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Identification of specific nuclear protein kinase C isozymes and accelerated protein kinase C-dependent nuclear protein phosphorylation during myocardial ischemia

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Abstract Protein kinase C (PKC) has been suggested to mediate, at least in part, multiple processes in the pathophysiological sequelae of myocardial ischemia. The present study demonstrates that the ε, η and ι isozymes of PKC are translocated to nuclei in response to brief intervals of global ischemia as well as reperfusion of ischemic rat myocardium. Concomitant with the translocation of PKC isozymes to nuclei during ischemia, increased PKC-mediated nuclear protein phosphorylation was observed. Taken together, the present results demonstrate that nuclear signaling mechanisms are activated during myocardial ischemia that include PKC translocation and PKC-mediated nuclear protein phosphorylation.

Key words: Protein kinase C; Myocardial ischemia; Protein phosphorylation

1. Introduction

Protein kinase C (PKC) is a ubiquitous enzyme that is involved in signal transduction pathways resulting in cell proliferation and differentiation [1–4]. In the heart, PKC is believed to play a role in ischemic preconditioning by a mechanism involving modulation of K_{ATP} channel activity [5–8]. In addition to the putative role of PKC in myocardial ischemic preconditioning, PKC may play an important role in the pathophysiological sequelae of myocardial ischemia including arrhythmogenesis and long-term myocardial dysfunction following recovery from ischemia. This latter effect mediated by PKC likely is coupled to the MAP kinase cascade and the activation of proto-oncogenes [9–11].

Several PKC isozymes, including the α, ε and η isozymes, have been shown to translocate to crude particulate fractions during myocardial ischemia [12,13]. However, the identification of the subcellular membrane pools that are enriched with specific PKC isozymes during myocardial ischemia is only partially known [14]. Additionally, although activated PKC has been detected in crude particulate fractions prepared from ischemic myocardium [15,16], the activation of PKC in specific subcellular membrane pools has not been determined in ischemic myocardium. Taken together, the subcellular membrane target(s) of activated PKC during myocardial ischemia is not clear.

Since it is likely that activated nuclear PKC isozymes have profound effects on post-ischemic myocardial function, the present studies were designed to determine the PKC isozymes that are translocated to nuclei during myocardial ischemia. The results herein demonstrate that during global myocardial ischemia the ε, η and ι isozymes of PKC translocate to the nucleus. Concomitant with increases in PKC isozymes translocated to the nuclei, nuclear protein phosphorylation increased during myocardial ischemia, which collectively demonstrate accelerated nuclear signaling during myocardial ischemia.

2. Materials and methods

2.1. Preparation of Langendorff-perfused rat hearts and induction of myocardial ischemia and reperfusion

Male Sprague-Dawley rats (200–250 g body weight) were injected with heparin (200 U i.p.) 30 min before being anesthetized with pentobarbital (25 mg i.p.) and the hearts were subsequently removed and placed in ice-cold saline prior to being perfused. Rat hearts were perfused retrogradely via the aorta (Langendorff-perfused) with modified Krebs-Henseleit buffer consisting of 137 mM NaCl, 4.7 mM KCl, 3 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 0.5 mM NaEDTA, 15 mM $NaHCO_3$, and 11 mM glucose equilibrated with O_2/CO_2 (95/5) (pH = 7.4) at 37°C for 15 min at a constant aortic perfusion pressure of 60 mm Hg. In selected experiments, hearts were pulse-chase radiolabeled with $^{32}P_i$ prior to experimental conditions. In brief, hearts were perfused for 45 min in a recirculating buffer mode with modified Krebs-Henseleit buffer that contained only 120 μM KH_2PO_4 as well as 625 μCi (9 Ci/μmol) of $^{32}P_i$. Following $^{32}P_i$ labeling, hearts were perfused with modified Krebs-Henseleit buffer containing 1.2 mM KH_2PO_4 for 15 min in a non-recirculating mode. In indicated experiments, 50 nM bisindolylmaleimide I (BIM I) was included in the perfusion buffer during the last 5 min of the 15 min chase interval. Following these perfusion protocols, Langendorff-perfused hearts were either control-perfused at 60 mm Hg (control), rendered globally ischemic for indicated intervals, or rendered globally ischemic for indicated intervals followed by reperfusion for indicated intervals as previously described [17]. At the end of each perfusion interval, ventricles were either rapidly freeze-clamped or directly homogenized for the preparation of cytosol and particulate or nuclei, respectively (see below).

2.2. Preparation of cytosolic, particulate and nuclear fractions from isolated perfused hearts

For the preparation of cytosolic and particulate fractions, freeze-clamped ventricular tissue was pulverized to a fine powder at the temperature of liquid nitrogen. Myocardial tissue (~0.8 $g_{wetweight}$) was then homogenized at 4°C in 20 ml of homogenization buffer A (20 mM Tris-HCl, 0.33 M sucrose, 5 mM EDTA, 0.5 mM EGTA, 1 mM PMSF and 0.005% leupeptin; pH = 7.4) utilizing a polytron (50% setting for 20 s) followed by Potter-Elvehjem homogenization with 5 strokes at a setting of 50%. Homogenates were then centrifuged at $24\,000 \times g_{max}$ for 20 min to collect cytosolic (supernatant) and particulate (pellet) fractions.

Rat heart nuclei were prepared as previously described with all steps maintained at 4°C [18]. In brief, ventricles from perfused hearts were immediately minced in 10 volumes of homogenization buffer B (10 mM Tris-HCl, 250 mM sucrose, 3 mM $MgCl_2$ and 0.1 mM

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Abbreviations: PKC, protein kinase C; PMSF, phenylmethylsulfonyl fluoride; BIM I, bisindolylmaleimide I; TnI, troponin I

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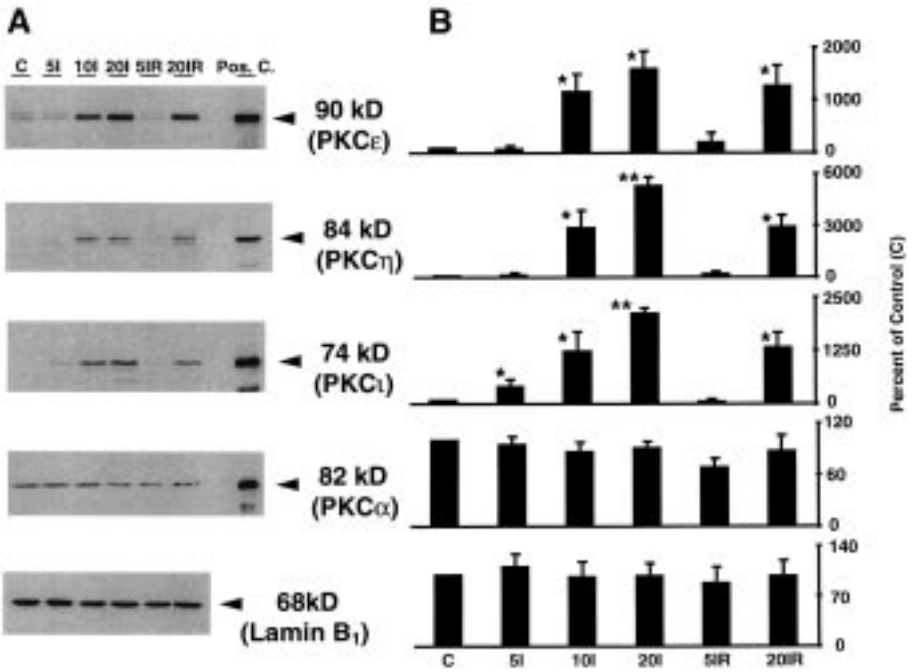

Fig. 1. The translocation of the ε, η and ι isozymes of PKC to nuclei during myocardial ischemia and reperfusion. Isolated adult rat hearts were Langendorff-perfused for 15 min with modified Krebs-Henseleit buffer and were subsequently subjected to either 20 min of perfusion (C, control), 5 min of global ischemia (5I), 10 min of global ischemia (10I), 20 min of global ischemia (20I), 5 min of global ischemia followed by 15 min of reperfusion (5IR) or 20 min of global ischemia followed by 15 min of reperfusion (20IR). Following each experimental interval, nuclei were prepared from ventricular tissue and nuclear proteins from each heart were then subjected to sequential SDS-PAGE and Western blot analysis as described in Section 2. Representative Western blots are shown in A. Blots (A) were probed with either anti-ε PKC (Sigma; rabbit, 4 μl/10 ml), anti-η PKC (Santa Cruz; rabbit, 5 μl/10 ml), anti-ι PKC (Transduction Laboratories; mouse, 20 μl/10 ml), anti-α PKC (Sigma; rabbit, 1 μl/10 ml), or anti-lamin B_1 (Zymed; rabbit, 5 μl/10 ml) as the primary antibodies. Positive controls (Pos. C.) on blots were the appropriate human recombinant PKC isozyme for ε, η, and α isozymes or rat brain lysate for the ι isozyme. The intensity of each band from each individual blot was quantitated utilizing NIH Image software and expressed as a percentage of the intensity of the control sample on each individual blot as described in Section 2 (B). Values for B are the means+S.E.M. from at least three individual independent experiments. $*P < 0.05$ and $**P < 0.01$, for comparisons between the indicated condition and the control condition.

PMSF; pH = 7.4) following experimental conditions and then homogenized utilizing a polytron (40% setting for 10 s). The homogenate was then centrifuged at $1000 \times g_{max}$ for 10 min and the pellet was subsequently resuspended in 10 volumes of homogenization buffer and re-homogenized with a Potter-Elvehjem homogenizer. The homogenate of the $1000 \times g_{max}$ pellet was filtered through nylon sieve mesh (100 μm) and the filtrate was then centrifuged at $1000 \times g_{max}$ for 10 min. The resultant pellet was resuspended in 10 volumes of homogenization buffer B supplemented with 0.1% Triton X-100. Again, the resuspended pellet was centrifuged at $1000 \times g_{max}$ for 10 min and subsequently the pellet was resuspended in 20 volumes of homogenization buffer B adjusted to 2.2 M sucrose. The resuspended pellet in sucrose was then underlaid with 5 ml of homogenization buffer B containing 2.2 M sucrose and then subjected to ultracentrifugation in a swinging bucket rotor (SW-28) at $113\,000 \times g_{max}$ for 1 h. The pellet (nuclei) was resuspended in homogenization buffer B without sucrose and immediately used for gel electrophoresis. The purity of the nuclear preparations was ascertained by immunoblotting with anti-lamin B_1 (Zymed) which demonstrated the enrichment of lamin B_1 in the nuclear preparations as compared to that of the cytosol and crude particulate.

2.3. Western blot analysis of PKC isozymes from subcellular fractions prepared from isolated perfused rat hearts

Cytosolic, nucleus-associated, and particulate-associated proteins prepared from isolated perfused rat hearts were quantitated by either a Bio-Rad protein assay or Lowry protein assay [19] and subsequently the samples were adjusted to equal protein concentrations prior to being subjected to SDS-PAGE in the presence of DTT utilizing 10% polyacrylamide gels (10 μg protein loaded per lane) with the exception that samples were subjected to SDS-PAGE in the absence of DTT for analysis of the SERCA2 ATPase and troponin I (TnI). Proteins were then quantitatively transferred to PVDF-plus membranes (Micron Separations Inc., Westborough, MA). The membranes were sequentially blocked for 1 h with 5% dry milk in Tris-buffered saline (pH = 7.6) at room temperature and then incubated for 1 h with primary antibodies at indicated concentrations in 5% dry milk in Tris-buffered saline (pH = 7.6) containing 0.05% Tween 20. It should be noted that each of these primary antibodies was determined to be isozyme-specific when screened for reactivity with recombinant human PKC isozymes. Next the membranes were washed with Tris-buffered saline containing 0.1% Tween 20 and then incubated 1 h with the appropriate horseradish peroxidase-conjugated secondary antibodies (Sigma goat anti-rabbit HRP, 1/7000 dilution or Bio-Rad goat anti-mouse HRP, 1/7000 dilution) in Tris-buffered saline containing 0.1% Tween 20 at room temperature at indicated concentrations. Immunoreactive bands were then visualized by chemiluminescence detected on X-ray film (Kodak X-OMAT AR) utilizing the ECL chemiluminescence system (Amersham Corp.). Multiple exposures of film to the blots were developed. Exposures that had linear levels of grain development were used for quantitation of band intensity utilizing NIH Image software following scanning and conversion of autoradiographic data to TIFF file formats using a Macintosh 5500/225 computer and a Linocolor-Hell Jade scanner. Quantitative analysis of the autoradiographic data was performed using the public domain NIH Image program (developed at the U.S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/). In all cases, the use of control peptides to these isozyme-specific PKC antibodies resulted in the loss of the PKC isozyme signal in Western blots.

2.4. Silver staining and autoradiography of SDS-PAGE gels

To ensure that both equal amounts of protein were loaded onto gels utilized for autoradiography and Western blot analysis and that subcellular fractions prepared from control and ischemic myocardium were similar in their individual protein profiles, parallel SDS-PAGE

gels were prepared and subsequently silver stained utilizing the Bio-Rad Silver Stain Plus kit. In experiments utilizing nuclei that were $^{32}P_i$ labeled, proteins were subjected to gel electrophoresis followed by autoradiography utilizing X-ray film (Kodak X-OMAT AR). Multiple exposures of film to the blots were developed.

2.5. Materials

Anti-α, -βI, -βII, -γ and -ε PKC were from Sigma. Anti-η and -ζ PKC were purchased from Santa Cruz. Anti-ι PKC was purchased from Transduction Laboratories. Anti-Na^+/K^+ ATPase β-1 and anti-SERCA2 ATPase were obtained from UBI and Affinity Bioreagents, respectively. Anti-TnI was purchased from Chemicon International. Secondary antibodies including goat anti-rabbit horseradish peroxidase and goat anti-mouse horseradish peroxidase were purchased from Sigma or Bio-Rad. Electrophoresis grade reagents for gel electrophoresis were purchased from ICN, Pharmacia and Bio-Rad. All other chemicals were purchased from either Sigma or Fisher. $^{32}P_i$ was purchased from NEN DuPont.

3. Results

3.1. Identification of the translocation of specific PKC isozymes to the nucleus during myocardial ischemia and reperfusion in the isolated perfused adult rat heart

Since it is likely that activated nuclear PKC isozymes have profound effects on post-ischemic myocardial function, experiments were performed to test the hypothesis that specific PKC isozymes are translocated to the nucleus during myocardial ischemia and ischemia-reperfusion protocols. Utilizing the isolated perfused rat heart model, 10 min of global ischemia resulted in significant increases in the levels of the ε, η and ι PKC isozymes associated with the nucleus which increased further with prolonged global ischemia (e.g. 20 min of global ischemia) and was maintained at an increased level in hearts subjected to 20 min of global ischemia followed by 15 min of reperfusion (Fig. 1). In addition, the ι isozyme of PKC was translocated to the nucleus within 5 min of global ischemia (5I in Fig. 1) while the ε and η isozymes of PKC were not significantly translocated to the nucleus following 5 min of ischemia. Also, the ε, η and ι isozymes of PKC were not present in the nucleus following 5 min of ischemia with subsequent reperfusion for 15 min (5IR) (Fig. 1). The presence of nuclei in the fractions used for these blots was confirmed by Western blotting utilizing anti-lamin B_1 (Fig. 1). It should also be noted that although the α PKC isozyme (Fig. 1) was also present in rat heart nuclei, the distribution of this isozyme was not altered following myocardial ischemia or ischemia followed by reperfusion. Additionally, the βI, βII, γ and ζ PKC isozymes were not detected in the nuclear preparations from the adult rat heart (data not shown). Concomitant with the increases in nucleus-associated PKC during 20 min of global ischemia, decreases in cytosolic ε and ι PKC isozymes were observed (Fig. 2). Additionally, the α, ε and ι PKC isozymes were translocated to the particulate fraction following 20 min of global ischemia (Fig. 2). It should be appreciated that in contrast to the ε, η and ι PKC isozymes, the α isozyme of PKC was translocated to crude particulate but was not translocated to the nucleus during global ischemia (Fig. 2). Furthermore, although the η PKC isozyme was translocated to the nuclei of ischemic hearts, this isozyme was not spatially translocated between the cytosol and particulate fractions in response to ischemia and was preferentially localized in the crude particulate fraction prepared from both control and ischemic hearts (Fig. 2).

3.2. Enzyme markers of nuclear and particulate fractions

Enzyme marker analysis was performed to demonstrate the purity of the nuclear preparation used to determine the translocation of PKC isozymes to the nucleus during myocardial

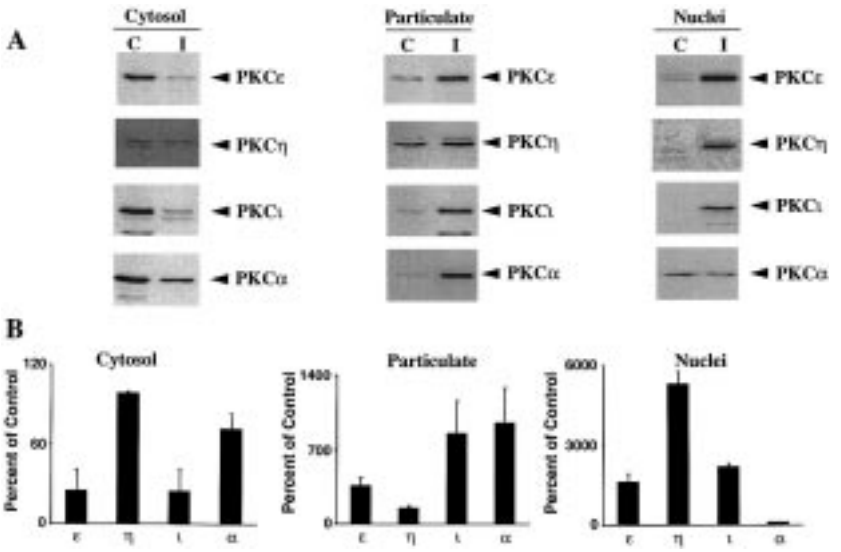

Fig. 2. Western blot analysis of PKC isozymes in cytosol, particulate and nuclei prepared from control and ischemic hearts. Isolated adult rat hearts were Langendorff-perfused for 15 min with modified Krebs-Henseleit buffer and were subsequently subjected to either 20 min of perfusion (C, control) or 20 min of global ischemia (20I) as described in Section 2. The indicated cytosolic and particulate fractions as well as nuclei were prepared and were sequentially subjected to SDS-PAGE and Western blot analysis as described in detail in MSection 2. In A, blots were probed with either anti-ε PKC (Sigma; rabbit, 4 μl/10 ml), anti-η PKC (Santa Cruz; rabbit, 5 μl/10 ml), anti-ι PKC (Transduction Laboratories; mouse, 20 μl/10 ml) or anti-α PKC (Sigma; rabbit, 1 μl/10 ml) as the primary antibodies as described in Section 2. The intensity of each band from each individual blot was quantitated utilizing NIH Image software and expressed as a percentage of the intensity of the control sample on each individual blot as described in Section 2 (B). Values for B are the means+S.E.M. from at least three individual independent experiments.

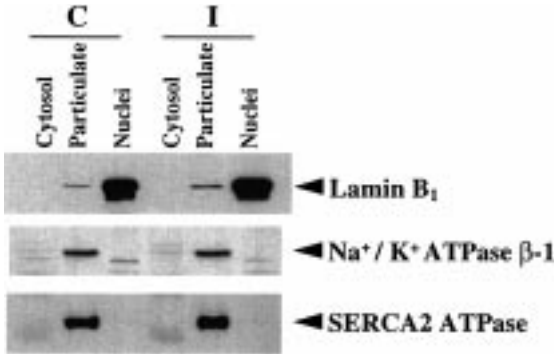

Fig. 3. Western blot analysis of subcellular markers in cytosol, particulate and nuclei prepared from control and ischemic hearts. Isolated adult rat hearts were Langendorff-perfused for 15 min with modified Krebs-Henseleit buffer and were subsequently subjected to either 20 min of perfusion (C, control) or 20 min of global ischemia (20I) as described in Section 2. The indicated cytosolic and particulate fractions as well as nuclei were prepared and were sequentially subjected to SDS-PAGE and Western blot analysis as described in detail in Section 2. Blots were probed with either anti-Na^+/K^+ ATPase β-1 (UBI; mouse, 9 μl/10 ml), anti-SERCA2 ATPase (Affinity Bioreagents; mouse, 5 μl/10 ml), anti-lamin B_1 (Zymed; mouse, 5 μl/10 ml) or anti-TnI (Chemicon International, mouse, 2.5 μl/10 ml) as the primary antibodies.

ischemia. Fig. 3 documents the purity of the nuclear preparation used in these studies since the cardiac sarcolemmal marker, Na^+/K^+ ATPase β-1 subunit, and the cardiac sarcoplasmic reticulum marker, SERCA2 ATPase, were only found in the crude particulate while the nuclear marker, lamin B_1, was highly enriched in the nuclear preparation. Furthermore, the nuclear preparation was not contaminated with the myofibrillar protein TnI (Fig. 3).

3.3. PKC-mediated protein phosphorylation during myocardial ischemia

To determine that the translocation of PKC isozymes to the nucleus during myocardial ischemia results in PKC-mediated phosphorylation of nuclear proteins, isolated perfused hearts were prelabeled with $^{32}P_i$ and then subjected to control perfusions and selected intervals of global ischemia with or without subsequent reperfusion. The autoradiogram of phosphorylated proteins from nuclei isolated from perfused hearts shown in Fig. 4A demonstrates that protein phosphorylation is increased following 20 min of global ischemia. In particular, a ~20 kDa protein was extensively phosphorylated in response to 20 min of global ischemia (20I) as well as 20 min of global ischemia followed by 15 min of reperfusion (20IR). Additionally, during reperfusion a ~28 kDa protein was extensively phosphorylated (Fig. 4A) which likely reflects different signaling mechanisms in the reperfused ischemic heart as compared to that in the ischemic heart (e.g. differences in the accumulation of specific PKC activators). Furthermore, the demonstration that nucleus-associated protein phosphorylation during 20 min of global ischemia was inhibited by treating hearts with the selective PKC inhibitor, BIM I (50 nM), prior to ischemia or control perfusions, suggests that the protein phosphorylation observed during ischemia is mediated by PKC (Fig. 4A). The addition of BIM I did not appreciably alter the translocation of the ε, η and ι PKC isozymes to the nucleus during global ischemia or ischemia followed by reperfusion (data not shown). In parallel experiments, nuclear preparations that were analyzed by autoradiography in Fig. 4A were also subjected to SDS-PAGE and subsequent silver staining to ensure that each nuclear preparation contained equal amounts of protein as well as similar protein composition (Fig. 4B). Taken together, these data support the hypothesis that PKC isozymes that are translocated to the nucleus during myocardial ischemia are active and mediate the phosphorylation of nuclear proteins.

4. Discussion

PKC has been suggested to mediate, at least in part, multiple processes in the pathophysiological sequelae of myocardial ischemia including preconditioning, arrhythmogenesis and long-term dysfunction [5–7,9–11,20]. Despite the potential importance of PKC in the myocardial response to ischemia, the

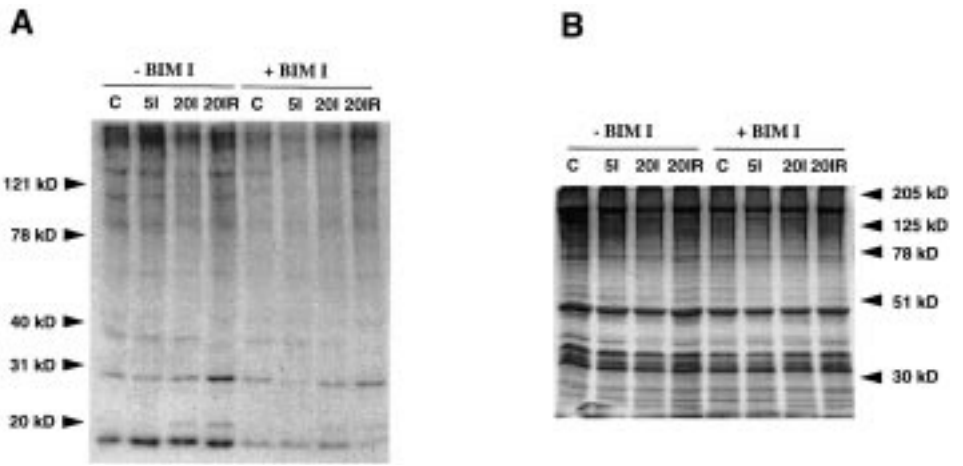

Fig. 4. PKC-mediated protein phosphorylation during myocardial ischemia. Isolated perfused hearts were pulse-chase radiolabeled with $^{32}P_i$ and subsequently subjected to either 20 min of perfusion (C, control), 5 min of global ischemia (5I), 20 min of global ischemia (20I) or 20 min of global ischemia followed by 15 min of reperfusion (20IR) in the presence (+BIM I) or absence (−BIM I) of the PKC inhibitor, BIM I (50 nM). Following each experimental interval, nuclei were prepared from ventricular tissue as described in Section 2 except that 50 mM NaF and 0.2 mM Na_3VO_4 were added to buffers. Purified nuclei were sequentially subjected to SDS-PAGE (10% gel) followed by either autoradiography (A) or silver staining (B) as described in Section 2.

subcellular localization of PKC isozymes during myocardial ischemia is not clear. In particular, the long-term responses of ischemic injury that could be a result of nuclear signaling involving PKC, have not been delineated. Accordingly, in the present studies the translocation and activation of PKC were determined at the nuclear subcellular level. The results herein demonstrate the selective translocation of the ε, η and ι PKC isozymes to the nucleus during global ischemia which is accompanied by accelerated PKC-mediated nuclear protein phosphorylation. Furthermore, reperfusion of ischemic hearts subjected to short intervals of ischemia (e.g. 5 min) resulted in the reversal of ischemia-induced PKC translocation to the nucleus. However, reperfusion did not reverse ischemia-induced PKC translocation to the nucleus in hearts subjected to prolonged ischemia (20 min) which results in irreversible myocardial damage (e.g. [21]).

Previous studies have demonstrated that the ε PKC isozyme is translocated to the nucleus in response to opiate stimulation [22]. The ε and η PKC isozymes have also been shown to translocate to crude particulate following global ischemia in the rabbit heart [13]. In contrast, the ι PKC isozyme has not been shown to translocate in the ischemic heart, although it has been detected in adult rabbit heart [13] and immature rat heart [23]. The present findings now demonstrate that the ι PKC isozyme is translocated to the nuclei during global ischemia in the adult rat heart. Since PKC isozymes do not possess a nuclear localization signal [24], it will be important to determine the biochemical mechanism responsible for the translocation of specific PKC isozymes to the nucleus during myocardial ischemia. It is likely that more than one mechanism is responsible for the translocation of these isozymes to the nucleus during ischemia since the ι PKC isozyme, unlike the ε and η PKC isozymes, does not possess a phorbol ester/diacylglycerol binding site [25].

The gross changes in the nuclear protein phosphorylation state during myocardial ischemia were striking and based on inhibition by the selective PKC inhibitor, BIM I, it appears that this protein phosphorylation is mediated by PKC. It should be noted that it is unlikely that the effects of this inhibitor are due to the inhibition of cAMP-dependent protein kinase since the concentration of BIM I employed in this study is 40-fold less than the K_i of cAMP-dependent protein kinase [26]. Importantly, the results herein demonstrate that the translocation of PKC to the nucleus during myocardial ischemia is accompanied by enhanced PKC-mediated phosphorylation of nuclear proteins. Accordingly, it will be important to identify the proteins which are phosphorylated by PKC during ischemia to thoroughly understand the role of PKC in the pathophysiological sequelae of myocardial ischemia.

Taken together, specific PKC isozymes are translocated to the nucleus during global myocardial ischemia resulting in the phosphorylation of nuclear proteins. The precise role of each of these PKC isozymes that are translocated during ischemia, as well as their protein substrates, remains to be resolved, but it is likely that one or more of these translocated isozymes and protein substrates are involved in the pathophysiological sequelae of myocardial ischemia. Furthermore, the delineation of the biochemical mechanism responsible for the translocation of each of these PKC isozymes to the nucleus during ischemia should provide new insights into the mechanism of PKC translocation and activation that could potentially lead to the development of novel mechanism-based inhibitors.

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