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The mechanisms by which the activities of phospholipase C-β (PLC-β) enzymes are negatively regulated have not been well defined. We used cardiac-derived rat myoblast H9c2 cells to investigate possible modes of regulation of PLC-β3 enzymes, the only endogenous β isoform abundantly found in these cells. The PLC-β3 population in these cells was detected almost exclusively associated with membrane with only trace amounts being cytosolic. H9c2 cells responded to vasopressin stimulation through the PLC-β pathway in a concentration-dependent manner. Direct activation of endogenous protein kinase C (PKC) by phorbol 12-myristate 13-acetate (PMA) significantly diminished vasopressin effects on PLC-β3-mediated PI hydrolysis. However, phosphorylation of PLC-β3 at the Ser1105 residue, the widely recognized primary phosphorylation site on PLC-β3, was not observed upon PKC activation. Other possible mechanisms by which the agonist-stimulated PLC-β3 activity is modulated were also tested. Short-term or prolonged stimulation of these cells with either vasopressin or stimulation of PKC by PMA did not promote translocation or down-regulation of the PLC-β3 population within the time frame
tested. Our observations imply that in H9c2 cells, activated PLC-β3 enzymes may be negatively regulated utilizing some other molecular mechanism not tested in this study.
Properties of Phospholipase C-β-mediated Signaling in H9c2 Cardiac Myoblasts

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Sun Hyung Kwon, Author
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Love to my parents who are my strength and who always see the best inside of me.
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This thesis is dedicated to my parents
Y. J. Kwon and J. S. Kim
Properties of Phospholipase C-β-mediated Signaling in H9c2 Cardiac Myoblasts

Chapter 1: Introduction
Signal transduction is a basic process in cell communication. A cell must interact with its surroundings in order to participate in the highly organized cellular network. Information from the external world is processed and transferred in the form of signals, which further elicit a series of coordinated events in the cell. Receptors and downstream effector proteins are key parts of the cell’s integrated information metabolizing machinery in initiation, transduction and modulation of the signals (37).

The physiological effects of many extracellular stimuli, such as hormones, growth factors and neurotransmitters, are mediated by activation of the phosphoinositide-specific phospholipase C (PLC) effector enzymes. PLC constitutes a large family of the multidomain hydrolytic phosphodiesterases that participate in lipid-signaling pathways in a calcium-dependent manner (43). From the simplest bacterial PLCs to the more complex mammalian forms, PLCs have been found at all stages in evolution. The mammalian PLC family, ranging in molecular masses from 85 to 150 kDa, currently consists of at least 13 separate isoforms in 6 sub-families (β, γ, δ, ε, ζ and η) that differ in their structural organization, mode of activation, cellular and tissue distribution, membrane-binding and catalytic properties (14, 43).

In response to a variety of agents, stimulation of seven transmembrane receptors coupled to the Go_q class of heterotrimeric G protein α-subunits specifically results in activation of PLC-β isoenzymes that catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), a rare membrane phospholipid. This hydrolysis generates two
important second messengers inositol 1,4,5-triphosphate (IP$_3$) and 1,2-diacylglycerol (DAG). IP$_3$ is soluble and releases calcium from endoplasmic reticulum calcium stores to activate a host of calcium-sensitive proteins and ion channels. DAG, in conjunction with this elevated intracellular calcium, activates protein kinase C (PKC). The second messenger-activated PKC in turn, functions by phosphorylating diverse downstream protein targets which control a variety of cellular responses such as proliferation, differentiation, apoptosis and cytoskeletal remodeling. These include proteins that regulate ion channels, growth factor receptors, structural and regulatory cytoskeletal proteins and calcium and calmodulin-binding proteins (14, 43, 69).

Activation of PLC-β enzymes is associated with normal physiological functions (e.g., cell growth and differentiation, smooth muscle contraction, hormone secretion, neuronal activation, and platelet aggregation) as well as pathophysiological processes (e.g. smooth muscle and cardiomyocyte hypertrophy and hyperplasia) (43). Studies using transgenic animal models lacking individual PLC-β isoforms have provided important insights into how these isoforms are implicated in physiological aspects. The absence of PLC-β1 in mice leads to spontaneous epileptic-like seizures and sudden death, suggesting that this isoform is necessary for the maintenance of the neuronal inhibitory pathways. Mice lacking PLC-β2, which is highly expressed in cells of the immune system, show no outward defects; however neutrophils from these animals failed to respond to chemoattractants. PLC-β4 knock-out mice show defects in motor coordination not related to muscle weakness or bone deformity, indicating
that the defects lie in the cerebellum. Also, PLC-β4 null models have defective visual responses in the retina. Interestingly, PLC-β3 seems more essential in the early developmental stages than other isoforms. Wang et al reported that disruption of the PLC-β3 gene in mice leads to lethality as early as within day 2.5 of development, showing disorganized development and low cell mass in the embryo (43). Other studies however, report survival of PLC-β3 deficient mice with defects in scratching behavior induced by histamine (15), or significantly enhanced responsiveness to morphine in antinociceptive tests (63).

Although absence of the individual isoforms may provide well-characterized models indicating that each isoform is critical to a select set of functions, many of the PLC-β isoforms are widely co-expressed in tissues, hence it is possible that some form of adaptation or redundancy is involved. A decisive step would be crossing of the null animal models for determination of this aspect.

On a cellular level, the molecular basis of PLC-β regulatory mechanisms have been intensively studied. These include studies questioning interaction of PLC-β with its physiological activators and membrane phospholipids, PLC-β subcellular distribution and translocation, down-regulation and phosphorylation mechanisms.

Stimulated G protein-coupled receptors promote the exchange of GTP for GDP on Gαq subunits, resulting in dissociation of the trimeric G protein into GTP-bound Gαq and Gβγ subunits. Activation of PLC-β occurs upon direct physical association with
the GTP-bound Gαq subunits or with free Gβγ (43, 54). The interaction is regulated reciprocally in that PLC-β possesses the inherent ability to inhibit Gαq through its inhibitory GTPase-activating protein (GAP) activity towards Gαq subunits (3, 36). Another modulator at the G protein level is the regulators of G protein signaling (RGS) protein whose function is to associate with Gαq and increase the rate of GTP hydrolysis, thereby inhibiting G protein signaling (19). PLC-β enzymes can also interact with proteins other than their activator G proteins. Calmodulin, a calcium-sensitive regulatory protein involved in multiple signaling pathways, is an interacting partner of PLC-β1 and -β3 and has been shown to potentiate Gβγ-mediated PLC-β3 activation upon direct binding to PLC-β3 (32). Evidence suggests that PLC-β also associates with diverse membrane structural proteins to form signaling complexes, which may play a role in modulating PLC-β pathway through mechanisms that are not yet completely understood (21, 54).

Although our understandings of how PLC-β-mediated signaling is regulated have increased since the early studies of these enzymes, still a plethora of questions remain unanswered to date. Cultured mammalian cells are recognized as useful models to study PLC-β signaling in cellular contexts; however, cell models often exhibit cell type-specificity and mechanisms involved may not be interpreted as universal. In search of a well-suited cell model to address questions that are not well-understood in regulation of PLC-β enzymes, here we report a cardiac-derived myoblast model,
H9c2 cells, which possess features of PLC-β subcellular distribution not found in any other cell line tested previously.

The study was performed to characterize the mechanisms by which the PLC-β enzyme activity is negatively regulated in hopes of better understanding how the PLC-β-mediated signaling pathway is modulated in this cardiac cell model.
Chapter 2: Literature Review
2.1. Phospholipase C-β isoenzymes and structural information

Currently, four mammalian PLC-β isoenzymes encoded by different genes and splice variants have been identified and characterized that differ in their tissue distribution and response to G proteins (43). PLC-β2 and PLC-β4 have limited tissue distributions, while PLC-β1 and PLC-β3 are widely distributed and co-expressed in a variety of tissues including brain, heart, liver, uterus, lung, parotid gland and platelets (43). In brain, PLC-β1 is the dominant isof orm expressed, whereas PLC-β3 is dominant in heart and smooth muscles. In many mammalian cell models, PLC-β1 and -β3 are co-expressed. PLC-β2 is expressed at highest levels in immune system, cells of hematopoietic origin, and PLC-β4 is abundant in the cerebellum and retina (9, 13, 16, 20, 29, 30, 33, 45, 51, 56). Despite studies that have attempted to define the importance of differential expression of PLC-β isoforms in tissues and in cell models, the relative contribution of each isoform to overall phosphatidylinositol hydrolysis remains largely undetermined.

Stimulation of the four PLC-β enzymes occurs by direct interaction with GTP-liganded Gαq subunits or with free Gβγ subunits which can separately activate effector molecules. Each PLC-β isoform is differentially sensitive to Gαq or Gβγ subunits; PLC-β1 and -β4 are most strongly activated by GTP-bound Gαq, but are relatively insensitive to activation by Gβγ, while PLC-β2 and -β3 are sensitive to activation by
both Go_q and Gβγ (44, 54). However, the in vivo consequences of this differential sensitivity are mostly unknown.

Structurally, PLC-β isoenzymes are multidomain calcium-binding proteins containing sequences with homology to several characterized protein structural motifs commonly found in membrane-associated, calcium and lipid binding proteins (Fig. 1). These motifs include a PH (pleckstrin homology) domain, four EF hand (elongation factor) domains, a C2 homology domain and a PDZ (PSD-95, DLG, and ZO-1) binding domain at the carboxy-terminus. The two catalytic X and Y domains are highly conserved among other PLC family members (14, 43). Multidoms of PLC-β isoenzymes and their functionality in regulation of the enzyme activity have been studied extensively; however, the exact roles these domains and motifs play in a large context are far from completely understood.

The PH domain is the putative module interacting with free Gβγ subunits and other protein partners whereas in some other PLCs, e.g. PLC-δ, the PH domain plays a crucial role in direct association with membrane phospholipids (14, 43, 59).

Like all PLCs, PLC-β requires calcium for activity; however, the exact calcium-interacting sites on PLC-β are still ambiguous. Calcium is required in the catalytic core for hydrolysis of substrates. In addition, the C2 domain in PLC-δ is a binding site of calcium and also serves to assist the catalytic site onto the membrane. In PLC-β, however, the function of the C2 domain is less well understood, and studies show that the domain may play a role in binding to activated GTP-bound Go_q proteins (14, 43, 58). Although this may be the case in biochemical binding studies, the recently
proposed crystal structure of PLC-β2 in complex with Rac1 GTPase suggests that the C2 domain makes no direct contact with the Rac1 GTPase (24). Moreover, the four EF hand domains, which function as calcium-binding sites in other structural contexts, are different in PLC-βs at key residues, suggesting that these regions may be involved in some other regulatory functions (42).

The carboxy-terminal (C-tail) domain of approximately 400 amino acids in PLC-β, by which PLC-βs are distinguished from other PLC family members, is involved in membrane association and activation by GTP-bound Goq (23, 27, 35, 49). This region also contains several putative phosphorylation sites that may modulate the extent of Goq activation and interactions with the membrane phospholipid substrates or multiple protein regulators (62, 64, 65). Additionally, the C2 domain in the catalytic X and Y region of PLC-β1 and -β2 appears to be involved in Goq interaction and transmission of the signals to the catalytic sites (58).

2.2. Subcellular localization of phospholipase C-β

2.2.1. Phospholipase C-β distribution between membrane and cytosol

Studies on the subcellular localization of mammalian PLC-β isoenzymes have revealed that PLC-βs do not show a uniform localization pattern in tissues or in various mammalian cell models. In brain, PLC-β1 and -β3 may be purified from
cytosolic tissue fractions as well as from particulate, membrane fractions, e.g. PLC-β1 from bovine brain (45, 46) and PLC-β1 and -β3 from rat brain (25), while PLC-β3 primarily partitions with cytosolic fractions from cardiac and uterine tissues (unreported data; S. H. Kwon). When CV-1 cells were overexpressed with PLC-β1, a majority of the population localized with membrane particulate (27). In differentiated rat adrenal pheochromocytoma PC12 cells, both endogenous and overexpressed PLC-β1 are found not only associated with the plasma membrane but a significant cytosolic population of PLC-β1 was also observed, whereas overexpressed Gαq in the same cells was almost entirely localized on the plasma membrane. This cytosolic PLC-β1 population was stable upon Gαq stimulation, suggesting that the cytosolic PLC-β1 does not serve as a reservoir in the event of agonist stimulation (8). Interestingly however, PLC-β2 distribution studies in overexpressing HEK 293 cells and COS-7 cells showed that overexpressed PLC-β2 mainly resides in the cytosol where upon cell stimulation it translocates to the plasma membrane (22), suggesting that isoform-specific mechanisms are involved in regulation of the PLC-β distribution. Cytosolic PLC-β1 has also been observed by cell fractionation in COS cells (27). In HEK 293 cells and 1321N1 astrocytoma cells expressing endogenous PLC-β1 and -β3, PLC-β3 was found in plasma membrane fractions as well as in cytosolic fractions in both cell types (see Results). PLC-β1, however, was detected mainly in plasma membrane fractions in 1321N1 cells, but displayed both cytosolic and membrane distribution in HEK 293 cells (unreported data).
Despite efforts to determine what differential roles these cytosolic and plasma membrane populations of PLC-β play, the basis of their subcellular localization has not been well understood currently and the molecular mechanisms by which the PLC-β distribution is governed are yet to be defined. Studies suggest that PLC-β can bind strongly and fairly nonspecifically to phospholipid vesicles of varying compositions, and localization of PLC-β due to membrane interactions may give rise to both cytosolic and membrane populations (8, 23). In addition, PLC-β interaction with other membrane-linked protein partners which may participate in the integration of signaling components is also probable. PLC-βs are shown to interact with a number of PDZ-containing scaffolding proteins through C-terminal PDZ-binding domains (1, 21, 54). These proteins are thought to play essential roles in promoting recruitment of membrane signaling components and forming signaling complexes in a tissue or cell type-specific manner. Shank2, for example, is a PDZ scaffolding protein which forms a signaling complex with the metabotropic glutamate receptor mGluR and other anchoring components in the neuron. Disruption of PLC-β3 interaction with Shank2 reduced the Gαq-coupled mGluR-mediated calcium release, implying that Shank2 may play a role in efficiently coupling the G protein receptors to PLC-β effectors (21). However, the physiological significance of this coupling remains undetermined. Although data suggest that PLC-β is organized with structural proteins and interactions with these components are possible parameters in modulating PLC-β
signaling presumably by means of determining localization, further information is necessary to fully appreciate the basis of PLC-β subcellular distribution.

To determine mechanisms that regulate subcellular localization and translocation of PLC-β isoforms in cell models, we studied the PLC-β isoform expression and subcellular distribution in HEK 293 cells and 1321N1 cells. More recently, we characterized a cardiac-derived cell model where PLC-β3 is the only isoform endogenously expressed at detectable levels and in which PLC-β3 is almost entirely membrane-localized, unlike other mammalian cell models studied. H9c2 cardiomyoblasts are originally derived from embryonic rat heart tissue and have adopted morphological and biochemical features of skeletal muscle and adult cardiac cells (18, 28). The cell model is unique in that almost no cytosolic PLC-β3 population is observed in the basal state when analyzed by biochemical methods (see Results). As detailed in Chapter 2.5, H9c2 cells are responsive to a variety of agonists including carbachol, oxytocin and arginine vasopressin, that are capable of stimulating the Ga<sub>q</sub>/PLC-β/PKC signaling pathway as we and others have reported (6, see Results). In our present study, PLC-β3 subcellular distribution and translocation in H9c2 cells were characterized as part of our larger goal to determine the molecular mechanisms by which mammalian PLC-β3 enzyme activity is regulated in vivo.

2.2.2. Phospholipase C-β association with plasma membrane subcompartments
Plasma membrane is recognized as a dynamic surface and is the primary site of PLC-β action. However the precise distribution of PLC-βs within the membrane is unclear and limited information is available on the membrane microdomain localization of these enzymes. During the past several decades, detergent-insoluble low density membrane compartments, also known as lipid rafts, have been studied, and the functional importance of these fine lipid-rich structures in a variety of cellular events, e.g. signal transduction, lipid transport, membrane internalization processes, has been established (38). The detergent-resistant membrane population is comprised of at least two types of domains: invaginated caveolae and flat rafts. Caveolin, an integral membrane protein, is the principal protein component of caveolae while flat rafts generally lack caveolin. Experimentally however, these two domains are typically isolated together during subcellular fractionation and often are undistinguished (38). Evidence shows that raft protein components not only play roles in determination and organization of the raft structure but can also interact with diverse membrane-linked signaling components as well. In support of this notion, caveolin was shown to interact with many growth factor receptors and downstream molecules leading to inhibition of cellular growth and proliferation (11, 12). Of interest, it was suggested that changes in caveolin expression in pathogenic rat hearts may play a role in cardiac hypertrophy through mechanisms involving regulation of the α1-adrenergic-mediated signaling (11).
It has been revealed that many signaling molecules accumulate in lipid rafts and these signaling components are able to relocate into or out of rafts upon stimulation (2, 53). Hence, it is conceivable that more efficient membranous signaling may occur in these regions than in non-raft membranes. More recent studies show that G protein-coupled receptors, G proteins and protein kinases are enriched in lipid rafts at rest and can translocate into non-raft membranes or to cytosol upon stimulation in PC-12 cells (53). In human platelets, stimulation with thrombin triggers relocation of PLC-β2 and -β3 from detergent-soluble to -insoluble actin-rich cytoskeletal fractions (2).

Head et al studied the expression and co-localization of diverse G protein-coupled receptor (GPCR) signaling components with the muscular form of caveolin (caveolin-3) in adult rodent cardiomyocytes and demonstrated that caveolin-3 co-localizes and interacts with multiple classes of GPCR components that regulate cyclic AMP production (e.g. β1- and β2-adrenergic receptors, M2 and M4 muscarinic receptors, μ-opioid receptor, adenylyl cyclase 5/6, Gαs and Gαi). However, the distribution pattern of each component with caveolin-3 was not uniform and was different from distributions reported for neonatal cardiocytes or some other non-cardiac cell models (17). The consequences of this differential distribution are not obvious.

Association of PLC-β3 with detergent-soluble as well as insoluble membrane fractions was reported in thrombin-stimulated platelets (2). However, Fujita et al have reported the exclusive accumulation of PLC-β subtypes (PLC-β1 and -β3) along with Gαq and α1-adrenergic receptors in caveolin-enriched membrane fractions from rat heart tissue.
using a non-detergent method to prepare raft membranes (12). Caveolar concentration of α1-adrenergic receptor was also demonstrated in H9c2 cells by Fujita et al, however, no data was available on G protein and PLC-β subtype co-localization with caveolin in these cells from their report (12). PLC-β1 was also shown to localize with raft membranes prepared from synaptic plasma membrane of rat brain (55).

Several inconsistencies were also reported in the literature on distribution of PIP₂ on the plasma membrane. PIP₂, as the primary substrate for PLC-βs and other PLC families, is thought to play multiple roles in coordinating membrane-related events such as vesicular trafficking, membrane movement and cytoskeletal dynamics (31). While Martin and Pike et al demonstrated that phosphoinositides, including PIP₂, are largely compartmentalized and enriched in lipid rafts, and that PIP₂ hydrolysis preferentially occurs in intact caveolar rafts (31, 38-40), a more recent report by Watt et al detected less than 10% of the total cell surface PIP₂ in caveolae (59). Interestingly, these studies were done on the same cell line, without explanation of the apparent discrepancies; reconciliation awaits further studies.

Although still being intensively studied, potential explanations for these discrepancies are discussed below. Lipid rafts are enriched in cholesterol and glycosphingolipids, as their low buoyant density comes from this characteristic lipid composition. However, lipid constituents in rafts often display heterogeneity leading to non-uniform raft populations. A number of biochemical and immunological methods to isolate lipid rafts have been developed and different methodologies to prepare raft membranes may produce different results. This observation has given rise to speculation that lipid rafts
are inherently heterogenous in protein and lipid composition, and thus are differentially sensitive to a variety of preparation methods (38). Thus, any analysis of lipid rafts must involve a clear understanding of the method being used.

In this context, and as discussed earlier, it is possible that PLC-β enzymes interact with components within the plasma membrane microdomains, such as scaffolding or cytoskeletal proteins, which are capable of modulating signaling proteins. Membrane microdomains are well known for harboring diverse anchoring proteins and forming complexes with signaling components (54). Further studies should provide deeper understandings of how such interactions can relate to modulation of PLC-β activity.

2.3. Phosphorylation of phospholipase C-β3

Protein phosphorylation can be a critical regulatory parameter in eukaryotes. The G protein-coupled receptor signal transduction network utilizes numerous phosphorylation events to either dampen or promote downstream signaling. In both cases, phosphorylation typically modulates the protein interaction with its binding partners.

Previously, Yue et al and Xia et al reported phosphorylation of PLC-β3 following activation of a variety of protein kinases in diverse mammalian cell models and have identified a number of specific phosphorylation sites of *in vitro*-phosphorylated PLC-β3 (62, 64-66). In tissues, PLC-β3 phosphorylation was observed in guinea pig longitudinal muscle myenteric plexus (LMMP) during chronic morphine treatment (5)
and in primary glomerular mesangial cultures in high glucose conditions (10). Phosphorylation of PLC-β3 appears to occur physiologically as a consequence of direct (negative) feedback regulation by increased intracellular calcium and DAG (65), of cross-pathway regulation by the cAMP-dependent PKA (64) or from the cGMP-dependent PKG pathway (62). Cellular studies demonstrated that G protein-stimulated PLC-β3 activity, as determined by the extent of phosphoinositide turnover, was inhibited when PKA catalytic subunits or PKG isoforms were overexpressed in COSM6 cells and COS-7 cells, respectively (62, 64). When assayed in whole cells transfected with PLC-β3, direct activation of endogenous PKC by phorbol 12-myristate 13-acetate (PMA) or activation of overexpressed PKG by the cGMP analogue 8-pCPT-cGMP showed marked decreases in agonist-stimulated production of total inositol phosphates along with increases in PLC-β3 phosphorylation (62, 65).

Although the correlation between protein kinase activation and inhibition of PLC-β3 activity was demonstrated in these studies, the exact biochemical mechanism that governs phosphorylated PLC-β3 interaction with other signaling components is not completely characterized. In CHO cells, Strassheim and Williams proposed that phosphorylation of PLC-β3 mediated by PKC diminishes the interaction of Gαq/11 with PLC-β3, thereby inhibiting the PLC-β3 activity (52).

PLC-β3 can be phosphorylated at multiple residues. The predominant phosphorylation site by PKC, which is also phosphorylated by PKA and PKG, is reported to be Ser1105. In above studies, mutation of this serine residue (Ser1105→Ala) removed
the inhibitory effects of all three kinases on the G protein-activated mutant PLC-β3 activity. Minor putative phosphorylation sites on PLC-β3 were also reported and identified as Ser26 and Ser474 residues which are likely to be phosphorylated secondarily by the above kinases (62, 64, 65). These three putative sites are located within different domains of PLC-β3; Ser26 lies on the PH domain which may play a role in interaction with Gβγ subunits, Ser474 in proximity to the XY catalytic domain and Ser1105 is within the C tail. As noted in Chapter 2.1, the PLC-β C-terminal extension is what structurally distinguishes PLC-β family from all other PLCs, and is known to be involved in interaction with the activated Gαq subunit, association with membrane phospholipids and other regulatory functions (23, 27, 35, 49). Phosphorylation of PLC-β3 by Ca²⁺/calmodulin-dependent protein kinase II (CAMKII) was reported to occur at Ser537 in the linker region between the catalytic X and Y domains, however, Ser537 phosphorylation is less well studied than other kinase effects (66). A number of phosphoserine-specific antibodies became commercially available which recognize the phosphorylated form of PLC-β3 at particular serine residues. For example, above-mentioned PLC-β3 studies in LMMP tissues and in glomerular mesangial cells both took advantage of the phospho-Ser1105 specific antibody to detect phosphorylated form of PLC-β3 (5, 10). Since the primary PLC-β3 phosphorylation site is located within the C-terminal domain, which is crucial in membrane association as well as interaction with the Gαq
subunit, it is tempting to speculate that phosphorylation in this region may affect PLC-β3 interaction with the membrane structure or phospholipids themselves.

Although Strassheim and Williams proposed the notion that PKC activation followed by phosphorylation of PLC-β3 negatively affects PLC-β3 association with activated Gαq/11 (52), other possibilities that may have contributed to PLC-β3 deactivation were not tested in their studies or elsewhere. In fact, their observation was based on immunological co-precipitation of PLC-β3 with activated Gαq/11 before and after PKC stimulation.

These observations led us to speculate that translocation of membrane-bound PLC-β3 to the cytosol upon PKC stimulation may serve to limit the activity of PLC-β3. In this study, we tested the hypothesis that PLC-β3 phosphorylation alters membrane localization of PLC-β3 using H9c2 cells, a cardiomyoblast model, in which PLC-β3 is predominantly confined to the plasma membrane.

2.4. H9c2 cardiomyoblasts- A model system to study phospholipase C-β3

H9c2 cells, a permanent cardiac cell line derived from embryonic rat heart tissue, were first studied and characterized by Kimes and Brandt (28) and their morphological, biochemical and electrophysiological properties were reinvestigated by Hescheler et al (18). Originally, Kimes and Brandt reported that these cells had adopted features of skeletal muscle as they express nicotinic receptors and can synthesize certain muscle-specific enzymes. Morphologically, H9c2 cells are more similar to immature
embryonic cardiocytes and can undergo differentiation as well as dedifferentiation depending on culture conditions. Early electrophysiological studies showed that H9c2 cells exhibit electrical characteristics of cardiac currents, therefore the cell line has been adopted in studies of cardiotonic or antiarrhythmic agents (18). The cell line also serves as an accepted model for cardiac hypertrophy research (4, 11, 12, 61).

H9c2 cells respond to a variety of GPCR signal-transducing agents and the presence of the corresponding receptors and downstream signaling pathways were identified. Like adult cardiomyocytes, H9c2 cells are sensitive to catecholamine stimulation (7, 48) and express acetylcholine receptors (28). In Chapter 4, Results, we show that H9c2 cells respond to carbachol, a muscarinic acetylcholine receptor agonist, in whole cell IP accumulation assays. Cellular effects of carbachol stimulation were not extensively studied in these cells except that an increase in glucose oxidation was reported in the energy metabolic pathway upon stimulation (60).

Arginine vasopressin, through the Gαq-coupled V1-vasopressin receptor, induces a dose-dependent increase in IP formation and arachidonic acid release which are both pertussis toxin-insensitive in H9c2 cells (6). In addition to its antidiuretic action on the renal Gs-coupled V2 receptors, vasopressin is involved in a number of cardiovascular activities (e.g. vasoconstriction and myocardial hypertrophy) mediated by the V1 receptors (4). Vasopressin is known to function as a growth factor that can induce cellular hypertrophy and tissue hyperplasia (57). Most notably, the cardiac-derived H9c2 cells were shown to undergo hypertrophic events upon stimulation of the V1
receptor (4, 61). Upon vasopressin stimulation, H9c2 cells displayed standard hypertrophic responses verified by increased cellular protein accumulation and morphology changes. However, other hypertrophic agents such as angiotensin II, phenylephrine and endothelin-1 that are known to induce hypertrophy in cardiomyocytes were ineffective under conditions that vasopressin promoted hypertrophy in H9c2 cells (4).

Cells of cardiac origin typically provide a suitable tool for electrical and hormonal signaling pathway studies; however, myoblasts like H9c2 cells may not have preserved all elements of mature cardiocytes, therefore may not precisely resemble pharmacological and physiological profiles of adult cardiocytes (28). Adrenergic receptor distribution between adult rat heart and H9c2 cells is an example. H9c2 cells express both isoforms of the $\beta$-adrenergic receptors; however, subtype distribution and up-regulation of these receptors is different from those expressed in rat heart tissue (7). In addition, although studies report that H9c2 cells express endogenous $G_\alpha_q$-coupled $\alpha_1$-adrenergic receptors (12), these cells were relatively insensitive to short-term phenylephrine stimulation, an $\alpha_1$-adrenergic receptor agonist (see Results), which normally causes vasoconstriction in the blood vessels and can induce hypertrophy in hearts. Isolated neonatal rat cardiac myocytes have been shown to respond to phenylephrine stimulation and further develop hypertrophy as characterized by activation of specific gene profiles, increased total protein expression and accumulation, and yielding significantly enlarged cell size (41). Lack of significant
phenylephrine response in H9c2 cells may be in line with the relatively low expression levels of α1-adrenergic receptors, as reported by Fujita et al (12). Interestingly however, in rodent cardiac tissues, α1-adrenergic receptors are shown to display selectivity for PLC-β1 isoforms over PLC-β3, in the presence of both isoforms (1). In this respect, since we report that PLC-β1 isoforms were not detected in H9c2 cells, the functionality of the α1-adrenergic signaling pathway remains questionable in this particular cell type (see Results).

In searching for an experimental cell system in which to study PLC-β regulation mechanisms in vivo, we find that PLC-β3 is the only PLC-β isoform expressed in H9c2 cells and is localized almost exclusively with membrane fractions. We also confirmed that arginine vasopressin increased [3H]IP formation in a concentration-dependent manner in these cells as previously reported (6, see Results). More interestingly, we observed that activation of endogenous PKC by phorbol 12-myristate 13-acetate (PMA) treatment significantly inhibited agonist-stimulated IP formations as previously reported in other non-cardiac cell models (65, see Results).

Together, these observations led to investigations characterizing molecular mechanisms responsible for modulating PLC-β3 activity in H9c2 cells.
Figure 1: *Structure and domain function of PLC-β isoenzyme.* The PH domain is the putative module interacting with free Gβγ subunits. Calcium is required in the catalytic core for hydrolysis of PIP₂. The carboxy-terminal (C-tail) domain is involved in membrane association and activation by GTP-bound Gα₉ proteins. Abbreviations for domains follow: Pleckstrin homology domain (PH), EF-hand domain (EF hands), X catalytic domain (X), Y catalytic domain (Y), C2 domain (C2), carboxy-terminus of the protein (C-Terminus) and PDZ (PSD-95, DLG, and ZO-1) binding domain (PDZBD).
Chapter 3: Materials and Method
3.1. Materials

Rabbit polyclonal antibody specific for the phosphorylated form of PLC-β3 (Ser1105) was obtained from Cell Signaling Technology, Inc. (Danvers, MA). Fluorophore-conjugated IRDye 680 conjugated goat anti-rabbit secondary antibody was purchased from Li-Cor Biosciences (Lincoln, NE). All other antibodies including isoenzyme-specific rabbit polyclonal antibodies raised against peptide sequences unique to PLC-β1, 2, 3 or 4, and horseradish peroxidase-conjugated goat anti-rabbit secondary antibody were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The Optiprep density gradient media was purchased from Axis-Shield PoC (Oslo, Norway). [³H]Inositol (1 mCi/ml) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and [γ-³²P]ATP (10 mCi/ml) was purchased from PerkinElmer (Waltham, MA). Purified PKC enzymes isolated from rat brain, mixture of isoforms α, β1, β2, and γ, were purchased from Calbiochem (Cat. No. 539494, San Diego, CA). All other reagent-grade chemicals and drugs used in this study including protease inhibitors and phosphatase inhibitors were purchased from commercial sources. Cell culture materials including fetal bovine serum were from HyClone (Logan, UT) and Cellgro Mediatech (Manassas, VA).

3.2. Purification of PLC-β from Sf9 insect cells
All purified PLC-β isoenzymes including the Ser1105→Ala mutant PLC-β3 were expressed in and purified from baculovirus-infected insect Sf9 cell cultures. The Ser1105→Ala mutant PLC-β3 was constructed by Yong Zhang in our lab. Proteins purified this way were kindly provided by Dr. Walter K. Vogel in our lab.

### 3.3. Cell culture

H9c2 cardiomyoblasts derived from embryonic rat heart tissue and human astrocytoma 1321N1 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). Human embryonic kidney (HEK) 293T cells were kindly provided by Dr. Mark Leid (College of Pharmacy, Oregon State University). H9c2 stock cultures were maintained in the myoblastic state in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS), and 10,000 U each of penicillin and streptomycin. For experiments, cells were plated onto tissue culture dishes or wells and grown to near confluency (70-90%) in a 37 °C humidified atmosphere with 5% CO₂. H9c2 cells above passage number 35 were not used for experiments in this study as we observe changes in cell growth rate and morphology above this passage number. HEK 293T and 1321N1 cell cultures were similarly maintained.

### 3.4. Separation of plasma membrane and cytosol in cultured cells
Methods for separation of membrane vesicles from cytosol was based on the Optiprep (60% iodixanol in water, Axis-shield application S28) buoyant density gradient (68). H9c2, HEK 293T or 1321N1 cells were harvested, washed twice with ice-cold phosphate-buffered saline (PBS) and Dounce homogenized in a hypotonic lysis buffer (25 mM HEPES pH 7.4, 2 mM EDTA and 1 mM DTT plus protease inhibitors; 0.1 mM PMSF, 1 μM Pepstatin A, 10 μM Leupeptin and 20 μM Benzamidine). In some experiments, phosphatase inhibitors (Complete Phosphatase Inhibitor mixture from Roche Molecular Biochemicals, Indianapolis, IN) were included in all buffers and solutions used. After centrifugation at 800 xg for 5 minutes to pellet nuclei and intact cells, the resulting supernatant was made isotonic (150 mM NaCl) and mixed with 50% Optiprep solution to make 400 μl of a final 30% solution. On top of the 30% solution, 500 μl of 25% and 100 μl of 5% solutions were carefully loaded using a syringe to achieve a 1ml discontinuous iodixanol gradient. Ultracentrifugation was performed in a Beckman TLA-120.2 rotor at 120,000 rpm for 1 hr at 4°C, and the 5%/25% interface (membrane vesicles), which appears as a sharp opaque layer, and the bottom 30% fraction (cytosol) were collected by manual displacement. Proteins from each fraction were extracted with chloroform and methanol and suspended in 2X SDS sample buffer. Equal-sized aliquots from each fraction were analyzed by SDS-PAGE and immunoblotting.

3.5. In vivo and in vitro phosphorylation of PLC-β3 by PKC
3.5.1. Agonist-induced activation of the Gαq/PLC-β3 pathway or direct activation of PKC by PMA in H9c2 cells

H9c2 cells plated in 100-mm culture dishes were grown to near confluency in DMEM media with 10% FBS and then serum-starved overnight at 37°C. On the day of experiment, cultures were exposed to PMA (1 μM) for direct activation of PKC, vasopressin (100 nM) or carbachol (100 μM) for stimulation of the Gαq/PLC-β3 pathway, for 25 minutes at 37°C. Cells were washed twice with ice-cold PBS containing 20 mM sodium fluoride, 5 mM sodium pyrophosphate and 50 nM calyculin A (phosphatase inhibitors), harvested and Dounce homogenized in hypotonic lysis buffer containing protease inhibitors and phosphatase inhibitors (Complete Phosphatase Inhibitor mixture from Roche Molecular Biochemicals). After a centrifugation at 800 xg for 5 minutes to pellet nuclei and intact cells, proteins from the resulting supernatant were extracted with chloroform and methanol. Equal-sized aliquots from each protein sample were analyzed by SDS-PAGE and immunoblotting. In some experiments, a time-dependent stimulation was conducted and cells were collected after the designated time period for stimulation. Cultures were either treated with PBS, PMA (1 μM) or vasopressin (100 nM) for 25 minutes, 12, 24 or 48 hrs. Harvested cells were subjected to the Optiprep separation method described in detail in 3.4 to separate membrane from cytosolic proteins. Proteins from both fractions were precipitated with chloroform and methanol before analysis by SDS-PAGE and immunoblotting.
3.5.2. In vitro phosphorylation of PLC-β3 by PKC

In vitro phosphorylation of PLC-β3 by PKC to obtain positive and negative Ser1105-phosphorylated controls for immunoblotting was carried out according to methods modified from Yue et al (65). Purified PLC-β3 or PLC-β3Ser1105→Ala proteins were incubated for 1 hr at 30°C with purified PKC enzymes isolated from rat brain, mixture of isoforms α, β1, β2, and γ, in the presence of 200 μM ATP in a total volume of 10 μl of PKC reaction buffer (20 mM TES pH 7.3, 4 mM CaCl₂, 5 mM MgCl₂, 3 mM EGTA, 100 μg/ml phosphatidylserine, 20 μg/ml diacylglycerol). Kinase reactions were stopped by adding SDS sample buffer. PLC-β3 phosphorylation by PKC was confirmed in a parallel experiment using [γ-32P]ATP. Known quantities of PLC-β3 were in vitro-phosphorylated in the presence of [γ-32P]ATP in reaction conditions described above and analyzed by SDS-PAGE. Incorporation of 32P was imaged using the PhosphorImager SI and ImageQuant (Molecular Dynamics, Sunnyvale, CA) and pmols of 32P associated with PLC-β3 bands were determined by comparison to pmols of 32P calibration standards, from which the stoichiometry (mol phosphate/mol PLC-β3) was calculated (see Results).

3.6. Western blot analysis

3.6.1. Electrophoresis
The sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed according to the modified Laemmli system. Sample proteins were routinely analyzed on 7.5-12% polyacrylamide gels. In some experiments, sample protein content was determined by protein assays (Bio-rad detergent-compatible protein assay, Hercules, CA) before electrophoresis.

3.6.2. Immunoblotting

Proteins were electrophoretically transferred onto nitrocellulose membranes (semidry electroblotting) and blocked with 5% non-fat milk in immunoblotting buffer (20 mM Tris pH 7.4, 500 mM NaCl, 0.05% Tween 20) for 1 hr. For anti-phospho PLC-β3 blots, 3% BSA in immunoblotting buffer was used instead. All primary and secondary antibodies were used diluted in immunoblotting buffer with 1% BSA as recommended by manufacturers. Typically, primary antibodies were diluted 1:2,000 and secondary antibodies were diluted 1:20,000. Protein bands were visualized using the western blot detection system from PIERCE (Rockford, IL) and length of film exposure time was optimized for each experiment.

3.6.3. Quantitative western blot analysis

In some experiments, PLC-β3 protein bands were quantified using the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln NE). After incubation of the
nitrocellulose membrane with anti-PLC-β3 rabbit polyclonal primary antibody (Santa Cruz Biotechnology) and subsequently with the IRDye 680 conjugated goat anti-rabbit secondary antibody (Li-Cor Biosciences), the membrane was scanned at 700 nm wavelength in a Li-Cor Odyssey Imager. Standard curves were generated from purified PLC-β3 and protein bands were analyzed and quantified using the Li-Cor Odyssey Software according to the manufacturer’s manual.

3.7. H9c2 whole cell IP accumulation assays

Phosphatidylinositol hydrolysis upon agonist stimulation in H9c2 cells was assessed by measuring $[^3]$H]inositol phosphate accumulation as described by Zhang et al (67) and McCullar et al (32). Typically, cells were grown to near confluency on 24-well plates and labeled with 1 μCi/well $[^3]$H]inositol during an overnight 20 hr-incubation period in inositol-free DMEM lacking serum. Where indicated, pertussis toxin (PTX) was added to 100 ng/ml per well. The following day, cells were adjusted for 10 min in serum-free 10 mM Hepes-buffered DMEM pH 7.4, containing 10 mM LiCl, prior to agonist stimulation for 90 sec or for 20 min in a 37°C waterbath in room air. In some experiments, cells were subjected to 1 μM PMA or 0.1% dimethyl sulfoxide (DMSO) pretreatment for 25 min before agonist stimulation. Reactions were stopped by adding 5% trichloroacetic acid to lyse cells for 5 min. Cell lysates were extracted three times with ethyl ether and inositol phosphate species were separated on Poly-prep columns containing a 0.5 ml bed volume of AG1-X8 anion exchange resin (Formate form, 200–
400 mesh, Bio-Rad Laboratories, Hercules, CA). Total IPₙ species (IP, IP₂, and IP₃) were collected and radioactivity was determined in a liquid scintillation counter. All assays were performed in triplicate and values reported represent at least two separate experiments.

### 3.8. Data Analysis

Data analysis was performed using GraphPad Prism 4 (GraphPad software, San Diego, CA). Data are presented as mean ± S.D with $p < 0.05$ considered significant as determined by Student's unpaired two-tailed $t$-test.
Chapter 4: Results
4.1. Identification of PLC-β isoenzyme expression and subcellular distribution in H9c2 cardiomyoblasts

The PLC-β family is comprised of four known isoforms (-β1 to -β4). Among them, PLC-β1 and -β3 are found ubiquitously expressed in a variety of tissues (see Literature Review). Expression and subcellular distribution of all four PLC-β isoenzymes were investigated in H9c2 cells by cell fractionation. Membranes were floated in an iodixanol buoyant density gradient to obtain improved resolution of membrane and cytosol (see Materials and Methods). Conventional centrifugation methods to pellet membranes frequently result in contamination of membranous pellets with cytosolic components and vice versa. For confirmation of efficient buoyant density separation, membrane and cytosolic fractions were also blotted against the selective plasma membrane marker, cadherin. We found that in H9c2 cells, PLC-β3 is the only isoform endogenously expressed at detectable levels and its population is predominantly confined to the membrane (Fig. 2.2). Many other mammalian cell models and tissues previously investigated in our hands (1321N1 cells, HEK 293 cells, RBL-2H3 cells, adult mouse heart and mouse brain) all showed a larger population of PLC-β3 in the cytosolic fraction as compared to H9c2 cells (data shown for 1321N1 cells and HEK 293 cells, Fig. 2.1, other data not shown). Moreover, except for RBL-2H3 cells, all other cells co-expressed PLC-β1 in both cytosol and membrane fractions (data not shown).
A rough estimate of the endogenous level of PLC-β3 expressed in a known number of H9c2 cells was determined with quantitative western analysis using the Odyssey Infrared Imaging System and IRDye 680 conjugated secondary antibody (see Materials and Methods). A standard curve was generated of purified PLC-β3, and integral fluorescent intensity values of PLC-β3 detected from H9c2 cells were determined. From a single determination, we detected approximately 50 ng of PLC-β3 /2.5x10^6 cells in the membrane fraction alone. However, we were not able to quantitate an amount of cytosolic PLC-β3 due to detection limits.

4.2. H9c2 cells respond to a variety of agonists that stimulate the PLC-β pathway

In H9c2 cells, the presence of the vasopressin V1-receptor/Gαq/PLC-β pathway was previously demonstrated by Chen and Chen (6), and the acetylcholine receptor pathway was detected by Kimes and Brandt (28). In an attempt to identify or confirm Gαq-coupled pathways that activate PLC-β3, H9c2 cells were stimulated with epinephrine, phenylephrine, carbachol, 2-methylthio-ATP, oxytocin or arginine vasopressin at concentrations indicated in Fig. 3.1A and assayed for [3H]inositol phosphate (IP_n) accumulation. Total [3H]IP_n formation was determined upon agonist stimulation for 20 minutes. To rule out other PLC isoenzyme (e.g. PLC-δ, PLC-ε) effects generating from increased intracellular calcium levels, agonists that were capable of inducing a significant increase in [3H]IP_n formation for 20 minutes were then also tested for 90 seconds. This 90 second-time course is known to restrict
activation to PLC-β effects on [3H]IPn accumulations (26, Fig. 3.2). Vasopressin, oxytocin and carbachol significantly induced [3H]IPn production in 20 minutes, while epinephrine and phenylephrine displayed modest increases. H9c2 cells were completely insensitive to 2-methylthio-ATP stimulation. For vasopressin, concentration-dependent IPn formation was additionally tested (Fig. 3.1B) and the half-maximal effective concentration (EC₅₀) obtained was approximately 2.0 nM. To our knowledge, an oxytocin signaling pathway was not previously identified in H9c2 cells. The concentration-response data indicate that the EC₅₀ of oxytocin is approximately 100 fold higher than that reported on oxytocin receptors in other cell types or tissues, and consistent with oxytocin exerting its effects through the structurally similar vasopressin receptor known to be present in these cells (data not shown).

Vasopressin and carbachol effects on H9c2 cells were not completely pertussis toxin (PTX)-insensitive in our experiments (Fig. 3.3). Since PLC-β3 can be stimulated either by Gα₉ or Gβγ subunits separately, and carbachol can also act on PTX-sensitive Gαi-coupled muscarinic receptors that release the Gβγ subunits upon stimulation, any Gβγ effects cannot be ruled out as contributing to total IPn accumulation. Pretreatment of cells with 100 ng/ml pertussis toxin overnight (20 hrs) slightly decreased vasopressin or carbachol-stimulated IPn formations by approximately 23.9 % and 40.6 %, respectively, indicating that Gα₉-coupling and potentially some Gαi receptors are involved in both vasopressin and carbachol actions to mediate PI hydrolysis in H9c2 cells. However, in the Chen and Chen study of H9c2 cells, the same concentration of
PTX treatment for 24 hrs had no effect on vasopressin-induced IP\textsubscript{n} formation or arachidonic acid release, a further downstream effect of vasopressin action, in these cells (6). We were not able to track the cause of this minor discrepancy between their PTX effects and ours. However, it is notable that cells in our study were subjected to serum-starvation during the overnight \[^{3}\text{H}]\text{inositol-labeling.} A simple explanation is that an overnight PTX treatment and serum-starvation alters other parameters, thereby resulting in a minor difference in the vasopressin-stimulated PI hydrolysis.

4.3. PMA inhibits vasopressin- or carbachol-stimulated IP\textsubscript{n} formation

Direct involvement of conventional PKC in PLC-\(\beta\)3 phosphorylation has been suggested in many different cell lines and in tissues. Yue \textit{et al} showed that PMA-activated PKC can inhibit agonist-stimulated IP formation in PHM1-41, HeLa, COSM6 and RBL-2H3 cells, and Strassheim and Williams observed the same deactivation of PLC-\(\beta\)3 in CHO cells (52, 65). It was proposed that the inhibitory effect was due to direct phosphorylation of PLC-\(\beta\)3 at the Ser1105 site by activated PKC, since PMA had no effect on Go\(_{q}\)-stimulated Ser1105→Ala mutant PLC-\(\beta\)3 activity when tested in co-transfected cells (65). In our study, we tested whether PKC activation could inhibit vasopressin- or carbachol-stimulated IP\textsubscript{n} formation in H9c2 cells endogenously expressing PLC-\(\beta\)3. Consistent with the prediction, pretreatment of cells with 1 \(\mu\)M PMA for 25 minutes significantly reduced the agonist-stimulated increase in IP\textsubscript{n} formation (Fig. 4). However, we did not pursue the same
Ser1105→Ala mutant study in these cells, as endogenous level of wild type PLC-β3 expression is high, thus it is difficult to measure the functional integrity of the transfected mutant PLC-β3 in this system.

4.4. Phosphorylation of PLC-β3 at a major phosphorylation site did not occur upon direct activation of PKC or stimulation with vasopressin in H9c2 cells

Ser1105, which is located within the C tail domain of PLC-β3, is now known as a major phosphorylation site on this enzyme (62, 64, 65). Although a number of GPCR pathways are fairly well studied in H9c2 cells, PLC-β3 phosphorylation by PKC was not previously reported in these cells. We used either PMA to directly activate endogenous PKC or vasopressin and carbachol to stimulate the Gαq-coupled receptors which in turn activate endogenous PKC via PLC-β3 stimulation. To our surprise, in both experiments we were not able to detect PLC-β3 phosphorylation at a major putative phosphorylation site, Ser1105, using commercially available anti-phosphoserine antibody for western blotting (Fig. 5). This antibody recognizes the phosphorylated form of PLC-β3 only when it is phosphorylated at Ser1105. To confirm that the antibody affinity or specificity for phosphorylated PLC-β3 was not the problem, we used positive and negative western blotting controls. Purified wild type and Ser1105→Ala site-directed mutant PLC-β3 were in vitro-phosphorylated by purified PKC enzymes as described in Materials and Methods. The wild type PLC-β3 was routinely phosphorylated with a stoichiometry of approximately 0.49 mol of
phosphate/mol protein (data not shown). As shown in Figure 5, wild type PLC-β3 phosphorylated this way was well recognized by our Ser1105 phosphoserine-specific antibody while the mutant was not. Since the antibody only recognizes one phosphorylation site, we were not able to conclude that PKC inhibition of agonist-stimulated PLC-β3 activity occurs through mechanisms that involve PLC-β3 phosphorylation.

4.5. Effects of short-term and long-term PMA or vasopressin treatment on PLC-β3 expression and subcellular distribution in H9c2 cells

Among the nine isoforms (α, β1, β2, γ, δ, ε, ζ, η and θ) of PKC, H9c2 cells endogenously express PKCα, β1, ε, δ and ζ (6). Chen and Chen showed that when H9c2 cells were exposed to 1 μM PMA for 10 minutes, a marked increase in membrane localization of PKC isoforms was observed which correlated well with the decrease in cytosolic population of PKC, suggesting translocation of PKC toward the membranous targets. Prolonged stimulation (24 hrs) with PMA caused complete down-regulation of the PKC isoforms in their study (6). In correlation with these observations, any changes of PLC-β3 behavior during PKC stimulation, which eventually leads to its diminished activity, were questioned.

When Strassheim and Williams revealed that PKC activation followed by agonist-stimulation diminished PLC-β3 co-immunoprecipitated with Gαq/11 (52), we questioned whether the decreased co-precipitation was truly a direct effect of altered association of Gαq/11 with PLC-β3. In fact, other regulatory mechanisms such as PLC-
β3 release from the membrane and translocation to the cytosol, or down-regulation cannot be ruled out since these events may also result in apparent decrease in Gαq/11 interaction with PLC-β3. We tested these possibilities in H9c2 cells, taking advantage of the exclusive membrane localization of the endogenous PLC-β3 population. We detected no changes in subcellular localization or expression level of PLC-β3 upon short-term and long-term stimulation of the pathway. H9c2 cells were treated with either 1 μM PMA or 100 nM vasopressin in serum-free growth conditions for 25 minutes, 12, 24 or 48 hrs before harvest and fractionated into membrane and cytosolic fractions using the buoyant density separation method described earlier. Figure 6 shows that short-term and long-term treatment with either PMA or vasopressin had no effect on membranous PLC-β3 translocation to the cytosol. Moreover, PLC-β3 expression levels remained unchanged compared to the PBS-treated controls, even upon long-term exposure to PMA or vasopressin, suggesting that PLC-β3 down-regulation did not occur during the time frame tested.

Although how activated PKC inhibits agonist-stimulated PLC-β3 activity is still questionable, the most widely accepted mechanism points to PLC-β3 phosphorylation by PKC at a major serine residue, ultimately altering its biochemical properties (5, 10, 65). However, we did not detect PLC-β3 phosphorylation at the primary phosphorylation site upon PKC activation in H9c2 cells.
Figure 2.1: *PLC-β3 subcellular localization in HEK 293 and 1321N1 cells.* HEK 293 (top panel) and 1321N1 (middle panel) whole cell lysates were fractionated into membrane and cytosolic fractions in a buoyant density Optiprep gradient. Membrane (M) and cytosolic (C) proteins were precipitated with chloroform and methanol and resuspended in 2x SDS sample buffer. Equal-sized aliquots were separated by 7.5% SDS-PAGE and analyzed by western blotting. To confirm that the buoyant density separation was efficient, membrane and cytosolic proteins from HEK 293 cells were also blotted for cadherin (bottom panel), a selective marker for plasma membranes. Arrows indicate migration of PLC-β3 and cadherin at expected sizes. Results shown are typical of three independent experiments.
Figure 2.2: PLC-β isoenzyme expression and subcellular localization in H9c2 cardiomyoblasts. H9c2 cell lysates were fractionated into membrane and cytosolic fractions in a buoyant density Optiprep gradient. Membrane (M) and cytosolic (C) proteins were precipitated with chloroform and methanol and resuspended in 2X SDS sample buffer. Equal-sized aliquots were separated by 7.5% SDS-PAGE. Western blotting was performed using isoenzyme-specific PLC-β antibodies. To confirm that the buoyant density separation was efficient, membrane and cytosolic proteins were blotted for cadherin, a selective marker for plasma membranes. β-Actin is also shown as a control for protein loading. Arrows indicate migration of each purified PLC-β protein positive controls, cadherin and β-actin at expected sizes. Results were confirmed in two independent experiments.
Figure 3.1: **H9c2 cells respond to a variety of agonists.** (A) H9c2 cells were seeded on 24-well plates and grown to confluency. Cells were labeled with $[^3]$H]inositol and stimulated with each agonist at the following concentrations for 20 min in Hepes-DMEM media containing 10 mM LiCl: control (PBS), phenylephrine (PE; 100 $\mu$M), epinephrine (10 $\mu$M), oxytocin (100 nM), carbachol (100 $\mu$M) and 2-MeS-ATP (1 $\mu$M). (B) Cells were labeled with $[^3]$H]inositol as described above and were stimulated with indicated concentrations of arginine vasopressin (AVP) for 20 min in Hepes-DMEM media containing 10 mM LiCl. Total $[^3]$H]IP$_n$ formed were measured as described in Materials and Methods. Results shown are the average of triplicates and representative of two independent experiments. Error bars give the range of triplicates. ** indicates p<0.01 and *** indicates p<0.001 compared to Control.
Figure 3.2: 90 Second agonist-stimulated IP₃ formation in H9c2 cells. PE, AVP and carbachol were tested for specific PLC-β-mediated IP₃ formation in H9c2 cells. Cells prelabeled as described were stimulated with either phenylephrine (PE; 100 μM), arginine vasopressin (AVP; 100 nM) or carbachol (100 μM) for 90 sec in Hepes-DMEM media containing 10 mM LiCl. [³H]IP₃ formed was measured by methods described in Materials and Methods. Results shown are the average of triplicates and representative of two independent experiments. Error bars give the range of triplicates. *** indicates p<0.001 compared to Control.
Figure 3.3: Effects of PTX on vasopressin- or carbachol-stimulated IP$_n$ accumulation in H9c2 cells. H9c2 cells were seeded on 24-well plates and grown to confluency. Cells were labeled with [³H]inositol and treated concurrently with PBS (control) or 100 ng/ml of pertussis toxin (PTX) overnight (20 hrs). Cells were then stimulated with either 100 nM arginine vasopressin (AVP) or 100 μM carbachol for 20 min in Hepes-DMEM media containing 10 mM LiCl. Total [³H]IP$_n$ formed were measured as described in Materials and Methods. Results shown are the average of triplicates and representative of two independent experiments. Error bars give the range of triplicates. * indicates p<0.05 and *** indicates p<0.001 compared to Controls.
Figure 4: Effects of PMA on agonist-stimulated IP₃ formation in H9c2 cells. Cells prelabeled as described were pretreated with either vehicle (0.1% DMSO) or 1 μM PMA for 25 min prior to agonist stimulation. Cells were then stimulated with either 100 nM arginine vasopressin (AVP) or 100 μM carbachol for 20 min (A) or 90 sec (B) in Hepes-DMEM media containing 10 mM LiCl. Total [³H]IP₃ formed were measured as described in Materials and Methods. Results shown are the average of triplicates and representative of two independent experiments. Error bars give the range of triplicates. ** indicates p<0.01 and *** indicates p<0.001 compared to Controls.
Figure 5: Phosphorylation of PLC-β3 at a major phosphorylation site did not occur upon direct activation of PKC or stimulation with agonists in H9c2 cells. H9c2 cells were grown to confluency and serum-starved overnight (20 hrs). Cells were treated with either PBS (lane 3), 1 μM PMA (lane 4), 100 μM carbachol (lane 5) or 100 nM arginine vasopressin (lane 6) for 25 min before harvest. After homogenization in lysis buffer containing complete phosphatase inhibitor cocktail, proteins from whole cell lysates were extracted with chloroform and methanol and resuspended in 2X SDS sample buffer. Samples were analyzed by 7.5% SDS-PAGE and blotted first with anti-Ser1105-phospho-specific PLC-β3 antibody, stripped and blotted with anti-PLC-β3 antibody. Purified wild type and Ser1105→Ala mutant PLC-β3 were in vitro-phosphorylated by PKC in reaction conditions described in Materials and Methods. Wild type (lane 2) and mutant PLC-β3 (lane 1) phosphorylated were used as positive and negative blotting controls for Ser1105-P-PLC-β3. Results were confirmed in three independent experiments.
Figure 6: No effects of short-term or prolonged stimulation of H9c2 cells with either PMA or vasopressin on PLC-β3 subcellular distribution and down-regulation. H9c2 cells were treated with either PBS (control), 1 µM PMA or 100 nM vasopressin for the time period indicated under serum-free growth conditions. At each time point, cells were harvested and membrane and cytosolic fractions were collected by Optiprep separation. Membrane (M) and cytosolic (C) proteins were analyzed as described previously. Results were confirmed in two independent experiments.
Chapter 5: Discussion
Although it is well recognized that PLC-β enzymes play key roles in Gαq-coupled transmembrane signaling, the mechanisms by which PLC-βs are regulated are not completely described. Past studies showed that phosphorylation of the PLC-β3 isoform by protein kinases negatively modulated its agonist-stimulated hydrolytic activity; still, how the phosphorylated state affects the enzyme’s catalytic property is not completely determined.

During our search for a well-suited mammalian cell model to study mechanisms of regulation of the Gαq/PLC-β3/PKC signaling pathway, we have characterized a myoblastic cell line derived from rat heart, H9c2 cells, which expresses relatively high levels of endogenous PLC-β3 in the absence of all other PLC-β isoforms. Moreover, PLC-β3 was detected exclusively associated with the membrane with only trace amounts found in the cytosol, a characteristic which was not seen in other mammalian cell models we had studied previously. Finally, GPCR-stimulated IP formation was previously detected in these cells.

We currently have little information that allows us to understand the basis of the different subcellular distribution of PLC-β3 in different cell types and tissues. Scarlata et al previously discussed that subcellular localization of PLC-β1 should be a function of intrinsic membrane binding affinity and the presence of interacting protein partners, upon observation of two populations of PLC-β1, cytosolic and membranous, in PC12 and HEK 293 cells (8). Accordingly, it is conceivable that PLC-β3 localization to the membrane is the result of both specific interactions with G proteins as well as interactions with membrane lipids and other membrane-binding proteins. Multiple
interactions of PLC-β3 with membrane-localized factors other than the direct GPCR signaling components, such as cytoskeletal or scaffolding proteins, are likely. As discussed in Chapter 2, PLC-β3 can interact with PDZ-containing scaffolding proteins, some of which are shown to potentiate PLC-β3-mediated signaling when overexpressed in cell systems. Interestingly, these scaffolding proteins are expressed tissue- or cell type-specifically (21, 54). Hence, it is tempting to speculate that differential localization of PLC-β3 in different cells or tissues may in part arise from differential distribution or expression of interacting membrane-surface structural proteins. Although likely, the exact mechanisms involved still remain to be elucidated.

While exploring the PLC-β3 pathway in H9c2 cells, we determined a number of agonists that were capable of stimulating PLC-β3 through Gαq-coupled receptors. Arginine vasopressin was shown to increase [3H]IP accumulation in H9c2 cells in a dose-dependent manner by Chen and Chen (6), and here we confirmed their observation. H9c2 cells also responded to carbachol stimulation through the Gαq-coupled pathway, however, both vasopressin- and carbachol-induced responses were mostly but not completely PTX-insensitive.

Although it was reported that H9c2 cells endogenously express α1-adrenergic receptors (12), these cells were relatively insensitive to short-term phenylephrine stimulation at concentrations and conditions that we used, if not completely ineffective. Unlike many other cell lines that endogenously express both PLC-β3 and -β1, H9c2 cells do not express detectable levels of PLC-β1. To our knowledge,
PLC-β1 isoforms in this cell line is not well understood, since there are studies that report the presence of receptors that primarily couple to PLC-β1 in other systems, rather than PLC-β3. α1-Adrenergic receptors were shown in rodent cardiac tissues to selectively couple to PLC-β1 (1), and the presence of the α1-adrenergic receptors in H9c2 cells was reported by Fujita et al, although receptor expression levels were low as determined by radioligand binding assays (12). Hence, both low expression of α1-adrenergic receptors and the preference to couple to PLC-β1 isoforms may serve as possible explanations for low responses to phenylephrine stimulation in H9c2 cells.

While mechanism and implications of α1-adrenergic receptor preference for PLC-β1 over PLC-β3 are not at all understood, a potential relevance to cardiac function or hypertrophy could be considered. In addition, potential specific coupling of V1-vasopressin receptors to PLC-β3 isoenzymes in H9c2 cells could be investigated. Further studies are required to pinpoint the mechanism involved.

Yue et al reported that direct activation of PKC by PMA can inhibit agonist-stimulated IP formation in diverse cell models. It was suggested that PKC-mediated phosphorylation of PLC-β3 at a major phosphorylation site contributed to the diminished activity of PLC-β3 (65).

Specific phosphorylation sites on PLC-β3 have been identified previously. A serine residue located within the C-terminal region, Ser1105, is recognized as the primary phosphorylation site on PLC-β3 by PKC and by some other protein kinases (62, 64,
When we tested this observation in our H9c2 cells, we were well able to reproduce substantial negative PMA effects on agonist-stimulated IP accumulation. Pretreatment of cells with PMA for 25 min significantly diminished carbachol- or vasopressin-stimulated IP₃ formations. However, the postulated phosphorylation of PLC-β3 at Ser1105 residue was not observed when PMA-treated cells were analyzed by immunoblotting. It is unlikely that the commercial phospho-specific antibody itself was the issue, which specifically recognizes the Ser1105 phosphorylated form of PLC-β3, since our in vitro-phosphorylated PLC-β3 by PKC was well recognized by the same antibody. Currently, we are not able to propose a reasonable explanation for this discrepancy and our understandings are challenged in this respect. At best, we can state that regulation of PLC-β3 in H9c2 cells is unique among the cell lines of which we are aware, both in regards to localization and phosphorylation.

Since the PLC-β3 population primarily and rather uniquely exists associated with the membrane in the unstimulated state in H9c2 cells, we hypothesized that short-term or prolonged stimulation of the pathway may lead to negative modulation of PLC-β3 activity in the form of translocation to the cytosol or down-regulation involving protein degradation.

We find that the membranous population of PLC-β3 is immobile upon either agonist stimulation or direct activation of PKC. Neither short-term (25 min) nor long-term (12, 24 and 48 hrs) exposure of cells to PMA or vasopressin was able to trigger PLC-β3 translocation to the cytosol. Moreover, we observe that PLC-β3 down-regulation,
as indicated by decreased expression of this protein or degradation of existing protein population, did not occur during the time-course tested. These results also indicate that PKC activation-induced inhibition of PLC-\(\beta\)3 activity in H9c2 cells does not involve translocation or down-regulation of PLC-\(\beta\)3. As suggested by Strassheim and Williams, it is possible that PKC diminishes PLC-\(\beta\)3 activity by altering PLC-\(\beta\)3 interaction with stimulated G\(\alpha_q\)/\(\alpha_{11}\) subunits in some molecular fashion (52). Although the widely recognized mode of regulation responsible for these events could be phosphorylation of PLC-\(\beta\)3 at a major putative phosphorylation site, we failed to detect any levels of PLC-\(\beta\)3 phosphorylation at Ser1105 residue upon stimulation in these cells.

A number of studies have reported the involvement of the vasopressinergic G\(\alpha_q\)/PLC-\(\beta\)3 pathway in hypertrophy in H9c2 cells (4, 61). H9c2 cells possess all basic features of myocardial V1-vasopressinergic pathway and continuous activation of this pathway leads to hypertrophic development of the myoblast as determined by standard morphologic and biochemical criteria (4). Brostrom et al reported that under serum-free conditions, prolonged vasopressin stimulation of H9c2 cells promoted hypertrophy of these cells whereas some other agonists such as angiotensin II, phenylephrine and endothelin-1 were ineffective under the same conditions (4). Later studies by Woischwill et al showed that Ca\(^{2+}\)/calmodulin-dependent processes downstream of the vasopressin stimulation play the most critical roles in this cardiomyoblast hypertrophic response (61).
Schnabel et al reported that in response to certain hypertrophic stimuli, such as growth hormone and insulin-like growth factor-1, PLC-β3 mRNA levels significantly increased within 30 minutes in rat neonatal cardiomyoblasts, through signaling mechanisms that do not involve direct activation of the G protein-coupled receptors. Up-regulation of PLC-β3 mRNA was not observed when the same cells were stimulated with norepinephrine (50).

Although it may be possible, currently we find no correlation between lack of phosphorylation at Ser1105 residue, down-regulation or translocation of PLC-β3, and the H9c2 cells’ programmed nature to undergo myocardial hypertrophic events upon activation of the PLC-β pathway by vasopressin, which take place within 24 hrs of stimulation and may continue up to 48 hrs (4). In our study, no further investigations on the behavior of PLC-β3 beyond 48 hrs were performed.

We recognize that our understandings of the PLC-β signaling in H9c2 cells are challenged in many respects. Nevertheless, we are the first to report that the H9c2 cell line provides the first cardiac-derived model system where PLC-β3 isoenzyme-selective GPCR coupling can be studied in the absence of other PLC-β expression without genetic manipulation.

We also report that V1-vasopressin receptors, despite many functional similarities to α1-adrenergic receptors to stimulate myocyte hypertrophy, do not display a preference for PLC-β1 isoenzymes, but activate PLC-β3 isoforms in H9c2 cells. Again, the
physiological consequences and mechanisms of this potential receptor selectivity for
PLC-β3 isoforms await further study.
Observations and interpretations discussed in the present study will provide much
information for future investigations.
Chapter 6: Conclusion
The H9c2 cell line derived from rat cardiac myoblasts was used as a model for investigations into the PLC-β-mediated signal transduction and modes of PLC-β regulation. We observed that the H9c2 model possesses characteristics advantageous over other cells previously used to study PLC-β3 regulation. H9c2 cells express relatively high levels of PLC-β3, exclusively localized at the membrane, but no other PLC-β isoforms at detectable levels when analyzed by immunoblotting. Although H9c2 cells did not show strong response to α1-adrenergic receptor stimulation, vasopressin- and carbachol-induced effects were significant. Vasopressin induced a concentration-dependent stimulation of IP formation in these cells, and this effect was significantly reduced by PKC activation with PMA pretreatment. However, we were not able to detect any changes in biochemical properties of PLC-β3, including phosphorylation at a major serine residue, which may be responsible for the negative modulation of the enzyme’s hydrolytic activity. In addition, when H9c2 cells were stimulated short-term (25 min) or long-term (12, 24, 48 hrs) with either vasopressin or PMA to activate PKC, no PLC-β3 translocation or down-regulation was observed. We did not extend our studies further into other possible mechanisms that might involve altered interaction of PLC-β3 with components of the membrane structure. Recently, a number of structural proteins have indeed emerged as potential PLC-β3 interacting partners and modulators of PLC-β signaling. The relevance of these interacting partners to regulate potential V1-vasopressin receptor-selective activation of PLC-β3 also awaits further study.
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