/or receptor-specific targets for regulation. Novel putative regulators of PLCβ isoenzymes identified recently include: NHERF2, which is postulated to interact with the 8-amino acid carboxyl terminus of PLCβ (19); de-polymerized tubulin, which interacts with PLCβ₁ and interferes with Go₃ activation (28, 29); and Rac1, which activates PLCβ₂ (30–32).

1321N1 cells are an established human astrocytoma cell line extensively used to study G protein-coupled inositol phospholipid hydrolysis. In 1321N1 astrocytoma cells, PLCβ₃ segregates mostly with cellular particulate rather than soluble fractions, whereas the cellular distribution of PLCβ₁ is such that it is primarily isolated from soluble fractions. In addition to different subcellular locations of these enzymes in 1321N1 cells, previous work in other cell lines describe differences in activation among the isoforms (33, 34) suggestive of unique mechanisms of regulation. Our laboratory has sought to identify novel regulators of PLCβ₁ and/or PLCβ₃ isoyme signaling.

Full-length CaM was identified as a possible PLCβ₃ interacting protein by yeast two-hybrid screening of a mouse brain cDNA library. Sequence analysis of the full-length PLCβ₃ protein reveals three putative CaM-binding sites, one in the amino terminus and two in the carboxyl tail of the protein. PLCβ₃ has a CaM-binding site in the NH₂-terminal with 90% identity to the PLCβ₁ CaM-binding site. The best described CaM binding motif is an IQ motif (35); however, other non-IQ CaM-binding domains, both Ca²⁺-independent and Ca²⁺-dependent, have 3 T. M. Fritz, unpublished observations.
been identified as short 16–35-amino acid regions that segregate hydrophobic residues from basic and polar residues on an α-helical wheel projection (36). Non-IQ CaM-binding domains are found in other membrane-associated proteins including G protein-coupled receptor kinases (37), regulators of G-protein signaling (RGS) proteins (38), and myristoylated alanine-rich C kinase substrate protein (39, 40), among others. The CaM-binding site identified in the NH2-terminal regions of PLCβ3 and PLCβ1 has a similar distribution of amphiphilic residues as these non-IQ motif CaM-binding proteins (Fig. 2B).

PLCβ enzymes are Ca2+/CaM-sensitive proteins (12) that associate with the plasma membrane, both of which are properties associated with CaM-regulated proteins such as phosphatidylinositol 3-kinase (41), RGS proteins (38), and myristoylated alanine-rich C kinase substrate (42). Ca2+/CaM regulates many signaling molecules that are also sensitive to Gβγ and phospholipids similarly to PLCβ3 and PLCβ1. Anionic phospholipid-binding sites and CaM-binding sites share some similarities and may overlap in certain proteins. Ca2+/CaM attenuates the ability of PIP3 to inhibit RGS4 intrinsic GTPase activity without activating RGS directly (38). Membrane association of G protein-coupled receptor kinases is required for activation and is enhanced by phosphatidylinositol bisphosphate and Gβγ subunits (43, 44); Ca2+/CaM inhibits several G protein-coupled receptor kinase subtypes as a consequence of reduced phospholipid binding/membrane association (24, 37, 45).

The presence of a CaM-binding site in the NH2-terminal region of PLCβ, and the isolation of N-PLCβ3 and PLCβ1, from cell lysates with CaM-Sepharose beads, suggest that CaM is a PLC regulatory protein that had not been previously recognized. We demonstrated by co-precipitation assays that CaM binds PLCβ3 through the NH2-terminal region. These data suggest that N-PLCβ3 is a Ca2+/CaM-independent CaM-binding peptide (Fig. 4). Data to support physiological significance of a CaM/PLCβ interaction is shown by the isolation of both PLCβ3 and PLCβ1 isomers from 1321N1 cell lysates by CaM-Sepharose (Fig. 5). Inhibition of CaM by W-13 was shown to reduce inositol phospholipid hydrolysis (Fig. 6, A and B) in vitro consistent with an inhibition of PLCβ activity. Inhibition was seen when cells were treated with W-13 prior to activation when intracellular Ca2+ levels are low, and when cells were treated concurrently with activation or 10 min post-activation when intracellular Ca2+ levels are changing because of the activation of PLCβ. These data suggest that CaM is integral in the muscarinic receptor-activated inositol phospholipid hydrolysis pathway, and the association of PLCβ with CaM may be independent of intracellular Ca2+ levels similar to the Ca2+ independence of CaM binding N-PLCβ3 (Fig. 4A). In neural tissues where concentrations of CaM and CaM-binding proteins are very high (46), many CaM-binding proteins are known to associate with CaM when intracellular Ca2+ levels are very low and even in the presence of chelators (47). Neurmodulin binds CaM in the presence of EGTA and Ca2+ disrupts binding (48), similar to the profile seen with N-PLCβ3 binding of CaM (Fig. 4A). Inducible nitric-oxide synthase is an example of a protein that binds CaM independent of Ca2+ levels and is then activated by CaM maximally at 0.1 nM free Ca2+ in vitro (49).

Data herein support a direct interaction between PLCβ and CaM and maintain that regulation of PLCβ activity by calmod-
CaMKII inhibitors in PLCβ/H9252. We were not able to repeat this finding in 1321N1 cells endogenously expressing PLCβ and PLCβ1 (Fig. 7 and data not shown). Regulation and phosphorylation of PLCβ2 may occur through a different mechanism in 1321N1 cells distinct from endogenously expressing transfected cells.

We propose a model whereby CaM facilitates PLCβ activity, possibly by increasing access to substrate. While cognizant that CaMKII is an activator of PLCβ activity, we are currently working to determine the differential effects of CaM on PLCβ2 and PLCβ1 activity, membrane association, or stimulation by G-proteins.

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