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Phospholipase C-β (PLCβ) is an important effector enzyme in G-protein-coupled signaling pathways. Activation of PLCβ by Gα 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 yielded potential PLCβ3-interacting proteins including calmodulin (CaM). Physical interaction between CaM and PLCβ3 was supported by a positive secondary screen and the identification *in silico* of a CaM binding site in the amino terminus of PLCβ3. Co-precipitation of *in vitro* transcribed and translated amino- and carboxy-terminal PLCβ3 fragments revealed CaM binding at a putative amino-terminal binding site. Direct physical interaction of PLCβ3 or PLCβ1 isoforms with CaM was supported by pull-down of both isoenzymes with CaM-sepharose beads from 1321N1 cell lysates. CaM inhibitors reduced muscarinic receptor stimulation of inositol phospholipid (IP) hydrolysis in 1321N1 astrocytoma cells consistent with a physiologic role for CaM in modulation of PLCβ activity. CaM inhibitors significantly reduced ^{3}H-PIP hydrolysis in 1321N1 cells under various activation conditions. There was no effect of CaM kinase II inhibitors on M1-

muscarinic receptor stimulation of IP hydrolysis, consistent with a direct interaction between PLCβ isoforms and CaM. CaM-Sepharose pull-down experiments with PLCβ3 fragments, deletion and truncation mutations show the CaM binding site residing within the putative EF-hand domains of PLCβ3. Fluorescent anisotropy data was used to calculate the binding affinity between CaM and PLCβ1 or PLCβ3 as 260 nM and 320 nM, respectively. The binding interaction between PLCβ3 and CaM appears to occur under physiologically relevant Ca^{2+} concentrations. There was no effect of CaM on basal- and Gαq-stimulated PLCβ1 or PLCβ3 activity. Instead, the interaction between CaM and PLCβ3 leads to potentiation of activation by Gβγ in an *in vitro* hydrolysis assay. CaM does not alter the affinity of PLCβ1 or PLCβ3 for the membrane phospholipids immobilized on nitrocellulose. However, these experiments revealed a previously unappreciated binding of PLCβ3 to PIP3 and specificity for lipid side-chain composition. This work furthers our understanding of the regulation of the PLC isozymes by identifying and characterizing the novel PLCβ activity potentiating protein, CaM.

Regulation of Phospholipase C-beta isozymes by Calmodulin

by

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Theresa M. Filtz discussed experimental analysis, and reviewed all manuscripts. Shana A. Larson helped with the preliminary calmodulin inhibition experiments in 1321N1 cells (Chapter 2). Ryan Millimaki helped with pull-down of phospholipase C-beta from whole cell lysates (Chapter 2). Kristi Crofoot aided in running dot blots (Chapter 3). Dr. Sonia Anderson and Dr. Dean A. Malencik provided purified calmodulin and discussed analysis of fluorescence anisotropy experiments (Chapter 3).

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Chapter 1: Regulation of Phoshoplipase C-beta isozymes by Calmodulin.

Introduction:

Any cellular response requires the activation of a signaling cascade, be it movement, death, cell division or secretion; all cells must be able to respond and react to their cellular environments. Many cellular responses are initiated through membrane receptors. These receptors span the plasma membrane and provide a mechanism by which signals from outside the cell are relayed to signaling complexes within the cell. Equally as important as the receptors is the lipid environment immediately surrounding the receptors. Localized areas in a plasma membrane, rich in receptors, associated signaling proteins, and specific membrane phospholipids known as lipid rafts [1], set the stage for efficient initiation of signaling.

The hydrolysis of membrane phospholipids is a key step in many cell signaling pathways. Enzymes that can catalyze cleavage of acyl or phosphoacyl moieties from membrane phospholipids are phospholipases (PL). PLs are important regulators of cell function, implicated both in the rearrangement of membrane phospholipids and as mediators of cell signaling and metabolism.

Phospholipases:

The four major families of PLs (A1, A2, C and D) are categorized by site of hydrolysis. Target recognition by PLs is influenced both by side chain and head group composition of the membrane phospholipids. Phospholipase C (PLC), also known as phosphoinositide-specific phospholipase, is a key intracellular signaling molecule that once activated, catalyzes the hydrolysis of phosphotidylinositol 4,5-bisphosphate

(PIP2) into inositol triphosphate (IP3), a regulator of intracellular calcium, and diacylglycerol (DAG), a well-characterized activator of protein kinase C (PKC) [2]. PIP2 is also a precursor molecule for the synthesis of phosphotidylinositol 3,4,5-triphosphate (PIP3), by the action of phosphotidylinositol 3-kinase [3], another membrane lipid that is integral to a variety of cell signaling pathways.

PLCs are among the most common effector enzymes activated by transmembrane receptors. Transmembrane receptors are the most common drug targets in the body. A better understanding of the complexity and peculiarities of regulation of these primary effectors enzymes may someday aid in the development of more specific drugs selectivities. To better understand PLC function, we require a complete description of the molecular partners, interacting proteins, subcellular localization and temporal activation and inhibition of these enzymes. Our current molecular understanding is described in the following pages.

Phospholipase C:

The 13 identified mammalian PLC isozymes have been classified into six families, β, γ, δ, ε, ζ, and η [4–7] with a common catalytic domain which hydrolyzes PIP2. There are multiple isoforms in several families of PLCs, as shown in Figure 1.1. Currently described are: β1-4; δ1, 3 and 4; γ1 and 2; η1 and 2; ε1 and ζ1. Additionally, two PLC-like proteins are identified as PLC-L1 and -L2. While PLC-Ls share the presence of a homologous catalytic domain with PLCβ, the amino acids

essential for hydrolysis are have been replaced and the ability to hydrolyze PIP2 eliminated [8, 9].

Structural motifs common to PLC isozymes:

Along with catalytic function, the PLCs share common molecular structural motifs. By better understanding the structural organization of the PLC isozymes, we have gained insights into the function of the proteins in cell signaling and additionally, the unique mechanisms of regulation of the different isozymes. All the PLC isoforms have a two part catalytic domain, designated the X domain and the Y domain, which structurally forms part of a TIM barrel motif. The X and Y domains are separated by a flexible linker 40-190 amino acids long in the PLCβ, δ, ε, η and ζ isoforms, and up to 400 amino acids long in PLCγ. The sequence intervening the X and Y domains in PLCγ contains structural motifs unique among PLCs, including src homology regions SH2 and SH3, which are characterized in other protein contexts as protein-protein interaction domains [10], and a split pleckstrin homology (PH) domain. All the PLC isoforms except PLCζ also have PH domains, but the split domain is unique to PLCγ.

PH domains are best characterized as phosphoinositide binding domains [11] or protein interaction domains. The PH domain in PLCδ has selectivity for PIP2 and is involved in targeting to the membrane. However, there are differences among isoforms of PLCδ in the strength of the PIP2-PH domain interaction [12], suggesting unique roles for the PH in each protein context. Experiments done with a green fluorescent protein fusion of the PLCγ PH domain show that both the amino-terminal

PH domain and the split PH domain are integral in membrane targeting of the enzyme [13]. Besides binding membrane phospholipids, PH domains also participate in protein-protein interactions. The PH domain of PLCβ2 strongly associates with Gβγ, whereas the PH domains of PLCβ1 and PLCδ only weakly associate with Gβγ [14].

In addition to the presence of a functional catalytic domain, PLC isozymes all have a C2 domain. C2 domains were first identified as a conserved sequence motif in Ca^{++}-dependant protein kinase C and are best characterized as regions that bind calcium and phospholipids [15]. However, binding properties of the C2 domain, like most characterized domains, varies in each protein context, as well as within families of proteins. For example, in PLCδ, the C2 domain of PLCδ1 and PLCδ3 exhibit Ca^{++}-dependant membrane targeting, whereas the C2 domain of PLCδ4 binds the plasma membrane independent of Ca^{++} [16]. Along with its lipid and Ca^{++} binding functions, the C2 domain has been found to bind free inositol [17] or other proteins. The C2 domain of PLCβ1 and PLCβ2 associates strongly with GTPγS-bound Gαq and weakly with the inactive, GDP bound form of Gαq. The C2 domain of PLCδ does not bind to Gαq, nor is PLCδ sensitive to activation by Gαq enzymes, unlike PLCβ1 and PLCβ2 [18]. The role of the C2 domain in the other PLC isozymes is still undetermined.

Four putative EF-hand domains adjacent to the PH domain were identified upon crystallization of PLCδ [19] and are another common motif in the PLCs. The EF-hand domain has been best characterized as a Ca^{++}-binding sequence [20], but the Ca^{++}-binding properties of putative EF-hand domains in the PLC isoforms have not

been completely determined. Sequence homology among the isoforms suggests that EF-hand domains are present in PLCβ, γ, ε, η and ζ family members. In PLCζ, the there are four identified EF-hand domains. The third EF-hand domain is responsible for Ca^{++}-sensitivity of the enzyme; the first and second EF-hands are important for overall activity of the enzyme [21]. Specific amino acid differences in the sequence of EF-hand domains in PLCβ isozymes suggests that Ca^{++} may or may not bind all or any of the EF-hand sequences in PLCβ.

Features unique to PLC families:

In addition to the common structural motifs, each of the PLC families has some unique structural characteristics and each has a different mechanism of activation. Knowledge of the unique features of each family of isozymes provides insight into the different methods of regulation and pathways in which the enzymes are integral. The basic signaling mechanism in the PLCγ family of isozymes is well established. PLCγ (PLCγ1 and PLCγ2) isozymes are key mediators of signaling by growth factors and immune responses. PLCγ enzymes are activated and phosphorylated by receptor tyrosine kinases (RTK), and by antigen or immunoglobin receptors that couple to non-receptor tyrosine kinases. Additionally, G-protein coupled receptor transactivation of RTKs can activate PLCγ. The SH2 and SH3 protein-protein interaction domains unique to the PLCγ isoform are sites for receptor or kinase interaction and phosphorylation. Tyrosine phosphorylation is required for activation of PLCγ isozymes. Translocation of the enzyme to the plasma membrane from the

cytosol involves PIP3 binding via the carboxy-terminal SH2 domain and the C2 domain [22]. Like PLCε, δ and ζ, PLCγ lacks the long carboxy-terminal tail seen in PLCβ and η.

There are two isoforms of the PLCε family, PLCε1 and PLCε2. PLCε are unique among PLC family enzymes in possessing guanine-nucleotide exchange factor activity towards Ras-like small GTPases (RasGEF activity) and containing Ras association (RA) domains, which suggest that PLCε is both an activator and an effector for Ras-like GTPases. [22]. The RA domain of PLCε binds preferentially to activated (GTP-bound) Ras; over-expression of Ras promotes translocation of PLCε to the membrane [6]. Other activators of PLCε include RhoA, Gα12, Gα13 and Gβγ [23-26], suggesting that PLCε may be a common switching station mediating a diversity of signaling inputs and cellular output functions. Recently, PLCε signaling has been implicated in oncogenesis [27, 28], and knockout mice were shown to have severe congenital malformations of both the aortic and pulmonary valves [29], illustrating the importance of this complex signaling molecule.

The PLCη (PLCη1 and PLCη2) and ζ families are the most recently discovered. Abundant in neural tissue, regulation of PLCη is still being determined, but it was shown to be very sensitive to calcium. PLCη was activated by 10 nM Ca^{++}, a level 10 times less than is required for PLCδ or PLCβ, suggesting a role for PLCη as a calcium sensor [4, 30, 31]. PLCη isoforms have an extended carboxy tail

characteristic of the PLCβ family of isozymes, the function of which is as yet undetermined.

PLCζ, a sperm-specific PLC that is the most similar in structure to PLCδ, but lacks the amino-terminal PH domain [7], is the shortest of the PLC isozymes. This highly localized, very Ca^{++}-sensitive phospholipase triggers Ca^{++} oscillations and oocyte activation upon fertilization. PLCζ is 100-fold more sensitive to Ca^{++} than PLCδ [32].

The PLCδ (δ1, δ3 and δ4) are the best characterized PLC family members in terms of structure, as they were the first family to be crystallized [19]. Structural information on other families of PLCs has been deduced by comparing sequence homology to PLCδ. PLCδ2 was recently found to be a bovine homolog of human PLCβ4 [33]. PLCδ have all of the common PLC domains and are most similar in structure to PLCβ except for the lack of the long carboxy tail domain. The mechanism for activation of PLCδ is still largely unknown. PLCδ binds inactive Gαh (transglutaminase II), suggesting a role for Gαh in inhibition of PLCδ [34-36]. High cellular Ca^{++} concentrations, polyamines, Gαo/i and phospholipids [37-39] have all been proposed as direct PLCδ regulators, but distinct signaling pathways are still being determined. For instance, a recent paper has shown that PLCδ1 is coordinately regulated by Ral, a member of the Ras superfamily of small GTPases, and calmodulin. A calmodulin-binding motif unique to PLCδ1 in the interdomain between the X and Y catalytic regions was identified and shown to bind CaM in a Ca^{++}-independent manner

in the context of the full-length protein [40]. Exogenous CaM inhibited Ca^{++}-stimulated PLCδ1 activity. However, the addition of Ral, a previously characterized Ca^{++}-dependant CaM binding protein [41], resulted in release of CaM inhibition of PLCδ1, but did not result in dissociation of the PLCδ/CaM complex. PLCδ1, Ral and CaM co-precipitated in elevated Ca^{++} buffers, suggesting that CaM binds and inhibits basal PLCδ1 activity. However, upon activation, Ca^{++} binding of CaM promotes Ral association and results in potentiation of PLCδ activity. This model of CaM/Ral PLCδ regulation is supported by the additional evidence that isoprenylation of Ral is required for binding to CaM [42]. Isoprenylation and membrane association of Ral could potentially function to keep Ral accessible to the PLCδ/CaM complex. One possible model for regulation of PLCδ activity through interaction of this complex is as follows: upon activation and increased intracellular Ca^{++}, saturation of the Ca^{++} binding sites in CaM causes a well characterized conformational change in CaM that allows for binding to membrane-associated Ral. Subsequently, CaM bound to Ral reorganizes the catalytic domain of PLCδ, resulting in increased affinity for substrate.

Yet another model for activation of PLCδ involves dimerization with PLCβ2 [43]. Researchers suggest that PLCβ2 binding to PLCδ1 inhibits PLCδ1 activity. Addition of Gβγ dimers resulted in a concentration-dependant increase in the activation of PLCβ2 and concomitant release of PLCδ1, giving rise to a synergistic effect of PLCβ2 and PLCδ on induction of a Ca^{++} response. However, we speculate that CaM may have played an unappreciated role in these whole cell experiments. The

interaction between transfected PLCδ and PLCβ2 in these experiments was monitored in the presence of increasing concentrations of transfected Gβγ. Since the Gβ subunit of the Gβγ dimer is a CaM binding protein and CaM binding to Gβγ was not shown to interfere with activation of PLCβ2 [44], the addition of Gβγ in the aforementioned experiments may have removed endogenous CaM inhibition of PLCδ, contributing to its activation. Despite these new insights, the complexities of PLCδ signaling are still being determined.

Phospholipase Cβ isozymes:

Of all the PLC isozymes, those most closely associated with receptor drug target activation are those of the PLCβ family. The PLCβ family consequently is the focus of our studies herein. PLCβ enzymes are key effectors in G-protein-linked receptor-mediated signaling cascades and are integral to a vast array of bodily functions requiring complex signaling pathways including inflammation, neurotransmitter release, hormone signaling, smooth muscle contraction, platelet aggregation, light sensing and membrane depolarization. The activity of the PLCβ family of isozymes is stimulated by G-protein coupled receptors (GPCR). GPCRs are heptahelical membrane receptors that couple to the heterotrimeric guanine nucleotide binding proteins (G-proteins) composed of Gα and Gβγ subunits. Upon activation of GPCR, the Gα subunit exchanges GDP (inactive state) for GTP (activated subunit) and dissociates from the Gβγ dimer. There are four mammalian families of Gα

proteins that are activated by GPCRs, Gαs, Gαi, Gαq, and Gα12/13. Once activated, Gαs and Gαi stimulate and inhibit (respectively) adenylate cyclase. The downstream target for Gα12/13 is still unknown, but may include PLCε. Upon receptor binding, the Gαq subunit activates PLCβ isozymes. PLCβs are also sensitive to activation by the Gβγ dimer to varying degrees. Currently, 6 Gβ subunits and 13 Gγ subunits have been identified. The specificities of the unique dimer pairs have only begun to be investigated, but when combined with the diversity of the Gα subunits, there is the potential for over 1500 different G-protein signaling trimers, coupling to a wide variety of GPCR receptors.

There are four identified and characterized mammalian isoforms of PLCβ, 1-4 [45-50]. PLCβ2 and PLCβ4 have limited tissue distributions. PLCβ2 is found in hematopoetic cells, brain and liver; PLCβ4 is limited to retina and brain. PLCβ3 and PLCβ1 are nearly ubiquitous in human tissues; PLCβ1 expression is dominant in brain and PLCβ3 is dominant in heart and smooth muscle [50-58]. In addition to tissue distribution, there are differences in cellular localization of PLCβs. With some isoenzyme differences, PLCβs may be purified from both cytosolic and particulate fractions of cells [59, 60] or translocate to the cell nucleus [61-65]. Translocation or maintenance of the enzyme in proximity to substrate at the membrane may be one means of regulation.

All four PLCβ isoenzymes are activated by Gαq and Ca^{++} to varying degrees [66-68], but only PLCβ2 and PLCβ3 are activated by Gβγ dimers [69]. Regional

deletions and chimeric molecules were used to determine the G-protein regulatory domains in PLCβs. Work done in multiple labs in 1993 showed that deletion of the carboxy-tail from PLCβ1 and PLCβ2 produced active phospholipases with Ca^{++}-sensitivity but no activation by Gαq [70-73]. In addition to the carboxy-tail, the C2 domain of PLCβ can bind GTP-Gαq, and an isolated C2 domain can competitively inhibit Gαq activation of PLCβ1 and PLCβ2 [18]. Experiments with chimeric molecules created from PLCβ2 and PLCδ1 suggest the PH domain is the necessary and sufficient for Gβγ regulation of PLCβ activity [14, 74]. The amino acids within the PH domain responsible for Gβγ activation have been identified in PLCβ3 [75]. PH domains interact with Gβγ subunits in other protein contexts and are a logical Gβγ interaction site on PLCβ [76-78]. However, the Y-domain of PLCβ2 has also been implicated in Gβγ interactions [79].

As a family, PLCβ proteins contain at least five distinct structural domains, identified by homology to the crystal structure of PLCδ1 [19]. The PH domain, important for Gβγ subunits interaction with PLCβ2 and β3 isoforms and discussed above [14, 75], does not demonstrate lipid selectivity in PLCβ isozymes 2, 3 or 4, or a membrane binding function in isolation [80]. In one experiment the isolated PH of PLCβ1domain was shown to translocate to the plasma membrane after receptor stimulation or microinjection of Gβγ subunits [81]. In other experiments, Gβγ did not alter membrane affinity of any of the PLCβ isozymes [14]. In all PLCβ isoenzymes, the PH domain is followed sequentially by four EF hand domains whose function in

PLCβs has yet to be characterized; it is not yet known which, if any, of the EF hands bind Ca^{++}. The secondary structure of the middle third of the PLCβ proteins, the X and Y domains, forms part of a TIM barrel, the activity of which requires a Ca^{++} co-factor. In PLCδ, the pocket formed by the catalytic domain binds Ca^{++} and coordinates PIP2 binding. The catalytic domain is closely followed by the C2 domain, also thought to be integral to membrane and Ca^{++} binding, associated with Gαq activation of PLCβ1, but largely undefined. Unique to PLCβ isozymes is the C-terminal portion of the isozymes. This region of the protein, comprising almost a third of its total sequence, was crystallized as an antiparallel dimer from the turkey PLCβ homologue. Three long alpha helices form an extensive dimer interface hypothesized to be responsible for dimerization of the full length PLCβ enzyme [82-84]. This region also contains a site for activation by Gαq subunits in all PLCβ isoenzymes[70, 71], and a four amino acid PDZ-binding domain in PLCβ1-3 at the most distal end of the carboxy terminus [85, 86]. PLCβ isozymes are also GTPase-activating proteins (GAPs) for Gαq [87]. Consequently, in addition to hydrolyzing PIP2, PLCβs also accelerate the hydrolysis of GTP-bound Gαq to GDP-Gαq, resulting in an increase in the rate at which signaling is terminated. The GAP activity of the protein lies within the C2/carboxy-tail. All PLCβ isoforms have GAP activity, but the PLCβ1 isoform is best characterized [87-91]. GAP activity of PLCβ1 is blocked by the addition of Gβγ. This is presumably because Gβγ binds GDP-Gαq, making Gαq unavailable. However, this data does not rule out a direct interaction of PLCβ1 with Gβγ [89]. The previously

described translocation of the PLCβ1-PH domain was directly stimulated by Gβγ [81]. Although Gβγ does not effectively activate PLCβ1, it is possible that in a specific signaling environment, there could be alternative mechanisms of interaction between PLCβ1 and Gβγ.

Phosphorylation of PLCβ is an important mechanism of regulation, but unlike PLCγ isozymes, is largely inhibitory. PLCβs are substrates for phosphorylation by cAMP-dependant protein kinase (PKA), cGMP-dependant protein kinases (PKG), CaM-dependant kinase II (CaMKII) and cyclic nucleotide-independent kinase (PKC) [92-97]. PLCβ2 and β3 were identified as *in vivo* and *in vitro* substrates for phosphorylation by PKG, which results in inhibition of G-protein activation of PLCβs. The serine residues Ser26 and Ser1105 in PLCβ3 were identified as targets of PKG, mutation of which to alanine, abolished phosphorylation and reduced the inhibitory effect of PKG on PLCβ3 [92]. Interestingly, Ser1105 in PLCβ3 has also been identified as a target for PKA phosphorylation. Similar to the PKG phosphorylation of PLCβ3, phosphorylation of PLCβ3 by PKA at Ser1105 inhibits Gαq-stimulated PLβ3 activity [95]. PLCβ2 is also a target for PKA phosphorylation. Phosphorylation of PLCβ2 by PKA inhibits Gβγ activation of PLCβ2 [98]. Similar to PKA, PKC inhibits Gαq stimulated PLCβ3 activity as a result of phosphorylation at Ser1105 [94]. CaMKII can phosphorylate Ser537, a residue in the X-Y linker of PLCβ3, but does not phosphorylate PLCβ1. However, mutation of Ser537 did not affect G-protein-stimulated activity of the PLCβ3, nor were any direct effects of CaMKII-dependant

phosphorylation determined [93]. While it is clear from the research above that phosphorylation regulates the activity of the PLCβ isozymes, it is also clear that much more work needs to be done in determining the specificities of the kinases and the physiological consequences of phosphorylation. If Ser1105 of PLCβ3 were indeed phosphorylated by PKA, PKC and PKG *in vivo*, then this would suggest that the signaling environment is integral to specificity of the enzyme/kinase interaction. The tools currently available to study crosstalk between receptor signaling pathways and differential regulation in specific signaling settings are limited, but provide a base of relevant information from which we can speculate as to the complexity of signaling pathways *in vivo*.

To study PLCβ regulation, we may first look to the most abundant PLCβ isozyme, PLCβ3. PLCβ3s importance in a variety of cellular processes is clear, but a complete profile of regulatory mechanisms is lacking. Aberrancies in expression of PLCβ3 can lead to tumorogenesis. Low or no PLCβ3 expression was found in endocrine tumors indicating that PLCβ3 could be involved in the tumorogenesis of a subset of endocrine tissues [99]. In addition, neuroendocrine neoplasias are low in PLCβ3 expression [100]. Transfection of PLCβ3 into neuroendocrine tumor cells reduced size and growth rates of the tumor [101]. PLCβ3 knockout mice show changes in μ-opioid response [102] or early embryonic lethality [103]. The importance of PLCβs in pathophysiological conditions is further illustrated by its role in diabetic cardiomyopathy, where expression and activity of the PLCβ3 isozyme is decreased

[104]. It is thought that the increase in Gαq protein seen during cardiac dysfunction associated with diabetes [105, 106] is an effort to compensate for the insufficient PLCβ3 mediated signaling. PLCβ3 also plays key roles in the pathogenesis underlying problems with premature uterine smooth muscle contractions [107-109], inappropriate platelet activation [110], and adaptation to chronic morphine [111, 112].

Regulatory Proteins:

To understand the regulation of PLCβ isozymes, we must know the regulatory partners that interact with the proteins. Cell signaling is increasingly described in terms of multi-protein complexes with activators, inhibitors and translocators all efficiently and tightly localized [113]. Regulatory proteins may employ a diverse number of mechanisms, examples of which include preventing or facilitating binding of activators or inhibitors, preventing or allowing phosphorylation, mediating cleavage or lipid modifications, exerting a conformational change or domain shift, or promoting translocation and providing scaffolding. With the sequencing of the human genome and the discovery of fewer genes than experts expected, it is becoming clear that human biology utilizes regulatory proteins for multiple roles in cell signaling that also incorporate temporal and spatial regulations of events to fine tune responses.

PLCβ regulatory proteins:

Although both PLCβ3 and PLCβ1 are ubiquitous, they clearly are differentially regulated, as illustrated by differential sensitivities to G-proteins, unique

roles of the defined domains and different patterns of subcellular localization. There is no data suggesting that one PLCβ isozyme can compensate for the loss of function in the other. Despite this, few proteins are known to differentially interact with PLCβ1 and PLCβ3. One protein family that differentially interacts with the PLCβ1 and PLCβ3 isozymes is the Na+/H+ exchanger regulatory factors (NHERF). NHERFs, NHERF1 and NHERF2, are scaffolding proteins that facilitate receptor signaling by linking receptors to effectors and regulatory proteins through their PDZ (PSD-95/Disk-large/ZO-1) domains. PDZ domains are protein-protein interaction domains that associate with specific carboxy-terminal PDZ binding domain sequences on target proteins. Both PLCβ1 and PLCβ3 have PDZ binding domains at the their carboxy-tail. NHERF2 binds specifically to PLCβ3, but not to PLCβ1, forming a ternary complex with the lysophosphatidic acid receptor 2 [114, 115]. Alternatively, reports regarding NHERF1 interacting with PLCβ have been conflicting. While it is generally agreed that NHERF1 binds PLCβ1, there is conflicting data as to whether NHERF1 binds PLCβ3 as well [85, 116].

Another scaffolding protein with an affinity for PLCβ is Shank. Shank is a member of a multimeric protein complex in dendritic spines that contains a variety of signaling and scaffolding proteins. This protein, like the NHERFs, contains a PDZ domain that interacts specifically with PLCβ3. Another protein, Homer1b, forms a complex with Shank and PLCβ3 [85]. Homer proteins are also scaffolding proteins. Homer 2 protein binds to PLCβ1 and regulates its GAP activity [117]. The role of

scaffolding proteins is to link many signaling intermediates, but this function may complicate efforts in determining binding specificities. Despite this difficulty, scaffolds are understood to play important roles in the organization of GPCR signaling pathways [113].

Calmodulin as a regulatory protein:

CaM is an established, ubiquitous and abundant Ca^{++}-sensitive regulatory protein associated with a vast array of cellular functions [118]. CaM is a member of the Ca^{++}-binding protein families that are characterized by four EF-hand motifs. EF-hand domains consist of an amino-terminal helix (the E helix) followed by a Ca^{++}-binding loop and a carboxy-terminal helix (the F helix). CaM, the most extensively studied of these Ca^{++}-binding proteins, binds four molecules of Ca^{++} cooperatively, and undergoes a significant conformational change upon Ca^{++} binding that is important for its many Ca^{++}-sensitive regulatory functions. The carboxy-terminal pair of EF-hand domains binds Ca^{++} with three- to fivefold higher affinity than the amino-terminal pair of EF-hands domains (reviewed in [118]). The Ca^{++} differential affinities of the amino- and carboxy-terminal ends of protein ensure that CaM is not saturated with Ca^{++} at resting intracellular Ca^{++} levels, and as a result, is sensitive to changes in intracellular Ca^{++} [119]. This differential sensitivity of the Ca^{++} binding sites in CaM adds to the complexity of the ways in which this protein can act. It is doubtful that CaM exists intracellularly without any of its Ca^{++} binding sites occupied, but it is clear that non-Ca^{++}-saturated CaM has a very active role in regulation [20]. For simplicity,

CaM that is not completely saturated in its Ca^{++} binding will be referred to as apocalmodulin (ApoCaM).

CaM mechanism of action:

In a 2000 review [118], Chin and Means classified proteins that bind CaM into six categories based on their modes of regulation and the Ca^{++} saturation state of CaM. The first group of effectors binds essentially irreversibly to CaM irrespective of Ca^{++}, making CaM more like a subunit, an example being phosphorylase kinase b (reviewed in [120]). The second category of effectors is proteins that bind apoCaM and dissociate from CaM reversibly in the presence of Ca^{++}. The best characterized example is neuromodulin which is thought to serve as an intracellular reservoir for CaM (reviewed in [121]). The next group of effectors forms low affinity interactions with apoCaM, and high affinity interactions at higher Ca^{++} concentrations, which activate the effector functions. Phosphotidylinositol 3-kinase is an example of a protein that is activated by Ca^{++}/CaM [122]. The fourth category of CaM binding proteins also binds in the presence of Ca^{++}, but the interaction results in inhibition of effector function. Examples of this group are the G-protein-receptor kinases (GRKs) [123-125]. The remaining two categories of proteins regulated by CaM include examples from the CaM phosphorylation cascade including proteins like the multimeric CaM kinase II (CaMKII). Unassociated with CaM prior to Ca^{++} saturation, CaMKII is activated by Ca^{++}/CaM, which makes it a target for phosphorylation by another CaM-regulated kinase (CaM kinase kinase). CaM kinase kinase comprises the sixth and final category of CaM interacting proteins, those that require CaM interaction for activation and

target recognition (reviewed in [126, 127]). CaM binding sites are amphipathic α-helices, typically 20-35 amino acids long, with basic and hydrophobic residues sorting to one side of the α-helical projection. CaM binding sites are nearly as diverse as the number of CaM binding proteins.

A recently emerging theme in the epic of CaM regulatory mechanisms is the paradigm of mutually exclusive binding of CaM and another regulatory protein or lipid to a signaling molecule. In this situation, CaM is neither an activating protein nor an inhibitor itself, but regulates by releasing inhibition by another protein or lipid, or by preventing activation by binding in a mutually exclusive fashion to the target site of an activating protein. The regulator of G-protein signaling protein 4 (RGS4) is an example whereby CaM releases inhibition by a lipid. RGS proteins are GTPase-activating proteins (GAPs) for Gα subunits in G-protein linked signaling. The GAP activity of these proteins can function not only to turn off signaling, but to localize and amplify signaling within a defined region of the cell surrounding the activated receptor. By hydrolyzing GTP, RGS proteins increase the local pool of available Gα-GDP substrate for activation by the receptor, and prevent diffusion of the activated Gα protein, keeping the signaling localized [128]. CaM binds RGS proteins in a Ca^{++}-dependant manner, suggesting a role for Ca^{++}/CaM association with RGS after GPCR/PLCβ-mediated intracellular Ca^{++} concentrations rise. Data supporting this hypothesis include the information that RGS4 inhibits Ca^{++} signaling in mammalian cells [129-133], but CaM does not activate or alter RGS signaling in any manner. RGS4 protein GAP activity is inhibited by dipalmitoyl-PIP3 binding (but not PIP3

with shorter lipid side-chains). Popov et al. found that CaM association with RGS4 released inhibition of GAP activity by PIP3 [134]. This model of PIP3/CaM regulation of RGS4 was monitored in experiments with G-protein sensitive cardiac potassium channels [135]. The structural determinants for protein binding of CaM binding are similar to these protein structures that bind phospholipids. Thus, mutually exclusive PIP3/CaM binding is a logical mechanism for protein regulation.

Another model of the mutually exclusive CaM/phospholipid binding occurs in the heterotrimeric complexes required for the Ca^{++}-dependant release of cellular vesicles containing neurotransmitters and neuropeptides. CaM and phospholipid bind at the carboxy-terminal portion of the vesicle protein synaptobrevin in a mutually exclusive fashion [136, 137]. Investigators have developed a model whereby Ca^{++} /CaM binding releases inhibition of the cis-lipid membrane and promotes association of synaptobrevin with trans-lipids required for vesicle formation [138].

There are also examples of mutual regulation of signaling proteins by CaM and other proteins instead of phospholipids. CaM and Gβγ proteins have been shown to bind in a mutually exclusive fashion to the presynaptic metabotropic glutamate receptor (mGluR) at partially overlapping domains [139]. mGluR functions, including Ca^{++} channel closure and inhibition of neurotransmitter release, are mediated by Gβγ subunits which bind the carboxy-terminus of the receptor. Ca^{++}/CaM displacement and release of Gβγ from the carboxy-tail of mGluR is required for mGluR-activated Gβγ functions [140].

CaM and Gβγ can work in a concerted manner in other protein contexts. The L-type Ca^{++}-channels bind apoCaM at a proposed split CaM binding domain on the amino- and carboxy-terminal intracellular portions of the channel [141]. Inhibited by GPCRs, the proposed mechanism for regulation of these channels follows: upon GPCR receptor activation, the Ca^{++}-channel/CaM complex binds a Gβγ subunit, resulting in partial inhibition of the channel. As Ca^{++} concentrations increase and CaM becomes saturated, the Ca^{++}/CaM and Gβγ interaction becomes tighter, completely inhibiting the channel. Thus, CaM and Gβγ have a cooperative effect on channel inhibition. The various mechanisms by which CaM regulates proteins are growing as fast as the list of CaM binding proteins. More roles for lipids and other proteins in CaM regulatory functions are expected to surface.

Calmodulin as a Ca^{++} buffer:

In addition to regulatory functions, CaM and the other Ca^{++}-binding proteins are important for intracellular Ca^{++} buffering. The intracellular concentration of Ca^{++} is in the 100 nM range, 10-20,000 times less than extracellular Ca^{++} concentrations, setting up a very powerful transmembrane gradient. Once inside a cell, it is estimated that a Ca^{++} molecule diffuses no more than 0.1 to 0.5 μm or ~50 μS before encountering a Ca^{++} binding protein (based on cell size and concentration and abundance of Ca^{++} binding protein [142]). During influxes of Ca^{++} from the endoplasmic reticulum or the extracellular milieu, the cellular concentration of Ca^{++} is estimated to reach 1 μM, but localized concentrations of Ca^{++} could be much higher

[143]. Ca^{++}-sensitive proteins localized near the areas of Ca^{++} influx would have a distinct advantage in competing for Ca^{++} to bind.

While CaM is clearly an ever-present and abundant regulatory protein, it is limited in availability as a binding partner. CaM participates in virtually all cellular processes, and the network of CaM-binding proteins is vast. Given the diversity in proteins that bind apoCaM, a significant portion of the CaM available in the cell will be bound before Ca^{++} entry; after intracellular Ca^{++} increases, all the CaM is predicted to be Ca^{++} saturated [144-146]. The limited diffusion of Ca^{++}, the vast number of Ca^{++} binding proteins, and the abundance of CaM binding sites relative to available CaM molecules, makes it logical to assume that protein signaling cascades regulated by Ca^{++}/CaM be preassembled in close proximity to sites of Ca^{++} entry. The initiation of signaling and increased localized Ca^{++} will set off a series of switches, protein domain shifting and conformational changes that result in transducing the initiated signal via a series of protein-protein, protein-lipid and protein-ion interactions. Crosstalk and preconditioning between signaling complexes is starting to be understood in temporal and coincidence effects of signaling.

What we describe in this thesis is evidence for a new CaM binding protein, PLCβ isozymes.

Chapter 2: Calmodulin is a Phospholipase C-Beta Interacting Protein

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Abstract:

Phospholipase C-β3 (PLCβ3) is an important effector enzyme in G-protein-coupled signaling pathways. Activation of PLCβ3 by Gα and Gβγ subunits has been fairly well characterized, but little is known about other protein interactions that may also regulate PLCβ3 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 carboxy-terminal PLCβ3 revealed CaM binding at a putative amino-terminal binding site. Direct physical interaction of PLCβ3 and PLCβ1 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 IP 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 IP hydrolysis, consistent with a direct interaction between PLCβ isoforms and CaM.

Introduction

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 (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 mediates an increase in cytosolic Ca^{++} by releasing intracellular stores, whereas DAG activates protein kinase C (PKC) [2]. Four isoforms of PLCβ, 1-4, have been identified in mammals. PLCβ2 and PLCβ4 have limited tissue distributions, whereas PLCβ3 and PLCβ1 are nearly ubiquitous in human tissues; PLCβ1 is dominant in brain and PLCβ3 is dominant in rat heart and smooth muscle [50-58]. All four mammalian PLCβ isoenzymes are activated by Gqα-type G-protein subunits to various degrees, but only PLCβ2 and PLCβ3 are activated by Gβγ dimers. PLCβs, with some isoenzyme differences, may be purified from both cytosolic and particulate fractions of cells [59, 60], 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 PLCδ1 [19]. The amino terminus contains a pleckstrin homology (PH) domain, a known protein interaction and membrane-binding domain that may be also a region for Gβγ subunits interaction with

PLCβ2 and β3 isoforms [14, 75]. In all PLCβ isoenzymes, the PH domain is followed sequentially by four EF hand domains, which frequently bind Ca^{++} 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 Ca^{++} co-factor. The C-terminal portion of PLCβ isozymes 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 [82]. This region also contains a site for activation by Gαq subunits [70, 71] in all PLCβ isoenzymes and a four amino acid PDZ-binding domain in PLCβ1-3 [85, 86]. While there is significant homology among mammalian PLCβ isotypes (35-55% in full length protein and 80-90 % in catalytic domains), the differences in G-protein selectivity for activation and subcellular localization among isotypes suggest that each has unique mechanisms of regulation.

Given the ubiquitous distribution of PLCβ3 and PLCβ1 and their differential regulation by Gβγ G-protein subunits, isoenzyme specific modifiers of PLCβ3 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 EF hand domain of PLCβ3. Several candidates have been identified, most intriguing being calmodulin (CaM). PLCβ3 is a Ca^{++}-sensitive protein whose activation leads to increases in cytosolic Ca^{++} levels. CaM acts as an intracellular Ca^{++} 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 PLCβ1 enzymes expression in 1321N1 human astrocytomas, and using this cell line as an *in vivo* model system, determined that CaM is a regulator of GPCR-stimulated PLCβ activity.

Materials and Methods:

Abbreviations:

Carbachol, carbamyl choline chloride; W-13, N-(6-Aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride; Fluphenazine, fluphenazine N-2 chloroethane dihydrochloride; KN-62, 2-[N-(4'-Methoxybenzenesulfonyl)]amino-N-(4'-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate; KN-92, 2-[N-(4 Methoxy-benzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine phosphate; KN-93, N-(2-[N-[4-Chlorocinnamyl]-N-methylaminomethyl]phenyl)-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide; TCA, trichloroacetic acid.

Materials:

KN-62, KN-93, KN-92, W-13, and Fluphenazine were obtained from Calbiochem (San Diego, California). Dulbecco's modified Eagle's medium (DMEM) was purchased from Mediatech Cellgro (Herden, VA). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, UT). ^{3}H-myo-inositol and protein-A sepharose were purchased from Amersham Biosciences (Piscataway, NJ). 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). ImmuneStar® chemiluminescent substrate was purchased from Bio-Rad (Hercules, California). CaM-Sepharose 4B was purchased from Pharmacia Biotech (Uppsala, Sweden). pBTM116 and pBL1 vectors, and L40 and PL3α *S. 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 [147, 148] with the following modifications. cDNA coding for the amino-terminal PH and EF hand domain region of PLCβ3 (amino acids 2-315; PH-EF-β3) was PCR amplified to incorporate 5' *EcoR1* and 3' *XhoI* restriction sites with the following primers: 5'-ATATATCTCGAGGCGGGCGC-3' and 3'-TATATACTCGAGTCAAGATCCAGGGCTTCCAGGG-5'. The PCR amplified fragment was subcloned into pBTM116 (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-primed 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 for prey selection. *S. cerevisiae* L40 reporter yeast strain with an integrated *lexA* operon in front of HIS3 and *lacZ* genes was doubly transformed with "bait" vector,

pBTM116-PH-EF-β3, and the mouse brain cDNA library in pACT2. Approximately 1 X 10^4 yeast transformants were screened for HIS3 reporter activity on His^- Leu^- Trp^- selection plates in the presence of 50 mM aminotriazole to suppress residual HIS3 expression in the absence of interaction. 150 positive clones were isolated and screened for β-galactosidase (β-gal) activity in liquid assay as described previously [147]. Positive clones with β-gal activities of at least twice background (defined by β-gal 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-gal-positive yeast were grown for three 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 (NIH, National Library of Medicine) for similarity to known sequences. Previously identified sequences were assessed in a secondary yeast two-hybrid screen for interaction with PH-EF-β3 in a different vector context. For secondary screening, positive clones in the pACT2 vector were co-transformed in the *S. cerevisiae* strain PL3α with pBL1 (a histidine-selectable yeast expression plasmid) containing the PH-EF-PLCβ3 fragment subcloned in frame with the estrogen receptor DNA binding domain (ER-Dbd). 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 [149]. 1321N1 cells were routinely subcultured in DMEM, 10% FBS and standard antibiotics (penicillin and streptomycin) in humidified 5% CO_2 at 37°C. For IP assays, 1321N1 cells were subcultured in 24 well plates at a density of $1x10^5$ cells/well and allowed to attach overnight. Cells were then labeled overnight with ^{3}H-myo-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 minutes (KN-62, KN-92, and KN-93) or 10 minutes (W-13 and Fluphenazine) in 20 mM Hepes-buffered DMEM, pH 7.4 (HDMEM) at 37°C in ambient air. After pretreatment, 10 mM LiCl was added for 10 minutes followed by stimulation of muscarinic receptors with 1 mM carbachol for 20 minutes. Following stimulation, cells were lysed with 0.5 ml cold 5% TCA and the soluble lysate was ether extracted thrice. Inositol phosphates (IP, IP2, IP3) were purified by anion exchange chromotography with ammonium formate. Lipids were solubilized with 1 N NaOH and collected to quantitate ^{3}H-inositol phospholipids. Samples were harvested into scintillation vials and radioactivity quantitated in a liquid scintillation counter. Percent (%) conversion calculated as (^{3}H-inositol phosphates (dpm))/(^{3}H-inositol phospholipids (dpm) + ^{3}H-inositol phosphates (dpm)). All assays were performed in triplicate and values reported reflect an average of at least three experiments ± SE. Students t-tests were performed to assess statistical significance where indicated.

Cell Fractionation and Immunoprecipitation:

1321N1 cells were plated at $3x10^6$ cells/10 cm 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 x g for 5 minutes. Cold lysis buffer (0.6 mls; 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM EDTA, 5 nM PMSF, 1 nM pepstatin A, 1 nM leupeptin) was added to each sample and incubated on ice for 10 minutes. Cells were lysed with 15 strokes of a Dounce homogenizer and a 50-μL fraction saved as crude lysate. The remainder of the lysate was centrifuged at 500 x g, 4°C for 5 minutes to pellet nuclei and intact cells. The low speed supernatant was centrifuged at 300,000 x g, 4°C for 25 minutes, separating cytosol and pelleted membranes. The 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 DTT, 5 nM, PMSF, 1 nM pepstatin A, 1 nM leupeptin) and incubated at 4°C for one hour with inversion. After extraction, the membrane fraction was centrifuged at 16,000 x g, 4°C for 30 minutes to pellet insoluble particulates. Supernatant was saved as membrane extract.

For immunoprecipitation, anti-PLCβ isozyme selective polyclonal antibodies were added to 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 Protein A sepharose that had been washed and equilibrated in extraction buffer (vol:vol) according to 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 (ImmunStar®) according to manufacturer's specifications.

For preparation of cell lysates for CaM-Sepharose 4B binding assay, 1321N1 cells were plated at $3x10^6$ cells/10cm plate and grown for 3 days at 37°C with 5% CO_2 in DMEM+10% fetal bovine serum and antibiotics. Plated cells were washed twice with HDMEM. Cells were scraped into tubes and pelleted at 500 x g for 5 minutes. Cold lysis buffer (0.6 mls; 10 mM Tris, pH 7.4, 5 mM $MgCl_2$, 2 mM CaCl, 5 nM PMSF, 1 nM pepstatin A, 1 nM leupeptin) was added to each sample and incubated on ice for 10 minutes. Cells were lysed with 15 strokes of a Dounce homogenizer. The lysate was centrifuged at 500 x g, 4°C for 5 minutes. to pellet nuclei and intact cells prior to incubation with CaM-sepharose 4B beads described below.

Transcription and translation of radiolabeled proteins:

^{35}S-methionine-labeled amino-terminal and carboxy-terminal PLCβ3 was prepared according to manufacturers instructions using the *in vitro* TnT® Quick Coupled Transcription/Translation System (Promega, Madison, WI). Briefly, 1 μg of pcDNA3 plasmid containing cDNA sequence for the amino- (amino acids 1-313, N-PLCβ3) or carboxy- (amino acids 670-1173, C-PLCβ3) terminal sequences of PLCβ3 was incubated with TnT® reticulocyte lysate, and 20 μCi ^{35}S-methionine in a total volume of 50 μl for 90 minutes at 30°C. Labeling efficiency and level of protein

expression were evaluated by SDS-PAGE and autofluororadiography (described below).

CaM-Sepharose 4B binding assay:

The CaM-Sepharose binding assay with ^{35}S-methionine-labeled proteins was performed according to the protocol of Chuang et al. [150] with the following modifications. CaM-Sepharose 4B resin was washed twice in 500 ul of dH_2O, 4°C, and equilibrated for 1 hour in binding buffers made with Calcium Calibration buffers (K_2EGTA and/or CaEGTA in varying amounts) from Molecular Probes (Eugene, OR) in 30 mM MOPS, pH 7.2, 100 mM KCl, 150mM NaCl, 2mM MgCl, 1 mM dithiothreitol, and 5 nM PMSF. Free Ca^{++} concentrations ranged from zero to 39 μM. Equivalent radioactive quantities of radiolabeled ^{35}S-N-PLCβ3 or ^{35}S-C-PLCβ3 were incubated with 50 μl of pre-equilibrated calmodulin-sepharose 4B resin in a total volume of 500 μl overnight at 4°C with inversion. Unbound 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 precipitants were resolved by 12.5% SDS-PAGE. For autofluororadiography the resultant gel was soaked in glacial acetic acid for 20 minutes, impregnated with 10% PPO (2,4-diphenyloxazole) scintillant for 15 minutes, and rinsed in ddH_2O for 10 minutes before being dried to Whatman #1 filter paper. The gels were exposed overnight to x-ray film and the ^{35}S-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 4B resin was equilibrated in extraction buffer (50 mM Hepes, 2.5 mM CaCl, 150 mM NaCl, 1% Triton X-100, 1 mM DTT, 5 nM, PMSF, 1 nM pepstatin A, 1 nM leupeptin) and added to each lysate sample. Samples were incubated at 4°C with inversion for 1 hour. After incubation the pelleted beads were washed three times in extraction buffer at 4°C; after the final wash, proteins were eluted from the beads with the addition of 50 ml 2x SDS-PAGE loading buffer and boiling for 5 minutes. Proteins in the supernatant were separated by 7.5% SDS-PAGE and transferred electrophoretically to nitrocellulose paper followed by Western blotting as described above with PLCβ-selective polyclonal antibodies.

Results

Identification of CaM/PLCβ3 interaction:

To identify novel protein interactions with PLCβ3, we utilized a yeast two-hybrid screening system. A mouse brain cDNA library was screened with the N-terminal region (PH and EF hand domain) of PLCβ3 as bait. Of the positive clones identified in this screen, full-length mouse calmodulin was isolated at least three times and interaction confirmed by a β-galactosidase assay (Table 2.1) and a secondary growth screen as described in Methods (Figure 2.1).

Binding site search and analysis:

The Calmodulin Target Database site (*http://calcium.uhnres.utoronto .ca/ctdb/pub_pages/general/index.htm*) was used for computer analysis of the PLCβ3 protein sequence. Three putative calmodulin binding sites were identified in the full length PLCβ3 protein sequence by hydropathy, alpha-helical propensity, residue weight, residue charge, hydrophobic residue content, helical class, and occurrence of key residues. Two of these sites are located in the C-terminal region and one in the N-terminal region of PLCβ3. The N-terminal CaM-binding site is shared by PLCβ1 and has 90% identity to the N-terminal binding site in PLCβ3 (Figure 2.2A). When compared to other CaM-binding domains, conserved hydrophobic and positively charged amino acids were identified in the PLCβ3 and PLCβ1 sequences consistent with putative calmodulin binding domains (Figure 2.2B).

PLCβ3 and PLCβ1 are expressed in 1321N1 cells:

1321N1 cells express Gq-coupled muscarinic receptors that are activated by carbachol to hydrolyze phospholipids [151]. To establish which PLCβ isoforms are expressed in 1321N1 cells to mediate this G-protein activated inositol phospholipid hydrolysis, we performed immunoprecipitation and western blot analysis using PLCβ isoenzyme selective antibodies. We determined that PLCβ3 and PLCβ1 isoenzymes are expressed in 1321N1 cells, whereas PLCβ2 and PLCβ4 are not expressed at detectable levels (Figure 2.3). Having confirmed the presence of PLCβ3 in 1321N1 cells, we investigated the possible interaction of the enzyme with calmodulin *in vitro*.

N-PLCβ3 physically interacts with CaM:

To verify that the CaM binding domains of PLCβ3 physically interact with CaM, a co-precipitation assay was performed using ^{35}S-radiolabeled N-terminal (N-PLCβ3) or C-terminal (C-PLCβ3) regions of PLCβ3 (see Figure 2.2A) and CaM-Sepharose beads. *In vitro* transcription and translation and ^{35}S-radiolabeling of N-PLCβ3 and C-PLCβ3 were verified by SDS-PAGE and autoradiography (not shown). Co-precipitation assays were performed with ^{35}S-N-PLCβ3 (Figure 2.4A) in the presence of binding buffers containing the indicated concentrations of free Ca^{++} (Figure 2.4A). N-PLCβ3 protein bound most strongly in the buffer containing no free Ca^{++} and bound less effectively with increased Ca^{++}. Co-precipitation of C-PLCβ3 (Figure 2.4B) with CaM-sepharose in the presence of binding buffers containing the indicated concentrations of free Ca^{++} revealed that C-PLCβ3 did not bind CaM-Sepharose under any of the above conditions (Figure 2.4B). Binding of N-PLCβ3 to CaM occurred in the absence of free Ca^{++} in the presence of varying NaCl concentrations up to 200 mM (data not shown).

Full length PLCβ3 and PLCβ1 are isolated from cell lysates by CaM-Sepharose beads:

To determine if the physical interaction between CaM and N-PLCβ3 identified by the *in vitro* co-precipitation assay occurred with the full-length protein, we used CaM-sepharose beads to precipitate PLCβ3 or PLCβ1 from 1321N1 whole cell lysates. Cell lysates (supernatant from a low speed centrifugation) were incubated

overnight with CaM-Sepharose beads. The beads were pelleted and the CaM-binding proteins eluted with SDS-PAGE loading buffer. The eluted proteins were separated by SDS-PAGE, and immunodetected with PLCβ3- or PLCβ1-specific antibodies. Figure 2.5 (Lanes 3 and 4) shows that both PLCβ3 (Top Panel) and PLCβ1 (Bottom panel) can be immunoprecipiated from cell lysates with CaM-Sepharose. Isolation of PLCβ3 and PLCβ1 from 1321N1 cell lysates with CaM-Sepharose supports an interaction between PLCβ and CaM, and suggests physiological relevance for CaM binding to PLCβ3 and PLCβ1.

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-dependant 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 73 and 85%, respectively, compared to carbachol stimulation of vehicle pre-treated cells (Figure 2.6A), 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 Ca^{++} concentration, we investigated effects of calmodulin inhibitor W-13 on IP hydrolysis concurrent with carbachol activation and 10 minutes 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 minutes post-activation (Figure 2.6B).

To address whether CaM kinase may mediate the effects of CaM on PLCβ activity, 1321N1 cells were treated with CaM kinase II (CamKII) inhibitors, 30 μM KN-93 or 2 μM KN-62 [152, 153]. In 1321N1 cells pre-treated with CaMKII inhibitors for 30 minutes, there was no effect on basal or carbachol-stimulated IP hydrolysis (Figure 2.7). 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 inositol triphosphate (IP_3) and diacylglycerol (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 in order 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 eight amino acid carboxyl terminus of PLCβ3 [85]; de-polymerized tubulin which interacts with PLCβ1 and interferes with Gqα activation [154, 155]; and Rac1 which activates PLCβ2 [156].

1321N1 cells are an established human astrocytoma cell line extensively used to study G-protein coupled IP hydrolysis. In 1321N1 astrocytoma cells, PLCβ3 segregates mostly with cellular particulate rather than soluble fractions, while the cellular distribution of PLCβ1 is such that it is primarily isolated from soluble fractions (unpublished observation, TMF). In addition to different subcellular locations of these enzymes in 1321N1 cells, previous work in other cell lines describes differences in activation among the isozymes [22, 157] suggestive of unique mechanisms of regulation. Our lab has sought to identify novel regulators of PLCβ3 and/or PLCβ1 isozyme 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 carboxy tail of the protein. PLCβ1 has a putative CaM binding site in the N-terminal with 90% identity to the putative PLCβ3 CaM binding site. The best-described CaM binding motif is an IQ motif [119]; however, other non-IQ CaM binding domains, both Ca^{++}-independent and Ca^{++}-dependent, have been

identified as short 16-35 amino acid regions that segregate hydrophobic residues from basic and polar residues on an α-helical wheel projection [158]. Non-IQ CaM binding domains are found in other membrane-associated proteins including G-protein-coupled receptor kinases (GRK), regulators of G-protein signaling (RGS) proteins [134] and myristoylated alanine-rich C kinase substrate (MARCKS) protein [159, 160], among others. The CaM binding site identified in the N-terminal regions of PLCβ3 and PLCβ1 have a similar distribution of amphiphilic residues as these non-IQ motif CaM binding proteins (Figure 2.2B).

PLCβ enzymes are Ca^{++}-sensitive proteins [60] that associate with the plasma membrane, both of which are properties associated with CaM-regulated proteins such as PI-3-kinase [161], RGS proteins [134] and MARCKS [162]. Ca^{++}/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. Ca^{++}/CaM attenuates the ability of PIP3 to inhibit RGS4 intrinsic GTPase activity without activating RGS directly [134]. Membrane association of G-protein-coupled receptor kinases (GRKs) is required for activation and is enhanced by PIP2 and Gβγ subunits [77, 163]; Ca^{++}/CaM inhibits several GRK subtypes as a consequence of reduced phospholipid binding/membrane association [150, 164, 165]. The presence of a CaM binding site in the N-terminal region of PLCβ, and the isolation of PLCβ3 and PLCβ1 from cell lysates with CaM-sepharose beads, suggest that CaM is a PLCβ regulatory protein that had not been previously recognized.

We demonstrated by co-precipitation assays that CaM binds PLCβ3 through the N-terminal region. These data suggest that the N-PLCβ3 is a Ca^{++}-independent CaM binding peptide (Figure 2.4). Data to support physiological significance of a CaM/PLCβ interaction is shown by the isolation of both PLCβ3 and PLCβ1 isoforms from 1321N1 cell lysates by CaM-Sepharose (Figure 2.5). Inhibition of CaM by W-13 was shown to reduce IP hydrolysis (Figure 2.6A and B) *in vivo* consistent with an inhibition of PLCβ activity. Inhibition was seen when cells were treated with W-13 prior to activation when intracellular Ca^{++} levels are low, and when cells were treated concurrent with activation or 10 min post-activation when intracellular Ca^{++} levels are changing due to the activation of PLCβ. These data suggest that CaM is integral in the muscarinic receptor-activated IP hydrolysis pathway, and the association of PLCβ with CaM may be independent of intracellular Ca^{++} levels similar to the Ca^{++}-independence of CaM binding N-PLCβ3 (Figure 2.4A). In neural tissues where concentrations of CaM and CaM binding proteins are very high [166], many CaM binding proteins are known to associate with CaM when intracellular Ca^{++} levels are very low and even in the presence of chelators [167]. Neuromodulin binds CaM in the presence of EGTA and Ca^{++} disrupts binding [168], similar to the profile seen with N-PLCβ3 binding of CaM (Figure 2.4A). Inducible nitric oxide synthase (iNOS) is an example of a protein that binds CaM independent of Ca^{++} levels and is then activated by CaM maximally at 0.1 nM free Ca^{++} *in vitro* [169].

Data herein support a direct interaction between PLCβ and CaM and maintain that regulation of PLCβ activity by calmodulin is not acting through CaMKII.

Previous work by others has shown an inhibition of carbachol-stimulated PLCβ3 activity with CaM-dependant kinase II (CaMKII) inhibitors in PLCβ3-transfected COS cells, suggesting that CaMKII is an activator of PLCβ3 activity [170]. We were not able to repeat this finding in 1321N1 cells endogenously expressing PLCβ3 and PLCβ1 (Figure 2.7 and data not shown). Regulation and phosphorylation of PLCβ3 may occur through a different mechanism in 1321N1 cells distinct from exogenously-expressing transfected cells.

We propose a model whereby CaM facilitates PLCβ activity, possibly by increasing access to substrate. While cognizant that the *in vivo* activity of CaM inhibitors to inhibit IP hydrolysis may result from effects on any member of the pathway from M1 receptor to G-protein to PLCβ, the association of CaM *in vitro* with N-PLCβ3 and the co-precipitation of PLCβ3 and PLCβ1 by CaM-sepharose suggest that PLCβ isozymes are at least one target affected by CaM. We are currently working to determine the differential effects of CaM on PLCβ3 and PLCβ1 activity, membrane association or stimulation by G-proteins.

Chapter 3: Calmodulin potentiates Gβγ activation of Phospholipase C-beta

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Abstract

Phospholipase C-β (PLCβ) isozymes hydrolyze key second messenger signaling molecules in response to receptor activation and other cellular stimuli. It was previously reported by our lab that the PLCβ3 and PLCβ1 isozymes bind calmodulin (CaM) a small ubiquitous intracellular Ca^{2+} sensitive molecule [171]. The CaM binding site is located within the putative EF hand domains of PLCβ3, as shown by deletion and truncation experiments. Fluorescent anisotropy data calculates the binding affinity of CaM with PLCβ1 and PLCβ3 as 260 nM and 320 nM, respectively, and that the binding interaction occurs at physiologically relevant Ca^{2+} concentrations. There was no effect of CaM on basal and Gαq stimulated activity assays for either isozyme. The interaction between CaM and PLCβ3 leads to potentiation of activation by the Gbetagamma (Gβγ) dimer of the heterotrimeric G-protein in an *in vitro* assay. 1321N1 cells treated with CaM inhibitors concurrent with activation of muscarinic receptors significantly reduced ^{3}H-PIP hydrolysis. CaM does not alter the affinity of PLCβ1 or PLCβ3 for the membranes phospholipids on PIP strips™, but these experiments did reveal a previously appreciated binding of PLCβ3 to PIP3.

Introduction:

Phosphoinositide-specific phospholipase C (PLC) is a key intracellular signaling molecule that once activated catalyzes the hydrolysis of PIP2 into IP3, which is a regulator of intracellular calcium and diacylglycerol (DAG), a well-characterized activator of protein kinase C (PKC) [2]. The identified PLC isozymes have been classified by sequence homology into six families, β, γ, δ, ε, ζ, and η [4, 6, 7]. Each family has unique mechanisms of activation and regulation. The activity of the PLCβ family of isozymes is stimulated by G-protein coupled receptors (GPCR). GPCRs are heptahelical membrane receptors that couple to the heterotrimeric G-proteins, Gαβγ. Upon activation of the receptor, the Gα subunit exchanges GDP (inactive state) for GTP (activated subunit) and dissociates from the Gβγ dimer.

There are four identified and characterized isoforms of PLCβ, 1–4 [45-50]. PLCβ2 and PLCβ4 have limited tissue distributions, whereas PLCβ3 and PLCβ1 are nearly ubiquitous in human tissues; PLCβ1 is dominant in brain and PLCβ3 is dominant in heart and smooth muscle [50-58]. PLCβ1 and PLCβ3 are both activated by calcium and Gαq [66-68], but the PLCβ3 isoform is additionally sensitive to activation by Gβγ [69]. The mechanism of regulation of PLCβ3 is still largely a mystery, despite the enzyme's importance in a variety of cellular processes [95, 102, 110, 172, 173]. Aberrancies in expression of PLCβ3 can lead to tumorogenesis [100, 173, 174], and PLCβ3 knockout mice show changes in μ-opioid response [102] or early embryonic lethality [103].

CaM is an established, ubiquitous and abundant Ca^{++} sensitive regulatory protein associated with a vast diversity of cellular functions [118]. CaM is a member of the Ca^{++} binding protein family that is characterized by four EF-hand motifs. This domain consists of an amino-terminal helix (the E helix) followed by a Ca^{++} binding loop and a carboxy-terminal helix (the F helix). CaM, the most extensively studied of these Ca^{++} binding proteins, binds four molecules of Ca^{++} cooperatively, and undergoes a significant conformational change upon Ca^{++} binding that is important for its many Ca^{++}-sensitive regulatory functions. CaM binding sites are nearly as diverse as the number of CaM binding proteins. CaM binding sites are amphipathic α-helices, typically 20-35 amino acids long, with basic and hydrophobic residues sorting to one side of the α-helical projection. These binding sites are similar in structure to some lipid binding domains. The list of various mechanisms by which CaM regulates proteins is growing as fast as the list of CaM binding proteins. More roles for lipids and other proteins in CaM regulatory functions are expected to surface.

It was previously reported by our lab that CaM directly interacts with PLCβ3 and PLCβ1, and that CaM inhibitors attenuate IP_3 hydrolysis in a whole cell assay [171]. Calmodulin is a small ubiquitous Ca^{++} binding protein that interacts with a great number of proteins involved in signaling pathways [44, 164, 168, 170, 171, 175-180]. The means by which CaM regulates activity of these proteins varies greatly. In the regulator of G-protein signaling 4 (RGS4) protein, CaM binding was shown to release a PIP3 mediated inhibition of activity [134, 135]. In neuronal L-type voltage dependant Ca^{++} channels, CaM binds with Gβγ to inhibit activity of the channel [141].

In this paper, we sought to characterize the nature of the PLCβ/CaM interaction by determining the effect of CaM on activity and activation of the isozymes, and to determine the binding affinity and the calcium state required for PLCβ/CaM interaction.

Materials and Methods:

Materials:

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). ImmuneStar™ chemiluminescent substrate was purchased from Bio-Rad (Hercules, CA). CaM-Sepharose 4B was purchased from GE Healthcare, formerly Amersham Biosciences (Piscataway, NJ).

Construction of PLCβ3 fragments:

cDNA coding for fragments of PLCβ3 was PCR amplified to incorporate 5' EcoR1 and 3' Xba1 restriction sites with the following primers: amino acids 132–471, 5'-AAAGAATTCTCTGAGGAGCTATTC-3', 3'-TGCGCTGGGTCGGTGTCTAGAAAA-5', and amino acids 191–471, 5'-AAAGAATTCGAATCCTGTGGCCTC-3', 3'-TGCGCTGGGTCGGTGTCTAGAAAA-5'. The PCR fragments were subcloned into pcDNA3 (a mammalian expression plasmid) and an in-frame insertion was confirmed by dideoxy sequencing using a T7 promoter-specific primer. Deletions of the putative

CaM binding regions were accomplished using the QuikChange® II site-directed mutagenesis kit (Stratagene, La Jolla, CA). The following deletion primers were designed to excise regions of the amino terminal CaM binding site of pcDNA3-N-PLCβ3 as described in Results: amino acids 164-170^186-192; 5'-GTGACCCAGGATGGTCGGATC^GTGGAGACTGCGCTGGAATCC-3' with 5'-GGATTCCAGCGCAGTCTCCAC^GATCCGACCATCCTGGTTCAC-3' and amino acids 144-148^164-169; 5'-CCGACCATCCTGGTTCAC^GGAGGCGTTCTGAGC-3' with 5'-GCTCAGAACGCCTCC^GTGAACCAGGATGGTCGG-3' (See reference [171] for construction of pcDNA3-N-PLCβ3 plasmid used in mutagenesis.)

Transcription and Translation of Radiolabeled Proteins:

[^{35}S]-methionine-labeled PLCβ3 fragments were prepared according to the manufacturer's instructions using the *in vitro* TNT™ quick-coupled transcription/translation system (Promega, Madison, WI). Briefly, 1 μg of pcDNA3 plasmid containing cDNA sequence for the mutants or fragments of PLCβ3 were incubated with TNT™ reticulocyte lysate and 20 μCi of [^{35}S]-methionine in a total volume of 50 μl for 90 minutes at 30°C. Labeling efficiency and level of protein expression were evaluated by SDS-PAGE and autofluororadiography (described below).

CaM-Sepharose 4B Binding Assay:

The CaM-Sepharose binding assay with [^{35}S]-methionine-labeled proteins was performed according to the protocol of Chuang et al. [150] with the following modifications. CaM-Sepharose 4B resin was washed twice in 500 µl of dH2O at 4°C, and equilibrated for 1 hour in binding buffers (30 mM MOPS, pH 7.2, 100 mM KCl, 100mM $CaCl_2$, 1 mM dithiothreitol, and protease inhibitors). Equivalent radioactive quantities of radiolabeled ^{35}S-PLCβ3 fragments, as estimated by SDS-PAGE, were incubated with 100 µl of a 50% slurry of pre-equilibrated CaM-Sepharose 4B resin in a total volume of 700 µl overnight at 4°C with inversion. Unbound material was removed by washing three times with 500 µl of binding buffer. SDS sample buffer was added to the final pellet and the CaM-Sepharose precipitants were resolved by 12.5% SDS-PAGE. For autofluororadiography, the resultant gel was soaked in glacial acetic acid for 20 minutes, impregnated with 10% 2,4-diphenyloxazole scintillant for 15 minutes, and rinsed in ddH_2O for 10 minutes before being dried to Whatman No. 1 filter paper. The gels were exposed overnight to x-ray film and the ^{35}S-radioactive bands were visualized.

Fluorescence anisotropy:

Fluorescence anisotropy and fluorescence emission spectra were measured with a Perkin Elmer LS-50 Luminescence Spectrophotometer. The fluorometer was connected to a water bath to maintain constant temperature (20°C). Measurements of fluorescence anisotropy for PLCβ1 and PLCβ3 with CaM were performed at an

excitation wavelength of 490 nm (band-pass 5mm) using a linear polarizer, and the fluorescence emission intensities at 525 nm (band-pass 20mm) were monitored through a second linear polarizer. Anisotropy R was calculated with

$r = \frac{I_{VV} - G \times I_{VH}}{I_{VV} + 2 \times G I_{VH}}$, where I_{VV} and I_{VH} are the intensity of vertically or horizontally polarized emitted light, respectively, obtained with vertically polarized exciting light. Four readings were taken for each measurement and averages calculated and recorded. The pertinent value of G, 0.982, which corrects for unequal transmission of vertically and horizontally polarized emitted light, was obtained from tables prepared by the Anderson laboratory (personal communication).

Reconstitution Assay:

The catalytic activity of PLCβ1 and PLCβ3 was quantitated using [^{3}H]-phosphostidylinositol 4,5 -phosphate (PIP_2) substrate as described previously [181]. Briefly, 45 ng of purified PLCβ3 or 15ng of PLCβ1 in 20 μl of 50 mM HEPES pH 7.2, 3 mM EGTA, 80 mM KCl (Buffer 1) and 1mg/ml fatty-acid free-BSA (FAF-BSA) was added to 20 μl of Buffer 1 containing 15 mM PIP_2, 135 mM phosphotidylethanolamine with 6-10,000 counts ^{3}H-PIP_2. Ten μl of 50 mM HEPES pH 7.2, 1mM EDTA, 3 mM EGTA, 5 mM $MgCl_2$, 100 mM NaCl, 1% cholate (Buffer 2) was added to each reaction containing either 30 ng Gαq protein or 50 ng Gβγ protein for G-protein stimulated activity of PLC or no added protein for assessment of basal PLC activity. Nine mM $CaCl_2$ in Buffer 1 was added to yield a final assay

volume of 60 µl. The reaction proceeded at 30°C for 10 minutes and was terminated by the addition of 375 µL of ice cold $CHCl_3$:CH_3OH:HCl (80:40:1). Followed by addition of 125 µl $CHCl_3$ and 125 µl 0.1N HCl with vigorous mixing. The aqueous and organic phases were separated by centrifugation for 5 minutes. at 2000 x g. [^{3}H] IP3 product release was quantitated by scintillation counting of 400 µl of the upper phase. Triplicate samples were run in three separate and representative experiments.

PIP Strips:

Protein-lipid overlay assays were performed as follows. Phospholipid membrane arrays (PIP-Strips®) were purchased from Echelon Research Laboratories (Salt Lake City, UT) or nitrocellulose membranes were spotted with 1 µl of 0, 50 or 100 pmol/µl concentration of the following lipids in chloroform: phosphotidylserine, phosphotidylethanolamine or PIP2 from Avanti Polar Lipids (Alabaster, AL). Membranes were blocked with 3% (wt/vol) FAF-BSA (Sigma-Aldrich; A-7030) in TBST [150 mM NaCl, 10 mM Tris·HCl (pH 8.0), and 0.1% (vol/vol) Tween-20] for 1 hour at 4°C. Blocked membranes were incubated overnight at 4°C with 0.5 µg/ml purified PLCβ3 or PLCβ1 in the presence or absence of 0.5 µg/ml Ca^{++}/CaM. The membranes were then washed three times for 10 minutes in TBST with 3% FAF-BSA, followed by incubation with PLCβ-selective polyclonal antibodies (1:2000 dilution in TBST with 1% FAF-BSA) overnight at room temperature with gentle agitation. Following three washes over 30 minutes in TBST with 1% FAF-BSA, the membranes were incubated for 1 hour with alkaline phosphatase-conjugated goat anti-rabbit

antibodies (1:20,000 dilution), and visualized with alkaline phosphatase chemiluminescent substrate (ImmuneStar™) according to the manufacturer's specifications.

Inositol phosphate assay:

Inositol phosphate (IP) assays were performed with 1321N1 cells as previously reported [149]. 1321N1 cells were routinely subcultured in DMEM, 10% FBS and standard antibiotics (penicillin and streptomycin) in humidified 5% CO_2 at 37°C. For IP assays, 1321N1 cells were subcultured in 24 well plates at a density of $1x10^5$ cells/well and allowed to attach overnight. Cells were then labeled overnight with ^{3}H-myo-inositol, 1 μCi/0.5 ml/well, prepared in sterile inositol-free, bicarbonate-buffered DMEM without additives. Following radiolabeling, cells were pre-treated for 10 minutes with W-13 (a CaM inhibitor) in 20 mM Hepes-buffered DMEM, pH 7.4 (HDMEM) at 37°C in room air. After pretreatment, 10 mM LiCl was added for 10 minutes to halt incorporation of free inositols into the membrane, followed by stimulation of muscarinic receptors with 1 mM carbachol for 20 minutes. Following stimulation, cells were lysed with 0.5 ml cold 5% TCA and the soluble lysate was ether extracted thrice. Inositol phosphates (IP, IP2, IP3) were purified by anion exchange chromatography with ammonium formate. Lipids were solubilized with 1 N NaOH and collected to quantitate ^{3}H-inositol phospholipids. Samples were harvested into scintillation vials and the radioactivity was quantitated in a liquid scintillation counter. Percent (%) conversion was calculated as (^{3}H-inositol phosphates

(dpm))/(^{3}H-inositol phospholipids (dpm) + ^{3}H-inositol phosphates (dpm)). All assays were performed in triplicate and values reported reflect an average of at least three experiments ± SE. Students t-tests were performed to assess statistical significance where indicated.

Results:

CaM binding to the PLCβ3 isozyme.

Previously, we demonstrated binding of an amino-terminal fragment of PLCβ3 to CaM. We now show that CaM binds to both PLCβ1 and PLCβ3 in the context of the full-length protein using a pull-down assay with CaM-sepharose resin and purified proteins (Figure 3.1, panels A and B, respectively). A putative CaM binding site present in the first EF hand domain of PLCβ3 was identified using the web-based CaM binding site search and analysis site, http://calcium.uhnres.utoronto.ca/ctdb/ctdb/sequence.html [171]. Updates to the site and search methods since publication of the previous paper have changed the sites identified in the amino-terminal portion of PLCβ3 and PLCβ1 to include a second putative binding site separated from the original site by a few amino acids (Figure 3.2, hatched boxes).

To determine the binding site of CaM within the amino terminal region of the PLCβ3, we used CaM-Sepharose pull-down experiments with PLCβ3 fragments deleted of the putative CaM binding regions. We also performed a series of CaM-

Sepharose pull-downs with a series of truncation mutants of the N-terminal portion of PLCβ3. As shown previously, CaM-Sepharose binds at N-terminal portion of PLCβ3 and deletion of everything but the carboxy-tail domain (Figure 3.2, rows 1 and 2) eliminated all CaM-Sepharose binding. In contrast, a fragment composed of only the EF-hand and X domain, devoid of PH, Y and carboxy-tail domains, bound as well as full-length PLCβ3 (Figure 3.2, row 3), defining the CaM binding site as residing somewhere within the EF hand domains or X domain. Deletion of either of the putative CaM binding sites in the first two EF hand domains in PLCβ3 greatly reduced, but did not eliminate, binding to CaM (Figure 3.2, rows 4 and 5). Deletion of both CaM binding sites (Figure 3.2, row 6) or deletion of three out of four of the EF-hand domains (Figure 3.2, row 7) eliminated nearly all CaM-Sepharose binding.

These data suggested that both CaM binding domains are involved in full length PLCβ3 binding to CaM. These data illustrate that binding between CaM and PLCβ3 occurs through the amino-terminal portion of the protein and is localized to the EF Hand domain region of PLCβ3 within defined CaM binding homology domains.

Fluorescent anisotropy of PLCβ3 and PLCβ1.

Having established direct binding in the context of the full-length protein and binding of CaM through EF hand domains in PLCβ3, we sought to determine the affinity of PLCβ for CaM. Using CaM conjugated to a fluorophore, Alexa-490, we measured the fluorescence anisotropy of the Alexa-CaM titrated with PLCβ1 and

PLCβ3 in the presence of calcium. We also measured the affinity of Alexa-CaM for PLCβ3 in the absence of calcium. These anisotropy titration curves are shown in Figure 3.3 (Panel A). We determined the K_d value of the Alexa-CaM/PLCβ1 or PLCβ3 complex by curve-fitting of the experimental titration data shown in Figure 3.3 (Panel A). The slight drift seen in the experimental data of PLCβ3 above the fit curve is a glycerol effect from the purified PLCβ storage buffer; this effect is corrected for in the curve-fitting. The dissociation constant (K_d value) for Alexa-CaM/PLCβ1 is 280 nM and the K_d value for Alexa-CaM/PLCβ1 is 320 nM in the presence of calcium, values that are on target with other membrane-associated proteins that bind CaM [137]. In the presence of EDTA, Alexa-CaM/PLCβ3 showed only small increases in anisotropy that failed to approach saturation over the concentration range covered. To further validate the calcium dependence of the interaction between PLCβ3 and CaM, we performed a calcium titration of Alexa-CaM/PLCβ3 using 10 nM Alexa-CaM and 1 μM PLCβ3 which showed binding between CaM and PLCβ3 over physiologically relevant calcium concentrations (Figure 3.3, Panel B).

Calmodulin potentiates Gβγ activation of PLCβ3 *in vitro.*

Previously, we reported that CaM inhibitors W-13 and Fluphenazine attenuated muscarinic receptor stimulated IP_3 hydrolysis in 1321N1 cells [171]. To determine whether the potentiation of IP_3 hydrolysis by CaM could occur through direct stimulation of PLCβ1 or PLCβ3, we performed reconstitution assays with purified

proteins and substrate *in vitro*. PLCβ3 can be activated *in vitro* by either Gαq or Gβγ dimers from heterotrimeric G proteins, whereas PLCβ1 is sensitive to Gαq stimulation but relatively insensitive to Gβγ dimers [182]. Calmodulin did not effect basal or Gαq-stimulated PLCβ3 or PLCβ1 activities at any concentration tested (Figure 3.4, Panels A and B, respectively). However, CaM did potentiate Gβγ stimulation of PLCβ3 activity (Figure 3.4, Panel A, hatched bars). A titration of increasing Gβγ in the presence of 10 μM CaM showed an increase in PLCβ3 activity of almost 30% (Figure 3.4, Panel C).

CaM does not affect lipid selectively of PLCβ isozymes.

CaM regulates the activity of other signaling proteins via changes in membrane or lipid interactions [134, 180]. We assessed the effect of CaM on the lipid selectivity of either PLCβ1 or PLCβ3 isozyme. CaM did not affect the lipid selectivity of 0.5 μg/ml of PLCβ1, assessed with PIP strips® (Figure 3.5, Panel A). With PLCβ3, the membranes were also incubated in the presence or absence of Gβγ proteins, to determine if a PLCβ3/Gβγ/CaM complex altered lipid selectivity (data not shown). Although no changes in selectivity of lipid were seen in the presence or absence of CaM or Gβγ, we found PLCβ3 binding to PIP3. Additionally, we found that PLCβ3 binding to synthetic lipids (single lipid species with 16:0 side-chains) did not mimic the binding to the brain-derived lipids (variety of chain lengths and saturations; Figure 3.5 panel C). Side-chain specificity in PLCβ lipid binding has not, to our knowledge, been previously reported.

CaM inhibitors attenuate IP_3 hydrolysis concurrent with muscarinic receptor activation.

To further dissect the mechanism by which CaM potentiates IP hydrolysis in whole 1321N1 cells, we attempted to assess whether CaM inhibitors have differential abilities to attenuate IP hydrolysis under varying cytosolic Ca^{++} conditions. We treated 1321N1 cells, prior to, concurrent with, and post activation of muscarinic receptors by carbachol (Cch). Concurrent addition of W-13 and Cch slightly increases the attenuation of IP_3 hydrolysis by W-13; W-13 is still able to elicit an effect in a post-activation cellular state.

Discussion:

Previously, we demonstrated the importance of CaM in regulating G protein-stimulated inositol phosphate accumulation by using CaM inhibitors in a whole cell assay. Additionally, we identified a CaM binding domain in the amino-terminal third of PLCβ3 suggesting that CaM interaction with this domain might account for the regulation of inositol phospholipid hydrolysis by CaM inhibitors in 1321N1 cells. With our current studies, we have further delineated the CaM binding site in PLCβ3, characterized the affinity of the direct interaction between CaM and PLCβ3, quantitated the effect of Ca^{++} on the interaction, defined the effects of CaM on PLCβ3 activity *in vitro*, and further investigated the effects of CaM inhibitors in whole cells

under varying conditions of activation. Cumulatively, this data continues to support and expand the role for CaM in potentiating G protein-stimulated PLCβ activity, particularly Gβγ-stimulated PLCβ3 activity.

Fluorescence anisotropy data demonstrated that mid-nanomolar concentrations of both PLCβ1 and PLCβ3 bound CaM in the presence of Ca^{++}. Because the number of known CaM binding proteins is vast and CaM binds proteins under both resting and Ca^{++} activated conditions, the total amount of CaM in a cell is limited [146]. Therefore, it is predicted that protein must bind to CaM with K_d values in the nanomolar range to be expected to form physiologically relevant complexes [144]. The K_d values for both PLCβ isozymes were below the micromolar range, suggesting the enzymes are forming complexes with CaM in a cellular context. The binding of CaM to PLCβ3 was sensitive to calcium concentrations over a physiological range with an apparent EC50 value of 1 μM (Figure 3.3), calculated using a non-saturating PLCβ3 concentration of 60 nM. This apparent EC50 is necessarily greater than the true EC50 of the complex because saturating concentrations of PLCβ3 were not achievable with limited amounts of purified protein available to use. The profile of anisotropy versus Ca^{++} concentration would be concentration dependent until PLCβ3 concentration was greater than the K_d. However, these limited data suggest that increasing calcium concentrations following initial activation of the enzyme may support further activation by increasing association with CaM as a potentiator of PLCβ activity.

Although we had previously identified the CaM binding domain of PLCβ3 as residing in the amino terminal third of the protein, and computer analysis of sequence revealed a putative CaM binding site in the first EF hand domain, we needed to directly substantiate the EF hand binding of CaM to PLCβ3. Surprisingly, deletion of the first and second EF hand domains of PLCβ3 reduced, but did not eliminate, binding of the PLCβ3 amino terminal fragment to CaM-sepharose. Instead, removal of all four EF hand domains was necessary for complete elimination of binding to CaM-sepharose. We speculate that structural similarity among the EF hand domains allowed different domains to substitute weakly for the EF hand domain involved in CaM binding. However, the data suggest that the strongest binding site for CaM is contained within the first EF hand domain as originally identified *in silico* by sequence analysis. Sequence identity between PLCβ1 and PLCβ3 in this EF hand domain is 80% and the same CaM binding site is identified for PLCβ1 *in silico*. However, we note that a second CaM binding site in the C-terminal tail domain of PLCβ1 and PLCβ3, contained wholly within the previously delineated Gαq activation domain, was also identified *in silico*. Data from CaM-Sepharose binding experiments using the isolated carboxy-tail domain of PLCβ3 suggested that this computer-generated CaM binding homology domain is not a true CaM binding site. Additionally, we demonstrated that CaM has no effect on Gαq regulation of PLCβ1 or PLCβ3, further suggesting that potential CaM interaction with the Gαq activation site is not of consequence.

We have shown a direct effect of CaM in potentiating Gβγ activation of PLCβ3 (Figure 3.4, Panels A and C). The CaM binding site in the EF hand domain of PLC-β3 overlaps with a site previously identified as important in Gβγ activation of the PLCβ [75]. Additionally, the beta subunit of Gβγ is a known CaM binding protein [44]. Potentiation of PLCβ3 activation by Gβγ in the presence of CaM suggests the existence of a signaling complex that involves all three proteins, Gβγ, CaM and PLCβ3. The ability of CaM to potentiate Gβγ activation of PLCβ3 is consistent with our data in whole cells whereby CaM inhibitors attenuated muscarinic G protein-coupled receptor-stimulated inositol phosphate accumulation [171]. While muscarinic receptor-stimulated inositol phospholipid hydrolysis in 1321N1 cells is characterized primarily as a Gαq /PLCβ1-mediated signaling event [183-185], the full response may still involve Gβγ and PLCβ3. 1321N1 cells express PLCβ1 and PLCβ3, with higher levels of the latter ([171] and Figure 2.3 herein). The potential for differential activation of PLCβ1 and PLCβ3 in varying spatial and temporal contexts is not at all well understood. Our data suggests that CaM is a co-factor in Gβγ-mediated PLCβ activation that may selectively increase PLCβ3 versus PLCβ1 activity in response to temporal increases in intracellular calcium concentration.

While full-length PLCβ3 may be found in both soluble and particulate cellular fractions, the submolecular domains responsible for PLCβ3 subcellular localization are not well-defined. We have observed that the PH domain of PLCβ3 does not localize to the membrane independent of full length protein (T.M. Filtz and S.R. Long,

unpublished data), unlike the well-characterized PLCδ PH domain which selectively interacts with PIP_2 [186]. Previous studies demonstrate that neither Gβγ, Gαq, nor Ca^{++} is capable of increasing the membrane binding affinity of PLCβ1 or PLCβ2 [187]. While membrane binding affinity was not increased for the full length proteins in this study, it possible that a domain shifting event in these enzymes results in bringing the catalytic domain in closer proximity to the membrane. We speculate that a CaM/Gβγ/PLCβ3 complex promotes membrane association of the catalytic region of PLCβ3. We are developing assays to quantitate the effects of disrupting the complex on PLCβ3 membrane association.

The role of CaM in regulating PLCβ1 remains elusive, despite our demonstration of direct binding. CaM binding could potentiate PLCβ1 activation, independent of Gβγ, by serving as a temporal scaffold or recruiter of other signaling regulatory proteins. The functions of CaM on proteins are not limited to direct effects on activity. For example, the CaM binding of Gβγ does not affect its activation of PLCβ2, but does affect its interaction with Gαo/i subunits [44]. While hydrolysis of PIP2 is the primary function of PLCβ isozymes, we did not look at the effect of CaM on GAP activities of the enzymes [89]. CaM binding to a protein can also simply act to prevent association of an inhibitory protein, as is in the case of RGS4, where CaM releases inhibition of PIP3 [134, 135].

If CaM were simply acting to pre-couple PLCβ isozymes to facilitate signaling upon activation, then CaM inhibitors should only be effected if added as a pre-

treatment. There should be no or little effect of W-13 when added concurrently with Cch or post-activation of muscarinic receptors. Our data (Figure 3.6) suggest that the function of CaM is not simply to scaffold PLCβ isozymes in close proximity to signaling events, but may have more of an active role in PLCβ signaling as a calcium sensor. The binding of CaM could maintain structural access to the Gβγ activation site on PLCβ3, or potentiate PLCβ3 activation by supporting the catalytic domain at the membrane interface.

Preliminary to developing a quantifiable assay for PLCβ3 membrane association, we determined that neither CaM nor Gβγ alters the lipid selectivity profile of PLCβ3 or PLCβ1 against phospholipids bound to nitrocellulose (PIP strips®) in overlay assays. However, the PIP strip® overlay assays did reveal a previously unappreciated affinity of PLCβ3 for PIP3. Quantification of the relative affinities of PLCβ3 for PIP, PIP2 and PIP3 is not possible with the overlay assay due to varying affinities of the phospholipids for the nitrocellulose, so only qualitative statements can be made. However, PIP3 is a membrane phospholipid that has a well-deserved reputation as a regulatory lipid in a variety of cell signaling pathways [188-192]. There have been reports of PIP_3 releasing inhibition by CaM [134, 135] in other protein contexts. Possibly, CaM will have a similar role in the PLCβ3 protein context, whereby it releases PIP3 mediated inhibition of PLCβ3 signaling or acts with PIP3 in regulating other functions of the protein.

In addition to demonstrating an affinity of PLCβ3 for PIP3, the PIP strip® overlay assay also revealed that PLCβ isozymes have different affinities for synthetic and wild-type lipids. The single saturated chain species of synthetic phospholipids that are available on the commercial PIP Strip® membranes is very different from the wide array of naturally derived lipids of varying chain lengths and saturation are present in a cell membrane [193]. Studies are underway to define the fatty acyl chain specificity of PLCβ binding to PIP, PIP2 and PIP3.

In conclusion, these studies further our understanding of the CaM/PLCβ interaction by showing that CaM can potentiate activation by the Gβγ dimer without affecting activation by Gαq. This work begins to detail the separate regulatory systems involving Gβγ and Gαq, and suggests cooperativity between Gβγ, CaM and PLCβ3. Further research is required to demonstrate the effect of lipids and membrane binding on the regulation of PLCβ3 activity. It is clear that temporal and subcellular aspects of cell signaling will need to be controlled for to understand the nuances of cellular responses.

Chapter 4: Conclusions and Future Directions:

Summary:

In the context of a limited number of sequenced human genes, multiple roles and functions for proteins are becoming increasingly appreciated. The studies described above further our understanding of the regulation of PLCβ isozymes by identifying and characterizing a novel interacting protein, CaM. Using co-precipitation assays with CaM-Sepharose and full-length, mutated, or truncated PLCβ3 proteins, we established a direct interaction between CaM and PLCβ1 or PLCβ3. We also defined the site of CaM interaction to the amino-terminal region of the PLCβ3 protein. In doing so, we have added PLCβ isozymes to a long list of CaM binding proteins, further illustrating the importance for CaM in nearly every cellular process. As more CaM binding proteins are discovered, models are being developed in which CaM functions as a Ca^{++}-sensitive switch or trigger that binds or dissociates with multiple proteins in a complex or in sequential fashion during signaling events.

The localization of the CaM interaction site in PLCβ3 is significant in that it resides at the juncture of the PH and EF hand domains where it can influence potential lipid binding properties of the PH domain or Ca^{++}-binding properties of the EF hand. These domains may also be binding domains for other PLCβ regulatory proteins, as their role in the context of PLCβ isozymes is not completely defined. The binding of CaM to this region in no way narrows the possibilities for other functional roles these domains may have, as CaM interacts reciprocally and concertedly with a number of lipids and proteins.

The finding that CaM potentiates Gβγ stimulation of PLCβ3 suggests that there may be a complex between the Gβγ dimer, CaM and PLCβ3, as binding by CaM did not interfere with PLCβ3 activation by Gβγ. The CaM site is within the region of PLCβ3 that is characterized as the site of Gβγ activation. Although, there is speculation that a region within the catalytic domain may also be required for Gβγ activation, this does not rule out a Gβγ/CaM/PLC complex. CaM may be acting in a variety of different ways to potentiate Gβγ activation of PLCβ3 some of which include affecting membrane association or substrate selectivity, eliciting a conformational change in the catalytic domain when activated by Gβγ that increases rate of activity, or shifting the amino-terminal region of the PLCβ3 such that Gβγ access to the protein is more efficient or maintained.

Due to the known interaction of PIP3 with CaM, we sought to determine whether CaM affected PLCβ phospholipid binding selectivity. We did not detect a change in PLCβ3 selectivity in the presence of CaM. However, in the course of those investigations, we did discover that PLCβ3 responds differently to lipids from different sources. Synthetic phospholipids have symmetrical, saturated, identical length acyl side chains and are bound with high affinity by PLCβ3. Phospholipids derived from natural sources have a diverse composition of acyl side chains of differing lengths and saturation states, and are differentially bound by PLCβ3 as compared to the synthetic lipids. This finding is of potentially great significance since the lipid binding profiles of PLC's and other proteins are frequently assessed with

synthetic lipids and these proteins are assumed to have the same binding properties for naturally derived lipids. Limitation on the availability of PIP3 with defined acyl sidechain composition of differing varieties have hampered our ability to assess qualitative differences in PLCβ3 binding to different phospholipids.

Additionally, we discovered that PLCβ3 binds to PIP3, a key signaling lipid and known CaM interacting lipid. PIP3 may act as a PLCβ regulatory lipid; there are other protein models in which PIP3 and CaM bind the same site on a protein in a mutually exclusive fashion thereby regulating its activity. Future studies outside the scope of this thesis will be directed at defining the potential regulatory role of PIP3 on PLCβ activation.

Our finding that CaM potentiates Gβγ stimulated activity but not Gαq-stimulated activity, speaks to separate cellular signaling pathways for Gβγ and Gαq. Regulation of PLCβ isozymes probably depends on the cellular and temporal context of activation. PLCβ has been described as a "coincidence detector" as activity responds to both G-proteins and increases in cytosolic Ca^{++}[194]. Researchers are finding that a signaling event through one receptor type can prime or inhibit subsequent signaling events initiated through completely different receptor types, resulting in the attenuation or potentiation of downstream effectors.

PLCβ isozymes are key intermediaries in a large number of cell processes and signaling pathways. Complete knock-outs of PLCβ3 are embryonic lethal and expression of only part of the enzyme results in severe defects in μ-opioid signaling.

Although PLCβ1 and PLCβ3 are both ubiquitous, there is no evidence to date that one will compensate for loss of function in the other. In addition, these enzymes are differentially activated by the heterotrimeric G-proteins, and have different, but overlapping sub-cellular localization. Together this information suggests different functions in the cell, such as coupling to specific receptor types at different levels of Ca^{++} concentrations. If the nuances of signaling pathways are not understood as fully as possible, we cannot fine-tune therapies for various disease states. Currently, many of the drugs the pharmaceutical industry has that target signaling pathways will stimulate or inhibit completely. These drugs do not take into consideration timing or concerted actions of multiple receptors involved in many pathophysiologies. By demonstrating not only that CaM is a PLCβ binding protein but that it also potentiates activation of PLCβ3 by Gβγ, we have shown for the first time that a protein other than a heterotrimeric G-proteins differentially regulates PLCβ3 and PLCβ1 activity. Additionally, we have established that PLCβ isozymes have specific lipid side chain specificities that have not been previouisly detected. This finding emphasizes the importance of replicating physiological conditions as accurately as possible in *in vitro* experiments.

Future directions for this research include the establishment of a lipid binding profile for the full-length PLCβ isozymes using phospholipids of varying and defined fatty acyl chain compositions, and potential lipid binding domains with PLCβ isozymes in isolation that mimic the profile of lipids derived from natural sources. This work would greatly alter our conception of phospholipid binding and hydrolysis

activity assays and potentially provide insight into membrane organization. One could then determine the role, if any, of CaM on lipid association of the PLCβ isozymes. How does PIP3 affect activation of PLCβ isozymes and does PIP3 alter the CaM potentiation of Gβγ activation of PLCβ3? Potential CaM/PIP3 binding interactions that apply to other proteins contexts may hold true for PLCβ isozymes. The binding of CaM to PLCβ isozymes has set the stage for elucidating a larger signal complex or transdusosome in which PLCβ isozymes participate. Eventually the mechanism of the entire signaling complex may be elucidated but will clearly vary in different cellular contexts. Physiological and structural emphases will be required to understand the nuances of cell signaling, and to put signaling in the appropriate cellular, spatial and temporal contexts. This work has added PLCβ isozymes to the body of CaM interacting proteins as key intermediaries in an incredible diversity of signaling pathways.

Appendix I: Figures and Tables

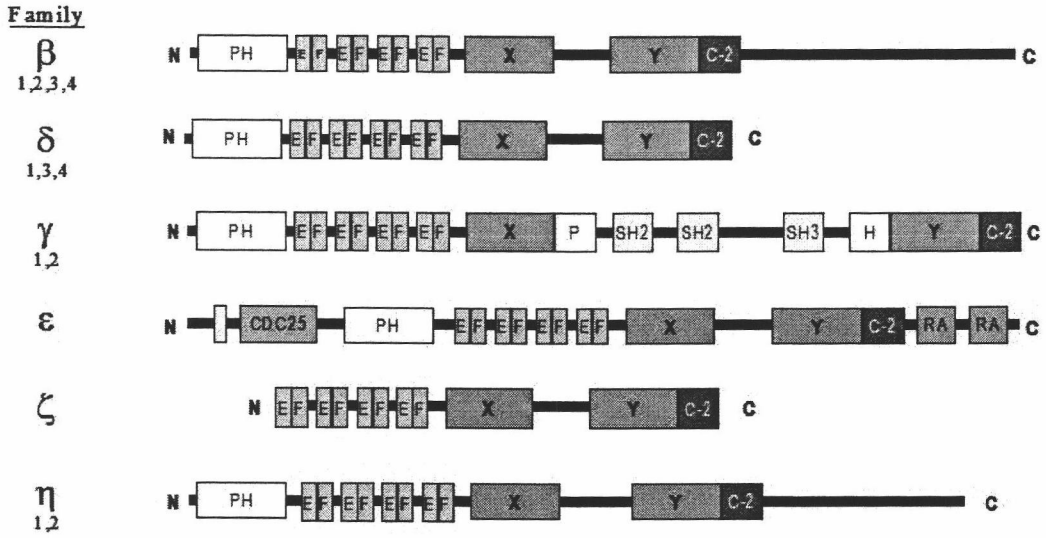


Figure 1.1: *Schematic of Mammalian Phospholipase C isozymes.* Family designated by β, δ, γ, ε, ζ and η. Numbers below family designator are the numbered isoforms within each family. Abbreviations for domains follow: amino-terminus of the protein (N), Pleckstrin homology domain (PH), EF-hand domain (EF), X catalytic domain (X), Y catalytic domain (Y), C-2 domain (C-2), src homology domains (SH2/3), ras association domain (RA), small G-protein guanine-nucleotide-exchange factor motif (CDC25), and carboxy-terminus of the protein (C).

Table 2.1: *Specific interaction of PLCβ_3 with CaM.* Table of β-galactosidase activity of representative growth-positive 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
14-3-3n	7.5
γ-actin	4.0
Cytochrome C oxidase*	3.5

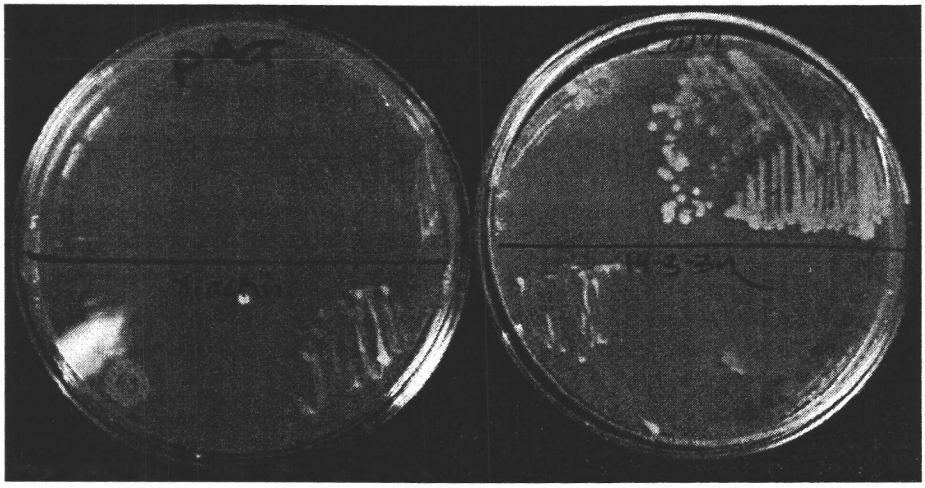
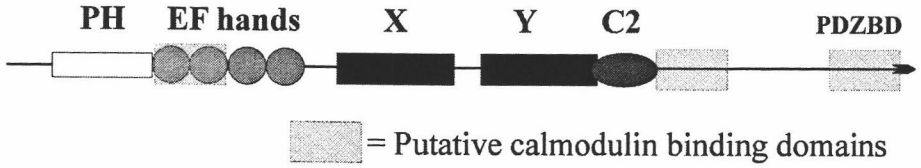


Figure 2.1: *CaM/PLCβ3 interaction in a secondary screen.* Plasmid DNA isolated from positive colonies from the N-PLCβ3 yeast two-hybrid library screening were retransformed into PL3α yeast expressing N-PLCβ3 in fusion with the ER-Dbd as described in Experimental Procedures. Shown are 72 hour growth plates of N-PLCβ3-pBL1 transformed PL3α yeast doubly transformed with pACT empty vector (pACT), pACT vector containing mouse calmodulin III (CaM), pACT with γ-actin (γ-actin), or pACT with 14-3-3η (14-3-3n) on Ura$^-$ His$^-$ Leu$^-$ minimal medium agar.

A



B

	Start	Sequence	End
PLCb3	(171)	RIPVKNILKMFSADKKRVETAL	(192)
PLCb1	(170)	RIPLKNIYRLFSADRKRVETAL	(191)
MARCKS	(151)	KKKKKRFSFKKSFKLSGFSFKKNKK	(175)
RGS4	(97)	EYKKIKSPSKLSPKAKKI	(114)

Figure 2.2: *Identification and sequence of putative CaM binding sites in PLCβ3.* (A) Linear schematic of PLCβ3 isozyme putative structural domains, including a PH domain, four EF hand domains, X and Y catalytic domains, a C2 domain and a PDZ binding domain (PDZDB). Location of putative CaM binding domains are indicated by shaded rectangles. Double-ended arrows delineate the fragments denoted as N-PLCβ3 and C-PLCβ3. (B) Amino acid sequences of the N-terminal PLCβ3 putative CaM binding domain (PLCβ3), PLCβ1 putative CaM binding domain (PLCβ1), CaM binding domain of MARCKS protein (MARCKS), and CaM binding domain of RGS4 (RGS4). Shaded residues are conserved hydrophobic and positively charged amino acids possibly integral to amphipathic helix formation and CaM binding. Starting (start) and ending (end) amino acids of the CaM binding domains are listed.

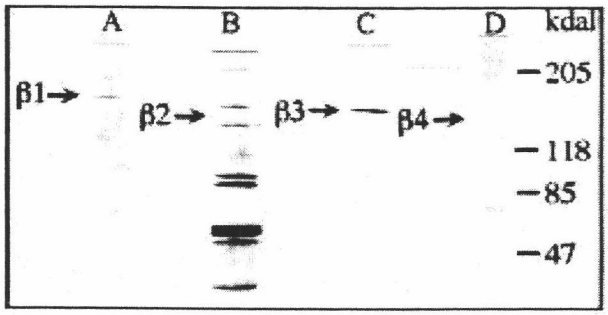


Figure 2.3: *PLCβ3 and PLCβ1 are expressed endogenously in 1321N1 cells.* 1321N1 whole cell lysates were separated by 7.5% SDS-PAGE and Western blotting was performed with anti-PLCβ specific antibodies; PLCβ1 (A), PLCβ2 (B), PLCβ3 (C), and PLCβ4 (D). Migration of molecular weight markers (kDal) is indicated on the right. Arrows indicate migration of purified protein standards for PLCβ1 and PLCβ2, or expected migration by size of PLCβ3 and PLCβ4.

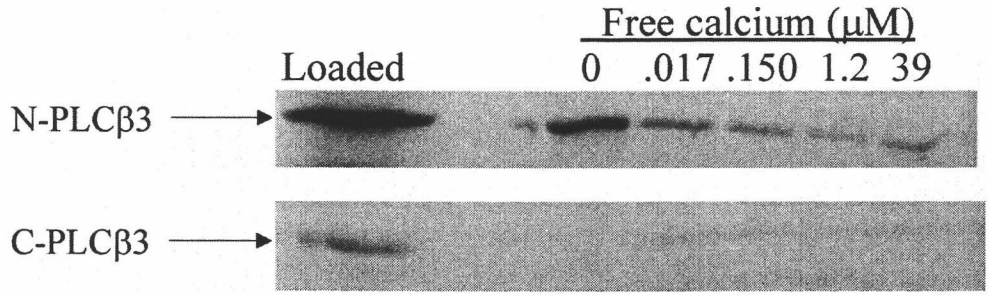


Figure 2.4: *^{35}S-labeled amino-terminal PLCβ3 but not carboxy-terminal PLCβ3 co-precipitates with CaM-Sepharose.* CaM-Sepharose resin was incubated with ^{35}S-labeled N-PLCβ3 (top panel) and ^{35}S-labeled C-PLCβ3 (bottom panel) in buffer containing 0 nM, 17 nM, 150 nM, 1.2 μM or 39 μM free Ca^{++} overnight at 4°C. The first lane represents the amount of ^{35}S-labeled N-PLCβ3 (top panel) and C-PLCβ3 (bottom panel) *in vitro* transcribed and translated product initially incubated with CaM-sepharose beads shown for comparison.

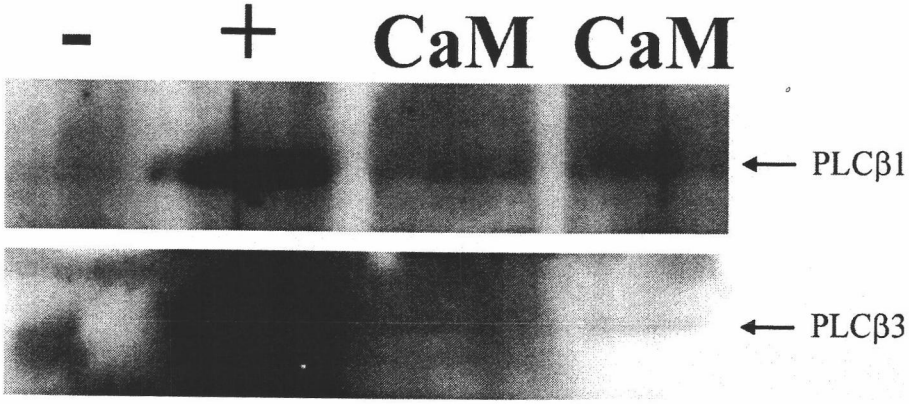


Figure 2.5: *Isolation of endogenous PLCβ isozymes from 1321N1 cells with CaM-Sepharose.* 1321N1 cell lysates were incubated with CaM-sepharose resin. The co-precipitated proteins were analyzed by western blotting with PLCβ-specific antibodies. Lane 1 (top and bottom panels) shows the results of precipitation of cell lysates with no primary antibody as a negative control. The band visible in the negative control lane of PLCβ3 is lower than the expected 133kDa seen in the positive and CaM-sepharose lanes. Lane 2 (Top and bottom panels) shows the results of immunoprecipation with PLCβ1- (Top panel) or PLCβ3- (Bottom panel) specific antibodies. Lanes 3 and 4 show PLCβ1 (Top panel) and PLCβ3 (Bottom panel) immunoprecipiation from cell lysates in the presence of CaM-Sepharose. Gels shown are representative of three separate experiments.

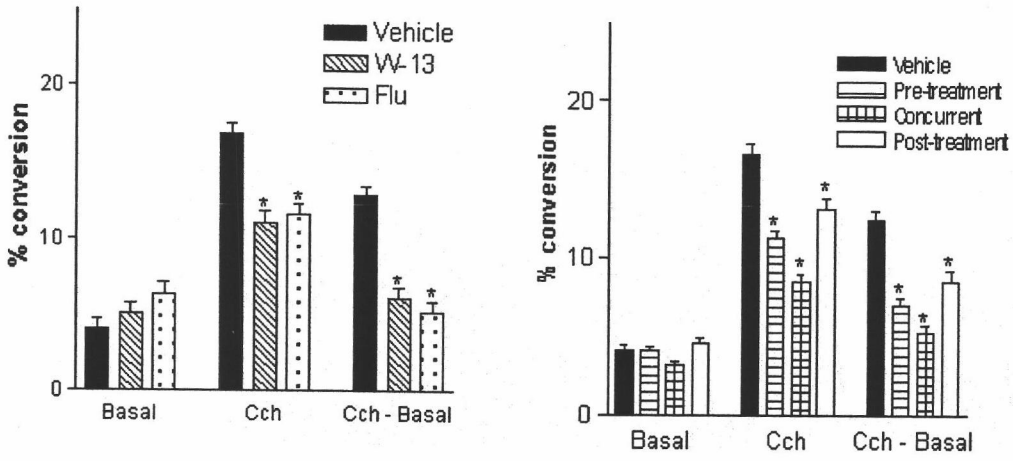


Figure 2.6: *CaM antagonists reduced carbachol-stimulated PLCβ activity in 1321N1 cells.* An IP hydrolysis assay was performed with 1321N1 cells. Results are reported as % conversion of ^{3}H-inositol phospholipids to ^{3}H-inositol phosphates. Data shown are % conversion in the absence of agonist (Basal), % conversion following 20 minutes treatment with 1 mM carbachol (CCh), and % conversion in carbachol-stimulated samples minus unstimulated samples representing net carbachol-stimulated IP hydrolysis (Cch-Basal). (A) 1321N1 cells were pretreated for 10 minutes with 1% DMSO (Vehicle), 100 μM W-13 (W-13), or 100 μM Fluphenazine. W-13 and Fluphenazine pretreatments significantly reduced total carbachol-stimulated (CCh) IP hydrolysis as well as net carbachol-stimulated IP hydrolysis (CCh-Basal) compared to vehicle pre-treated controls (*$p<0.001$). (B) Cells were treated with 1% DMSO 10 minutes pre-stimulation (Vehicle), with 100 mM W-13 10 minutes pre-stimulation (pre-treatment), with 100 μM W-13 concurrent with stimulation (concurrent), or with 100 μM W-13 10 minutes post-stimulation (post-treatment). W13 treatments significantly reduced total carbachol stimulated (CCh) IP hydrolysis as well as net carbachol-stimulated IP hydrolysis (CCh-Basal) compared to vehicle pre-treated controls (*$p<0.001$) by 42% with pretreatment, 58% with concurrent treatment, and 32% with post-treatment. Experiments were performed in triplicate with n=3.

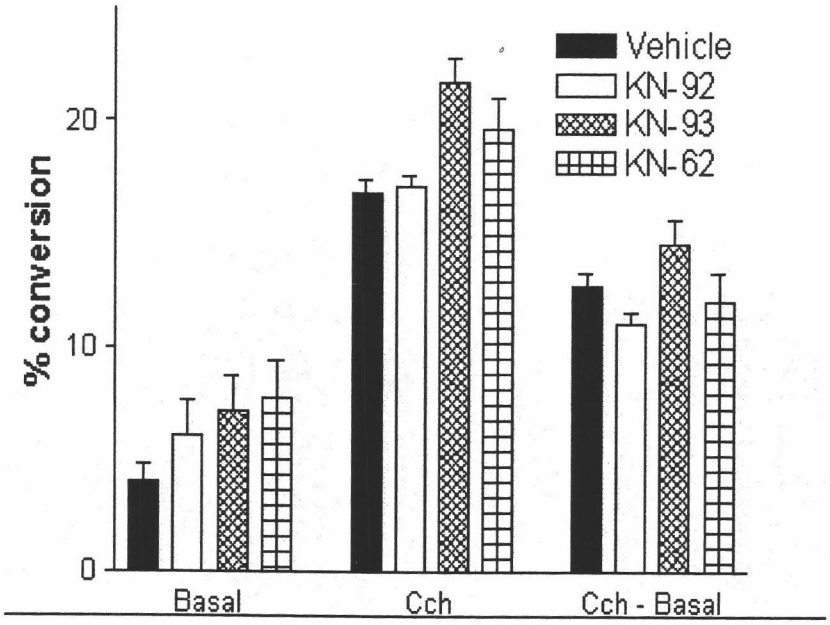


Figure 2.7: *CaMKII antagonists have no effect on the carbachol-stimulated IP hydrolysis in 1321N1 cells.* An IP hydrolysis assay was performed with 1321N1 cells. Results are reported as % conversion of ^{3}H-inositol phospholipids to ^{3}H-inositol phosphates. Data shown are % conversion in the absence of agonist (Basal), % conversion following 20 minutes treatment with 1 mM carbachol (CCh), and % conversion in carbachol-stimulated samples minus unstimulated samples representing net carbachol-stimulated IP hydrolysis (Cch-Basal). Cells were pretreated for 30 minutes with 1% DMSO (Vehicle), 30 μM KN-62 (KN-62), 30 μM KN-92 (KN-92), or 30 μM KN-93 (KN-93). Neither basal nor carbachol-stimulated % conversion was significantly different in KN-62, KN-92, or KN-93 pretreated cells compared to vehicle pretreated cells ($p>0.1$).

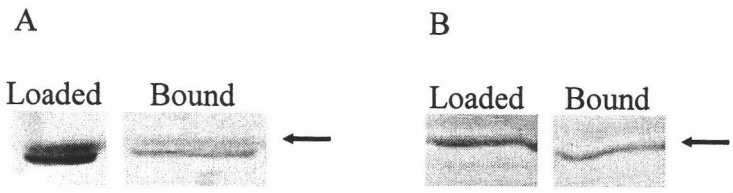


Figure 3.1: *Precipitation of full-length PLCβ1 and PLCβ3 with CaM-Sepharose beads.* (*A*) Purified full length PLCβ1 or (B) purified full length PLCβ3 were incubated with CaM-Sepharose beads as described in Methods. Shown are Coomassie blue-stained, 7.5% SDS-PAGE-separated samples of the amount of PLCβ protein added to CaM-Sepharose beads (loaded) and the resultant CaM-Sepharose-bound precipitate (bound). Data is representative of three similar experiments.

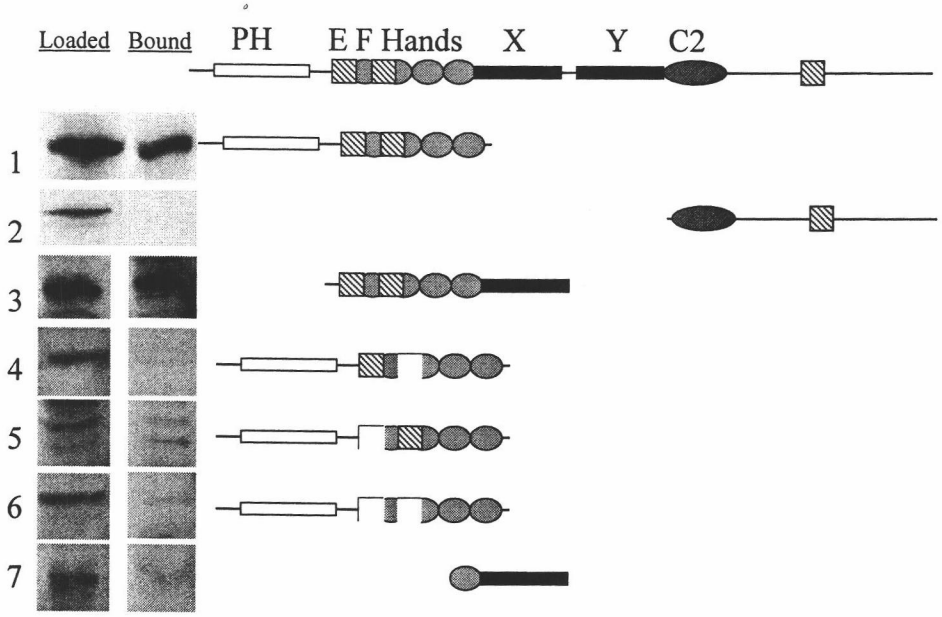


Figure 3.2: *Precipitation of PLCβ3 fragments with CaM-Sepharose beads. In vitro* transcribed and translated, [^{35}S]-labeled PLCβ3 fragments were incubated with CaM-Sepharose beads as described in Methods. Shown are autoradiograms of 7.5% SDS-PAGE-separated samples of the amount of PLCβ3 fragment added to CaM-Sepharose beads (loaded) and the resultant CaM-Sepharose-bound precipitate (bound). Truncated fragments are identified by the adjacent linear schematic illustration of the putative protein subdomain features of PLCβ isozymes. Hatched boxes define CaM binding sites. Elimination of hatched boxed refers to deletion of CaM binding sites detailed in Methods.

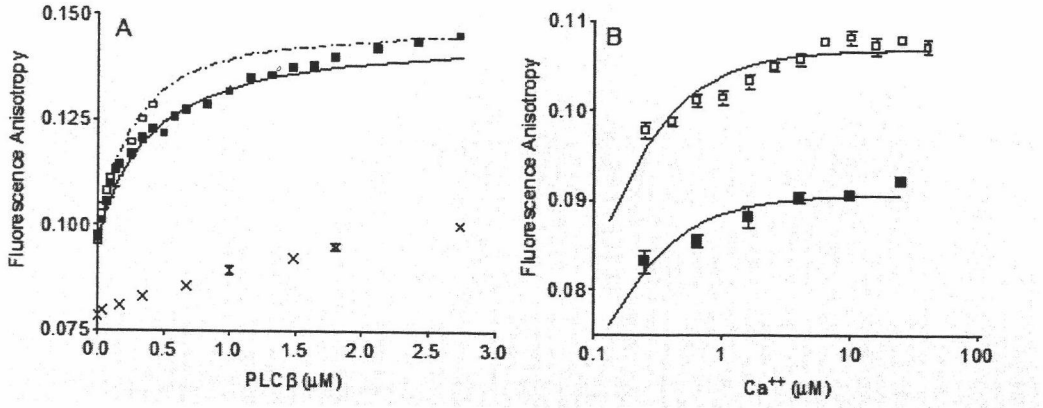


Figure 3.3: *Binding of fluorescently-tagged CaM to PLCβ1 and PLCβ3 monitored by fluorescence anisotropy.* (A) Fluorescence anisotropy monitoring of 10 nM Alexa-CaM binding to increasing concentrations of PLCβ3 (■) or PLCβ1 (□) in the presence of 1 mM Ca^{++}_{free}, or increasing concentrations of PLCβ3 in the presence of 1 mM EDTA (x). Lines were curvefit as described in Methods. K_D values calculated are 260 nM for CaM-PLCβ1 interactions and 320 nM for CaM-PLCβ3 interactions. (B) Effect of increasing Ca^{++} concentrations on fluorescence anisotropy emission intensity with 10 nM Alexa-CaM (■) or 10 nm Alexa-CaM plus 60 nM PLCβ3 (□). Fluorecence anisotropy (emission intensity) was measured at 525 nm (20 nm bandpass) with excition at 490 nm (5 nm bandpass) at 20°C in buffer containing 0.1 M KCl and 30 mM MOPS, pH 7.2. Shown is representative data from experiments repeated once. An apparent EC_{50} value of 1 μM was calculated for Ca^{++} effects on PLCβ3/CaM interactions, but is expected to underestimate the true value as PLCβ3 was not saturating.

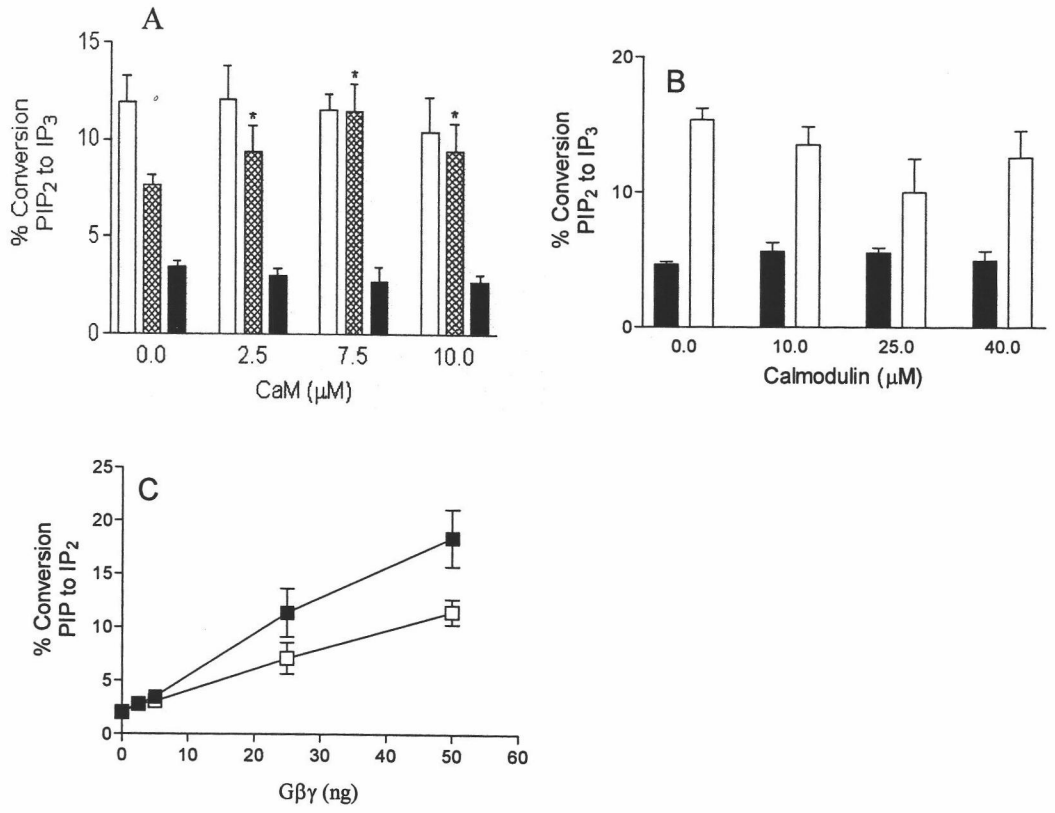


Figure 3.4: *Effect of CaM on basal and G-protein-stimulated PLCβ activity in vitro.* (A) Basal (solid bars), 50 ng Gβγ-stimulated- (hatched bars), and 30 ng GTPγS-Gαq-stimulated PLCβ3 activity was quantitated in the presence of increasing concentrations of CaM. (B) Basal (solid bars) and 30 ng GTPγS-Gαq-stimulated PLCβ1 activity was quantitated in the presence of increasing concentrations of CaM. Basal PLCβ activity was measured using PIP2 substrate in detergent/phospholipid micelles. G-protein-stimulated PLCβ activity was measured in the presence of purified G protein subunits against PIP2 substrate in detergent/phospholipid micelles. PLCβ activity was quantitated as percent conversion of [^{3}H]PIP2 substrate to [^{3}H]IP3 as described in Methods. (C) PLCβ3 activity was measured in the presence of increasing concentrations of Gβγ subunits with (■) or without (□) 10 μM CaM. Data shown are mean ± SEM of 3 to 5 separate experiments performed in triplicate. Asterisk (*) indicates treatment results significantly different from vehicle at $p > 0.001$.

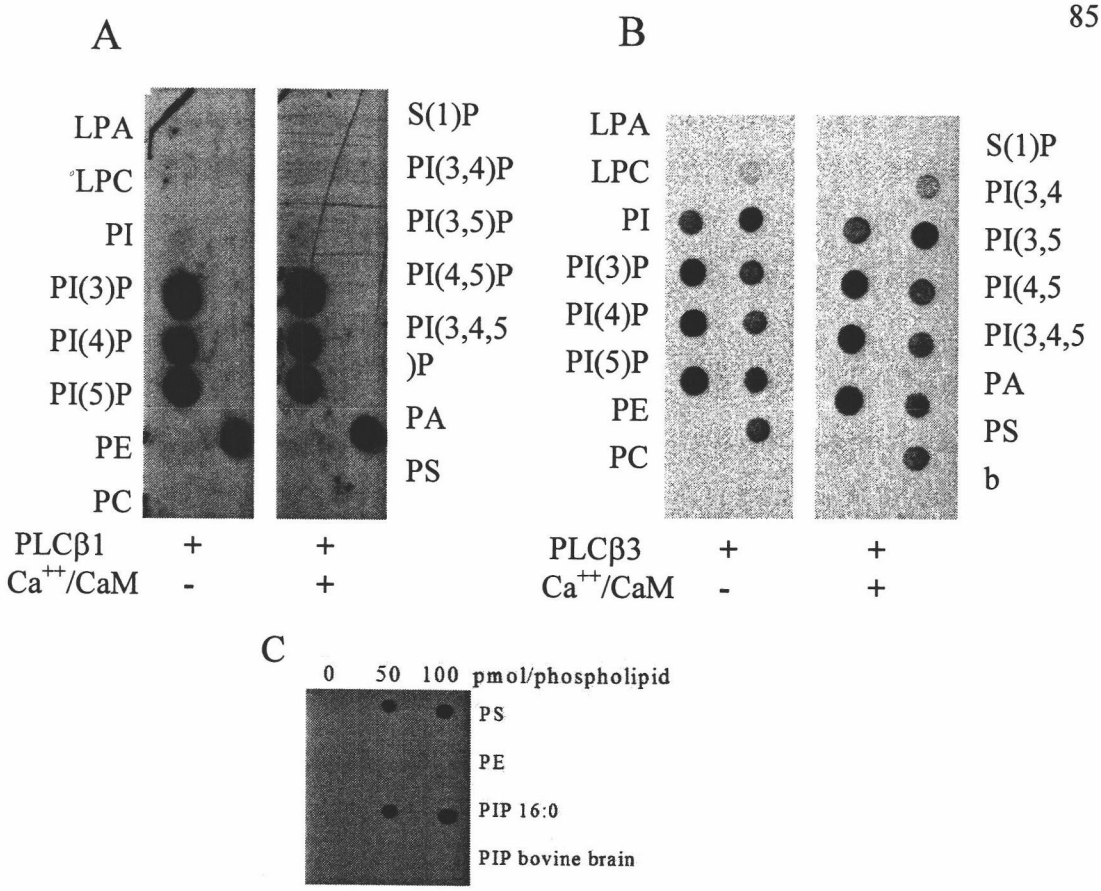


Figure 3.5: *CaM effects on PLCβ binding to immobilized phospholipids.* (A) Phospholipids immobilized to nitrocellulose (commercial PIP Strips®) were incubated with 0.5 μg/ml of PLCβ1 in the absence (left blot) or presence (right blot) of 0.5ug/ml calmodulin. (B) PIP strip® membranes were incubated with 2.5 μg/ml of PLCβ3 in the absence (left blot) or presence (right blot) of 0.5 μg/ml calmodulin. (C) Nitrocellulose membranes were spotted with indicated amounts of the listed phosphospholipids and incubated with PLCβ3. PIP Strips® and nitrocellulose membranes were washed, incubated with anti-PLCβ isoenzymes, and detected by Western blotting procedures as described in Methods. Positions of phospholipids (100 pmol/spot) are indicated in the figures: lysophosphatidic acid, LPA; lysophosphatidylcholine, LPC; phosphotidylinositol, PI; phosphotidylinositol-3-phosphate, PI(3)P; phosphotidylinositol-4-phosphate, PI(4)P; phosphotidylinositol-5-phosphate, PI(5)P; phosphotidylethanolamine, PE; phosphotidylcholine, PC; sphingo-1-phosphate, S(1)P; phosphotidylinositol-3,4-phosphate, PI(3,4)P; phosphotidylinositol-3,5-phosphate, PI(3,5)P; phosphotidylinositol-4,5-diphosphate, PI(4,5)P; phosphatidylinositol-3,4,5-triphosphate, PI(3,4,5)P; phosphatidic acid, PA; phosphotidylserine, PS; blank, b; synthetic phosphotidylinositol-4-phosphate with symmetric 16:0 saturated fatty acyl chains, PIP 16:0; natural bovine brain-derived phosphotidylinositol-4-phosphate, PIP. Data shown is representative of three similar experiments.

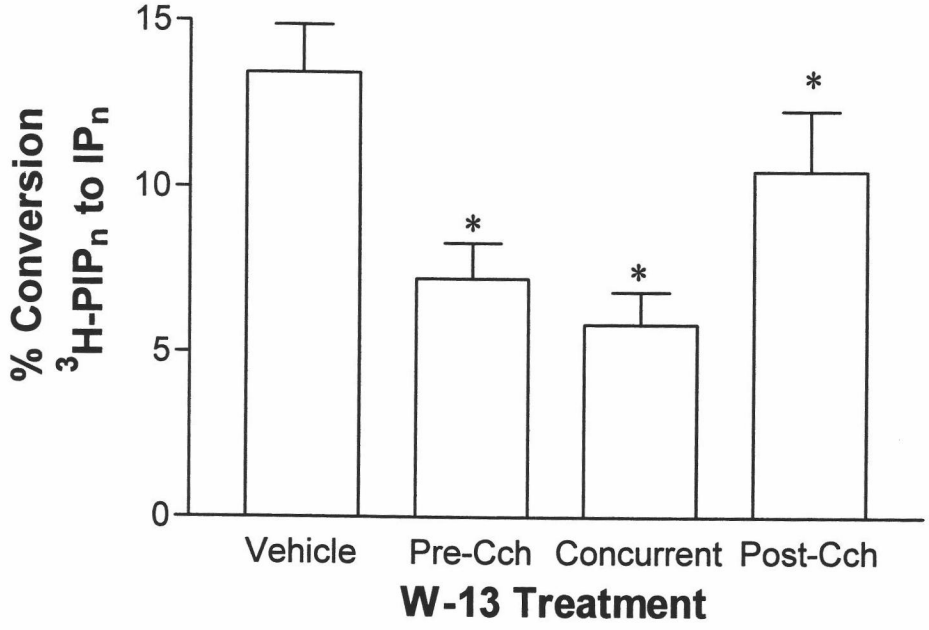


Figure 3.6: *CaM antagonists effects on carbachol-stimulated inositol phosphate accumulation under varying conditions of cell activation.* 1321N1 cells were stimulated for 20 minutes with 1 mM carbachol in the presence of 10 mM LiCl. Additionally, cells were either pretreated with DMSO (Vehicle) or 10 nM calmodulin inhibitor W-13 for 10 minutes prior to carbachol addition (Pre-Cch), treated with 100 mM W-13 for 20 minutes concurrent with carbachol addition (Concurrent), or treated for 10 minutes with 100 mM W-13 10 minutes after carbachol addition (Post-Cch). PLCβ activity was measured as percent of ^{3}H-PIP$_n$ converted to IP$_n$ in whole cells, collected and quantitated as described in Methods. Shown is cumulative data from three experiments performed in triplicate. Asterisk (*) indicates treatment results significantly different from vehicle at $p > 0.001$.

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