
UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

WANG, JUAN, REGULATION AND CHARACTERIZATION OF CARDIAC PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C ISOENZYMES. Master of Biomedical Science, Dec., 1997, 79pp., 20 illustration, bibliography, 62 titles.

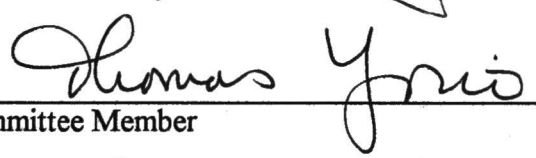
It is hypothesized that myocardial phosphoinositide-specific phospholipase C (PLC) isoenzymes are regulated by physiological intracellular Ca^{2+} and by cytosol-membrane translocation. The regulation and identification of PLC isoenzymes in rat and dog ventricular subcellular fractions.were studied. PLC-β_1, PLC-β_3 and PLC-δ_1 were identified in rat and dog cytosol and microsomal membranes by chromatographic separation, enzyme assays and western blotting. Truncated PLC-β isoforms with molecular weights of 69 kDa and 114 kDa were isolated from rat and dog cytosol, respectively. Species differences in the relative distribution of PLC isoenzymes were evident as PLC-δ dominant in rat whereas PLC-β isoenzymes were dominant in dog. A 91 kDa cytosolic protein which did not contain PLC activity alone markedly led to PLC activation when combined with microsomes. The activator protein was immunoprecipitated with an anti-PLC-δ identifying this activator as an inactive PLC-δ isoenzyme. These studies indicate that cytosolic PLC-δ may be activated by translocating to membranes. In addition, proteolysis may be involved in long term activation of cytosolic PLC isoenzymes. Further studies will be required to resolve the physiological significance of these modes of cardiac PLC activation.

REGULATION AND CHARACTERIZATION OF CARDIAC PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C (PLC) ISOENZYMES

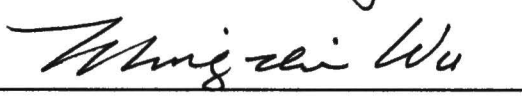
Juan Wang,

APPROVED:

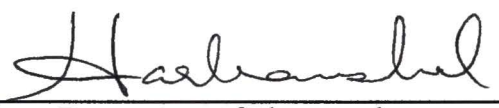
Major professor



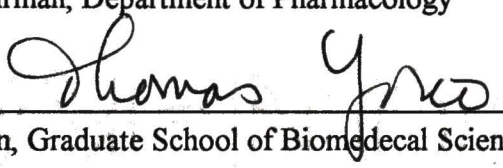
Committee Member



Committee Member



Chairman, Department of Pharmacology



Dean, Graduate School of Biomedecal Sciences

REGULATION AND CHARACTERIZATION OF CARDIAC PHOSPHOINOSITIDE-SPECIFIC PHOSPHOLIPASE C (PLC) ISOENZYMES

THESIS

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas Health Science Center at Fort Worth

in Partial Fulfillment of the Requirements

for the Degree of

MASTER OF SCIENCE

By

Juan Wang

Fort Worth, Texas

December, 1997

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

LIST OF ABBREVIATIONS

DAG	Diacylglycerol
HR	High resolution
IP3	Inositol 1, 4, 5-trisphospphate
PLC	Phosphoinositide-specific phospholipase C
PI	Phosphatidylinositol
PIP	Phosphatidylinositol 4-monophosphate
PIP2	Phosphatidylinositol 4, 5-bisphosphate
PMSF	Phenylmethanesulphonyl fluoride
TCR	T cell antigen receptor
TSH	Thyroid stimulating hormone
PTK	Protein tyrosine kinase

CHAPTER I

INTRODUCTION

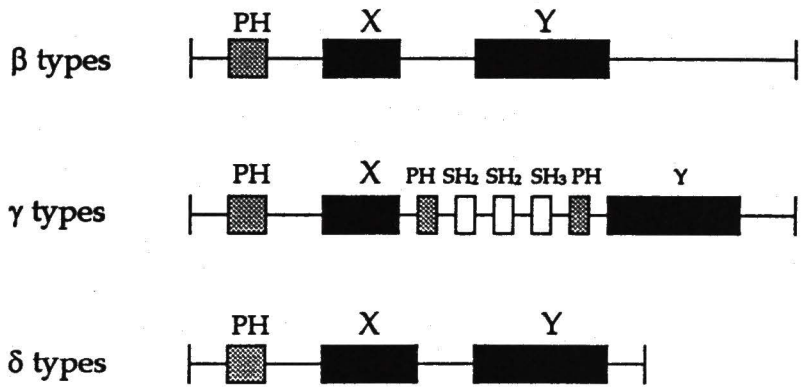
Activation of phosphoinositide-specific phospholipase C (PLC) isoforms result in the breakdown of phosphatidylinositol 4, 5-bisphosphate (PIP2) and the production of second messengers diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP_3). DAG is a direct activator of protein kinase C, whereas IP_3 causes a transient release of Ca^{2+} into the cytoplasm which is involved in the regulation of variety of cellular functions (1, 2). Myocardial phospholipase C (PLC) plays an important role in signal transduction by α_1-adrenergic receptor agonists (3), muscarinic agonists (3), endothelin (4), thrombin (5) and angiotensin II (6). It is also an important mechanism for mediating positive inotropic effects and possibly plays a role in the pathophysiology of myocardial hypertrophy (1,2, 7-10). Numerous investigators have reported that cardiac membranes contain endogenous PLC activity (11-13). However, the identities of PLC isoforms in myocardium and the relationship between membrane-associated and soluble PLC has been controversial (12,13,14). Some investigators have reported that phosphodiesteric hydrolysis of PI is restricted

to soluble forms of myocardial PLC and that membrane-associated PLC is highly selective for polyphosphoinositides (12, 14). In contrast, others have reported that the enzymatic properties are very similar (11). So it is important to identify and study myocardial PLC isoenzymes to delineate mechanisms for transmembrane signaling in heart.

Classifications of phospholipase C isoforms--Like many other proteins important in signal transduction. PLC exists in multiple isoforms. Initially, three isoforms that are distinct with respect to size, immunological reactivity, and deduced amino acid sequence were purified from bovine brain and named PLC-β_1, PLC-γ_1, PLC-δ_1 (15). Subsequently, a total of 14 isoenzymes have been identified. All PLCs are single-polypeptide enzymes and can be divided into three types (β, γ and δ) exemplified by the 150-kDa PLC-β_1, the 145 kDa PLC-γ_1, and the 85 kDa PLC-δ_1 on the basis of size and amino acid sequence (16, 17).

Structures and function--Although the overall amino acid sequence similarity between the different PLC types is low, significant similarity is apparent in two regions: one of ~170 amino acids and the other of ~260 amino acids, designated X and Y, respectively (Fig. 1). All PLC isoforms contain an amino-terminal region of

Fig. 1. Linear display of three types of mammalian PLC isozymes. All members contain highly homologous X and Y regions. Src homology domains, SH2 and SH3, and pleckstrin homology (PH) are indicated.


β types
PH
X
Y
γ types
PH
X
PH
SH2
SH2
SH3
PH
Y
δ types
PH
X
Y

~300 amino acids that precedes the X-region. PLC-β and PLC-δ isoenzymes contain short sequences of 40-110 amino acids that separate the X and Y regions whereas PLC-γ isoenzymes have an additional long sequence of ~400 amino acids that contains the so-called Src homology (two SH_2 and one SH_3) domains. The functions of the X, Y and SH domains in PLC-γ isozymes have been studied by constructing various plasmids that encode truncated PLC-γ molecules and expressing these proteins either in Escherichia coli (PLC-γ_2) or transiently in Cos-1 cells (PLC-γ_1) (18). PLC activity was still detected after deletion of the SH domain indicating that this region is not the catalytic site. However, deletion of either the X or Y region resulted in a complete loss of activity. It appeared, therefore, that X and Y regions are essential for PLC activity. Furthermore, the observation that mutation of either of the two histidine residues, which are conserved in the X domain of all PLC isoenzymes, resulted in the complete loss of catalytic activity (19) and further strengthened the notion that the X and Y domains constitute the catalytic site of PLC. However the recent discovery of a highly truncated form of PLC (20), designated PLC-βx, lacked the amino-terminal region and X domains characteristic of other PLC isoenzymes, whereas all 260 amino acids of Y-domain were conserved. Given that the Y domain is essentially conserved throughout evolution, it would appear to be a fundamental requirement for catalytic function. Both PLC-γ_1 and PLC-γ_2 also contain a pleckstrin homology (PH) domain that has recently been shown to be present in many signaling

proteins (21-24). In PLC-γ, the PH domain is split by the SH domain. The function and structure of PLC-γ PH domain may be retained because the amino and carboxyl termini of the SH_2 and SH_3 domains are close to each other in three-dimensional structures and because SH domains can be inserted into different regions of host proteins without disrupting the overall protein structure (25). Reevaluation of PLC sequences recently revealed an intact PH domain in amino-terminal regions, preceding the X domain, in all mammalian PLCs (25).

Catalytic properties of PLC—All three types of PLC catalyze the hydrolysis of the three common inositol-containing phospholipids: phosphatidylinositol (PI), phosphatidylinositol 4-monophosphate (PIP), and phosphatidylinositol 4,5-biphosphate (PIP_2). However, differences or preferences for these substrates exist. The selectivity for PIP_2 over PI decreases in the order PLC-β_1 > PLC-δ_1> PLC-γ_1 (15). Hydrolysis of PI, PIP and PIP_2 by all three types of PLC yields cyclic and noncyclic inositol phosphates (26). More recently, Schwartz and Halverson found that PLC activity which degraded PIP and PIP_2 was primarily located in a crude total membrane preparation from rat ventricles, whereas the PLC-PI was completely cytosolic (3,4). Such diversity among the PLC isoforms suggests that distinct mechanistic and functional differences exist between PLC families.

Activation of PLC-β isozymes by $G_q\alpha$ subunits—Exton et.al. (27) purified a mixture of Gqα and $G\alpha_{11}$ which activated partially-purified PLC from bovine liver membranes. When reconstituted in the presence of GTPγS and various isoenzymes of PLC, the mixture of bovine liver Gqα and $G\alpha_{11}$ specifically activated PLC-β_1 but not PLC-γ or PLC-δ (27-28). The activation is achieved mainly by increasing the intrinsic activity (Vmax) of PLC, and not by lowering the concentration of Ca^{2+} required for the activity of the enzyme (28-30). Subsequently studies have delineated a hiearchy for the sensitivity of PLC-β subtypes to the Gαq and $G\alpha_{11}$ subunits: PLC-β1> PLC-β3> PLC-β2 (31, 32). This hierarchy suggests a degree of specificity in the interaction of these Gα subunits with different PLC-β isozymes. The receptors that are known to activate PLC-β via Gαq or $G\alpha_{11}$ include those for thromboxane A_2, bradykinin, thrombin, bombesin, angiotensin, histamine, vasopressin, acetylcholine and thyroid stimulating hormone (TSH) (33-35). It is hypothesized that interaction of Gq/11 with the ligand-occupied receptor causes dissociation of the heterotrimeric GDP-bound Gq to yield GTP-bound Gqα, which remains in the membrane. PLC-β_1 then binds to the GTP-bound Gqα probably via the carboxyl-terminal regions of both proteins, resulting in the activation of PLC-β_1 (Figure 2A). The sites through which G proteins interact with receptor have been elucidated at the molecular level (36,37). Transfection experiments revealed that the last three

residues of Gαq were critical for transduction of a signal from the α_2-adrenergic receptor. However, the sites of Gαq that interact with PLC-β are not known.

Activation of PLC-β isozymes by $G_{\beta\gamma}$ subunits --The function of the $G_{\beta\gamma}$ dimer was originally thought to be restricted to reassociation with the inactive, GDP-bound form of the Gα subunit so as to recycle it to the plasma membrane. However, it is now clear that in addition to this function, the $G_{\beta\gamma}$ also activates effector molecules (38) (Fig.2B). Two laboratories simultaneously described the differential activation of PLC-β subtypes by $G_{\beta\gamma}$ dimers (39, 40). These studies were subsequently extended to show that $G_{\beta\gamma}$ purified from bovine brain activates PLC-β isozymes (41) according to the hiearchy PLC-β_3 >PLC-β_2> PLC-β_1. The dimer does not activate PLC-β_4 (42). The specificity of $G_{\beta\gamma}$ dimers in the activation has also been examined. Wu et. al. (43) and Lee et. al. (44) demonstrated that the region of PLC-β that interacts with Gαq differs from that responsible for interaction with $G_{\beta\gamma}$ dimer. The amino-terminal two-third of PLC-β_2 was required for activation by $G_{\beta\gamma}$. The site of PLC-β interaction has been recently localized to the amino-terminal region preceding the X domain (45) that also contains the PH domain.

Activation of PLC-γ isoenzymes by growth factor receptor protein-tyrosine kinases—Growth factor-induced stimulation of PLC appears to be independent of

G proteins and requires the intrinsic PTK activity of the corresponding receptor. Mutant PDGF and EGF receptors that lack PTK activity bind their respective growth factors but fail to stimulate the hydrolysis of PIP_2 (46). Treatment of a number of cells with EGF, PDGF, or NGF results in an increase in the phosphorylation of PLC-γ, but not of PLC-β or PLC-δ (47-51). The increased phosphorylation occurs on both serine and tyrosine residues. Tyrosine phosphorylation is rapid and correlates well with the stimulation of PIP_2 hydrolysis (48). The major sites of phosphorylation by EGF, PDGF appear to be identical (49) and comprise tyrosine kinase residues 771, 783 and 1254. Further studies suggest that phosphorylation of Tyr-783 is essential for PLC-γ activation. It is possible that SH_2 domains of PLC-γ interact intramolecularly with the phosphorylated tyrosine residues. This leads to a conformational change that allows the SH_3 domain to bind to the membrane cytoskeleton and thereby position the catalytic X and Y domains at the cytoplasmic face of the cell membrane (Fig. 2C).

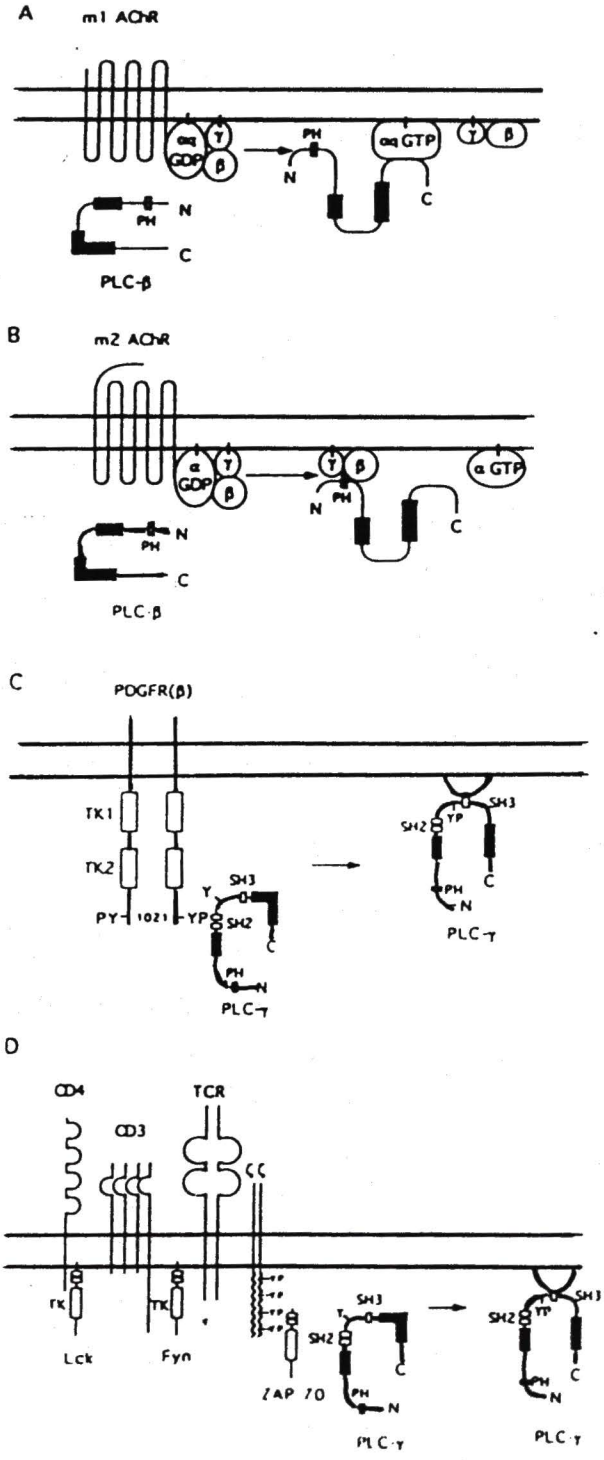
Activation of PLC-γ isozymes by nonreceptor protein-tyrosine kinases—Several nonreceptor PTKs also phosphorylate and activate PLC-γ isozymes. Such receptors include membrane immunoglobin M (mIgM) in B lymphocytes, the T cell antigen receptor (TCR). The TCR complex, which recognizes antigen and transduces signals across the plasma membrane, consists of an antigen-binding

heterodimer of α and β chains that is associated with the nonpolymorphic polypeptides γ, δ, and ε (collectively termed CD3) and with a homodimer (heterodimer) ζ family polypeptides. Although none of the components of the TCR complex is a protein kinase, the ligation of the TCR complex activates PTK activity. Candidates for the TCR-regulated PTK include Fyn and Lck (Src family), and Zap-70 (Syk family of PTKs). These polypeptides appear to phosphorylate downstream targets such as PLC-γ_1 and PLC-γ_2 (45) (Fig. 2D). TCR stimulation results in a rapid and transient phosphorylation of PLC-γ on both serine and tyrosine residues (52-56).

The mechanism of regulation of PLC-δ is not clear, but its activity is not affected by either the G-protein subunits or receptor tyrosine kinases. Recent evidence has suggested that there are other proteins involved in the activation or maintenance of sustained activity of PLC. These include a high molecular weight GTP-binding protein, G_h (56). In our preliminary studies, cardiac microsomes were found to possess a Ca^{2+} activated PLC activity which could be stimulated by a cytosolic protein (57). It is hypothesized that myocardial PLC isoenzymes are regulated by physiological Ca^{2+} and by cytosol-membranes translocation. The specific aims of this thesis are to: 1). Identify and characterize PLC isoforms in rat and dog myocardium. 2). Characterize the cytosolic protein responsible for

stimulation of membrane PLC activity or the translocation and activation of the cytosolic PLC isoforms to membranes.

Fig. 2. Four distinct activation mechanisms for PLC isozymes. Activation of PLC-β by Gq α subunits (A) and Gβγ subunits (B) is illustrated using the m1 and m2 AChR, respectively, as examples. GTP-bound Gq α and Gβγ subunits served as docking sites to bring cytosolic PLC-β to membranes. The carboxyl-terminal region of PLC-β interact with Gqα, whereas the amino-terminal region containing PH domain is important for the binding of Gβγ. The activation of PLC-γ by receptor protein-tyrosine kinases (C) and nonreceptor protein-tyrosine kinases (D) is illustrated using PDGF receptor and T cell antigen receptor complex, respectively, as examples. Binding of the tyrosine phosphorylated PLC-γ to a proline-rich protein located at the cytoplasmic face of the cell membrane causes its translocation from cytosol. TK, tyrosine kinase domain; YP, phosphotyrosine.


A
m1 AChR
αq GDP
γ
β
PH
N
αq GTP
C
PLC-β
B
m2 AChR
α GDP
α GTP
C
PLC-β
C
PDGFR(β)
TK1
TK2
PY
1021
YP
SH3
SH2
PH
N
PLC-γ
D
CD4
CD3
TCR
Lck
Fyn
ZAP 70
PLC-γ

CHAPTER II

MATERIAL AND METHODS

Materials — Phosphatidylinositol (PI), phosphatidylinositol-4, 5-biphosphate (PIP_2), leupeptin, aprotinin and phenylmethanesulphonyl fluoride (PMSF) were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A. Pre-swollen microgranular anion exchanger DE-52 was from Whatman BioSystems Ltd, England. Sephacryl S-200 was from Pharmacia AB Biotechnology, Uppsala, Sweden. Monoclonal antibody anti-bovine PLC-$\delta 1$ and polyclonal antibody anti-bovine PLC-γ_1 were purchased from Upstate Biotechnology Inc., Lake Placid, NY, U.S.A. Monoclonal antibodies anti-bovine PLC-β_1, PLC-β_2 and PLC-β_3 were gracious gift from Dr. P. Sternweis, Southwestern medical center, Dallas, TX, U.S.A. Anti-bovine PLC-γ_2 was a gracious gift from Dr. Carpenter, Nashville, TN. Protein A-Sepharose 4B was purchased from ZYMED Laboratories, Inc., San Francisco, CA, U.S.A. Anti-rabbit (or mouse) Ig, horseradish peroxidase linked whole antibody was purchased from Amersham life Science. [^{3}H]-PI (specific radioactivity 1 Ci /m mol) was from American Radiolabeled Chemicals Inc., St. Louis, MO, U.S.A. [^{3}H]-PIP_2 (specific

radioactivity 2.4 Ci / mmol) obtained from Dupont New England Nuclear, Boston, MA, U.S.A. All other chemicals used were of reagent grade.

Preparation of microsomes and cytosol from myocardium – Male and female Sprauge-Dawley rats were euthanized by CO_2 asphyxiation. The mongrel dogs were anesthetized with intravenous surital. Hearts were quickly removed and placed in cold 0.1 mM EGTA and isotonic saline solution. Tissue was minced with scissors, suspended in 10 mM HEPES, pH, 7.4, 2.0 mM $MgCl_2$, 0.5 mM dithiothreitol, o.1 mM PMSF and 0.1 mM EGTA and homogenized with a polytron. Microsomal membranes and cytosol were separated by differential centrifugation as described by Quist et al. (59). Cytosol and microsomes were stored at -80 °C for further assays or chromatography. Microsomal and cytosolic proteins were measured by the method of Bradford (60) using BSA as a standard.

PLC assay – PLC activity was routinely assayed by measuring the information of radioactive inositides from [^{3}H] PI or [^{3}H] PIP_2. The reaction mixture, in a final volume of 50 ml, contained 25 mM HEPES, pH7.4, 0.1 mM EGTA, 40 mM KCl, 10 mM $MgCl_2$, 25 mM NaCl, 1.0 mM NaN_3 and 10 mM LiCl. The reaction was initiated by adding 20 μM [^{3}H]-phosphatidylinositol or [^{3}H]phosphatidylinositol-4,5-biphosphate. The calcium concentration was varied in this medium. ATP (1 mM) was added

where indicated to assess ATP dependence of PLC activity in samples. Deoxycholate (final concentration 1.0 mM) or alamethicin (0.5 %) were added where indicated. Incubation was 20 min for [^{3}H] PI or 10 min for [^{3}H] PIP_2 at 37°C. Approximately 5-10 μg of protein was used in the assays of PLC activity in cytosol and microsomes. Reactions were stopped with 1.0 ml of $CHCl_3$: CH_3OH:HCl (100:100:0.6). After the addition of 0.3 ml of H_2O, 0.5 ml of upper phase was counted in 3.0 ml of ecolume. In reconstitution experiments the protein concentration in assay samples was diluted to achieved less than 12 % hydrolysis of substrate in a 10 min incubation. Hydrolysis of PIP_2 was a linear function of protein concentration under these condition.

DE-52 column chromatography —Freshly prepared cytosol, 5 ml, was loaded onto a 1 x 2.5 cm of DE-52 anion exchanger column that had been equilibrated in 10 mM Tris-HCl buffer, pH 8.0. After loading of cytosol, the column was washed with 10 ml of 20 mM NaCl, 20 mM Tris-HCl buffer, pH 8.0 twice to remove unbound protein. The eluant (10 ml each) were collected and the column was further washed with 100 mM, 200 mM and 500 mM NaCl, also containing 20 mM Tris-HCl, pH 8.0 and 0.01 mM EGTA. The eluted fractions were assayed for PLC activity alone or in combination with microsomes.

Sephacryl S-200 chromatography —Cytosol or freshly prepared cytosolic DE-52 fractions were applied to Sephacryl S-200 column (1.5x 48 cm) equilibrated with 100 mM NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 10 mM PIPES, pH 6.8 and 0.02 mM PMSF. The enzyme was eluted with the same buffer at a flow rate 0.14 ml / min and the fractions (0.28 ml each) were collected and assayed for PLC activity in fractions alone or in combination with microsomes.

Immunoblotting of PLC — PLC isoenzymes were detected in microsomal and cytosolic fractions. Membrane proteins were solubilized with one volume of Laemmli 10% SDS buffer and heated for 10 min at 90°C. Cytosolic fractions were separated on a anion exchanger DE-52 column with 100 and 200 mM NaCl, prior to solubilization with SDS Laemmli buffer (58). Approximately 50 μg of proteins was electrophoresed on 7% SDS-polyacrylamide mini-gels (1.5mm) and transferred to nitrocellulose membranes. Prestained molecular weight markers (Bio Rad) was electrophoresed simultaneously. Nitrocellulose membranes were blocked in 5% dry milk, 0.05% Tween 20, and Tris-Buffer-Saline-Tween 20 (TBST) pH 7.5 for one hour at room temperature and washed with same medium. The membrane was cut into strips (0.5x9 cm) containing microsomal or cytosolic proteins were incubated one hour with rocking at 5 °C with 1° antibodies (1: 500-1: 1500) to PLC-β_1, PLC-β_2 PLC-β_3 (obtained from Paul Sternweis, Southwestern medical center at Dallas), PLC- δ_1 (UBI), PLC-γ_1(

UBI), PLC-γ_2 (from Graham Carpenter). To ensure specific binding, antibodies were preincubated with 50μg / ml of preimmune antigen to PLC-β_1 and PLC-β_3 isoenzyme prior to incubation. Membranes were washed with TBST and incubated 1.5 hours with rabbit (or mouse for PLC-δ) Ig, horse radish peroxidase linked whole antibody (Amersham, 1:5000) and further washed according to the protocol in an enhanced chemiluminescence (ECL) kit from Amersham. Bound antibody antigen complexes were detected with ECL reagents and ECL Hyperfilm according to the manufactures instructions.

Immunoprecipitation —PLC-δ was immunoprecipitated from cytosolic DE-52 100 mM NaCl / 20 mM Tris-HCl buffer, pH 8.0 fractions. Cytosolic fractions were diluted to protein concentration roughly, 1 μg / μl. Approximately 200 μg of cytosolic protein was incubated with 2.5 μg anti-bovine PLC-δ_1 (UBI) overnight at 4 °C, with gentle rocking. To capture the protein-antibody complex, 50 μl of Protein A-Sepharose 4B (50% suspension) was added and tubes were further incubated for 2 hours. The microtubes were pulsed 5 second in a microcentrifuge at 4°C. The supernatant were collected for assaying PLC activity.

Statistical Treatment of Data – Data here contained is presented as the mean $\pm$ the standard error of the mean of at least 3 independent observations.

CHAPTER III

RESULTS

PLC activity in cytosol--The substrate selectivity of cytosolic and the effect of 1 mM deoxycholate on phosphoinositide-specific phospholipase C activity were determined. Dog and rat cytosol were assayed either in the presence of 20 μM [^{3}H]-PI and 5 mM $CaCl_2$ or 20 μM [^{3}H]-PIP_2 and 0.2 mM $CaCl_2$ at pH 7.0. Dog and rat cytosolic PLC hydrolyzed both phosphoinositides, however, differences in the substrate specificity and relative PLC activities were evident between the two species (Fig. 3). Dog and rat cytosolic PLC activities in the presence of PI were increased about 4 fold by 1 mM deoxycholate. Cytosolic PLC activity with PIP_2 as substrate was only slightly stimulated by 1 mM deoxycholate. Rat cytosolic PLC activity was about 5 times higher than dog when PI was used as the substrate. In contrast, PLC activity in rat cytosol was only 1.3 times higher than in dog cytosolic PLC if PIP_2 was used as the substrate. The relative ratios of PLC activities in the presence of PIP_2 versus PI were 5.8 in dog and 1.37 in rat cytosol indicating PLC isoforms in dog cytosol prefer PIP_2 over PI as substrate. This difference in substrate specificity may suggest that rat and dog cytosol possess different PLC isoenzymes.

Calcium requirement of cytosolic PLC –The calcium concentration dependence of dog and rat cytosolic PLC activity was investigated by using a Ca^{2+}/EGTA buffer system in the standard mixture. As shown in Fig. 4, rat cytosolic PLC activity plateaued at about 1 mM free Ca^{2+} with a Vmax of 0.95 nmoles/mg /min when assayed in the presence of PI and 1mM deoxycholate. Half-maximal activation of PLC activity occurred at approximately 0.4 mM Ca^{2+}. Dog cytosolic PLC activity also reached maximal activation at 1.0 mM Ca^{2+} with a Vmax of 0.22 nmoles /mg /min. in the presence of PI and deoxycholate. When PIP_2 was used as the substrate, rat and dog cytosolic PLC showed similar calcium curves with Vmax values of 0.95 nmoles /mg in rat and 0.52 nmoles /mg in dog (Fig. 5). Plateaus were observed in rat and dog at approximately 10 μM Ca^{2+}. Deoxycholate increased PLC activity about 2 fold in both rat and dog cytosol. The activity plateaued at approximately 5-10 μM Ca^{2+}in the presence of DOC and the Ka values for Ca^{2+} were approximately 1.0 μM .

Separation of cytosolic PLC activity by Sephacryl-200 column – Rat and dog cytosol were applied to a Sephacryl-200 column to separate PLC isoenzymes according to apparent molecular weights. From dog cytosol, two major peaks designated PLC-I (150 kDa,), PLC-II (114 kDa) and one minor peak PLC-III (83 kDa) were separated (Fig. 6). From rat cytosol, a minor peak designated PLC-I (150 kDa) and two major peaks designated PLC-II (85 kDa) and PLC-III (69 kDa) were eluted (Fig. 8). Protease

inhibitors were included in the homogenization medium and column buffer to reduce proteolysis to minimize generation of proteolytic fragments. As a further precaution, studies were performed with large and small volume columns to compare the effect of chromatography separation times on the molecular distribution of PLC isoenzymes. Generally, the distribution of PLC isoenzymes was similar if cytosol was chromatographed 17 hours versus 6 hours (not shown).

Ca^{2+} affinity of cytosolic PLC isoforms—The Ca^{2+} affinities of the dog and rat cytosolic PLC activities separated on the Sephacryl-200 columns above was studied using EGTA/ Ca^{2+} buffers in the presence of PI. The objective was to determine if activities in separated peaks represented different PLC isoenzymes. Dog and rat cytosolic fractions from Sephacryl-200 column above were activated with increasing concentrations of Ca^{2+} (Figs. 7 and 9). In fractions separated from dog cytosol (Fig. 7), PLC isoforms in the 150 kDa and 114 kDa peaks were maximally activated at about 0.4 mM Ca^{2+} but with different V_{max} values. The calcium concentration needed for half-maximal PLC activation was approximately 0.05 and 0.1 mM for these peaks, respectively. The activity eluting at 85 kDa plateaued at approximately 2 mM Ca^{2+}. In rat cytosolic fractions (Fig. 9), peak-I PLC, eluting with an apparent molecular weight of 150 kDa was maximally activated at about 0.4 mM Ca^{2+}. The Ca^{2+} concentration needed for half-maximal activation was 0.05 mM Ca^{2+}. PLC activity in peak II

(87 kDa) plateaued at approximately 1.0 mM Ca^{2+}. Activity in peak III (69 kDa) reached a plateau at about 0.2-0.6 mM Ca^{2+}.

Separation of cytosolic PLC isoenzymes by anion exchange chromatography— Experiments were also performed to separate PLC isoenzymes by anion exchange chromatography. Rat and dog cytosol were applied on DE-52 anion exchange column, washed with 20 mM NaCl containing 20 mM Tris-HCl buffer, pH 8.0 and then eluted stepwise with 100, 200 and 500 mM NaCl containing 20 mM Tris-HCl buffer, pH 8.0. As shown in Fig.10, rat cytosolic PLC activity was eluted primarily in the 200 mM NaCl fraction when assayed in the presence of 20 μM PI, 5 mM $CaCl_2$ and 1 mM deoxycholate. Dog cytosolic PLC activity was relatively lower and separated with 100 and 200 mM NaCl. Further experiments were conducted to determine the recovery of cytosolic PLC activity eluted with 200 mM NaCl from DE-52 columns. It was observed that less than 33.0 % of dog cytosolic PLC activity was recovered in the 200 mM NaCl DE-52 column fraction (Fig. 11a). In contrast, 100 % of rat cytosolic PLC activity was recovered in the 200 mM NaCl DE-52 fraction (Fig. 11b). In addition, dog cytosolic fractions were more highly activated by DOC than rat cytosolic fractions in the presence of PI and PIP_2. The results suggests that dog cytosolic PLC is more subject to denaturation or co-factor removal on the DE-52

columns than for rat cytosol. This difference and the effect of DOC further indicate that different PLC isoenzymes predominate in dog and rat cytosol.

Immunochemical properties of cytosolic PLC from rat and dog myocardium – Western blotting was utilized to further identify the PLC isoenzymes in rat and dog cytosol. Fractions from DE-52 anion exchange columns were subjected to SDS-PAGE and transferred to nitrocellulose paper for probing with monoclonal antibodies against PLC-β_1, PLC-β_2, PLC-β_3, PLC-γ_1, PLC-γ_2 and PLC-δ_1 from bovine brain. Antibodies to PLC-β_1 and PLC-β_3 reacted with a 150 kDa protein in rat and dog cytosolic 200 mM NaCl fractions (Fig. 12). PLC-δ_1 reacted with the 87 kDa protein present in rat but not dog cytosolic fractions. No PLC isoforms were detected in 500 mM NaCl fraction (rat) and 100 mM NaCl fraction(dog). PLC-β_2, PLC-γ_1 and PLC-γ_2 were not detected in rat and dog cytosolic fractions.

Fig. 3. Comparison of dog and rat cytosolic PLC activity when assayed with PI or PIP_2 as the substrate. The cytosol (5-10 ug) from rat and dog were assayed with 20 μM [^{3}H]-PI in the presence of 5 mM $CaCl_2$ or 20 μM [^{3}H]-PIP_2 in the presence of 0.2 mM $CaCl_2$ as described in MATERIAL AND METHOD. 1 mM deoxycholate was used as indicated.

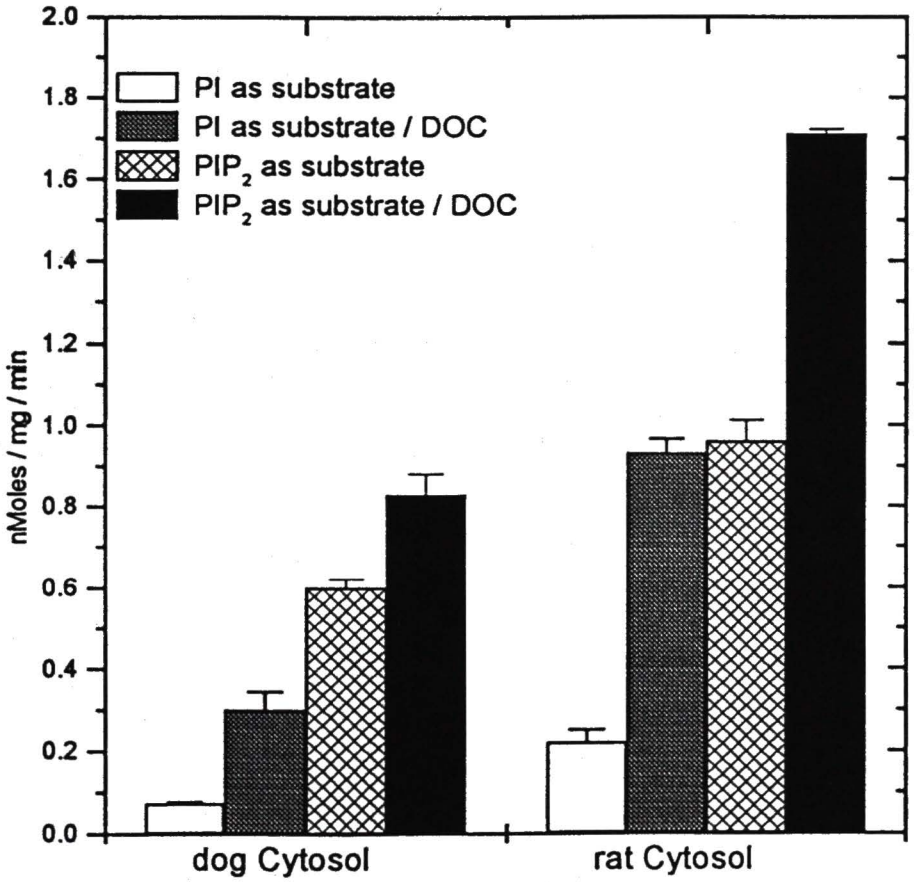

2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
nMoles / mg / min
PI as substrate
PI as substrate / DOC
PIP2 as substrate
PIP2 as substrate / DOC
dog Cytosol
rat Cytosol

Fig.4. Calcium concentration dependence of rat and dog cytosolic PLC activity when using PI as a substrate. Rat and dog cytosol (5-10 μg) were assayed in the standard mixtures with 20 μM [^{3}H]-PI in the presence of 1 mM deoxycholate as described in MATERIAL AND METHODS. The calcium concentration range was 0-2.0 mM.

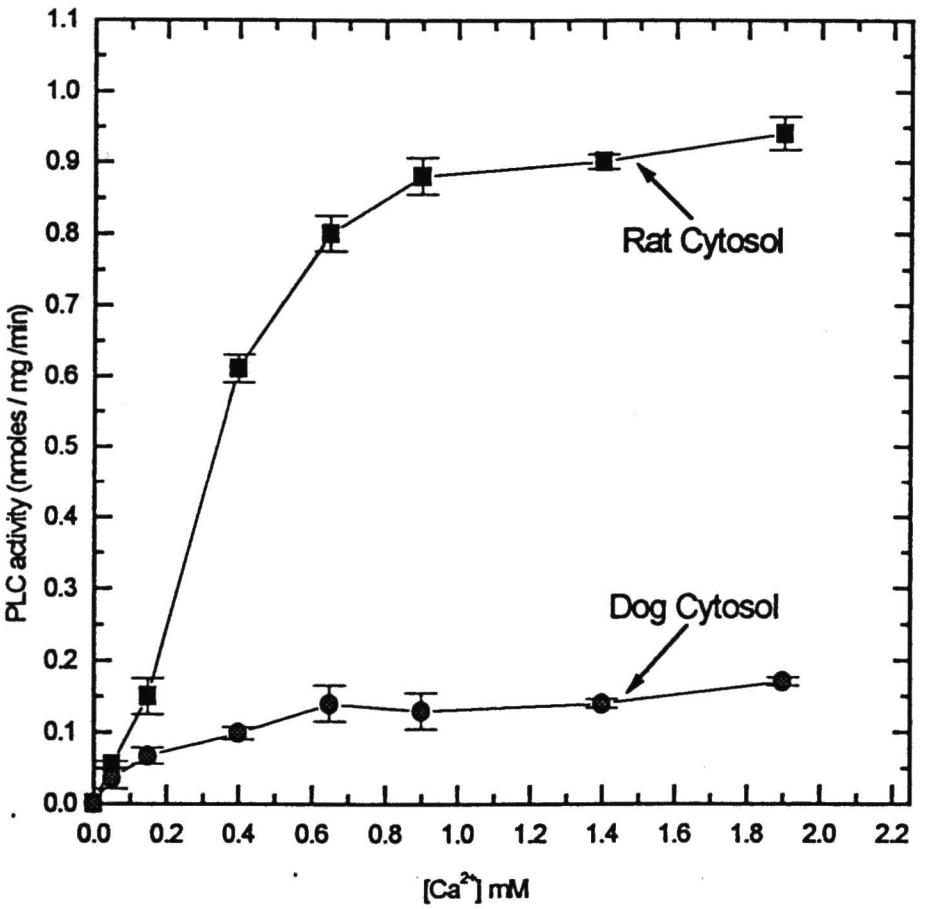
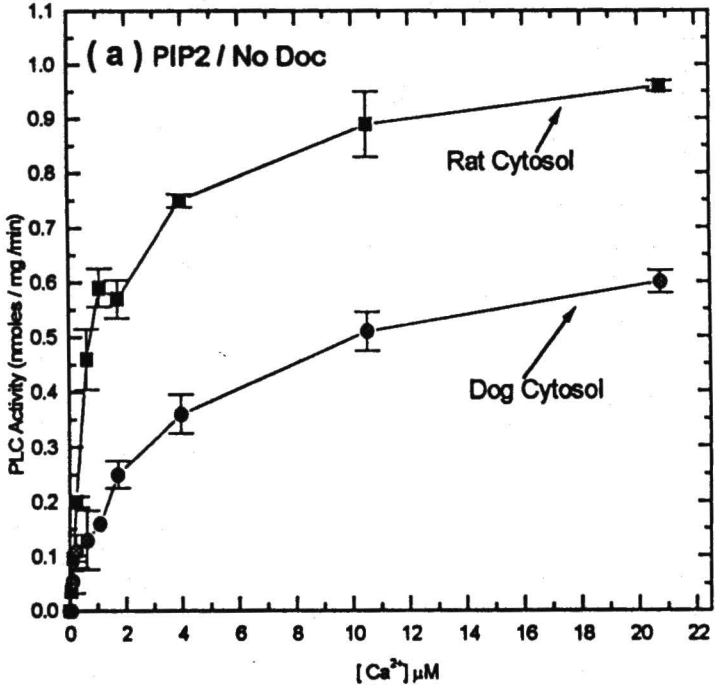

PLC activity (nmoles / mg /min)
[Ca2+] mM
Rat Cytosol
Dog Cytosol
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.1
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2

Fig.5. Calcium concentration dependence of rat and dog cytosolic PLC activity when using PIP_2 as a substrate. Rat and dog cytosol were assayed in the standard mixture with 20 μM [^{3}H]-PIP_2 in the absence (a) or presence (b) of 1 mM deoxycholate, as decribed in MATERIAL AND METHODS, The experimental calcium concentration was 0-22 μM.


(a) PIP2 / No Doc
Rat Cytosol
Dog Cytosol
PLC Activity (nmoles / mg /min)
[Ca2+] µM
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
0
2
4
6
8
10
12
14
16
18
20
22

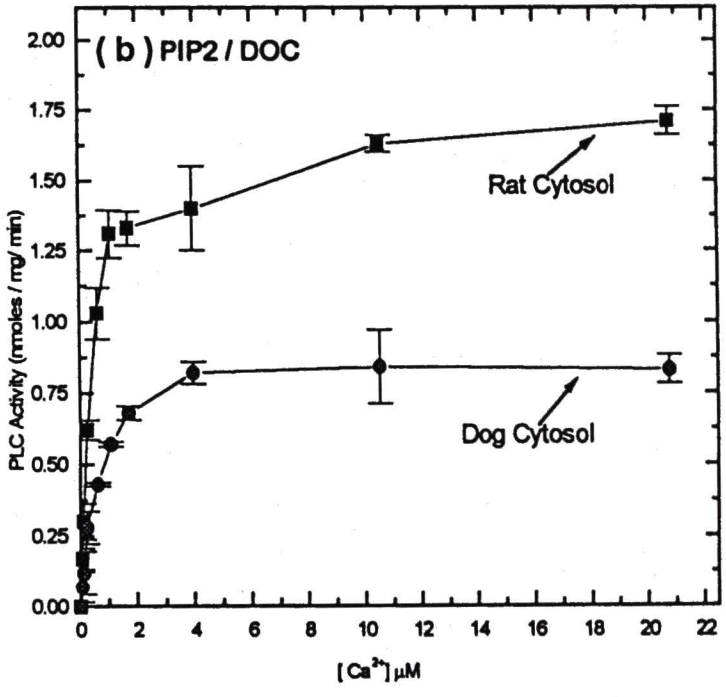

(b) PIP2 / DOC
Rat Cytosol
Dog Cytosol
PLC Activity (nmoles / mg/ min)
[Ca2+] µM
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
0
2
4
6
8
10
12
14
16
18
20
22

Fig. 6. Sephacryl-200 chromatography of dog myocardial cytosol. Approximately 1000 μg of myocardial cytosol, containng 6.0 nmoles/mg/min of PLC activity, were loaded onto a sephacryl-200 column and separated as described in MATERIAL AND MTHODS. Selected fractions were assayed for PLC activity with PI as a substrate in the presence of 5 mM $CaCl_2$ and 1 mM deoxycholate.

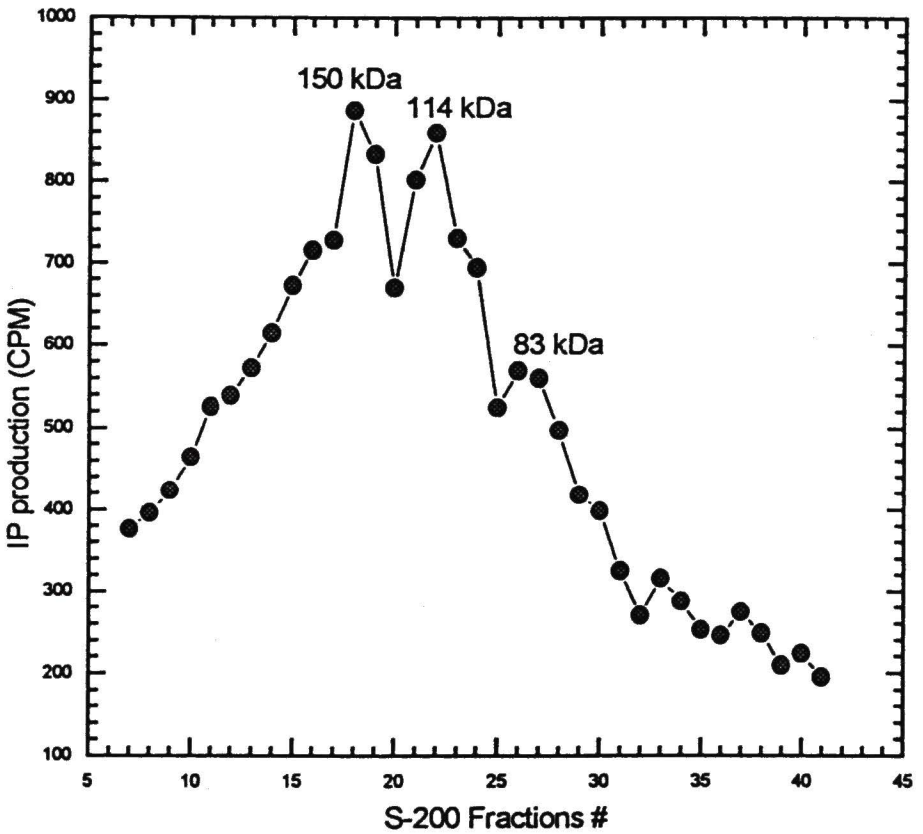

150 kDa
114 kDa
83 kDa
IP production (CPM)
S-200 Fractions #
1000
900
800
700
600
500
400
300
200
100
5
10
15
20
25
30
35
40
45

Fig. 7. Calcium concentration dependence of dog cytosolic PLC isoforms separated from sephacryl-200 column. Peak I (150 kDa), Peak II (114 kDa), Peak III (83 kDa) were assayed with PI as a substrate in the presence of 1mM deoxycholate. The calcium concentration was in the range of 0.0-2.0 mM.

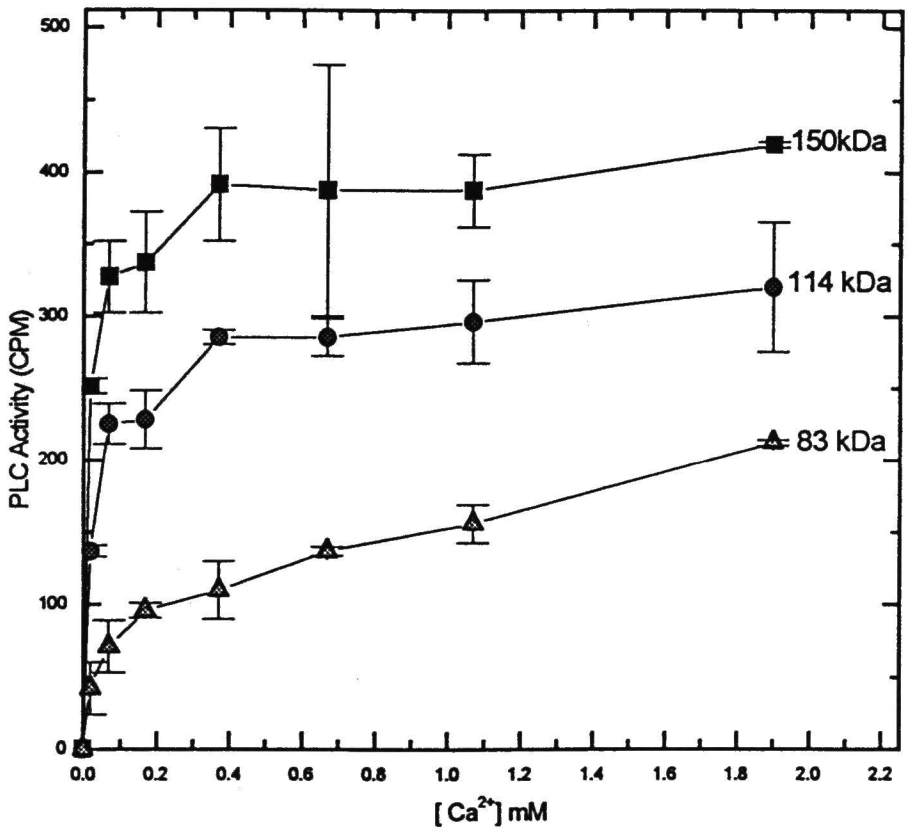

PLC Activity (CPM)
500
400
300
200
100
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
1.8
2.0
2.2
[Ca2+] mM
150kDa
114 kDa
83 kDa

Fig. 8. Sephacryl-200 chromatography of rat myocardial cytosol. Approximately 1.5 mg of myocardial cytosol, containng 11.0 nmoles/mg/min of PLC activity, were loaded onto a sephacryl S-200 column (1.5x 48) and separated as described in MATRIAL AND MTHODS. Selected fractions were assayed for PLC activity with PI as a substrate in the presence of 5 mM $CaCl_2$ and 1 mM deoxycholate.

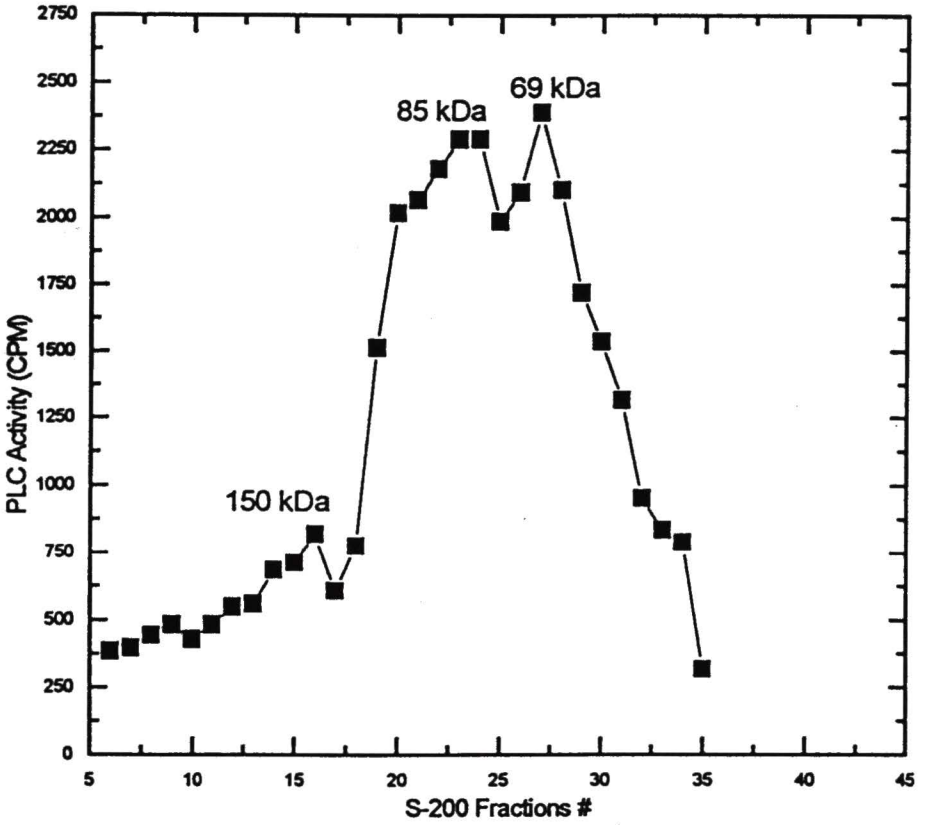

PLC Activity (CPM)
S-200 Fractions #
150 kDa
85 kDa
69 kDa
0
250
500
750
1000
1250
1500
1750
2000
2250
2500
2750
5
10
15
20
25
30
35
40
45

Fig. 9. Calcium concentration dependence of rat cytosolic PLC isoforms separated from sephacryl-200 column. Peak I (150 kDa), Peak II (85 kDa), Peak III (69 kDa) were assayed PLC activity with PI as a substrate in the presence of 1mM deoxycholate. The calcium concentration was in the range of 0.0-2.0 mM.

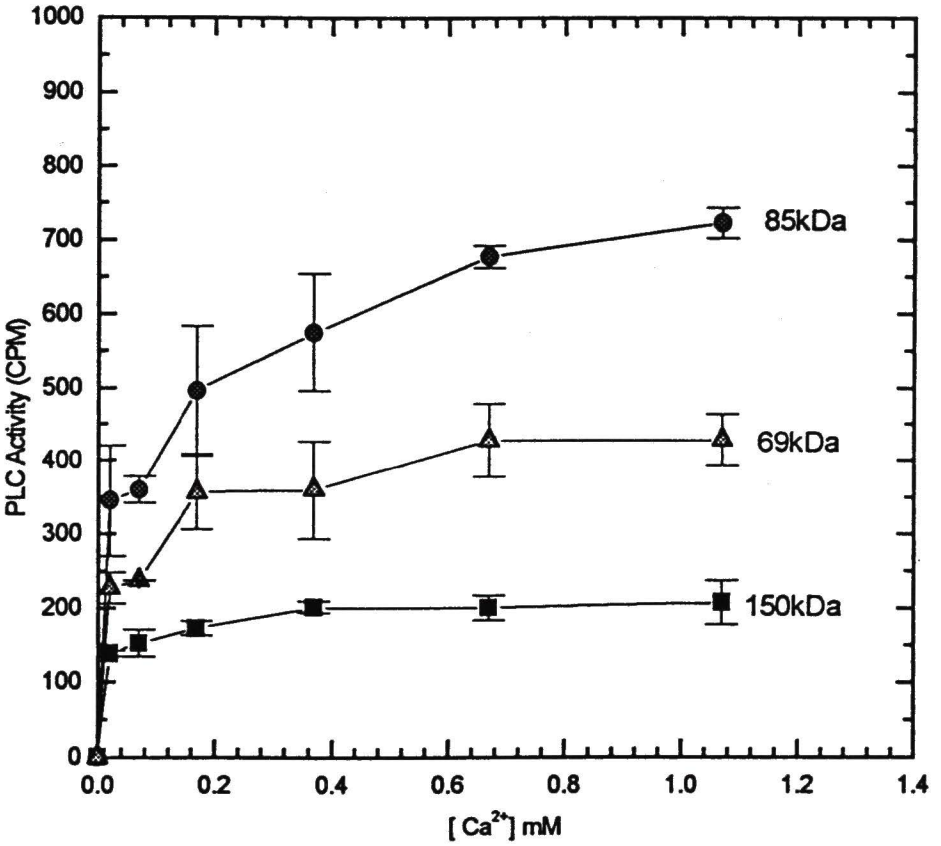

1000
900
800
700
600
500
400
300
200
100
0
PLC Activity (CPM)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
[Ca2+] mM
85kDa
69kDa
150kDa

Fig. 10. DE-52 anion exchange chromatography of rat and dog myocardial cytosol. Approximately 3 mg of cytosol were separated on DE-52 anion exchange column as described in MATERIAL AND METHODS. Eluted fractions (10 μl) from column were assayed for PLC activity with 20 μM [^{3}H]-PI in the presence of 5 mM $CaCl_2$ and 1 mM of deoxycholate.

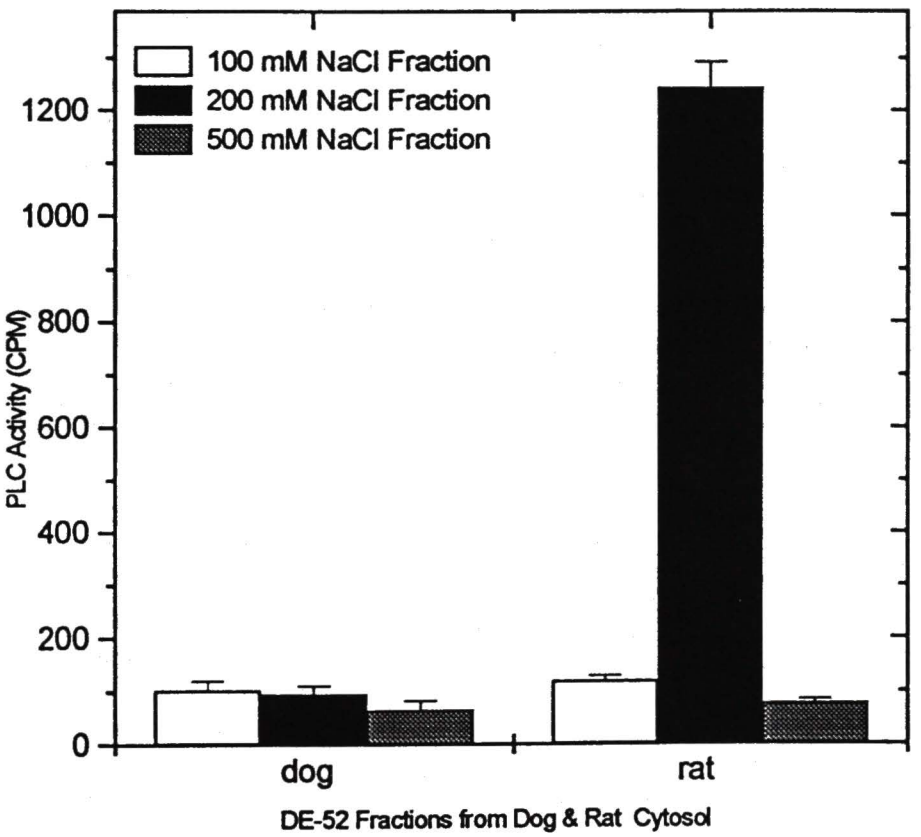
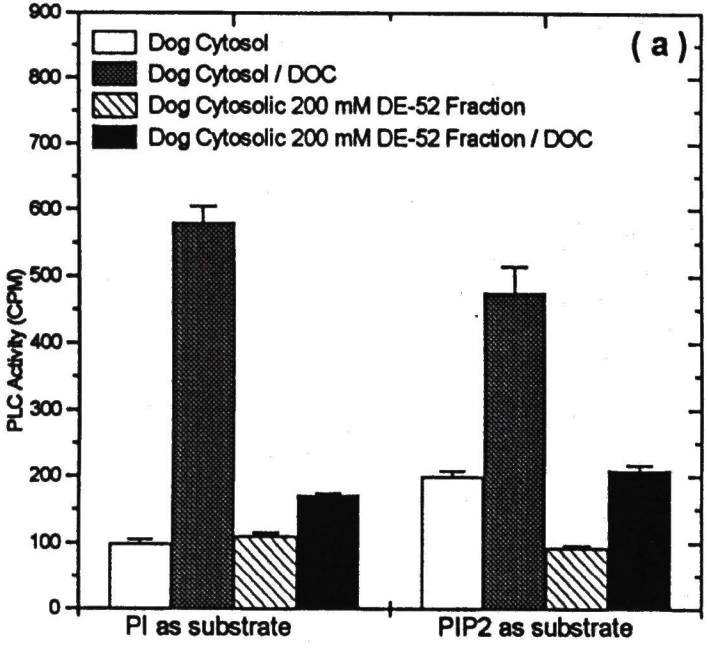

100 mM NaCl Fraction
200 mM NaCl Fraction
500 mM NaCl Fraction
PLC Activity (CPM)
0
200
400
600
800
1000
1200
dog
rat
DE-52 Fractions from Dog & Rat Cytosol

Fig. 11. Comparison between rat/dog PLC activity in cytosol and their 200 mM NaCl fractions from anion exchange DE-52 column. PLC activity was assayed with 20 μM [^{3}H]-PI or 20 μM[^{3}H]-PIP_2 in the presence and absence of 1 mM deoxycholate as described in MATERIAL AND METHODS. PLC activity from dog (a) and rat (b) were expressed in CPM.


(a)
Dog Cytosol
Dog Cytosol / DOC
Dog Cytosolic 200 mM DE-52 Fraction
Dog Cytosolic 200 mM DE-52 Fraction / DOC
PLC Activity (CPM)
0
100
200
300
400
500
600
700
800
900
PI as substrate
PIP2 as substrate

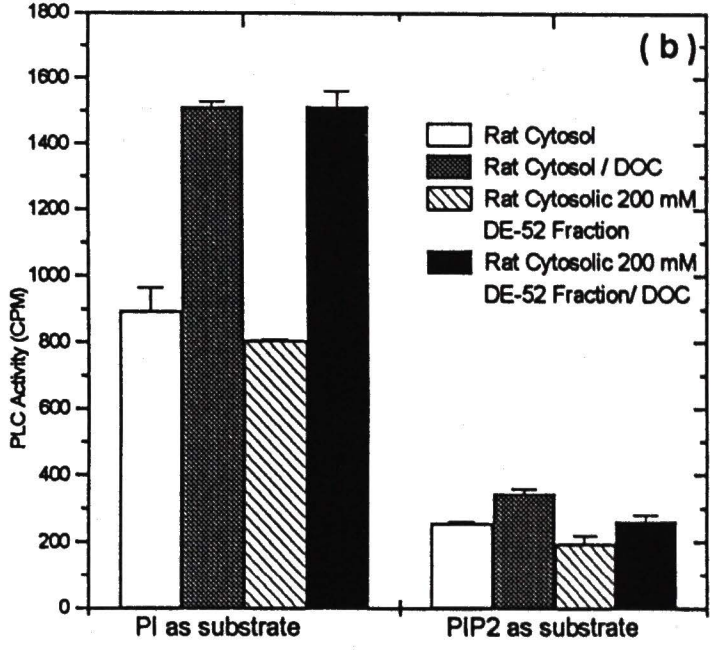
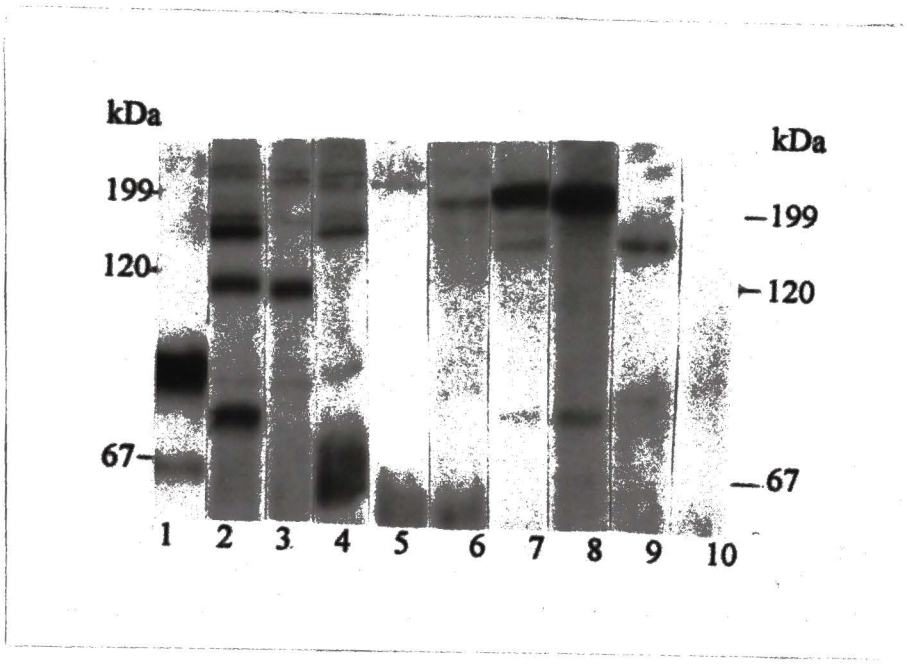

(b)
Rat Cytosol
Rat Cytosol / DOC
Rat Cytosolic 200 mM DE-52 Fraction
Rat Cytosolic 200 mM DE-52 Fraction/ DOC
PLC Activity (CPM)
0
200
400
600
800
1000
1200
1400
1600
1800
PI as substrate
PIP2 as substrate

Fig. 12. Immunoproperties of PLC isoforms in rat and dog cytosol. Rat and dog cytosolic fractions from DE-52 anion exchange column were applied to lanes 1-5 and 6-10 of an SDS/polyacrylamide gel (7%), respectively. Molecular makers corresponding to myosin (199 kDa), β-galactosidase (120 kDa), BSA (67 kDa) and ovalbumin (48 kDa) were in a separate lane. The proteins were subjected to SDS/PAGE, transferred to nitrocellulose membranes and then probed with antibodies against PLC-δ_1(lanes 1 and 6), PLC-β_3 (lane 2-3 and 7-8) and PLC-β_1 (lane 4-5 and 9-10). Peptides to PLC-β_3 were simultaneously applied on lane 3 and 7. Peptides to PLC-β_1 were simultaneously applied on lanes 5 and 10.


kDa
199
120
67
1 2 3 4 5 6 7 8 9 10
kDa
199
120
67

Microsomal PLC activity in rat and dog myocardium—The Ca^{2+} concentration requirements of rat and dog microsomal PLC in the presence of PI and PIP_2 was investigated by using EGTA/ Ca^{2+} buffers. The objective was to determine if the properties of the microsomal PLC activities differed in these species and if the properties of cytosolic versus membrane bound PLC differed. In the presence of PI, rat and dog microsomal PLC showed an absolute requirement for Ca^{2+} (Figures 13). Half-maximal and maximal activities were obtained at about 0.4 *m*M free Ca^{2+} and 1.0 mM Ca^{2+} in both species. The V_{max} values were approximately 1.8 and 0.9 n moles /mg/min in rat and dog, respectively. With PIP_2 as the substrate, rat and dog microsomal PLC showed slightly different calcium affinities but displayed similar V_{max} (6.2-6.6 n moles /mg/min) values. The half-maximal activity was attained at 0.5 μM Ca^{2+} in rat and 1.2 μM Ca^{2+} in dog microsomes. The relative rates of hydrolysis (PIP_2 / PI) were 6.9 in dog and 3.7 in rat microsomes indicating PIP_2 is a better substrate for dog microsomal PLC isoforms. No major differences in the Ca^{2+} affinities were noted between the microsomal and cytosolic PLC activities with PI or PIP_2 as substrates.

Immunochemical properties of PLC isoforms from rat and dog myocardial membranes – The microsomal membranes were subjected to SDS-PAGE, transferred to nitrocellulose paper and then probed with monoclonal antibodies raised against PLC-β_1, PLC-β_2, PLC-β_3, PLC-γ_1, PLC- γ_2 and PLC-δ_1 from bovine brain. As can be

seen from Fig.15, PLC-β_1 and PLC-β_3 reacted with the 150 kDa protein present in rat and dog myocardial microsomes. PLC-δ_1 reacted with a 87 kDa protein in rat but not dog microsomal protein. PLC-β_2, PLC-γ_1 and PLC-γ_2 were not detected in rat and dog microsomes. This results showed that same PLC isoforms detected in cytosol were expressed in rat and dog membranes.

Fig. 13. Ca^{2+} requirement of rat and dog microsomal PLC activity when PI as the substrate. The PLC activity was assayed in the presence of 0.5 % alamethicin and 1 mM of deoxycholate. The time of incubate was 20 min at 30° C. The calcium concentration range was 0-5.9 mM.

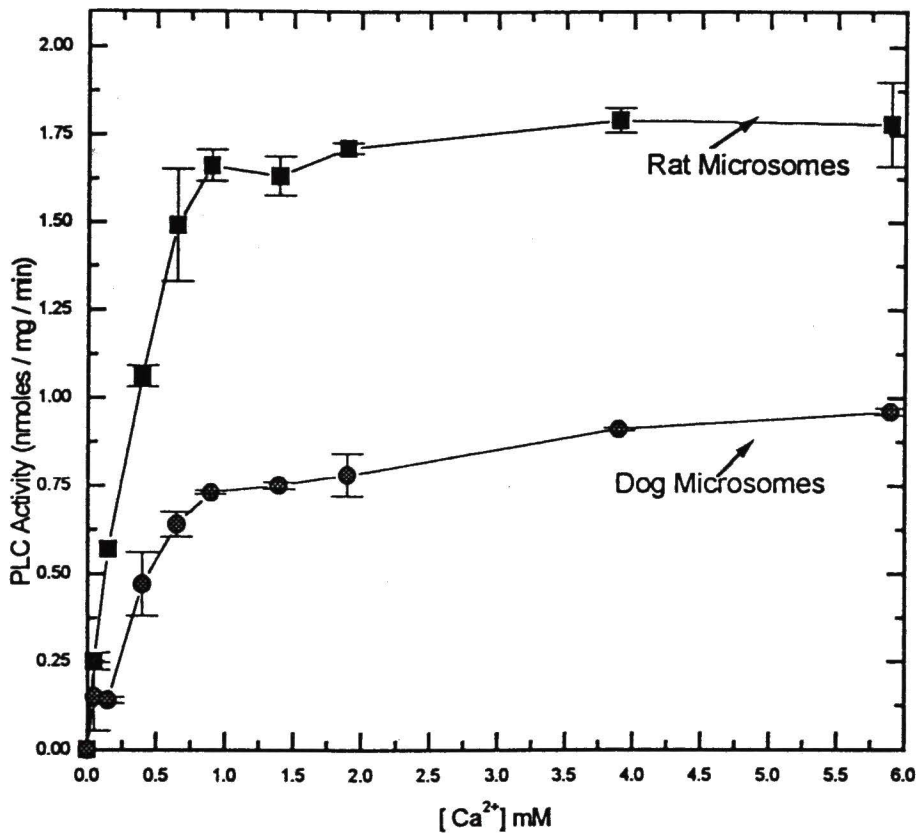

2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
PLC Activity (nmoles / mg / min)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
5.0
5.5
6.0
[Ca2+] mM
Rat Microsomes
Dog Microsomes

Fig. 14. Ca^{2+} concentration dependence of rat and dog microsomal PLC activity when PIP_2 as the substrate. The PLC activities were assayed in the presence of 0.5% alamethicin without 1 mM of deoxycholate. The time of incubate was 10 min at 30°C. The calcium concentration range was 0-12 μM.

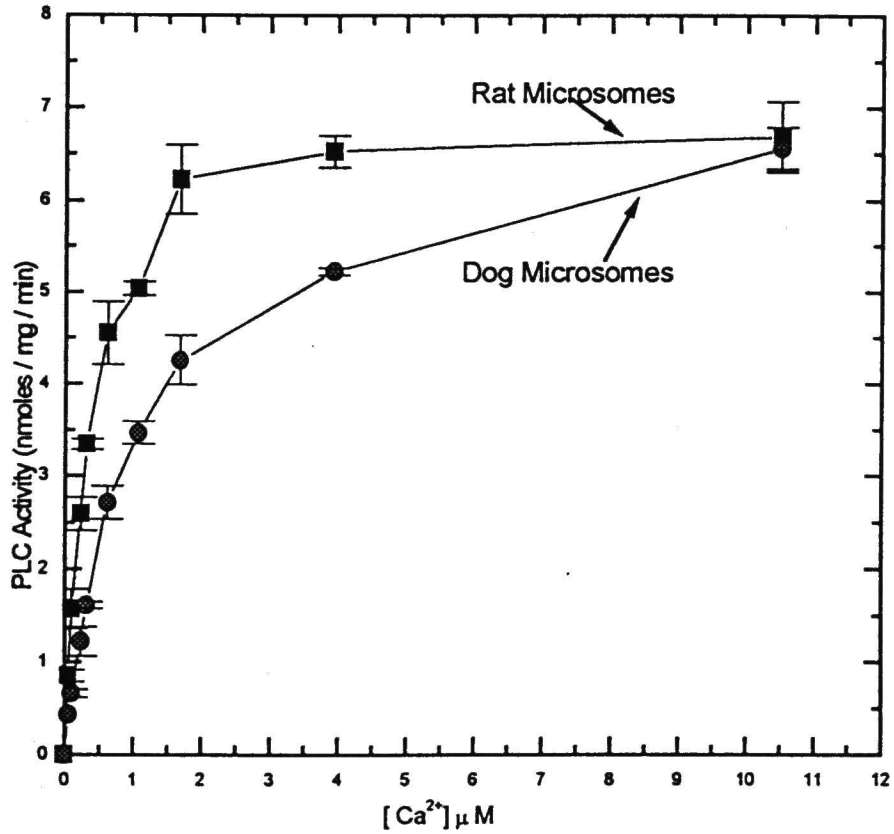
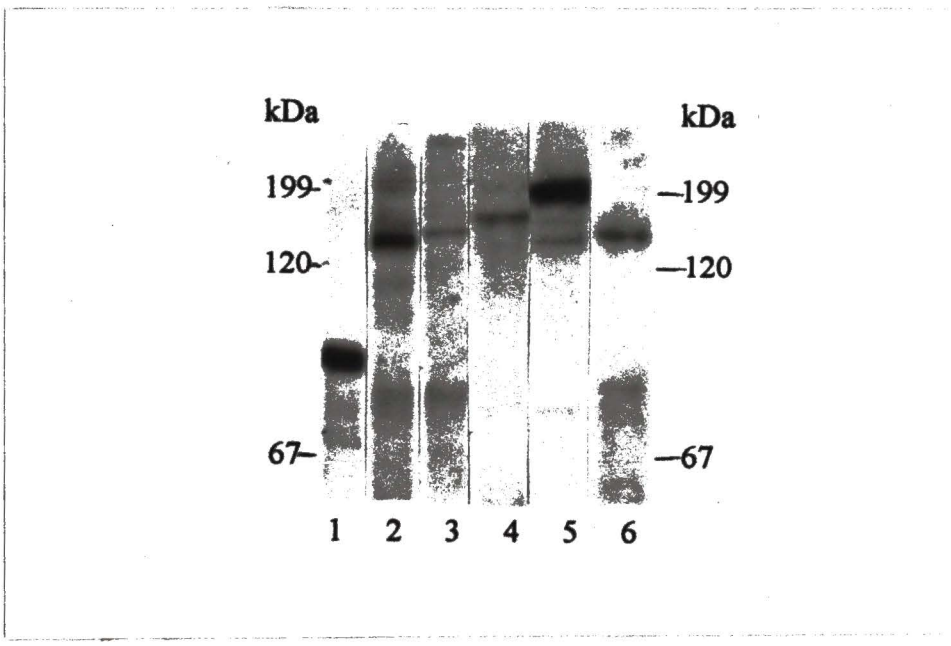

Rat Microsomes
Dog Microsomes
PLC Activity (nmoles / mg / min)
[Ca2+] μ M
0
1
2
3
4
5
6
7
8
9
10
11
12

Fig. 15. Westernblotting of microsomal PLC isoforms with monoclonal antibodies raised against bovine PLC-δ_1, PLC-β_1, PLC-β_2, PLC-β_3, PLC-γ_1. Fractions from rat and dog microsomes were applied to lanes 1-3 and 4-6 of an SDS/polyacrylamide gel (7%), respectively. Molecular makers were applied to an indicated lane. The proteins were subjected to SDS/PAGE, transferred to nitrocellulose papers and then probed with monoclonal antibodies against PLC-δ_1 (lanes 1 and 4), PLC-β_3 (lane 2 and 5) and PLC-β_1 (lanes 3 and 6).


kDa
199
120
67
kDa
199
120
67
1 2 3 4 5 6

Stimulation of microsomal PLC activity by a cytosolic protein—Experiments were undertaken to determine the effect of reconstituting cytosolic fractions with microsomal membranes on PLC activity. Studies were done by using PIP_2 as a exogenous substrate and a cytosolic 100 mM NaCl fraction from DE-52 anion exchange column. The rat cytosolic fraction alone did not contain PLC activity. Addition of this fraction with rat or dog microsomal membranes led to a 4 fold increase in PLC activity (Fig.16). Increasing the amounts of the DE-52 100 mM fraction increased the stimulation. The dog cytosolic 100 mM NaCl fraction from a DE-52 anion exchange column had very low PLC activity (Fig.4) but resulted in a 3 fold increase in rat and dog microsomal PLC activity (Fig.17). Interestingly, the stimulation was not obtained when PI was used as a substrate (not shown).

Molecular weight of cytosolic activator protein—The 100 mM NaCl fractions from DE-52 anion exchange columns were concentrated on a Amicon ultrafiltration concentrator and then fractionated on a S-200 Sephacryl-200 HR column. The reconstitution experiment demonstrated that a cytosolic protein, eluting with an apparent molecular weight of 91 kDa was responsible for the stimulation of microsomal PLC activity as shown in Fig. 18 (Dog) and 19 (rat).

Immunochemical properties of the cytosolic activator protein—In this study, the cytosolic fraction eluting with 100 mM NaCl from DE-52 anion exchange columns was immunoprecipitated by anti-bovine PLC-δ_1 isoforms. The rat microsomal PLC activity was assayed in the presence of rat or dog cytosolic 100 mM NaCl fractions before and after the immunoprecipitation. As shown in Fig 20, the stimulation of rat DE-52 100 mM fraction was completely abolished by the immunoprecipitation. The stimulation of the dog cytosolic 100 mM fraction on rat microsomes was decreased more than 50%. This species difference suggests that the bovine antibody is more selective for rat than dog PLC-δ. The cytosolic activator protein from rat and dog therefore appears to be an inactive PLC-δ isoform.

Fig. 16. Dependence of rat cytosolic DE-52 100 mM NaCl fraction on stimulation of microsomal PLC activity. Rat cytosolic fraction were prepared in 100 mM NaCl from DE-52 anion exchange column as described in MATERIAL AND METHODS. The protein content of 10 µl of the cytosolic fraction was 1.08µg. Both rat and dog microsomal PLC activities (1-2 µg) were assayed with 0, 2.5, 5 and 15 µl of the cytosolic fraction.

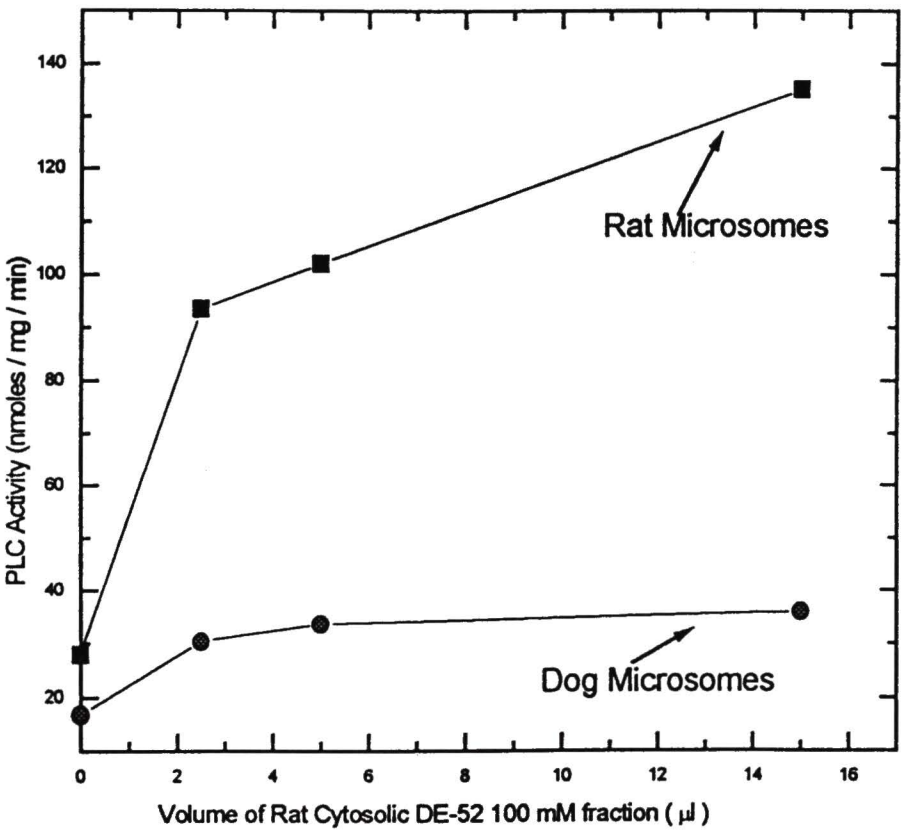

PLC Activity (nmoles / mg / min)
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
Volume of Rat Cytosolic DE-52 100 mM fraction (µl)
Rat Microsomes
Dog Microsomes

Fig. 17. Dependence of dog cytosolic DE-52 100 mM NaCl fraction on the stimulation of microsomal PLC activity. dog cytosolic fraction were prepared in 100 mM NaCl from DE-52 anion exchange column. The protein content of 10 μl of the cytosolic fraction was 1.0μg. Both rat and dog microsomes PLC (1-2 μg) activities were assayed in the presence of 0, 2.5, 5, and 15 μl of the cytosolic fractions.

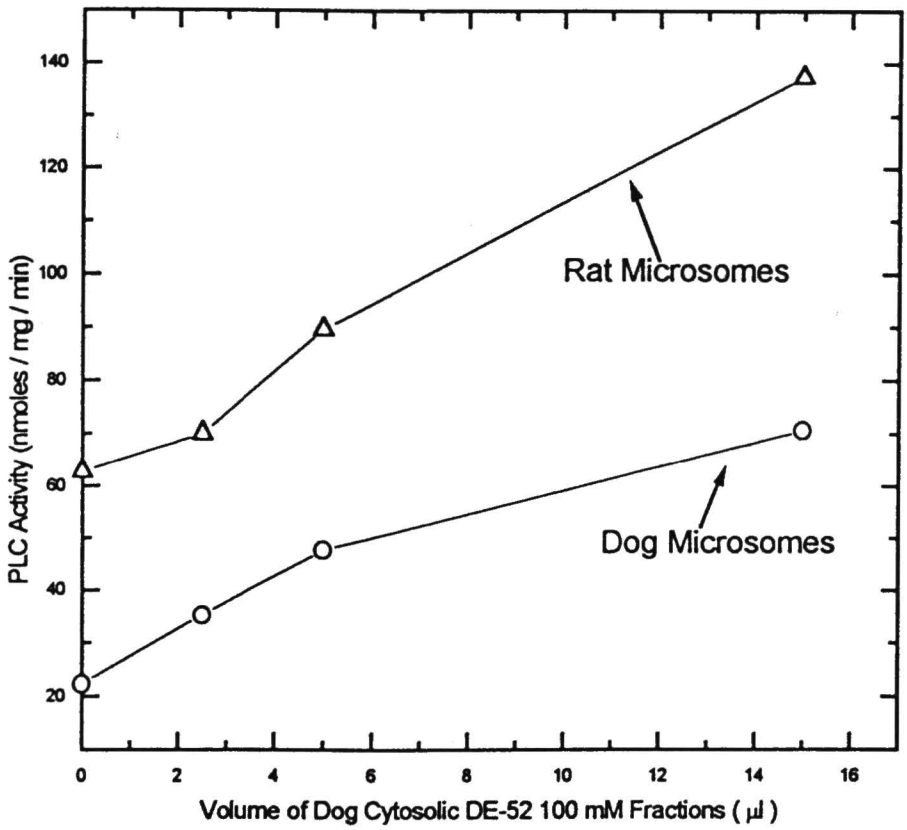

PLC Activity (nmoles / mg / min)
140
120
100
80
60
40
20
0
2
4
6
8
10
12
14
16
Volume of Dog Cytosolic DE-52 100 mM Fractions (µl)
Rat Microsomes
Dog Microsomes

Fig. 18. Molecular weight of 100 mM DE-52 fraction from dog cytosol on sephacryl-200 column. Dog cytosolic 100 mM NaCl fraction eluted from DE-52 anion exchange column was concentrated about 5.7 times on Amicon ultrafiltration cell, and then separated on a sephacryl-200 column as described in MATERIAL AND METHODS. The every second collected fraction was assayed with 20 μM [^{3}H]-PI in the presence of about 1-2 μg of dog microsomes.

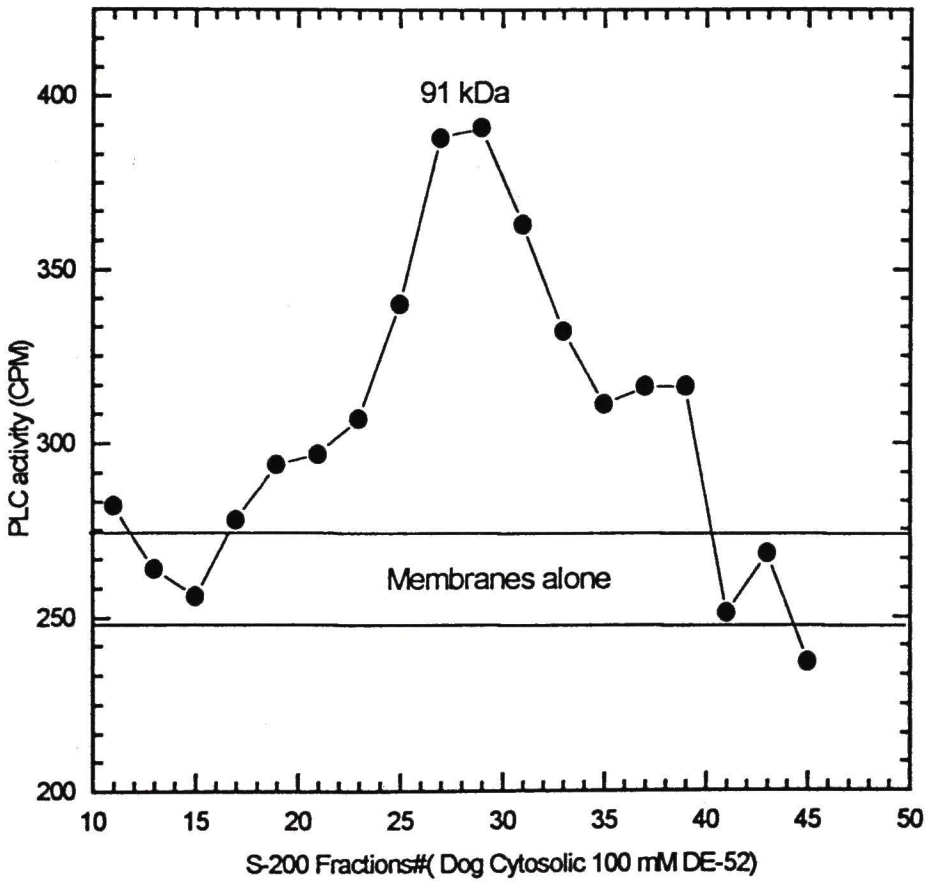

91 kDa
Membranes alone
PLC activity (CPM)
400
350
300
250
200
10
15
20
25
30
35
40
45
50
S-200 Fractions#(Dog Cytosolic 100 mM DE-52)

Fig. 19. Molecular weight of rat cytosolic protein eluting in 100 mM DE-52 fraction. Rat cytosolic 100 mM NaCl fraction eluted from DE-52 anion exchange column was concentrated about 6 times on Amicon Ultrafiltration Cell, and then, separated on a sephacryl S-200 column as described in MATERIAL AND METHODS. The every second collected fractions were assayed with 20 μM [^{3}H]-PI in the presence of about 1-2 μg of rat microsomes.

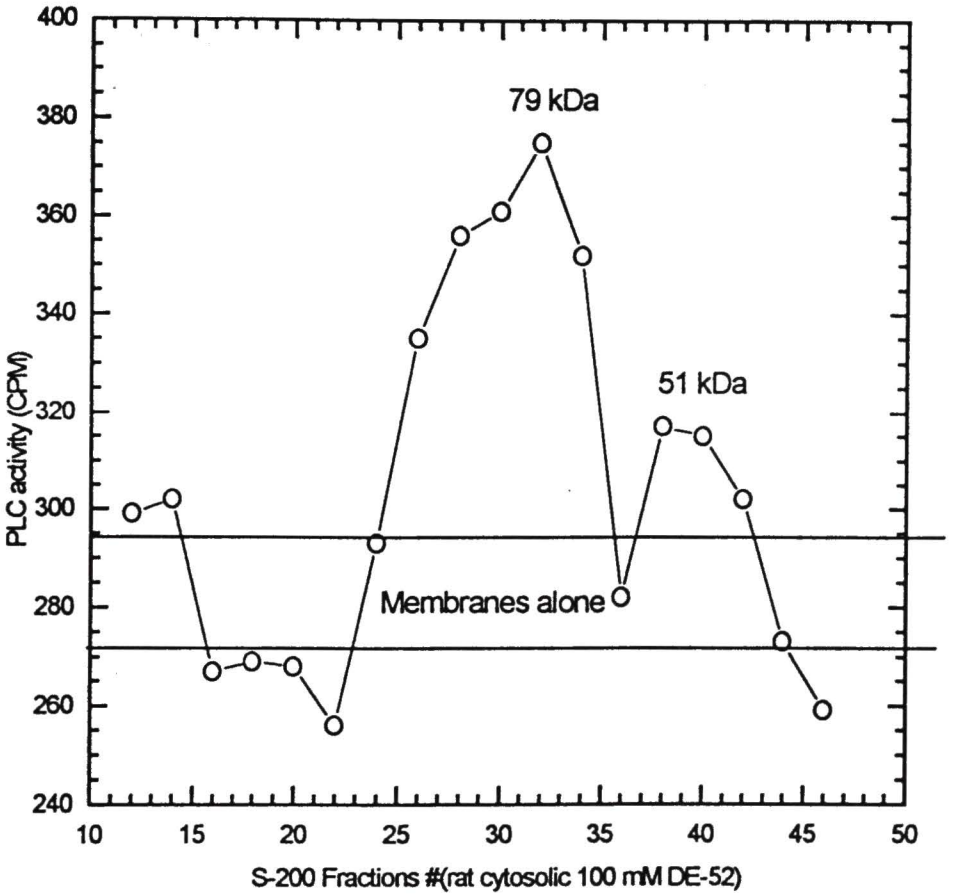
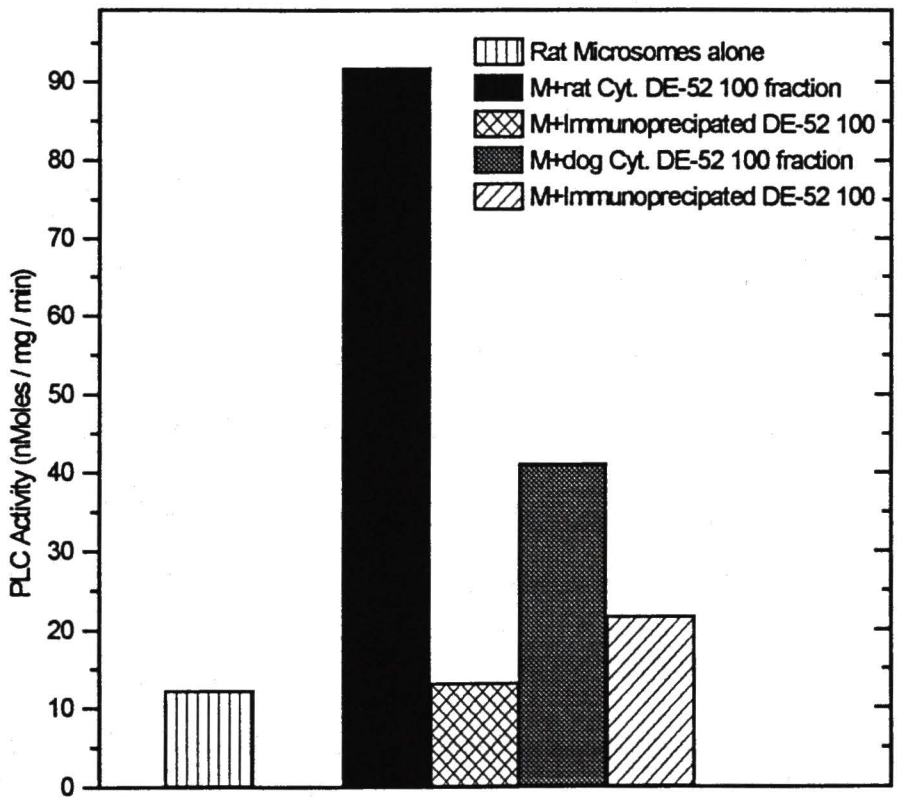

400
380
360
340
320
300
280
260
240
PLC activity (CPM)
79 kDa
51 kDa
Membranes alone
10
15
20
25
30
35
40
45
50
S-200 Fractions #(rat cytosolic 100 mM DE-52)

Fig. 20. Immunoproperties of 100 mM DE-52 fractions from rat and dog cytosol. Rat cytosolic 100 mM NaCl fraction was prepared from DE-52 anion exchange column as described in MATERIAL AND METHODS. The fractions were concentrated about 5 folds on amicon ultrafiltration cell, and then pretreated by immunoprecipitating with anti-bovine PLC-δ_1. Rat and dog microsomal (1-2 μg) PLC activities were assayed with 20 μM of [^{3}H]-PIP_2 in the presence of the pretreated or untreated (control) 100 mM NaCl fraction from rat cytosol.


Rat Microsomes alone
M+rat Cyt. DE-52 100 fraction
M+Immunoprecipated DE-52 100
M+dog Cyt. DE-52 100 fraction
M+Immunoprecipated DE-52 100
PLC Activity (nMoles / mg / min)
0
10
20
30
40
50
60
70
80
90

CHAPTER IV

DISCUSSION

Over the past fifteen years, PLC has been suggested to play an important role in myocardial function (3-10), Positive inotropic effects by several agents are accompanied by PLC activation (3-5). Myocardial phospholipase C (PLC) plays an important role in signal transduction by α_1-adrenergic agonists (4,8,9), muscarinic agonists (3), endothelin (5), thrombin (6), and angiotensin II (7). However, the identities of PLC isoforms in myocardium and the subcellular distribution of PLC isoforms were controversial (11-14,32,57). The transmembrane signaling mechanisms regulating myocardial PLC isoenzymes and relationship between soluble cytosolic PLC isoenzymes and membrane-bound PLC isoforms has not been delineated (14). These enzymes are difficult to study since hormonal activation is not evident in subcellular fractions. The purpose of the present study was to characterize the expression of isoenzymes of PLC in rat and canine myocardial cytosol and membranes and to determine the relationship of membrane PLC to soluble PLC in homogenates of ventricular myocardium. A species comparison may lead to functional insights into PLC

isoenzymes because of the large differences in the myocardial functional properties of rat and dog. Possibly relevant to this statement, marked differences in PLC activities were observed in dog and rat heart.

The present study demonstrated that rat myocardial cytosol contained three isoforms of PLC, designated PLC-δ_1, PLC-β_1, PLC-β_3 (Fig. 12). PLC-δ was the major isoform in rat cytosol and separated with an apparent molecular weight of approx. 85 kDa from a Sephacryl S-200 HR column (Fig.9). A 150 kDa isoform composed of PLC β_1 and PLC β_3 was detected in rat cytosol but activity in this peak was much lower than the 85 Kda PLC (Fig.9). In addition a PLC activity separating with an apparent molecular weight of 69 kDa from rat cytosol, expressed high activity and was identified by western blotting as a PLC- β_3 fragment (Fig.9 and 12). Dog myocardial cytosol contained only two detectable isoforms of PLC, designated PLC-β_1, PLC-β_3 by western blotting (Fig. 12). PLC-β activity eluted with appraent molecular weigts of 150 and 114 kDa (Fig.7). A PLC activity separating with an apparent molecular weight of 83 kDa could be separated from dog cytosol on a Sephacryl-200 column (Fig. 7). This peak has properties of PLC-δ (Fig.8) but was not recognized by the anti-PLC-delta from bovine brain (fig.12). Therefore the PLC-δ antibody only detects rat but not canine PLC-δ isoform. PLC-γ_1, PLC γ_2 and PLC-β_2 were not detected in either rat

or canine myocardial cytosol. The results further showed that rat and dog cytsolol contained similar PLC isoenzymes but that isoforms were present in different proportions in these species. In rat cytosol, PLC-δ and a 69 kDa PLC- β_3 fragment accounted for the majority of PLC activity. In canine cardiac cytosol, PLC-β isoenzymes were predominant. PLC-β_1 could not be separated from PLC-β_3 on S-200 column since PLC-β_1 and β_3 have similar molecular weights. This has been confirmed in several mamalian animal tissues (32) and corresponded well to the current results.

These studies confirmed that the Ca^{2+} affinities and the sensitivity of PLC δ and PLC-β isoenzymes were different. PLC isoforms have a very low activity in dog cytosol under physiologic conditions unless assayed in the presence of high (mM) Ca^{2+} and 1 mM deoxycholate (Fig. 3). Calcium concentration dependence studies demonstrated that PLC-β isoforms showed a higher calcium affinity reaching a plateau at about 0.4 mM Ca^{2+} whereas PLC-δ showed a relatively lower calcium affinity (fig 7 and 9). The properties of these PLC isoenzymes correspond well to the properties established for PLC- β_1 and PLC-δ, respectively (32, 57,58). In addition, PI was a relatively better substrate for PLC-δ in comparison to PLC-β which ustilizes PIP_2 better than PI (Fig.3,4,5). The study also showed that the same PLC isoenzymes detected in cytosol were

expressed in myocardial membranes in rat and dog, respectively but their specific activities were much higher in membranes than in cytosol (Fig.13,14). The membrane bound activities also did not required deoxycholate for maximal activity. This could suggest that membrane binding alters the structure of PLC similarly to DOC, leading to biological activation. Otherwise the Ca^{2+} affinities of the membrane bound and cytoslic activities were similar in the presence of PI or PIP_2. Although total PLC activity was relatively higher in rat than dog, microsomal PLC activities from both species were maximally activated by similar concentration of Ca^{2+} (Figs. 13 and 14).

This study was the first to identify PLC-β isoforms in canine myocardial microsomal membrane and cytosol eventhough many second previous investigators have mentioned multiple PLC forms exist myocardium (14, 32,58,). In contrast to the current results, PLC-δ with an apparent molecular weight of 85 kda and PLC-γ_1 with an apparent molecular weight of 145 kda were previously identified in canine myocardium cytosol by Wolf (14). These workers also detected PLC-δ in myocardial membranes and of further difference, detected no PLC-β in cytosol or membranes. The reasons for these differences are unclear but are likely due to differences in the antibodies used. The PLC- β antibodies used here were provided by Dr. P. Sterneweis and these antibodies were used

successfully by these workers to identify PLC- β_1 and PLC-β_3 in rat (31). Dr Petra Schnabel (59) also detected PLC-β_1 in human myocardium by RT-PCR and reported its activation by G-protein. PLC-β_1 and β_3 were also detected by Jhon, D.Y. et al (32) in rat heart homogenates. All evidence indicates that PLC-β isoforms are expressed in myocardium regardless of species . The relative expression of of PLC-β in myocardial tissue appears to be much lower than other tissues in rat (32). In the present study, PLC-β isoforms in cytosol were detected by westerblotting only after a 10-fold concentration. In comparison, the relative amount of PLC-β_1 and PLC-β_3 in microsomes was relatively higher than in cytosol. No concentration step was needed to detect PLC-β in microsomes. The distribution of PLC-β isoforms may possibly br related to its regulation mechanism by G-protein coupled to membrane receptors. In addition, a 69 kDa PLC isoform was detected in rat liver which regulated by a high molecular weight G protein and coupled to α_1-adrenergic receptors (54). This 69 kDa PLC isoform detected in rat liver may correspond to the PLC-β_3 fragment present in rat myocardium from my study. An interesting possibility is that the 69 kDa PLC-β_3 may be a naturally occurring PLC-β_3 isoform in rat liver and heart. The further study need to be done to determine if this fragment is denatured naturally from the 150 kDa PLC-β_3 isoenzyme.

It is of interest that McDonal and Mamrack also has identified PLC-δ in bovine cytosol (58) which is in partial agreement with my finding in rat cytosol (Fig.12) and microsomes (Fig.15) and in canine microsomes and cytosol (14). Thus PLC-δ appears to be an major isoenzyme present in myocardium of all species tested so far. The discrepancies in the relative amounts of PLC isoenzymes present in various species will still have to be resolved by further studies.

The present study is the first to show that myocardial cytosol contains a cytosolic protein with an apparent molecular weight 91 kDa (Fig.18,19) which is activated when combined with microsomal membranes (Fig.16,17). This protein eluted with 100 mM NaCl from an anion exchanger column and had no or very low PLC activity in assays using PI or PIP_2 as a substrate (Fig.10). When combining the protein to rat and canine myocardial membranes, there was a 3-4 fold of stimulation of PLC activity (Fig.16,17). Most interestingly immunoprecipatation with anti-bovine PLC-δ antibody completely abolished the ability of the rat cytosolic protein to stimuate PLC activity when combined with rat microsomal membranes (Fig.20). The activator protein from canine cytosol was only partially blocked by the antibody mediated immmunoprecipitation (Fig.20) further indicating the poor affinity of this

bovine antibody for canine PLC-δ. This results together suggested that the cytosolic activator protein was closely related with PLC-δ family in myocardial membranes and cytosol.

The most important aspect of this study is that rat and dog cytosol possess an inactive isoenzyme of PLC-δ which is activated by combining with membranes. Activation of the inactive cytosolic PLC-δ following membrane translocation may involve a conformational change or proteolysis by a membrane-bound enzyme. This could suggest that physiological activation of this PLC δ isoenzyme is due to both membrane binding and increases in intracellular Ca^{2+}. As we know, PLC-δ isoforms have been identified in most of mamalian tissues but the mechanism of PLC-δ isoenzyme activation has not been resolved. Results from this study provide further insights toward resolving the mechanism of PLC-δ. It would be interest to learn if PLC-δ binds to specific site on membrnaes. In addition, rat cytosol also possesses an active 85 kDa PLC-δ which can be activated by Ca^{2+} without combining with membranes. The physiologic reason for the presence of these different PLC-δ isoenzymes is not yet clear.

It is hypothesized that proteases may remove a small peptide from the inactive cytosolic PLC-δ isoform to produce an activated cytosolic PLC-δ. Proteases may act during the process of preparation of membranes and cytosol and result in active PLC-δ which can be detected by westernblotting. But the present study can not exclude in vivo specific protease. Otherwise the further experiments are necessary to find the relationship between the 91 kDa cytosolic protein immunoprecipated by anti-bovine PLC-δ and PLC-δ family. Studies are also needed to determine the factors involved in potential translocation and the activation of PLC-δ in vivo.

The data suggests an important role for PLC-δ in generation of PIP_2-derived second messengers in cardiovascular tissue because this isoenzyme was also expressed in cardiac membranes highly enriched in the substrates and enzymes necessary for local synthesis of PIP_2. The activation of cytosolic inactive PLC-δ by translocating to membranes were only observed in the assays using PIP_2 as a exogenous substrate (Fig.16,17). No activation has been noticed in assays using PI as a exogeneous substrate (data not shown). Furthermore, a physiological role for PLC-δ in cardiovascular tissue is again addressed by recent observation that aortic smooth muscle cells from spontaneously

hypertensive rats contain genomic DNA that point mutate in the catalytic domain of PLC-δ by (62).

The data in the present study help to reconcile conflicting reports concerning the relationship between soluble PLC to membrane-associated PLC in cardiac tissue. It explains why some investigators report that soluble PLC is distinct from membrane-associated PLC, whereas others report similarities between soluble and membrane-associated PLC. Assay conditions and choice of substrate can markedly influence the enzymatic activity of PLC-δ. Assays sensitive to PLC-δ activity, such as those with PIP_2 as substrate are likely to detect similarities in membrane-associated and soluble PLC in cardiac tissue. Because PLC-δ is present in both cytosol and membrane. In contrast, assays in which PI is substrate at high calcium concentrations are more selective for other PLC and are likely to detect differences between membrane-associated and soluble PLC.

There are several important limitations to characterize the myocardial compartmentalization of PLC isoenzymes in subcellular fractions. Isoenzymes that are associated with the membrane in vivo may be removed from this membrane during isolation of membrane vesicles from myocardial homogenate.

Additional isoenzymes may be expressed in myocardium that can not be detected by our enzyme assay or by our immunoblotting technique. Thus these data do not exclude the possibility that additional isoenzymes of PLC are associated with membrane. Truncated PLC-β isoforms with apparent molecular weight of 69 kDa may come from protease in vitro or in vivo. It is also not possible to conclude that the PLC isoform with an apparent molecular weight 114 kDa is a PLC-β fragment since analysis has not been performed from immunobloting. So far, we can not exclude it from a unknown PLC isoform with similar Ca^{2+} affinity with PLC-β isoform. Further study need to be done to find out if it can been recognized by anti- PLC-β antibodies or if it can be activated by G-protein.

BIBLIOGRAPHY

1. Berridge MJ, (1993) Inositol triphosphate and calcium signaling, Nature 361: 315-325.

2. Nishizuka Y, (1992) Intracellular signaling by hydrolysis of phospholipids and activation of protein kinase C. Science 258: 607-614.

3. Quist, E.E. and Satumtira, N.S.(1987) Muscarinic receptor stimulated phosphoinositide turnover in cardiac atrial tissue. Biochem. Pharmacol. 36, 499-505.

4. Brown, J.H., I. L. Buxton, and L. L. Brunton. α_1-adrenergic and muscarinic cholinergic stimulation of phosphoinositide hydrolysis in adult rat cardiomyocytes. Circ. Res. 57: 532-537..

5. Vigne, P., M. Lazdunski, and C. Frelin.(1989) The inotropic effect of endothelin-1 on rat atria involves hydrolysis of phosphatidylinositol. FEBS Lett, 249: 143-146.

6. Jones, L. G., P.M. McDonough, and J. H. Brown. (1989) Throbin and trypsin act at the same site to stimulate phosphoinositide hydrolysis and calcium mobilization. J. Pharmacol. Exp. Ther. 36: 142-149

7. Baker, K.M., and H. A. Singer.(1988) Identification and characterization of guinea pig angiotensin II ventricular and atrial receptors: coupling to inositol phosphate production. Circ. Res. 62: 896-904.

8. Terzic A, Puceat M, Vassort G, Vogel SM, (1993). Cardiac α_1-adrenoceptors: an overriew. Pharmacol Rev 45: 147-175.

9. Simpson P. C. (1983). Norepinephrine-stimulated hypertrophy of cultured rat myocardial cells is an alpha-1 adrenergic response. J. Clin Invest 72:732-738.

10. Johanna T.A. Meij, Vincenzo P., Nasrin M., John L. P and Naranjan S. D. (1997). Identification of changes in cardiac phospholipase C activity in congestive heart failure. J. Mol cell cardiol 29, 237-246.

11. Edes, I., and E.G. kranias. (1990) Characterization of cytoplasmic and membrane-associated phosphatidylinositol-specific phospholipase C enzymes activities in guinea pig ventricles. Basic Res. Cardiol. 85: 78-87.

12. Schwertz, D.W., and J. Halverson (1989). Characterization of phospholipase C-mediated polyphosphoinositide hydrolysis in rat heart ventricle. Arch. Biochem. Biophs. 269: 137-147.

13. Wolf, R.A.(1989). Cardiolipin-sensitive phospholipase C in subcellular fractions of rabbit myocardium. Am. J. Physiol. 257 (Cell Phsiol. 26): C929-C935.

14. Wolf, R.A. (1992). Association of phospholipase C-δ with a highly enriched preparation of canine sarcolemma. Am. J. Physiol. C1021-C1028.

15. Ryu,S.H., P.G. Suh, K.S. Cho, K.Y. Lee, and S.G.Rhee(1987). Bovine brain cytosol contains three immunological distinct forms of inositol phospholipid-specific phospholipase C. Proc. Natl. Acad. Sci. USA 84:6649-6653.

16. Rhee, S. G., and Choi, K.D.(1992) Adv. Second Messenger Phosphoprotein Res. 26, 35-61.

17. Kriz, R., L.L.Lin, L.Sultzman, C. Ellis, C.H. Heldin, T. Pawson, and J. Knopf.(1990). Phospholipase C isozymes: structural and functional similarities. Inc: CIBA Foundation Symposium, edited by G. Bock and J. Marsh. Cichester, UK: Wiley and Sons, vol. 150, p113-127.

18. Suh, P. G., S. H. Ryu, K. H. Moon, W. S. Suh, and S. G. Rhee (1988). Cloning and sequence of multiple forms of phospholipase C. Cell 54: 161-169.

19. Emori, Y., Homma, Y., Sorimachi, H., Kawasaki, H., naikanisi, O., Suzuke, K. And Takenawa, T. (1989) A second type of rat PLC containing a src-related sequence not essential for phosphoinositide-hydrolysis activity. J. Biol. Chem. 264, 21885-21890.

20. Smith, M. R., Liu, Y.-L., kim, H., Rhee, S.G. and kung, H.-F.(1990). Inhibition of serum- and ras-stimulated DNA synthesis by antibodies to phospholipase C. Science 247, 1074-1077.

21. Su, X., Chen, F. And Hokin, L.E. (1994). Cloning and expression of a novel highly truncated phosphoinositide-specific PLC cDNA from embryos of the brine shrimp, artenia. J. Bio. Chem. 269, 12925-12931.

22. Mayer, B.J., ren, R., Clark, K.L. and Baltimore, D. (1993) A putative modular domain present in diverse signaling proteins. Cell 73, 629-630.

23. Haslam, R.J., Koide, H.B. and Hemmings, B.A. (1993) Pleckstrin domain homology. Nature 363, 309-311.

24. Touhara, K., Inglese, J., Pitcher, J. A., Shaw, G. and lefkowitz, R.J. (1994) Binding of G protein βγ-subunits to pleckstrin homology domains. J. Biol. Chem. 269, 10217-10220.

25. Noh, D.-Y., Shin, S.H. and Rhee, S.G. (1995). Phosphoinositide-specific phospholipase C and mitogenic signaling. Biochem Biophys Acta. 1242, 99-114.

26. Kim, J.W., Ryu, S.H. and Rhee, S.G.(1989) Cyclic and noncyclic inositol phosphates are formed at different ratios by phospholipase C isozymes. Biochem. Biophys. Res. Commun. 163,177-182.

27. Taylor, S.J., Chae, H.Z., Rhee, S.G. and Exton, J.H. (1991) Activation of the β_1 isozyme of phospholipase C by α subunits of the Gq class of G proteins.Nature 350, 516-518.

28. Blank, J.L., Ross, A.H. and Exton, J.H. (1991) Purification and characterization of two G-proteins that activate the β_1 isozymes of phosphoinositide-specific phospholipase C. J. Biol. Chem. 266, 18206-18216.

29. Smrcka, A.V., Hepler, J.R., Brown, K.O. and Sternweis, P.C. (1991). Regulation of phosphoinositide-specific phospholipase C activity by purified Gq. Science 250, 804-807.

30. Waldo, G., boyer, J., Morris, A and Harden, T.K. (1991) Purification of an AlF_4^- and G-protein βγ- subunit-related phospholipase C -activity protein. J. Biol. Chem. 266, 14217-14225.

31. Smrcka, A.V. and Sternweis, P.C. (1993). Regulation of purified subtypes of phosphatidylinositol-specific phospholipase C β by G protein α and βγ subunits. J. Biol. Chem. 268,9667-9674.

32. Jhon, D.Y., Lee, H.H., park, D., lee, C.W., lee, K.H., Yoo, O. J. and Rhee, S.G. (1993). Cloning, sequencing, purification and Gq-dependent activation of phospholipase C-β_3. J. Biol. Chem. 268, 6654-6661.

33. Shenker,A., Goldsmith, P., Unson, c.G. and Spiegel, A.M. (1991) The G protein coupled to the thromboxane A2 receptor in human platelets is a member of the novel Gq family. J. Biol. Chem. 266, 9309-9313.

34. Wange, R.L., Smrcka, A.V., Sternweis, P.C. and Exton, J.H.(1991) Photoaffinity labeling of two rat liver plasma membrane proteins with [^{32}P]-γ–Azidoanilido GTP in response to vasopressin. J. Biol. Chem. 266, 11409-11412.

35. Gutowski, S., smrcka, A., Nowak, L., Wu, D., Simon, M.I. and sternweis, P.C. (1991). Antibodies to the αq-subfamily of guanine nucleotide-binding regulatory protein α subunits attenuate activation of PIP_2 hydrolysis by hormones. J. Biol. Chem. 266, 20519-20524.

36. Allgeier, A., Offermanns, S., van Sande, J., Spicker, K., Schultz, G. And Dumont, J. E.(1994). The human thyrotropin receptor activates G proteins Gs and Gq/11. J. Biol. Chem. 269, 13733-13735.

37. Conklin, B.R. and Bourne, H.R. (1993) Structural Elements of Gα subunits that interact with Gβγ, receptors and effectors. Cell 73,631-641.

38. Conklin, B.R., Farfel, Z, Lustig, K.D., julius, D. And Bourne, H.R.(1993) Substitution of three amino acids switches receptor specificity of Gqα to Giα. Nature 363, 274-276.

39. Clapham, D.E. and Neer, E. J., (1993) New roles for G-protein βγ dimers in transmembrane signalling. Nature 365, 403-406.

40. Camps, M., Carozzi, A., Schnabel, P., Scheer, A., Parker, P.J. and Gierschik, P. (1992). Isozyme-selective stimulation of phospholipase C-β_2 by G protein βγ subunits. Nature 360, 684-686.

41. Katz, A., Wu, D. And Simon, M.I. (1992) Subunits βγ of heterotrimeric G protein activate β_2 isoform of phospholipase C. Nature 360, 686-689.

42. Park, D., John, D.Y., Lee, C.W., Lee, K.H. and Rhee, S.G. (1993). Activation of phospholipase C isozymes by G protein βγ subunits. J. Biol. Chem. 268, 4573-4576.

43. Wu, D., Katz, A and Simon, M.I. (1993) Activation of phospholipase C β_2 by the α and βγ subunits of trimeric GTP-binding protein. Proc. Acad. Sci. USA 90, 5297-5310.

44. Lee, S.B., Shin, S.H., Hepler, J.R., Gilman, A.G. and Rhee, S. G. (1993) Activation of phospholipase C β_2 mutants by G protein αq and $\beta\gamma$ subunits. J. Biol.Chem. 268, 25952-25957.

45. Lee, C.W., Lee, K.H., Lee, S.B., park, D. And Rhee, S.g. (1994) Regulation of PLC-β_4 by ribonucleotides and the α subunit of Gq. J. Biol. Chem. 269, 25335-25338.

46. Touhara, K., Inglese, J., pitcher, J.A., Shaw, G. and Lefkowitz, R.J. (1994) G protein $\beta\gamma$ subunits. J. Biol. Chem. 269, 4388-4395.

47. Kim, U.H., Fink, D., Kim, H.S., Park, D.J., Contreras, M.L., guroff, G. and Rhee, S.G. (1991). Nerve growth factor stimulates phosphorylation of phospholipase C-γ in PC12 cells. J. Biol. Chem. 266, 1359-1362.

48. Huckle, R., Hepler, J.R., Rhee, S.G., Harden, T.K. and Earp, H.S.(1990) Endocrinology 127, 1697-1705.

49. Wahl, M.I., Nishibe, S., Suh, P.G., Rhee, S.G. and Carpenter, G. (1989) Epidermal growth factor stimulates tyrosine phosphorylation of phospholipase C-II independently of receptor internalization and extracellular calcium. Proc. Natl. Acad. Sci. USA 86, 1568-1572.

50. Koch, C.A., Anderson, D.A., Moran, M.F., Ellis, c. and Pawson, T.(1991). SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins. Science 252, 668-674.

51. Rotin, D., Honegger, A.M., Margolis, B.L., Ullrich, A and Schlessinger, Presence of SH2 Domains of phospholipase C g1 enhances substrate phosphorylation by increasing the affinity toward the epidermal growth factor receptor. J. (1992) J. Biol. Chem. 267, 9678-9683.

52. Meisenhelder, J., Suh, P.G., Rhee, S.G., and Hunter, T. (1989) Phospholipase C-γ is a substrate for the PDGF and EGF receptor protein-tyrosine kinases in vivo and in vitro. Cell 57, 1108-1122.

53. Kim, H.K., Kim, J.W., Zilberstein, A., Margolis, B., Kim, J.G., Schlessinger, J. And Rhee, S.G. (1991) PDGF Stimulation of inositol phospholipid hydrolysis requires PLC-γ_1 phosphorylation on tyrosine residues 783 and 1254. Cell 65, 435-441.

54. Kolanus, W., Romeo, C. And Seed, B. (1993) T cell Activation by clustered Tyrosine Kinases. Cell 74, 171-183.

55. Park, D.J., Rho, H.W., and Rhee, S.G. (1991) CD3 stimulation causes phosphorylation of phospholipase C-gamma-1 on serine and tyrosine residues in a human T-cell line. Proc. Natl. Acad. Sci. USA 88, 5453-5456.

56. Tanya, D., kwang, J.B., Caroline, G. And Mie-jae, I. (1993). Evidence that the Gh protein is a signal mediator from α_1-adrenerceptor to a phospholipase C. J. Biol. Chem. 268, 27398-27405.

57. Quist, E.E., Powell, P., Quist, C. and Page, S. (1989). Biochem. Biophys. Res. Comm. 163, 567-573.

58. Bradford, M.M.(1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72:248-254.

59. Low, M.G and Weglicki,w.B.(1983). Resolution of myocardial phospholipase C into several forms with distinct properties. Biochem J. 215:325-334.

60. McDonald, L.J. and Mamrack, M.D. (1988). Aluminium Affects phosphoinositide Hydrolysis by phospholipase C. Biochem Biophy Res Commun, 155: 203-208.

61. Schnabel,P., Gas,Heidi, Nohr,T., Camps,M and Bohm,M (1996). Identification and Characterization of G rotein-Regulated Phospholipase C in Human Myocardium. J.Mol Cell cardiol 28:2419-2427.

62. Yagisawa,H., tanase H and Nojma,H (1991). Phospholipase C-δ gene f the spontaneously hypertensive rat harbors point mutations causing amino acid substitutions in catalytic domain. J. Hypertens. 9:997-1004.