Nuclear translocation of PKCζ during ischemia and its inhibition by wortmannin, an inhibitor of phosphatidylinositol 3-kinase

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Abstract Protein kinase Cζ (PKCζ), a member of the atypical PKC subgroup, is insensitive to Ca^{2+}, diacylglycerol, and phorbol esters, but is activated by phospholipids such as phosphatidylinositol-3,4,5-triphosphate, a product of phosphatidylinositol 3-kinase (PI3-kinase). Here we show that PKCζ translocates from the cytosol to the 1000×g pellet (nuclear-myofibrillar) fraction during ischemia for 40 min in Langendorff-perfused rat hearts. In addition, immunohistochemical observation shows that ischemia induces the translocation of PKCζ to the nucleus. The nuclear translocation during ischemia is inhibited in a dose-dependent manner by wortmannin (10^{-9}–10^{-7} M), an inhibitor of PI3-kinase.

Key words: Protein kinase Cζ; Ischemia; Wortmannin; Rat heart

1. Introduction

The protein kinase C (PKC) family consists of 12 PKC isoforms classified into three subgroups [1,2]. Conventional PKCs (cPKCs) (α, βI, βII, and γ) are activated by both Ca^{2+} and diacylglycerol (DG) or phorbol esters; novel PKCs (nPKCs) (δ, ε, η, θ, and μ) are activated by phosphatidylserine (PS) and DG but lack the domain that confers Ca^{2+} sensitivity; atypical PKCs (aPKCs) (ζ and ι/λ) are unaffected by Ca^{2+}, DG, or phorbol ester but activated by phospholipids such as PS or phosphatidylinositol-3,4,5-triphosphate (PIP3) [2–7]. PIP3 is formed from phosphatidylinositol-4,5-bisphosphate (PIP2) by phosphatidylinositol 3-kinase (PI3-kinase) in response to insulin, platelet derived growth factor, or other agonists [8,9]. Recently, Akimoto et al. have shown that PKCι/λ is activated through PI3-kinase [10]. However, it is still unclear whether PIP3 participates in the activation of PKCζ isoform in vivo.

In the heart, PKC regulates the stretch of contraction [11], gene expression [12,13], and ion channel function [14]. Accordingly, potential PKC substrates exist in various subcellular compartments: myofibrils (troponin and myosin light chain) [15,16] and membranes (Ca^{2+} channels and phospholamban) [17,18]. PKC also seems to play an important role in ischemic heart. It has been reported that ischemia-reperfusion injures the myocardium by Ca^{2+} influx through a Na^+/H^+-exchanger whose activity appears to be regulated by PKC [19–21]. Furthermore, cycles of brief ischemia and reperfusion (designated preconditioning) protect the myocardium against infarction caused by subsequent sustained ischemia. The preconditioning effect is suppressed by selective PKC inhibitors [22]. Although PKC isoforms are known to be differentially expressed in various cells and regulated by distinct activators [23,24], the role of PKC isoforms in ischemic heart has been poorly elucidated. We have recently shown that PKCα, δ, and ε isoforms translocate during ischemia, and suggested that the Ca^{2+}-dependent protease calpain was involved in the activation [25]. However, the activation of PKCζ and its activator(s) during ischemia is unknown at present although PKCζ has been shown to be involved in signal transduction in various cell types [26,27].

In this study, we show that PKCζ in the cytosol translocated to the nucleus during ischemia and that the nuclear translocation was inhibited by wortmannin, an inhibitor of PI3-kinase.

2. Materials and methods

2.1. Materials

Anti-PKCα antibody and anti-PKCζ antibody (CV) against amino acids 394–590 of human PKCζ were bought from Transduction Laboratories Inc. (Kentucky, CA, USA), anti-PKCβ1 and β2 antibody, anti-PKCδ antibody, anti-PKCε antibody, and anti-PKCζ antibody (V) against amino acids 577–592 of rat PKCζ from Life Technologies Inc. (Gaithersburg, MD). Anti-histone H1 antibody was from Leinco Technologies Inc. and anti-MEK-1 antibody was from Update Biotechnology Inc. The enhanced chemiluminescence (ECL) reaction kit assay, anti-mouse and anti-rabbit immunoglobulin G antibodies coupled to horseradish peroxidase, and protein A-Sepharose were brought from Amersham Co.; the Vecstain ABC kit was from Vector Laboratories (Burlingame, CA); the biotin blocking system was from Dako Co. (Carpenteria, CA). All other reagents were commercially available.

2.2. Perfusion protocol

Male Wistar rats weighing about 200 g were anesthetized with sodium pentobarbital. The aortas were cannulated and attached to a Langendorff apparatus. The hearts were perfused as previously reported [28], and then were subjected to global ischemia for 10–40 min at 37°C. The hearts were quickly frozen in liquid nitrogen and stored at −70°C for later biochemical analyses.

2.3. Subcellular fractionation

Subcellular fractionation was performed at 4°C essentially as previously described [21,29]. The frozen hearts were minced and homogenized in 2 volumes of STE buffer (0.32 M sucrose, 10 mM Tris-HCl, pH 7.4, 1 mM EGTA, 2 mM EDTA, 5 mM NaN_3, 10 mM β-mercaptoethanol, 20 μM leupeptin, 0.15 μM pepstatin A, 0.2 mM PMSF, 50 mM NaF, and 1 mM orthovanadate and 0.4 nM microcystin as protein phosphatase inhibitor) with a Polytron homogenizer, mixed with 2 volumes of STE buffer, and centrifuged at 1000×g for 10 min to obtain pellet (P1: nuclear-myofibrillar) and supernatant fractions.

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Abbreviations: PKC, protein kinase C; cPKC, conventional PKC; nPKC, novel PKC; aPKC, atypical PKC; DG, diacylglycerol; PI3-kinase, phosphatidylinositol 3-kinase; PIP3, phosphatidylinositol-3,4,5-triphosphate; PIP2, phosphatidylinositol-4,5-bisphosphate; PS, phosphatidylserine; WT, wortmannin; PBS, phosphate buffered saline; ECL, enhanced chemiluminescence; MEK-1, mitogen-activated protein kinase/extracellular regulated protein kinase kinase-1

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The supernatants were further centrifuged at 100 000×*g* for 60 min to obtain supernatant (S: cytosolic) and pellet (P2: membrane) fractions. The P1 and the P2 fractions were suspended in the STE buffer.

2.4. Electrophoresis and immunoblotting

The samples and prestained molecular mass standard (Bio-Rad) were electrophoresed in 7.5% polyacrylamide gels in the presence of SDS, and transferred to nitrocellulose membrane (0.45 μm). The blots were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.02% Tween-20, and incubated with one of the antibodies diluted in 1% BSA in the same buffer. After washing the blots, the antigens were visualized with the ECL reaction kit.

2.5. Immunohistochemistry

Rat hearts were immersed in OCT compound (Miles) and rapidly frozen in liquid nitrogen. After sectioning at 6 μM, the specimens were fixed on glasses with PLP solution (0.02 M $NaIO_4$/0.075 M phosphate buffer/2% paraformaldehyde) for 10 min at 4°C and then incubated with 0.3% H_2O_2 in phosphate-buffered saline for 30 min to quench the endogenous peroxidase activity. The specimens were blocked with 1.5% normal serum in PBS, then incubated with anti-PKCζ antibody (V), anti-MEK-1 antibody, or anti-histone H1 antibody in 1% BSA in PBS for 1 h and immunostained with the avidin-biotin peroxidase complex method using a Vectastatin kit. The peroxidase label was visualized by exposing the sections to diaminobenzidine.

3. Results and discussion

3.1. Identification and translocation of PKCζ in ischemic heart

It has been reported that the mRNA for PKCζ is expressed in the cardiomyocytes of neonatal and adult rats [30], but the

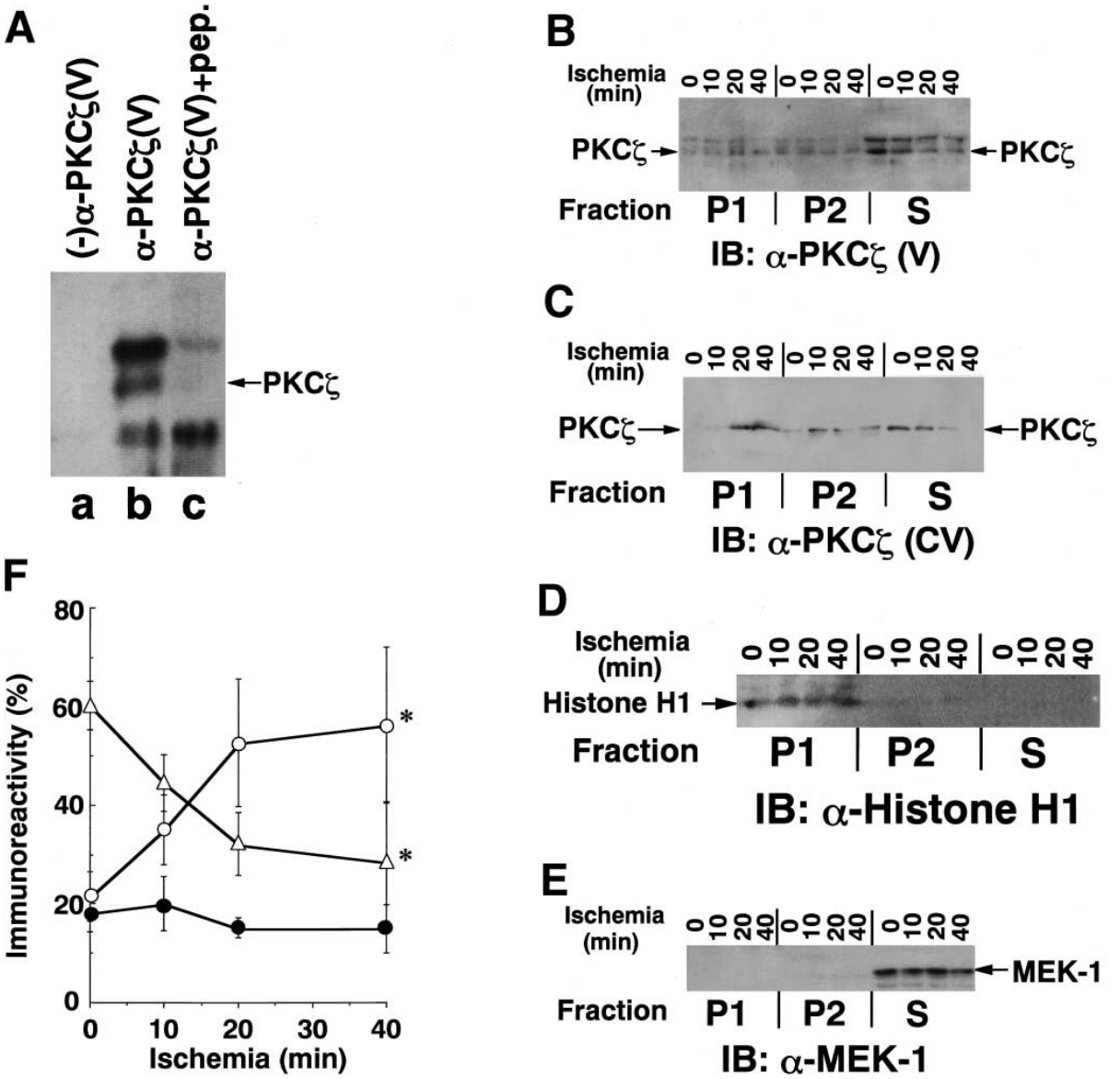

Fig. 1. Subcellular localization of PKCζ in the ischemic heart. The subcellular fractions were prepared from hearts rendered ischemic for 0–40 min, and subjected to immunoblotting with anti-PKCζ antibody. A: A representative immunoblot without anti-PKCζ antibody (V) against amino acids amino acids 577–592 of rat PKCζ (lane a), with anti-PKCζ antibody (V) (lane b), and with anti-PKCζ antibody (V) and the immunizing peptide (lane c). B–E: Representative immunoblots with anti-PKCζ antibody (C) (B), anti-PKCζ antibody (CV) against amino acids 394–590 of human PKCζ (C), anti-histone H1 antibody (D), and anti-MEK-1 antibody (E). These figures show representative immunoblots from three independent experiments. F: Amounts of PKCζ (%: the sum of P1, P2, and S fractions of control heart, $n=3$) densitometrically determined from the immunoblots using anti-PKCζ antibody (CV) (*$P<0.05$ vs. control, mean ± S.E., P1: nucleus-myofibril (○), P2: membrane (●), S: cytosol (△)).

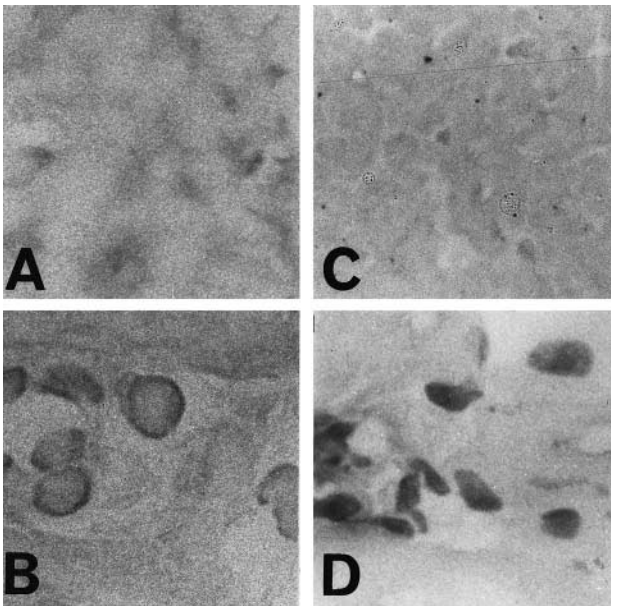

Fig. 2. Immunohistochemical localization of PKCζ. Hearts were subjected to ischemia for 0 min (A, C, and D) or 40 min (B), and then cryosectioned, fixed with PLP solution (0.02 M $NaIO_4$/0.075 M phosphate buffer/2% paraformaldehyde), and stained with anti-PKCζ antibody (A and B), anti-MEK-1 antibody (C), and anti-histone antibody (D) as described in Section 2. The figures show representative stainings from three independent experiments (magnification before reduction to column width: ×800).

protein is hardly detectable in the adult cardiomyocytes [23]. As shown in Fig. 1A,B, an anti-PKCζ antibody (V) against amino acids 577–592 of rat PKCζ recognizes a protein with an approximate molecular mass of 75 kDa by immunoblotting, a finding consistent with a previous report in bovine kidney [31]. The staining was prevented by preadsorption of the anti-PKCζ antibody (V) with immunizing peptide (Fig. 1A). The 82 kDa band appears to cross-react with PKCα, as previously described [32], since the band corresponding to the molecular weight of PKCα is strongly present in the nuclear-myofibrillar fraction after PMA treatment (data not shown), and the epitope against anti-PKCζ antibody (V) has moderate homology (31%) in the carboxy-terminal region of PKCα. PKCβ1 and β2, which have the same homology (31%) for the epitope, were not detectable in the rat heart by immunoblotting using anti-PKCβ1 and β2 antibody (data not shown). To confirm the expression of PKCζ, we carried out immunoblotting using an anti-PKCζ antibody (CV) against amino acids 394–590 of human PKCζ, distinct from the epitope against anti-PKCζ antibody (V). Fig. 1C shows that the immunoblot with the anti-PKCζ antibody (CV) was similar to that with the anti-PKCζ antibody (V) (Fig. 1B). These observations show that PKCζ is expressed in the heart of adult rats weighing up to 200 g. The density of visualized protein by ECL was proportional to the amount of the protein in each fraction up to 100 μg under the assay conditions (relative coefficient: >0.990). Electron microscopic observation showed that the $1000\times g$ pellet (P1: nuclear-myofibrillar fraction) contained the nuclei and myofibrils, and that the $100\,000\times g$ pellet (P2: membrane fraction) included membrane vesicles and mitochondria (data not shown). Histone H1, a nuclear protein, was mainly localized in the nuclear-myofibrillar fraction (82.3 ± 3.1%, mean ± S.E., $n=8$), and the recovery remained almost constant during ischemia (Fig. 1D). MEK-1 used as a marker of cytosol protein was present in the cytosol fraction (S fraction), and could not be detected in the P1 fraction and the P2 fraction (Fig. 1E). In untreated hearts, PKCζ predominates in the S fraction (60.2 ± 4.9%, mean ± S.E., $n=3$); the P1 fraction contains only 21.6 ± 5.1% ($n=3$, Fig. 1B,C,F). The amount of PKCζ in the P1 fraction increased significantly after 40 min of ischemia, with a concomitant decrease in the amount of PKCζ in the S fraction. PKCζ in the membrane fraction (P2 fraction) remained at control levels during ischemia for 40 min. PKCζ in the P2 fraction might not reflect cytosol contamination but may be associated with the membrane, since a cytosol protein, MEK-1, could not be detected in the P2 fraction (Fig. 1E) and PKCζ was released to the supernatant by Triton X-100 treatment of P2 fraction (data not shown). The immunohistochemical observation by anti-PKCζ antibody (CV) shows that PKCζ is localized in the cytosol and nucleus, with the nuclear staining enhanced after 40 min of ischemia (Fig. 2A,B), consistent with those by PKCζ (V). The staining was similar to that by anti-histone H1 antibody (Fig. 2D). The staining of PKCζ (V) was specifically blocked by preincubation with the immunizing peptide, and by only secondary antibody (data not shown). These results were similar to those in three independent experiments. The findings show that PKCζ translocates from the cytosol to the nucleus in ischemic heart. Since the translocation of PKC is a well-established parameter for its activation, the findings also suggest that PKCζ might be activated by activator(s) generated during ischemia.

3.2. *Nuclear translocation of PKCζ during ischemia is blocked by a PI3-kinase inhibitor, wortmannin*

It was recently reported that PIP3, a PI3-kinase product, activates PKCζ in vitro [33]. A fungal metabolite, wortmannin, inhibits PI3-kinase and contributes to determining the

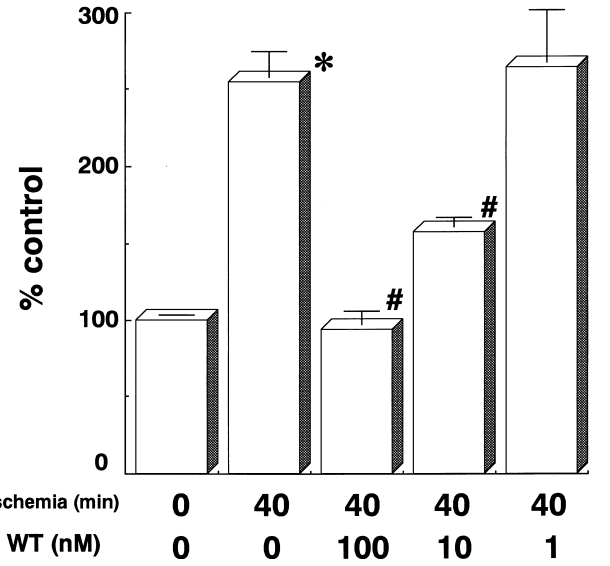

Fig. 3. Effect of PI-3 kinase inhibitor on PKCζ translocation during ischemia. The P1 fractions were prepared from hearts subjected to ischemia for 40 min in the presence ($n=3$) of wortmannin at the indicated concentration, and subjected to immunoblotting using anti-PKCζ antibody. The amounts of PKCζ were determined from the immunoblots by densitometric analysis (mean ± S.E.). (*$P<0.05$ vs. control; $P<0.05$ vs. ischemia 40 min, mean ± S.E.).

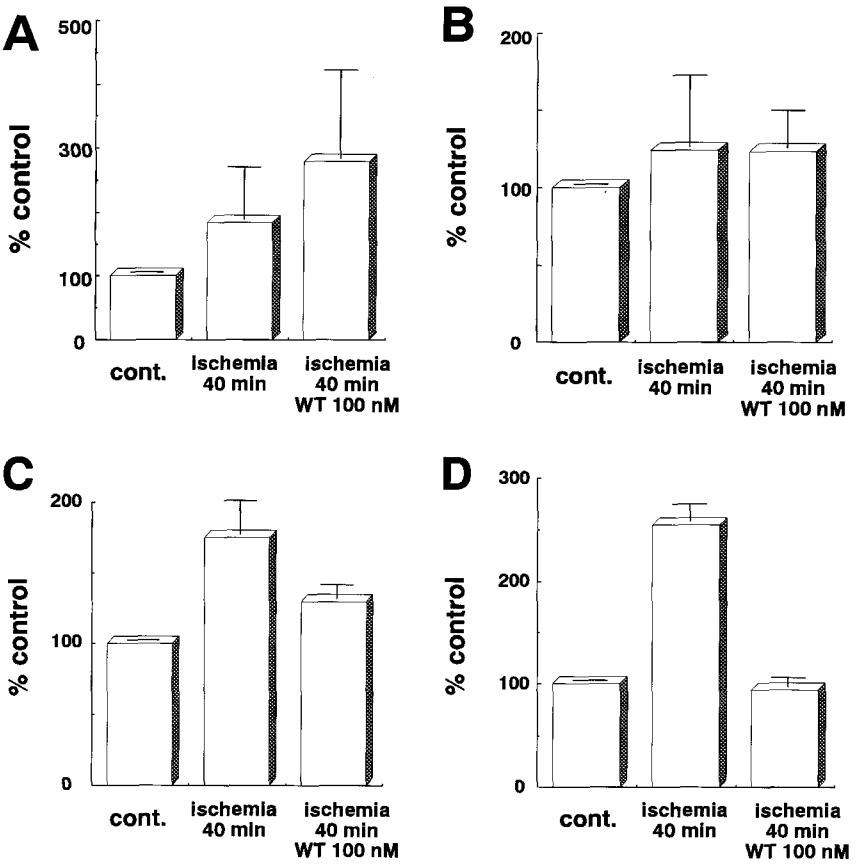

Fig. 4. Effect of PI-3 kinase inhibitor on the translocation of PKC isoforms during ischemia. The P1 fractions were prepared from hearts subjected to ischemia for 40 min in the presence ($n=2$) or absence ($n=2$) of wortmannin (100 nM), and subjected to immunoblotting with anti-PKCα (A), δ (B), ε (C), and ζ (D) antibodies. The amounts of PKC isoforms were determined from the immunoblots by densitometric analysis (mean ± S.E.).

precise role of PI3-kinase in whole cells (e.g. O_2^- generation and glucose transport) [34]. We found that wortmannin inhibits the nuclear translocation of PKCζ during ischemia in a dose-dependent manner (10^{-9}–10^{-7} M) (Fig. 3). The finding suggests that PKCζ may be activated by a PI3-kinase product, PIP3, during ischemia. PS also stimulates PKCζ several-fold in vitro, but it is unlikely that PS activates PKCζ in vivo since phosphatidylethanolamine, which is present in excess of PS in the membrane, is known to block the stimulation of PKCζ by PS at an equal molecular ratio in vitro [33].

3.3. *Effect of wortmannin on the translocation of PKC isoforms during ischemia*

PIP3 activates not only PKCζ but also PKCδ and PKCε in vitro, whereas the activity of PKCα is unaffected [35]. Therefore, we examined the effect of wortmannin on the translocation of PKCα, δ, and ε isoforms after 40 min of ischemia (Fig. 4). Ischemia induces the redistribution of PKCα and PKCε from the S fraction to the P1 fraction. Wortmannin (100 nM) partially inhibits the translocation of PKCε, but actually enhances the translocation of PKCα. This observation can be explained as follows: since wortmannin blocks the metabolism of PIP2 to PIP3 by PI3-kinase during ischemia, the accumulated PIP2 may be rapidly converted to DG, a potent activator of PKCα, by catalysis of phospholipase C. Recently, Cross et al. have reported that wortmannin inhibited phospholipase A_2 activity in vivo but not in vitro [36]. It is also possible that wortmannin may inhibit the release of arachidonic acid, another activator of PKCζ during ischemia [31]. To further clarify the involvement of PIP3 in the PKCζ activation, it might be necessary to determine the PIP3 level in vivo.

3.4. *Physiological implications*

In cardiomyocytes that have lost mitogenic activity, mitogenic stimuli such as growth factors and mechanical stretch are known to lead to hypertrophy [37,38]. The PKCζ isoform has recently been shown to be critical for mitogenic signal transduction [39]. Therefore, PKCζ activation during ischemia may be involved in the hypertrophy that is often seen in patients with ischemic heart disease [40]. On the other hand, tumor necrosis factor activates sphingomyelinase, generating ceramide [41]. Ceramide induces apoptosis and DNA fragmentation [42], and activates PKCζ in vitro [26]. Moreover, PKCζ is involved in the nuclear transport of nuclear transcription factor κB, which is activated by the sphingomyelinase pathway [27]. Since ischemia-reperfusion has been shown to induce apoptotic cell death [43], PKCζ might be involved in the signaling pathway of apoptosis in the ischemic heart. Recently, we also reported that PKCα, δ, and ε isoforms differentially localized during ischemia in the heart [25]. It will be of interest to clarify the biological role of PKC isoforms during ischemia.

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