A Selective ε-Protein Kinase C Antagonist Inhibits Protection of Cardiac Myocytes from Hypoxia-induced Cell Death*

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Protein kinase C activation is thought to protect cardiac tissue from subsequent ischemic injury by a process termed preconditioning. The protein kinase C isozyme that mediates preconditioning has not yet been identified. Using a cell culture model of hypoxic preconditioning, we found that cardiac myocyte viability after 9 h of hypoxia was increased by more than 50% over control. Preconditioning activated protein kinase C isozymes as evidenced by translocation from one cell compartment to another as follows: there was a 2.1-fold increase in ε-protein kinase C activation, a 2.8-fold increase in δ-protein kinase C activation, and no increase in β-protein kinase C activation. 4β-Phorbol 12-myristate 13-acetate mimicked hypoxic preconditioning, increasing myocyte survival after prolonged hypoxia by 34% compared with control. We previously identified an ε-protein kinase C-selective antagonist, εV1-2 peptide, that inhibits ε-protein kinase C translocation and function in cardiac myocytes (Johnson, J. A., Gray, M. O., Chen, C.-H., and Mochly-Rosen, D. (1996) J. Biol. Chem. 271, 24962–24966). εV1-2 peptide abolished hypoxic preconditioning and phorbol ester-mediated cardiac protection. Therefore, preconditioning can be induced in this culture model, and activation of ε-protein kinase C is critical for cardiac myocyte protection.

Identification of novel therapeutic targets for the prevention of ischemia-induced cardiac injury has been an area of intense investigation for the past 10 years. First described in a canine heart model (1), preconditioning of cardiac tissue with one or more brief episodes of ischemia remains one of the most potent experimental means of reducing irreversible tissue injury during subsequent prolonged ischemia. However, the cellular and molecular mechanisms underlying this protective phenomenon remain obscure. Numerous molecules such as adenosine, α1-agonists, angiotensin II, bradykinin, and opioids have been invoked as potential mediators of ischemic preconditioning. Activation of PKC correlates closely with the cardiac protection mediated by ischemic preconditioning in rat whole heart models. For example, PKC stimulation with dioctanoylglycerol protected adult rat heart during 45 min of regional ischemia, whereas PKC inhibition with chelerythrine abolished protection mediated by ischemic preconditioning (16). In addition, activation of PKC by transient ischemia or α1-agonists in an isolated rat heart model induced protection against global ischemia/reperfusion injury that was inhibited by PKC antagonists (17).

Studies of ischemic preconditioning in whole heart models have been limited by technical issues such as the relatively low specificity of available antagonists for PKC versus other kinases, the inability of agonists and antagonists to discriminate among multiple PKC isozymes, and the difficulty of examining signal transduction mechanisms at the level of the individual cell. Cardiac myocyte culture models may provide complementary approaches to the investigation of signal transduction in preconditioning. In one such culture model, a 25-min exposure to hypoxia followed by reoxygenation protected cardiac myocytes against membrane damage for up to 6 h of severe hypoxia, and pretreatment of cells with phorbol ester mimicked the protective effects of hypoxic preconditioning (18).

In this study, we identified two hypoxic preconditioning protocols that substantially improved the viability of cultured neonatal rat cardiac myocytes following several hours of profound hypoxia. We found that both protocols induced a selective activation of PKC isozymes as evidenced by translocation and that direct activation of PKC with a phorbol ester mimicked the protective effect of hypoxic preconditioning. Moreover, we used a novel 8-amino acid antagonist of εPKC, εV1-2 peptide, to identify the isozyme that mediates this protective effect. We previously proposed the use in general of such isozyme-selective peptide inhibitors of PKC translocation and function (19). εV1-2 peptide is derived from the first unique region (V1) of εPKC (amino acids 14–21), and its action as a selective antagonist of εPKC translocation and function in cardiac myocytes has been characterized in detail (20). Introduction of this peptide into myocytes selectively inhibited PMA-induced translocation of εPKC but not the translocation of other PKC isozymes (20). Furthermore, there was a selective loss of regulation of signal transduction pathways underlying protein kinase C activation involving protein kinase C (PKC). At least six different PKC isozymes have been identified in rat cardiac myocytes (7, 8), where PKC regulates a number of functions such as force of contraction (9), atrial natriuretic factor secretion (10), and gene expression (11). Individual isozymes translocate to characteristic intracellular sites following activation (12, 13) with each isozyme playing a different role in myocyte function (14, 15). Activation of PKC correlates closely with the cardiac protection mediated by ischemic preconditioning in rat whole heart models. For example, PKC stimulation with dioctanoylglycerol protected adult rat heart during 45 min of regional ischemia, whereas PKC inhibition with chelerythrine abolished protection mediated by ischemic preconditioning (16). In addition, activation of PKC by transient ischemia or α1-agonists in an isolated rat heart model induced protection against global ischemia/reperfusion injury that was inhibited by PKC antagonists (17).

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1 The abbreviations used are: PKC, protein kinase C; LDH, lactate dehydrogenase; PMA, 4β-phorbol 12-myristate 13-acetate.
contraction rate in the presence of eV1-2 that was not seen with βC2-4, a translocation inhibitor selective for the ePKC isoforms (20, 21). Using eV1-2 in a different cell culture model, we also showed that ePKC mediates, at least in part, glucose-induced insulin secretion in pancreatic cells (22). In the present study, we introduced eV1-2 peptide into cardiac myocytes and showed for the first time that isozyme-selective inhibition of ePKC translocation and function inhibited the protective effect of preconditioning. These data indicate a major role for the ePKC isoform in cardiac myocyte protection by hypoxic preconditioning.

EXPERIMENTAL PROCEDURES

Ventricular Myocyte Preparation—Primary cultures were prepared as described previously (23). After removal of non-myocytes in a pre-plating step, isolated myocytes (90–95% of the cells) were seeded at 800 cells per mm² into 8-well glass chamber slides (Nunc) for cell viability assays and immunofluorescence staining, into 6-well plastic culture plates (Fisher) for lactate dehydrogenase (LDH) assays, or into 100-mm glass culture dishes for Western blot analysis. On culture day 4, myocytes were placed in defined medium (23) then fed with fresh defined medium on the evening prior to treatment (days 6–8).

Additional Materials—PKC V1-2 in a different cell culture model, we also showed that hypoxic preconditioning does not cause irreversible cellular damage, an assumption that we verified using a cell viability assay (see below). In all cases, myocytes were subjected to a prolonged hypoxic challenge by transfer into the plexiglass chamber followed by incubation in glucose-supplemented medium tolerated up to 2 h of hypoxia without reduction of viability (24). The number of cycles was based on the study of preconditioning in canine myocardium in which four brief coronary artery occlusions protected cardiac tissue from prolonged ischemia (1). The protective effect of our protocol was determined using an assay that discriminated between living and dead myocytes upon completion of a prolonged hypoxic challenge. This method has been validated with a variety of adherent cell types (27) as well as with primary cultures of neonatal rat cardiac myocytes (28).

The proportion of viable (green fluorescent) cardiac myocytes under normoxic conditions was consistently greater than 95% (Fig. 1A). Following transfer, non-viable (red fluorescent) cardiac myocytes exceeded 50% after 7–9 h of exposure to the hypoxic environment (Fig. 1A). In two independent experiments, 20 random microscopic fields per condition (600 cells each) were scored just after the final preconditioning cycle. The proportion of viable cells in the preconditioned group was different from that of control cells (96.5 ± 0.7 versus 97.2 ± 0.8%). Therefore, hypoxic preconditioning had no immediate effect on myocyte survival.

In contrast, hypoxic preconditioning had a profound effect on expression of the inducible 72-kDa member of the heat shock protein 70-kDa family (Hsp70). In control myocytes Hsp70 was barely detectable by Western blot analysis, whereas precondi-
cytes can then be scored as basal or activated, and the
locates from the cytosol into the nucleus (8). Individual myo-
cytes maintained under normoxic conditions.

PKC translocates from the nucleus to perinuclear structures.

Hypoxic preconditioning increases Hsp70 protein levels and reduces cell death from subsequent prolonged hypoxia. A, cardiac myocytes underwent four 90-min periods of hypoxia alternating with 60-min normoxic incubations. Western blot analysis of control (C) and preconditioned (PC) cell lysates with anti-Hsp70 antibodies revealed an increase in Hsp70 expression induced by hypoxic preconditioning. Result is representative of two independent experiments. B, myocytes underwent 4 cycles of hypoxic preconditioning (PC). Control (C) and preconditioned myocytes were subjected to 9 h of hypoxia followed by determination of cell viability. 20 random fields per condition in each of four independent experiments were scored. Preconditioning increased the proportion of surviving myocytes by 52% compared with control. The number in each bar represents the total number of cells scored per condition. *p < 0.05 versus control.

Proportions of each were compared with those of cell populations stimulated with known activators of PKC such as norepinephrine. This immunofluorescence technique is extremely sensitive in demonstrating translocation because it does not disrupt cell structure and because dissociation from the translocation site does not occur in cells fixed immediately after treatment. We found that hypoxic preconditioning produced a 2.1-fold increase in ePKC activation (49.9 ± 2.8 versus 23.6 ± 2.6% for control) and a 2.8-fold increase in δPKC activation (58.4 ± 2.9 versus 20.7 ± 2.8% for control), an effect comparable to that induced by stimulation with 2 μM norepinephrine under normoxic conditions. No activation of βδPKC by hypoxic preconditioning was observed (Fig. 3). Therefore, hypoxic preconditioning selectively activated δ- and ePKC isozymes in cultured cardiac myocytes. A recent study using a similar myocyte culture model reported redistribution of ePKC in response to hypoxia using Western blot analysis (31), thereby providing independent corroboration of our observations.

We also determined whether direct activation of PKC with phorbol ester could protect cardiac myocytes from hypoxic damage. We stimulated myocytes with 10 nM PMA for 10 min since this concentration and incubation period induced a robust, selective activation of δ- and ePKC by Western blot analysis (Fig. 4). As we previously reported, there was no activation of ePKC and minimal activation of δPKC using <10 nM PMA (14). In contrast, where activation of these isozymes has been observed, the concentration of PMA used was 100 nM to 1 μM (7, 32, 33). This pattern of PKC activation by 10 nM PMA was comparable to that observed with hypoxic preconditioning as determined by immunofluorescence staining (Fig. 3). Furthermore, PMA-mediated preconditioning protected cardiac myocytes, increasing the proportion of surviving myocytes by 34% compared with control (59.3 ± 1.9 versus 44.1 ± 2.1%) following 9 h of hypoxia (Fig. 5A). PMA-mediated preconditioning was
FIG. 3. Hypoxic preconditioning results in selective activation of δ- and ePKC isozymes. A, myocytes underwent 4 cycles of hypoxic preconditioning or were stimulated with 2 μM norepinephrine for 2 min under normoxic conditions and then were fixed and stained with antibodies to ePKC (panels 1–3), δPKC (panels 4–6), or ββPKC. B, myocytes displaying activated ePKC (cross-striated staining), δPKC (perinuclear staining), or ββPKC (intracellular staining) in 15 random fields per condition per PKC isozyme in two independent experiments were scored. Preconditioning (PC) produced a 2.1-fold increase in ePKC activation over control (C) and a 2.8-fold increase in δPKC activation. No activation of ββPKC by hypoxic preconditioning was observed. For comparison, activation of these isozymes by 2 μM norepinephrine (NE) was also determined. The number in each bar represents the total number of cells scored per condition. *p < 0.05 versus control. **p < 0.05 versus control. C, specificity of ePKC immunostaining was confirmed by incubating primary antibody with either recombinant ePKC (10 ng) or recombinant δPKC (10 ng) expressed in Sf9 cells and spotted on nitrocellulose. Specific staining was almost abolished in control (panel 1) and norepinephrine-stimulated (panel 2) myocytes following preincubation with recombinant ePKC. Staining was unchanged in stimulated myocytes following preincubation with recombinant δPKC (panel 3).

An 8-amino acid peptide derived from eV1, termed eV1-2 (EAVSLKPT, ePKC(14–21)), displayed almost identical properties as an antagonist of ePKC translocation and function (20). Since ePKC is activated by preconditioning, we used this isoform-selective peptide antagonist to examine the role of ePKC in the signal transduction pathway underlying protection against hypoxic injury.

We used a transient, saponin-based permeabilization method developed in our laboratory to introduce eV1-2 and control peptides into cells. This technique has been characterized extensively in cultured neonatal rat cardiac myocytes and does not compromise cell viability, spontaneous or stimulated contraction rates, basal or hormone-induced expression of c-fos mRNA, or growth factor-induced hypertrophy (26). We found that the protective effect of PMA-mediated preconditioning was completely abolished by eV1-2 and unaffected by control peptides such as eV1-3 (Fig. 5A). (Note that eV1-3 is a peptide derived from the ePKC V1 region that does not act as an ePKC translocation inhibitor (20).) Therefore, ePKC mediates, at least in part, PMA-induced preconditioning in cultured cardiac myocytes.

Because activation of ePKC appeared to be critical to the protective effect of PMA-mediated preconditioning, we optimized our hypoxic preconditioning protocol by using ePKC translocation as a marker of cardiac protection. PKC translocation has traditionally been monitored by Western blot analysis, a method that may be less sensitive than immunofluorescent detection.
**FIG. 4. Selective activation of PKC isozymes by a short exposure to 10 nM PMA.** Myocytes were incubated with 10 nM PMA for 10 min and then harvested immediately (lane 2) or washed with fresh medium and harvested 12 h later (lane 3). Western blot analysis of control (lane 1) and PMA-treated cellular fractions revealed no translocation of αPKC and minimal translocation of βPKC from the cell soluble to the particulate fraction. Translocation of δPKC and εPKC in response to PMA was robust. Twelve hours after PMA stimulation (lane 3), the amount and distribution of PKC isozymes in the two cell fractions was indistinguishable from control cells (lane 1). Result is representative of two independent experiments.

**DISCUSSION**

In this study we describe a neonatal rat cardiac myocyte culture model in which two distinct hypoxic preconditioning protocols protected myocytes from death during a subsequent prolonged period of hypoxia. Two lines of evidence establish a central role for PKC in cardiac protection. First, hypoxic preconditioning selectively activated PKC isozymes. Second, direct activation of PKC with phorbol ester mimicked both the pattern of PKC isozyme activation and the protective effect of hypoxic preconditioning. These *in vitro* observations complement work described earlier with whole heart models in which PKC agonists mimicked the protective effect of ischemic preconditioning, and PKC antagonists inhibited myocardial protection (16, 17). Our findings also support the hypothesis that components of the hypoxic preconditioning signal transduction pathway necessary for cardiac protection are present in ventricular myocyte culture models.

The principle finding of this study is that the role of a particular PKC isozyme in the preconditioning response can be probed with isozyme-selective PKC antagonists. Activation of PKC is associated with its translocation from the cell soluble to the particulate fraction (34) and with its binding to specific anchoring proteins within the particulate fraction termed RACKs (19, 35, 37). Activation of a particular PKC isozyme can be assayed by determining its relative distribution between cell fractions by Western blot analysis or by immunofluorescence staining. However, assignment of a biological function to a particular PKC isozyme on the basis of these techniques is limited because it is based on correlation. We have previously shown that transient permeabilization of the εV1 fragment or εV1-2 peptide into cardiac myocytes resulted in inhibition of PMA-induced εPKC translocation without concurrent alteration of α-, β-, or δPKC translocation (20). Furthermore, PMA-mediated negative chronotropy, known from earlier work to correlate closely with εPKC translocation (14), was inhibited in cardiac myocytes permeabilized with the εV1 fragment or εV1-2 peptide but not with control peptide (20). In the present study εV1-2 peptide inhibited both preconditioning induced translocation of εPKC and protection of cardiac myocytes from hypoxic injury. In contrast, a selective translocation inhibitor of the C2-containing PKC isozymes did not affect hypoxic preconditioning, supporting a critical role for εPKC in this biological function (Fig. 5B).

Transient permeabilization rather than transfection was chosen as the method of introduction of PKC inhibitor into cardiac myocytes because of well-documented transfection efficiencies of less than 5% in this culture model (38, 39). For determination of changes in cell viability it is essential that the majority of myocytes contain the inhibitor. However, in a separate study we showed that stable expression of the εV1 region in PC12 cells caused a similar selective inhibition of PMA-induced εPKC translocation and function (36).

As evidenced by Western blot analysis, expression of εV1 fragment resulted in approximately 60% inhibition of εPKC translocation following exposure to 30 nM PMA and no change in δPKC translocation. As a result of this isozyme-selective inhibition of PKC translocation, responses to nerve growth factors were altered (36). These data suggest that regardless of the method of introduction, these PKC antagonists selectively inhibit the translocation and function of their corresponding isozymes.
Both δ- and ePKC translocate in cardiac myocytes upon stimulation with PMA or following hypoxic preconditioning (Figs. 3–5). However, because introduction of eV1-2 abolished the protective effect of preconditioning during subsequent prolonged hypoxia (Fig. 5), we conclude that activation of ePKC is necessary for PMA-induced and hypoxic preconditioning and predict that activation of δPKC is unlikely to mediate this acute form of myocyte protection. Instead, activation of δPKC may be an upstream event in the signal transduction pathway underlying the delayed form of myocyte tolerance to profound hypoxia known to occur 24 h after preconditioning (40). Alternatively, activation of δPKC may actually contribute to hypoxic injury. Studies in whole heart preparations suggest that PKC inhibition may at times reduce damage to cardiac tissue following severe ischemia (41). This controversy over the role of PKC in preconditioning may reflect opposing effects of δ- and ePKC.

**Fig. 5.** Activation of ePKC is required for PMA- and hypoxic preconditioning-induced protection of cardiac myocytes from subsequent prolonged hypoxia. A, myocytes were permeabilized to introduce a peptide inhibitor of ePKC (eV1-2), control peptide (eV1-3), or no peptide (–). Cells were then stimulated with 10 nM for 10 min (Phorbol Ester) or vehicle (Control), washed, and subjected to 9 h of hypoxia. 10 random fields per condition in each of four independent experiments were scored for cell viability. Preconditioning increased the proportion of surviving myocytes by 34% versus control. Protection was abolished by the ePKC-selective antagonist eV1-2. The number in each bar represents the total number of cells scored per condition. †, p < 0.05 versus control cells. ††, p < 0.05 versus PMA-treated cells permeabilized in the absence of peptide (–) or in the presence of ePKC-derived control peptide (eV1-3). B, myocytes were permeabilized to introduce eV1-2, control (βC2-4), or no peptide (–) and then exposed to 30 min of hypoxia in the absence of glucose (Preconditioned). After 30 min of recovery under normoxic conditions, preconditioned and control cells were subjected to 9 h of hypoxia. 20 random fields in each of two independent experiments were scored for cell viability. Preconditioning increased the proportion of surviving cells by 86% versus control. Protection was abolished by eV1-2. The number in each bar represents the total number of cells scored per condition. ††, p < 0.05 versus control cells. **, p < 0.05 versus preconditioned cells permeabilized in the absence of peptide (–) or in the presence of control peptide (βC2-4). C, left, myocytes were either maintained in glucose-supplemented medium under normoxic conditions (Nx) or incubated in glucose-free medium under hypoxic conditions (Hypoxia) for the times indicated. Western blot analysis revealed translocation of ePKC from the soluble to the particulate fraction following 30–120 min of hypoxia. Result is representative of two independent experiments. Densitometry revealed a 70% reduction in signal intensity at 120 min compared with normoxia in the soluble fraction and a 42% increase in the particulate fraction. C, right, myocytes were permeabilized in the presence of eV1-2 (1) or in the absence of peptide (–) and then either incubated under normoxic conditions (Nx) or exposed to 1 h of hypoxia (Hx) in glucose-free medium. Hypoxia induced translocation of ePKC from the soluble to the particulate fraction that was inhibited by the ePKC-selective peptide antagonist. Result is representative of two independent experiments. By densitometry, hypoxia caused reductions of 40 and 45%, respectively, in signal intensity in the second and third lanes of the soluble fraction compared with normoxia. The corresponding increase in the particulate fraction in the second lane was 47%, which was reduced by eV1-2 to less than half (21% increase).
ePKC activation is required for full protection from hypoxic cell death and the mechanisms underlying its down-regulation remain undefined. In addition, the stability of eV1-2 in cells is unknown. We have previously shown that the initial intracellular concentration of peptide is approximately one-tenth that present in the extracellular permeabilization buffer (26). The marked potency of a potentially unstable peptide might be explained by protection from proteolysis following binding to ePKC-specific RACKs. Alternatively, because ePKC translocation in response to preconditioning does not persist (Fig. 4), even the transient presence of eV1-2 peptide may have a profound effect on the downstream cellular events responsible for cardiac myocyte protection. Finally, although ePKC mediates negative chronotropic effects in cardiac myocytes (14, 20), it is not known whether slowing of contraction rate is necessary for protection from hypoxic injury. In a similar culture model, preconditioned myocytes ceased to contract earlier than paired control cells during a prolonged hypoxic challenge (18). However, the relationship between the earlier contractile failure of preconditioned myocytes and protection from hypoxic injury could not be determined from the available data.

In summary, we have shown that introduction of an ePKC isoyme-selective antagonist into cultured cardiac myocytes inhibits the protective effects of preconditioning during subsequent prolonged hypoxia. These observations suggest that investigation of PKC isoyme-selective agonists and antagonists, or drugs that mimic the effects of these peptides, may result in new approaches to the treatment of cardiac and vascular disease.

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