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Phospholipase C-δ1 rescues intracellular Ca^{2+} overload in ischemic heart and hypoxic neonatal cardiomyocytes

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Abstract

Ischemia and simulated ischemic conditions cause intracellular Ca^{2+} overload in the myocardium. The relationship between ischemia injury and Ca^{2+} overload has not been fully characterized. The aim of the present study was to investigate the expression and characteristics of PLC isozymes in myocardial infarction-induced cardiac remodeling and heart failure. In normal rat heart tissue, PLC-δ1 (about 44 ng/mg of heart tissue) was most abundant isozymes compared to PLC-γ1 (6.8 ng/mg) and PLC-β1 (0.4 ng/mg). In ischemic heart and hypoxic neonatal cardiomyocytes, PLC-δ1, but not PLC-β1 and PLC-γ1, was selectively degraded, a response that could be inhibited by the calpain inhibitor, calpastatin, and by the caspase inhibitor, zVAD-fmk. Overexpression of the PLC-δ1 in hypoxic neonatal cardiomyocytes rescued intracellular Ca^{2+} overload by ischemic conditions. In the border zone and scar region of infarcted myocardium, and in hypoxic neonatal cardiomyocytes, the selective degradation of PLC-δ1 by the calcium sensitive proteases may play important roles in intracellular Ca^{2+} regulations under the ischemic conditions. It is suggested that PLC isozyme-changes may contribute to the alterations in calcium homeostasis in myocardial ischemia.

Keywords: Phospholipase C-δ1; Ischemic heart; Hypoxic neonatal cardiomyocytes

1. Introduction

Alterations in cellular calcium homeostasis have long been recognized in association with contractile, metabolic, electrical and ionic changes resulting from myocardial ischemia and anoxia, as well as in hibernation myocardium, with stunning and with reperfusion-induced mitochondrial dysfunction [1]. During ischemia the early changes in cytosolic calcium do not correlate with the rapid decline in contractility. Rather, it has been suggested that the desensitization of contractile proteins was caused by ischemia-induced acidosis and increases in magnesium and inorganic phosphate [2,3]. In response to stimuli that simulate ischemia, such as hypoxia, serum and nutrient deprivation, and metabolic inhibition, with or without reperfusion cultured cardiac myocytes also undergo the same changes as infarcted myocardium in vivo [4–7]. Ischemia and simulated ischemic conditions cause Ca^{2+} overload in the myocardium and the increase in intracellular calcium activates a number of cytosolic proteins, including phospholipases, protein kinases, proteases and endonucleases [8].

Phospholipase C (PLC) hydrolyzes the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP_2) to generate diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP_3). DAG and IP_3 mediate the activation of protein kinase C (PKC) and intracellular Ca^{2+} mobilization, respectively [9]. The 11-mammalian PLC isozymes identified to date are all single polypeptides and can be divided into four types: PLC-β, PLC-γ, PLC-δ, and PLC-ε. PLC-β, -γ, and -ε are activated by G-protein-coupled receptors, receptor tyrosine kinase, and ras pathway, respectively [10]. Recently, it has been reported that PLC-δ1 is directly activated by a new class of GTP-binding protein (G_h, transglutaminase II) through coupling with a_1-adrenergic receptor [11]. This PLC-δ1–G_h pathway thus may be an important player in the

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signaling pathway that regulates calcium homeostasis and modulates physiological processes, such as smooth muscle tone (i.e. blood pressure) and neurotransmitter release [12]. All PLC isozymes contain C2 domain that is sensitive to Ca^{2+}-activation. Among the PLC isoforms, PLC-δ1 is the most sensitive to activation by intracellular Ca^{2+}. Based on consideration of the three-dimensional structure of PLC-δ, an increase in the intracellular concentration of Ca^{2+} to a level sufficient to fix the C2 domain of PLC-δ1 isozymes might trigger its activation. Thus, the PLC-δ-activation may be secondarily to receptor-mediated activation of other PLC isozymes or to activation of Ca^{2+} channels [13].

Although the δ1 and γ1-PLC isozymes seem to be predominant forms in normal cardiac cells [14,15], little is known about the expression and characteristics of PLC isozymes in myocardial ischemia. When PLC isozymes are activated in the ischemic heart, the initial products of PLC-mediated hydolysis of PIP_2, IP_3 and DAG, may play pivotal roles as intracellular second messengers through the mobilization of calcium from intracellular stores and activation of protein kinase C, respectively. Increased cytosolic free calcium activates a number of enzymes, including protein kinases and other degradative enzymes, such as calcium-activating proteases. Activation of these proteases could lead to proteolysis of proteins involved in the regulation of intracellular calcium levels and, thus, to decreased calcium responsiveness.

In this study, the expression and characteristics of PLC isozymes were investigated in ischemic rat heart. In addition, PLC isozymes were evaluated in neonatal cardiomyocytes subjected to hypoxic conditions that mimic ischemia.

2. Materials and methods

2.1. Experimental procedure

All experimental procedures for animal studies were approved by the Committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine, and perform in accordance with the Committee's Guidelines and Regulations for Animal Care. Myocardial infarction was produced in male Sprague–Dawley rats (200 ± 30 g) by surgical occlusion of left arterior descending coronary artery, according to the method of Haisong et. al. [16] with minor modifications. Briefly, after induction of anesthesia with ketamine (10 mg/kg) and xylazine (5 mg/kg), cutting the third and fourth ribs opened the chest, and the heart was exteriorized through the intercostal space. The left coronary artery was ligated 2–3 mm from its origin with 5-0 prolene suture (ETHICON, UK), and then the heart repositioned in the chest. The wound was closed with a pulse-string suture. Throughout the operation, animals were ventilated with 95% O_2 and 5% CO_2 using Harvard ventilator. Sham-operated animals were treated similarly, except that the coronary suture was not tied. Operative mortality was 10% within 48 h. At different times after surgery, the hearts isolated were immersed in 2% triphenyltetrazolium chloride (TTC) stain for 20 min at 37 °C to determine the infarct size. Finally, the viable LV (non-infarcted LV free wall remote from infarct and septum), border zone (∼2 mm viable tissue and ∼2 mm scar tissue), and scar region were isolated for determination of PLC isozymes concentration and localization.

2.2. Culture of neonatal cardiomyocytes

Neonatal rat cardiomyocytes were isolated and purified by the modifications of previously described methods [17,18]. Briefly, hearts of 1–2-day-old Sprague–Dawley rat pups were dissected, and the ventricles washed with Dulbecco's phosphate-buffered saline solution (pH 7.4, Gibco BRL) lacking Ca^{2+} and Mg^{2+}. Using micro-dissecting scissors, hearts were minced until the pieces were approximately 1 mm^3 and treated with 10 mL of collagenase II (0.8 mg/mL, 262 U/mg, Gibco BRL) for 15 min at 37 °C. The supernatant was then removed and the tissue was treated with fresh collagenase II solution for an additional 15 min. The cells in the supernatant were transferred to a tube containing cell culture medium (a-MEM containing 10% fetal bovine serum, Bibco BRL). The tubes were centrifuged at 1200 rpm for 4 min at room temperature, and the cell pellet was resuspended in 5 mL of cell culture medium. The above procedures were repeated 7–9 times until there little tissue was left. Cell suspensions were collected and incubated in 100 mm tissue culture dishes for 1–3 h to reduce fibroblast contamination. The non-adherent cells were collected and seeded to achieve a final concentration of 5×10^5 cells/mL. After incubation for 4–6 h, the cells were rinsed twice with cell culture medium and 0.1 mM BrdU added. Cells were then cultured in a CO_2 incubator at 37 °C. Simulated ischemia was induced using deoxygenated DMEM containing 1% FBS and then incubated in an anaerobic chamber (Thermo Forma Anaerobic System Model 1025, USA), or by treating myocytes for several hours at 37 °C with modified Krebs buffer (137 mM NaCl, 3.8 mM KCl, 0.49 mM $MgCl_2$, 0.9 mM $CaCl_2$, 4 mM HEPES) supplemented with 1 mM 2-deoxyglucose, 20 mM sodium lactate, 12 mM KCl, 1 mM sodium dithionite, and 0.2% FBS, pH 6.5.

2.3. Isolation of PLC isozymes from rat heart tissue

Tissue was homogenized in 5 volumes of homogenization buffer (10 mM Tris–HCl buffer (pH 7.4) containing 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 1 mM PMSF, 10 mg/mL leupeptin, 10 mg/mL aprotinin, and calpain inhibitors I and II [each at 4 mg/mL]), and then the homogenate centrifuged at 100,000 × *g* for 1 h. The supernatant was adjusted to 2 M KCl by the addition of solid KCl, stirred for 2 h at 4 °C, and then centrifuged at 35,000 × *g* for 30 min. The resulting supernatant was dialyzed overnight against 4 L of homogenization buffer and re-centrifuged. The supernatant (∼80 mg of protein) was applied to a heparin–sepharose CL-6B

column (20 mL of gel packed in a 1.5 cm × 15 cm Econo column) that had been equilibrated with 20 mM HEPES–NaOH (pH 7.0) containing 1 mM EGTA and 0.1 mM DTT. Bound proteins were eluted at a flow rate of 4 mL/min with equilibration buffer containing 1.2 M NaCl. Fractions (16 mL) were collected and assayed for PLC activity. Essentially all detectable PLC activity eluted in six fractions (~40 mg protein), which were pooled and concentrated in a stirred ultrafiltration cell fitted with a YM30 membrane (Amicon, Danvers, MA). After the final salt concentration was adjusted to 50 mM NaCl, the concentrate was centrifuged at 100,000 × *g* for 10 min. Proteins (20 mg, unless otherwise indicated) from the supernatant were injected onto a TSK gel heparin-5PW HPLC column (7.5 mm × 75 mm) that had been equilibrated with 20 mM HEPES–NaOH (pH 7.0), 1 mM EGTA, and 0.1 mM DTT. Proteins were eluted with equilibration buffer, at a flow rate of 1 mL/min, for 15 min, followed by a stepwise linear NaCl gradient of 0–0.64 M for 40 min and from 0.64 to 1 M NaCl for 10 min. The column was then washed with equilibration buffer containing 1 M NaCl. Fractions (0.5 mL) were collected and assayed for PLC activity (50 and 5 mL of each fraction were used to assay PI- and PIP_2-hydrolyzing activity, respectively).

2.4. PLC assay

PLC activity was determined using [^{3}H]-PI or [^{3}H]-PIP_2 as substrate. PIP_2-hydrolyzing activity was determined with mixed lipid vesicles of phosphatidylethanolamine and PIP_2 in a molar ratio of 4:1. The lipids in chloroform were dried under a stream of nitrogen gas, resuspended in 50 mM HEPES–NaOH (pH 7.0), 120 mM KCl, 10 mM NaCl, and 1.6 mM sodium deoxycholate, and sonicated. Assays were performed for 10 min at 30 °C in a 100 μL reaction mixture containing lipid micelles (12 μM [^{3}H]PIP_2, 12,000 cpm), 50 mM HEPES–NaOH (pH 7.0), 0.1% sodium deoxycholate, 120 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 2 mM EGTA, and 1.4 mM $CaCl_2$ (to give a final free Ca^{2+} concentration of 1 mM). PI-hydrolyzing activity was measured in a 200 μL reaction mixture containing 150 mM [^{3}H]PI (20,000 cpm), 50 mM HEPES–NaOH (pH 7.0), 3 mM $CaCl_2$, 2 mM EGTA, and 0.1% sodium deoxycholate. The reaction mixture was incubated at 37 °C for 10 min. Reactions were terminated with a mixture of chloroform, methanol, and HCl, and ^{3}H radioactivity in the aqueous phase determined as described previously [19].

2.5. Transfection

Transfections of PLC-δ1 cloned into the eukaryotic expression vector pcDNA3.1-HA were performed using LIPOFECTAMIN PLUS™ reagent (Gibco-BRL) [17]. Briefly, neonatal rat cardiomyocytes cultured in a 60 mm culture plate (5 × 10^5 cells/plate) were washed twice with serum-free DMEM. LIPOFECTAMIN PLUS™ reagent was diluted with serum-free DMEM and combined with 5 mg of DNA for the each plate. The DNA and LIPOFECTAMIN PLUS™ reagent was added into the each plate containing fresh medium on cells. After 12 h incubation in a CO_2 incubator at 37 °C, the medium were exchanged with 10% FBS–DMEM. The cells were further incubated for 48 h at 37 °C. For simulated ischemia, the cells transfected with PLC-δ1 were treated with deoxygenated DMEM containing 1% FBS and then incubated in an anaerobic chamber.

2.6. Confocal microscopy and fluorescence measurements

The measurement of cytosolic free Ca^{2+} concentration was performed by the confocal microscopy analysis [20]. Neonatal rat cardiomyocytes were plated on glass coverslips coated with laminin (5 mg/cm^2) for 1 day in cell culture medium (a-MEM containing 10% fetal bovine serum, Bibco BRL) and 0.1 μM BrdU. After incubation, the cells were washed with modified Tyrode's solution containing: 0.265 g/L $CaCl_2$, 0.214 g/L $MgCl_2$, 0.2 g/L KCl, 8.0 g/L NaCl, 1 g/L glucose, 0.05 g/L NaH_2PO_4, and 1.0 g/L $NaHCO_3$. Cells were then loaded with 5 μM of the acetoxymethyl ester of Fura-2 (Fura-2 AM, Molecular Probes, Eugene, OR) for 20 min, in the dark and at room temperature, by incubation in modified Tyrode's solution. Fluorescence images were obtained using an argon laser confocal microscope (Leica, Solms, Germany). Fluorochrome was excited by 488 nm line of argon laser and emitted light was collected through a 510–560 nm bandpass filter. Relative changes of free intracellular Ca^{2+} were determined by measuring fluorescent intensity.

2.7. Immunoblot analysis

Immunoblotting was performed as described previously [11]. Proteins were fractionated by sodium dodecyl sulfate (SDS)–polyacrylamide gel (10–12%) electrophoresis and then electrotransfered to methanol-treated polyvinylidene difluoride membranes. The blotted membranes were washed twice with water and blocked by incubation with 5% non-fat dried milk in PBS buffer (8.0 g NaCl, 0.2 g KCl, 1.5 g NaH_2PO_4, 0.2 g K_2HPO_4 per liter). After a 1 h incubation at room temperature, the membranes were probed overnight at 4 °C with monoclonal antibodies to PLCβ1, PLCδ1, and PLCγ1 followed by goat anti-rabbit IgG-peroxidase and detected by ECL.

2.8. Immunohistochemistry

For the immunohistochemical studies, serial 5 mm sections from paraffin-embedded heart tissue were treated with EZ-DeWax solution to remove the paraffin and then immersed in boiling citric acid for 5 min to block nonspecific endogenous peroxidases. After washing with distilled water, sections were immersed in hydrogen peroxide (H_2O_2) for 5 min and washed with phosphate-buffered saline (PBS) (pH 7.4). Sections were then incubated for 1 h in a moisture

chamber with a specific anti-PLC-δ1antibody at a dilution of 1:200. After incubation, slides were washed three times with PBS and incubated with streptavidin reagents in the LSAB kit (Dako, Denmark). Immunohistochemical staining was repeated three times to augment the intensity of staining and to obtain the reproducible results. The sections were reacted with DAB as a chromogen and counter stained with hematoxylin. Photographs were taken using an Olympus BH2 light microscope (Oympus Opical Co., Tokyo, Japan).

2.9. Statistical analysis

The data are expressed as mean ± S.E. *P*-values <0.05 were considered to be statistically significant (ANOVA and Student's *t*-test).

3. Results

3.1. PLC isozymes in normal rat heart tissue

Total heart extract containing about 200 mg protein was subjected to conventional heparin column chromatography (Fig. 1). Fractions, 35–55, containing the peak PLC activity were pooled and concentrated for the next step (about 70 mg of protein). As determined from activity counts in the crude extract and pooled fractions, it was estimated that more than 90% of PLC activity that was present in the crude extract was recovered in the pooled PLC fractions, a loss that was considered minor. Pooled PLC fractions eluted following conventional heparin column chromatography were fractionated further by heparin-5PW HPLC chromatography. Each fraction was assayed for PLC activity. Three main PLC activity peaks were identified. A prominent peak of PI-hydrolyzing activity was eluted in fractions 42–44 and small but definite peaks were also eluted in fraction 38 and 53. Minor broad peaks in fraction 46, 50 and 66 were also observed. Fractions containing the PLC activity were immunoblotted with isozyme specific antibodies to PLC-β1, -γ1 and δ1. Immunoblotting results indicated that the peak activity centered on fraction 43 was attributable to PLC-δ1 (85 kDa). The peaks around fraction 38 and 53 were found to be PLC-γ1 (145 kDa) and PLC-β1 (150 kDa), respectively. The amounts of PLC isozymes estimated to be present in rat heart was determined by comparing the immunoblotting intensity to known standards were PLC-δ1 [19], about 44 ng/mg of heart tissue; PLC-γ1, 6.8 ng/mg; and PLC-β1, 0.4 ng/mg.

3.2. Changes in cardiac PLC isozymes in infarcted heart tissue

Infarcted heart was characterized by TTC staining. In the representative photograph (Fig. 2A), the scar regions and border regions are shown by the white and pink colors, respectively. Quantitative assessment of the expression of PLC isozymes in infarcted regions in the border zone and in normal tissue was determined by Western blot analysis. Fig. 2B shows a representative autoradiograph demonstrating the differential expression of PLC isozymes. These data

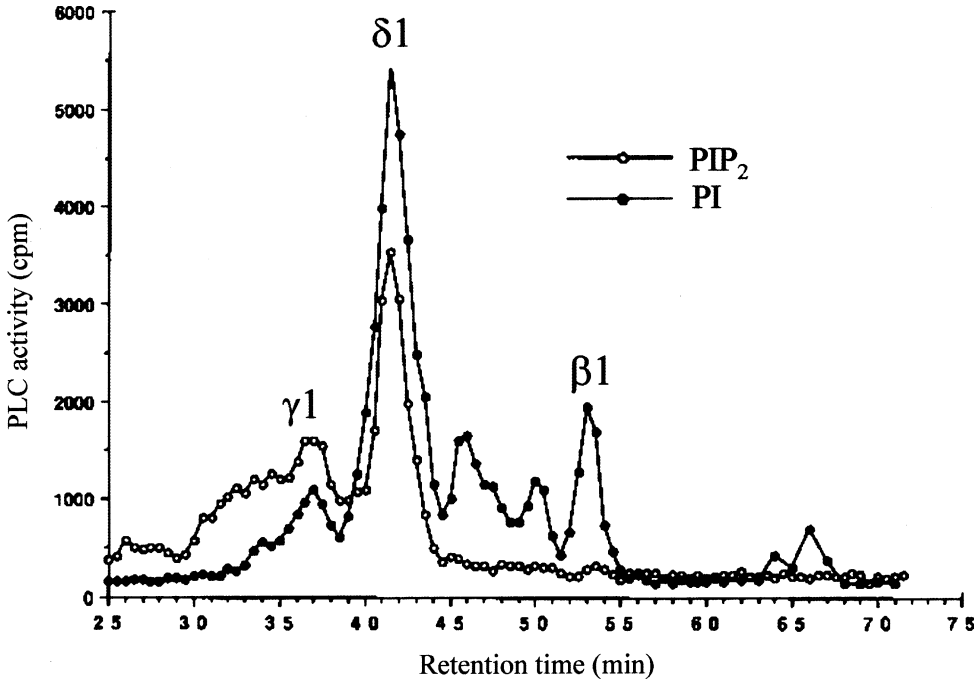

Fig. 1. Analysis of PLC isozymes in normal rat heart. The pooled PLC fraction from the heparin–sepharose chromatography was resolved by TSK gel heparin-5PW HPLC. Fractions (0.1 mL) were collected and assayed for both PI and PIP_2 hydrolysis.

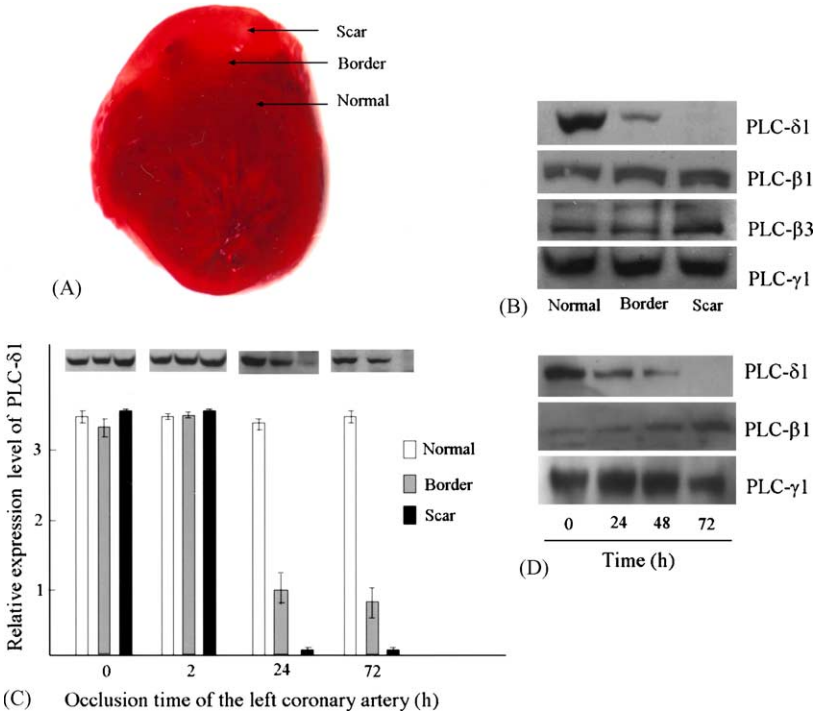

Fig. 2. Change in cardiac PLC isozymes in ischemic heart and cardiomyocytes. (A) Photograph of a heart slice stained with triphenyltetrazolium chloride. (B) Representative Western blots of PLC isozymes from normal, border, and scar regions of infarted heart. Myocardial infarction was produced in Sprague–Dawley rats by surgical occulusion of the left coronary artery for 72 h before sampling the tissue. (C) Western blot of PLC-δ1 in normal, border, and scar regions sampled at different time after ligation of the left coronary artery. (D) PLC isozymes in hypoxic neonatal cardiomyocytes. Confluent neonatal cardiomyocytes (approximately 7×10^6/10 cm diameter dish) were subjected to hypoxia (N_2:CO_2:H_2 = 85:10:5) in DMEM containing 1% FBS for the times indicated. (A) shows representative Western blots obtained in three separate experiments.

indicate that after 3 day of coronary artery ligation PLC-δ1 was degraded in both scar and border regions, whereas PLC-β1, PLC-β3 and PLC-γ1, were not. We investigated whether the abundance of PLC-δ1 was related to ligation time in scar, border, and normal regions. After ligation for 1 day, PLC-δ1 was almost absent in the scar and border regions whereas the amount of PLC-δ1 in normal regions was unchanged (Fig. 2C). Ischemia is a complex physiological process involving hypoxia, serum and nutrient deprivation, and metabolic inhibition. To study the effects of each of these components on PLC-δ1 degradation, primary neonatal cardiomyocytes were cultured in the presence of the non-metabolizable glucose analogue 2-deoxy-D-glucose (1 mM) or in deoxygenated DMEM containing 1% FBS in an anaerobic chamber. Under hypoxic conditions, the PLC-δ1 was degraded in a time-dependent manner, whereas other PLC isozymes were not affected (Fig. 2D), a pattern identical to that observed in infarcted myocardiums. The infarcted heart tissue was examined using two methods of staining: immunostaining with an anti-PLC-δ1 antibody, and hematoxylin and eosin (H&E) staining. Fig. 3A–C show the normal, border, and scar regions by H&E staining, respectively, which corresponded to the degree of tissue damage as characterized by the presence of dense eosinophilic staining. PLC-δ1 was uniformly present in the contractile elements of normal myocytes and was not observed in necrotic myocytes. In the lateral border of infarcted tissues, PLC-δ1 immunoreactivity was only shown in viable myoctes (Fig. 3D–F).

3.3. Effect of calpain and/or caspase inhibitors on hypoxia-induced PLC-δ1 degradation

Ischemia and simulated ischemic conditions cause Ca^{2+} overload in the myocardium and an increase in intracellular calcium activates cytosolic proteases [8]. To investigate the effect of calcium-activating proteases on the degradation of PLC-δ1, neonatal cardiomyocytes were treated with 100 nM calpastatin, a calpain inhibitor, and/or 10 mM zVAD-fmk, a caspase pseudosubstrate inhibitor. Both inhibitors prevented the degradation of PLC-δ1 in hypoxic neonatal cardiomyocytes (Fig. 4).

3.4. Role of PLC-δ1 on the cytosolic Ca^{2+} overload by hypoxic conditions

To address the role of PLC-δ1 on Ca^{2+} homeostasis in ischemic conditions, *PLC*-δ1 gene cloned into pcDNA3.1-HA was expressed in neonatal rat cardiomyocytes and the cells overexpressing PLC-δ1 were subsequently treated in anaerobic chamber. Fig. 5 shows a representative confocal microscopic image demonstrating the different intracellular

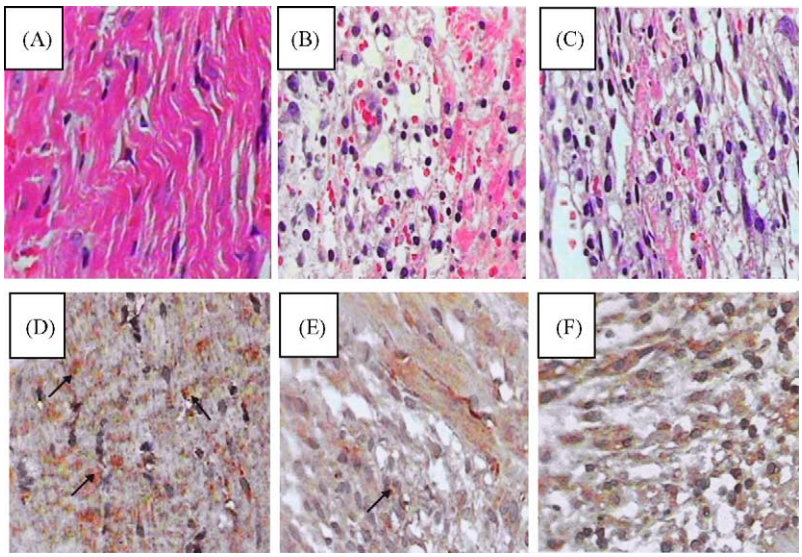

Fig. 3. Immunohistochemical localization of PLC-δ1 in normal (A and D), border (B and E), and scar (C and F) regions after ligation of the left coronary artery. Panels A–C was stained with hematoxylin and eosin (1000×). At Panels D–F, immunoactive PLC-δ1 protein appears as brown stained material (arrow). Magnification: 400×.

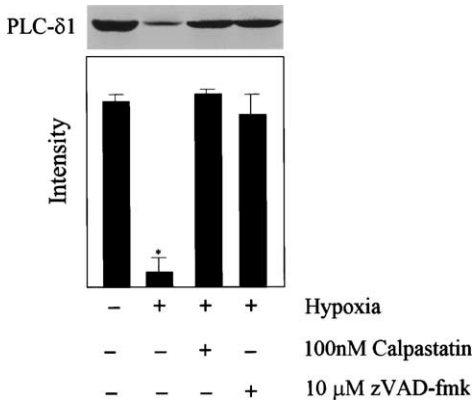

Fig. 4. Effect of protease inhibitors on PLC-δ1degradation in hypoxic neonatal cardiomyocytes. Confluent neonatal cardiomyocytes (approximately 7×10^6/10 cm diameter dish) were subjected to hypoxia (N_2:CO_2:H_2 = 85:10:5) in DMEM with 1% FBS for 48 h. Calpastatin (100 nM) or zVAD-fmk (10 mM) was added into the medium. Figures show a representative Western blot and relative intensity of PLC-δ1 for three separate experiments.

Ca^{2+} concentrations. In hypoxic conditions, both cells overexpressing PLC-δ1 and treating the calpastatin led to the normal fluorescence intensity compared with the cells transfected with pcDNA3.1-HA vector only. However, the hypoxic cells show a significant increase of the fluorescence intensity by four-fold, indicating that intracellular Ca^{2+} overload was induced by hypoxic conditions.

4. Discussion

Our study first demonstrates that in normal rat heart tissue PLC-δ1 was most abundant isozymes compared to PLC-γ1 and PLC-β. More importantly, we found that in ischemic heart PLC-δ1 was selectively degraded without changes in the other isozymes. In hypoxic neonatal cardiomyocyte, PLC-δ1 degradation was similarly observed. In addition, degradation of PLC-δ1 could be completely inhibited by the calpain inhibitor, calpastatin, or the caspase inhibitor, zVAD-fmk, suggesting an increase in degradation rather than an effect on synthesis of PLC-δ. By overexpression of the PLC-δ1 in hypoxic neonatal cardiomyocytes, intracellular Ca^{2+} overload induced by ischemic conditions was dramatically rescued.

Cytosolic calcium accumulation has been proposed as a mediator of the pathogenic changes that occur during myocardial ischemia [21]. Whether the rise in cytosolic calcium is a result of influx or redistribution from internal stores has not been elucidated. We questioned PLC isozymes are affected by the Ca^{2+} influx in ischemic heart. Ca^{2+} in vitro activates all PLC isozymes, but PLC-δ1 isozymes are more sensitive to Ca^{2+} than the other isozymes. The increase in cytosolic Ca^{2+} during ischemia activates phospholipase C isozymes, which further increase Ca^{2+} to form a vicious cycle. An increase in the intracellular concentration of Ca^{2+} to a level sufficient to fix the C2 domain of PLC-δ might therefore trigger its activation. Thus, activation of PLC-δ isozymes might be secondary to receptor-mediated activation of other PLC isozymes or the result of Ca^{2+} channels activation. PLC, upon stimulation, cleaves IP_3 and DAG from the membrane-bound phosphatidylinositol. Interestingly, Ca^{2+} overload itself causes IP_3 generation, potentially initiating a positive feedback relationship between Ca^{2+} and IP_3 which would further aggravate any electrophysiological damage to the heart [22]. DAG acts as a hydrophobic factor, and increases the binding of PKC

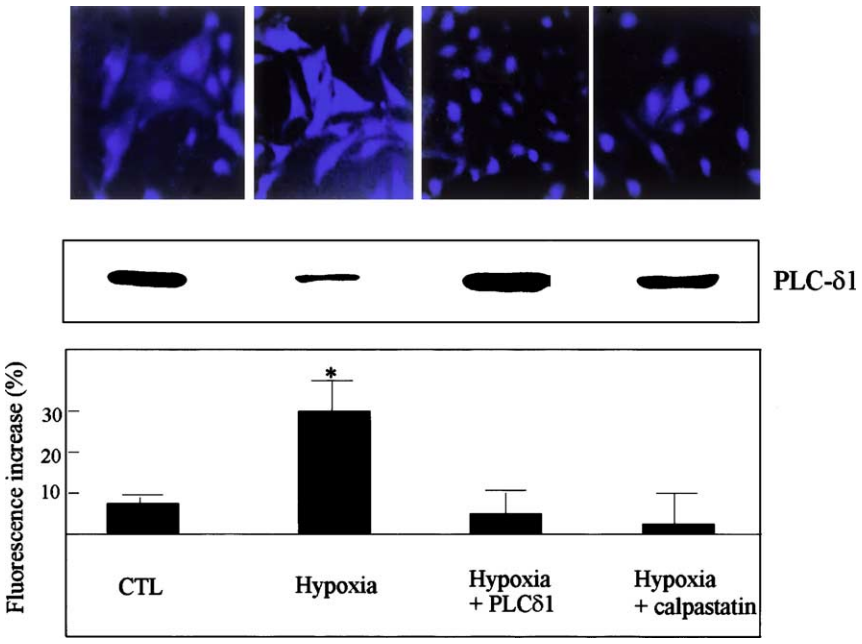

Fig. 5. Effect of PLC-δ1 on intracellular Ca^{2+} concentration. Confocal fluorescent image of neonatal rat cardiomyocytes was obtained loading with fura-2 AM. The cells with pcDNA3.1-HA (CTL) or pcDNA3.1-HA + PLC-δ1 were further incubated in anaerobic chamber for 24 h. Mean increases of the normalized fluorescent level (expressed as: % of increase ± S.E.M.) in individual cell ($n = 8$) observed in the each condition.

by membrane-integrated structures within subcellular compartments. In cardiomyocytes, direct involvement of PKC in this process was confirmed for genes encoding cardiac myosin light chain 2, atrial natriuretic factor, and the cardiac isoform of sarcoplasmic Ca^{2+}-ATPase [23–26]. Removal of Ca^{2+} from the cytosol is accomplished by several transports, including the sarcolemmal Na^{+}/Ca^{2+} exchanger and Ca^{2+}-ATPases of the sarcolemma and the sarcoplasmic reticulum (SR) [27]. Recent evidence suggests that calcium overload may activate calpains, resulting in selective proteolysis of myofibrils [8]. These proteolytic events are responsible for contractile depression, as they result in decreased responsiveness of contractile filaments to calcium. In our rat infarct model, we showed selective degradation of PLC-δ1 without other PLC isozymes being affected (Fig. 2B). This observation points to the primacy of alterations in the PLC-δ1 pathway of Ca^{2+}-activated inositol phosphate responses. In hypoxic neonatal cardiomyocytes, degradation of PLC-δ1 was completely inhibited by the calpain inhibitor, calpastatin, and by the caspase inhibitor, zVAD-fmk. This indicates that activation of Ca^{2+}-sensitive proteases by an increase in free cytosolic calcium is directly responsible for PLC-δ1-selective degradation.

Immunohistochemistry of the paraffin-embedded heart tissue obtained from infracted heart was used to explore the subcellular localization of PLC-δ1 (Fig. 3). PLC-δ1 was found to be universally present in the contractile elements of normal myocytes. It was only in viable myocyte in lateral border of infarcted tissue. These results indicate that the degree of PLC-δ1 degradation is directly related to the loss of viable myocytes in three different regions after myocardial infraction.

To further investigate the role of PLC-δ1 in ischemia, PLC-δ1 was overexpressed in hypoxic neonatal rat cardiomyocytes (Fig. 5). The PLC-δ1-overexpressed overcame the intracellular Ca^{2+} overload by hypoxic conditions, suggesting that the expression level of PLC-δ1 may contribute the intracellular Ca^{2+} homeostasis in patho-physiological circumstances linked to the altered cardiac performance from ischemia.

In conclusion, PLC-δ1 may play a role cytosolic calcium homeostasis in normal hearts, but its selective degradation after myocardial infarction may alter calcium balance. Based on these observations, we hypothesize that a Ca^{2+}-dependent protease activated by myocardial infarction-induced Ca^{2+} overload results in selective degradation of PLC-δ1.

Acknowledgements

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