Preconditioning cultured human pediatric myocytes requires adenosine and protein kinase C

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Ikonomidis, John S., Toshizumi Shirai, Richard D. Weisel, Bogdan Derylo, Vivek Rao, Catherine I. Whiteside, Donald A. G. Mickle, and Ren-Ke Li. Preconditioning cultured human pediatric myocytes requires adenosine and protein kinase C. *Am. J. Physiol.* 272 (*Heart Circ. Physiol.* 41): H1220–H1230, 1997.—We showed previously that 20 min of low-volume anoxia ("ischemia") and 20 min of "reperfusion" preconditions quiescent pediatric myocyte cultures against damage resulting from 90 min of subsequent prolonged ischemia and 30 min of reperfusion. The purpose of this study was to assess the roles of adenosine and protein kinase C (PKC) in this preconditioning model. Our results suggest that *1*) preconditioned myocytes secrete a protective mediator(s) into the "ischemic" supernatant that is transferable to other cells, and adenosine is released into the supernatant in quantities sufficient for adenosine-receptor activation; (*2*) preconditioning is inhibited by adenosine-receptor antagonism, and myocyte protection similar to preconditioning can be achieved with exogenously administered adenosine or adenosine-receptor stimulation; (*3*) brief ischemic and adenosine-induced myocyte preconditioning is mimicked by the phorbol ester 4β-phorbol 12-myristate 13-acetate (PKC agonist) and inhibited by PKC antagonists; and (*4*) brief ischemic and adenosine-induced myocyte preconditioning both induce PKC translocation to myocyte membranes and increase the PKC phosphorylation rate. These data suggest that adenosine released from ischemic human pediatric myocytes mediates preconditioning through activation of PKC.

cardiomyocytes; signal transduction; cell viability

THE MECHANISM of ischemic preconditioning, a powerful myocardial protective strategy of potential clinical relevance, is not yet well understood. Evidence is accumulating to suggest that the capacity for preconditioning exists in human hearts, although very few data have been obtained from human tissue studies (23, 29). Therefore, the elucidation of the mechanism of preconditioning could permit the development of new cardioprotective strategies for medical or surgical treatment of high-risk patients.

The rapid time course for the development of myocardial tolerance to ischemia with preconditioning mandates that the preconditioning "signal" is rapidly conveyed to an ultimate cellular effector. This rapid response is typical of signaling initiated by interactions between membrane receptors and an extracellular ligand. Among other authors, Przyklenk and colleagues (52) provided evidence of a humoral mediator of preconditioning. Although the mediator(s) has not been conclusively identified, adenosine, released into extracellular fluid in substantial quantities from myocardial cells shortly after the onset of ischemia (5), is the leading candidate (38). Adenosine has been demonstrated to be involved in the preconditioning effect (38), and through release from cardiomyocytes in response to brief ischemia, adenosine may be an "autocrine" regulator of the acute response to myocardial ischemia through its interaction with sarcolemmal adenosine receptors.

After a receptor-ligand interaction, signals may be conveyed directly to effector mechanisms, as with channels coupled to membrane receptors, or through so-called second messenger systems often regulated by membrane "transducers" such as guanosine 5′-triphosphate-binding proteins. Of the many signal transduction pathways known to exist, the inositol phospholipid pathway has received particular attention with respect to preconditioning. Evidence has accumulated from several authors using different animal species that membrane translocation and activation of protein kinase C (PKC), the result of inositol phospholipid hydrolysis, is required for preconditioning (8, 39, 44, 57, 66). In addition, a number of studies in different tissues including myocardium documented activation of this pathway after exposure to adenosine or adenosine-receptor agonists (25, 54, 58, 63).

We recently described a unique model of preconditioning using simulated ischemia in monolayer cultures of human pediatric myocytes (21). We hypothesized that adenosine released from ischemic myocytes may confer autologous protection through activation of PKC. The results of the present study demonstrate that preconditioning supernatant protects nonpreconditioned myocytes from damage due to simulated ischemia and simulated reperfusion, and that adenosine, identified in the supernatant, is an important mediator. We also provide evidence that suggests that PKC plays a major role in the process of preconditioning signal transduction and that preincubation of cells with adenosine induces myocyte protection, which also requires activation of PKC.

MATERIALS AND METHODS

Human Pediatric Myocyte Cell Culture

Cultures of human pediatric myocytes obtained during cardiac surgery were grown as described previously (21, 33). Five- to twenty-milligram ventricular biopsies were obtained from sixteen tetralogy of Fallot patients undergoing corrective cardiac surgery. Each patient provided one biopsy for study. After being washed in phosphate-buffered saline (PBS) containing (in mmol/l) 136.9 NaCl, 2.7 KCl, 8.1 Na_2HPO_4, and 1.5 KH_2PO_4, pH 7.3, the connective tissue was removed and the remaining myocardial cells were separated using enzy-

matic digestion with a mixture of 0.2% trypsin (Difco Laboratories, Detroit, MI) and 0.1% collagenase (Worthington Biochemical, Freehold, NJ). The separated cells were then seeded onto cell culture dishes and cultured at 37°C and 5% CO_2 in Iscove's modified Dulbecco's medium (GIBCO Laboratories, Life Technologies, Grand Island, NY) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, and 0.1 mmol/l β-mercaptoethanol. Purification was achieved using the dilution cloning technique (15). Processing of one biopsy typically yielded 100–1,000 cells for seeding, which was done at a low density (50–100 cells per 9.0-cm-diameter culture dish). With this low seeding, individual myocytes were distinguished morphologically by their rectangular shape and large size (40 × 80 μm) from contaminating cells such as fibroblasts and endothelial cells, which are smaller and elongated in shape. Rod-shaped cells were never isolated. All cells on the plate were allowed to divide, and by 7–14 days myocyte and fibroblast colonies formed. Single myocyte colonies were then transferred, using a Pasteur pipette, to another culture dish. Culture purity >95% was demonstrated for each cell passage with fluorescent monoclonal antibody staining for actin (Ref. 33; Enzo Biochemical, New York, NY) and human ventricular myosin heavy chain (Rougier Bio-Tech, Montreal, Canada) (33). The resultant cultures were inspected daily, and any culture dishes seen to be contaminated with any additional cell types were discarded. Cell homogenates have been shown by Northern blot analysis to contain significant ventricular myosin heavy chain mRNA (11). In addition, the cells stain positively with human cardiac-specific monoclonal antibodies against myoglobin, troponin T, troponin I, and the MB isozyme (CK-MB) of creatine kinase (CK) (31). The CK-MB activity of these cells is high to passage 9 and is not present in cultured endothelial cells (32). Electron microscopy of these cells shows abundant mitochondria, extensive but disrupted sarcomeric networks, and intercalated disks (33).

The cell plates used for this study aged (mean ± SE) 46 ± 6 days with two to six passages and were allowed to grow for 3–4 days from the last passage before use. Cells used for all microscopic assessments were grown in 9.0-cm diameter culture plates and were nonconfluent (~225,000 cells/plate). Cells used to study in situ phosphorylation were grown to confluence on plates with 96 flat-bottom wells (~20,000 cells/well). Except where indicated, each intervention was assessed with 8 plates or 10 wells. Cells from at least three patients were used for any given set of experiments.

Experimental Protocols

Myocyte preconditioning model. After stabilization in 15 ml of perfusion PBS (PBS as described above with the addition of 0.49 mmol/l $MgCl_2$, 0.68 mmol/l $CaCl_2$, and 3.0 mmol/l glucose) at 37°C for 30 min, preconditioning ischemia was simulated by placing the cells into a Plexiglas chamber flushed with 100% nitrogen followed by addition of a low volume (1.5 ml for 9-cm plates, 10 μl/well for 96-well plates) of anoxic (Po_2 = 0 mmHg) perfusion PBS at 37°C for 20 min. This technique of inducing cell "ischemia," as well as the method of obtaining anoxic PBS, was previously described in detail (59). "Reperfusion" was accomplished by exposure to 15 ml of normoxic, 37°C perfusion PBS for 20 min. This period of ischemia and reperfusion was shown previously to optimize the protective effect of preconditioning in this model (21). After this stimulus, the cells were subjected to a further 90 min of ischemia and 30 min of reperfusion (21).

Supernatant and adenosine studies. We investigated the protective effect of ischemic supernatant and the role of adenosine in four studies. In the first set of experiments, nonpreconditioned cell plates stabilized for 30 min in perfusion PBS at 37°C were incubated for 20 min with 15 ml of supernatant gathered from 10-cell plates (1.5 ml each) exposed to 20 min of ischemia. After this exposure, the cells underwent 20 min of reperfusion before prolonged ischemia and reperfusion. In one group of cell plates, this supernatant was supplemented with the adenosine-receptor antagonist 8-(*p*-sulfophenyl)theophylline (SPT; Research Biochemicals International, Natick, MA) at a concentration of 100 μmol/l. SPT, a nonselective adenosine-receptor antagonist with six times the affinity for A_1 over A_2 receptors (3), was shown previously to inhibit preconditioning at a concentration of 100 μmol/l in isolated rabbit cardiomyocytes (2).

In the second set of experiments, "hypoxic" preconditioning was assessed by exposing the cells to 15 ml of anoxic supernatant for 20 min with a subsequent 20 min of reperfusion before prolonged ischemia and reperfusion.

In the third set of experiments, cell plates were incubated with perfusion PBS containing varying concentrations of adenosine (0.1–400 μmol/l) for 20 min with a subsequent 20 min of nonadenosine reperfusion before prolonged ischemia and reperfusion.

In the fourth set of experiments, cell plates were incubated with perfusion PBS containing 100 μmol/l of the adenosine-receptor agonist *R*(−)-N^6-(2-phenylisopropyl)adenosine (PIA, Research Biochemicals International) for 20 min with a subsequent 20 min of non-PIA-containing reperfusion before prolonged ischemia and reperfusion. PIA is a nonselective adenosine-receptor agonist that possesses 100-fold selectivity for A_1 over A_3 receptors (3). At a concentration of 100 μmol/l, this compound was demonstrated by Armstrong and Ganote (3) to mimic preconditioning in rabbit cardiomyocytes. In addition, separate cell plates were treated with 100 μmol/l SPT during the 30-min stabilization period and the ischemic component of preconditioning before prolonged ischemia and reperfusion. Both PIA and SPT were dissolved in perfusion PBS.

PKC studies. To assess the role of PKC in preconditioning, cell plates were incubated with perfusion PBS containing 1 μmol/l of the phorbol ester 4β-phorbol 12-myristate 13-acetate (PMA) or its inactive relative 4α-phorbol 12,13-didecanoate (PDD) for 20 min with a subsequent 20 min of reperfusion before prolonged ischemia and reperfusion. PMA is known to activate most of the isoforms of PKC and was shown, at a dose of 1 μmol/l, to protect cultured cardiomyocytes from simulated ischemia in a manner similar to preconditioning (4). In addition, separate cell plates were stabilized for 30 min in perfusion PBS containing the specific PKC antagonist calphostin C [200 nmol/l (Ref. 2), in the presence of fluorescent light (Ref. 9)] or chelerythrine (1 μmol/l; Ref. 18) (these reagents from Calbiochem Biochemicals, La Jolla, CA) before preconditioning and during the ischemic component of preconditioning followed by antagonist-free reperfusion and subsequent prolonged ischemia and reperfusion. Calphostin C, isolated from *Cladosporium cladosporioides*, inhibits PKC through interaction with lipid cofactor binding sites to the enzyme (24) and was shown previously to prevent preconditioning at a concentration of 200 nmol/l in isolated rabbit cardiomyocytes (2). In contrast, chelerythrine, an isolate from the leaves and stems of *Macleaya cordata* and *M. microcarpa*, interferes with the catalytic site (18). The dose of chelerythrine selected is approximately two times the published effective dose (Calbiochem). Chelerythrine was dissolved in perfusion PBS, and PMA, PDD, and calphostin C were dissolved in dimethyl sulfoxide (DMSO) to give 2 mmol/l stock solutions before

dilution in perfusion PBS to give the specified concentration. The final concentration of DMSO never exceeded 0.05%.

To assess the relationship between adenosine and PKC, cell plates stabilized for 50 min in perfusion PBS containing calphostin C (200 nmol/l) were exposed to 50 μmol/l of adenosine during the last 20 min, followed by prolonged ischemia and reperfusion.

Assessment of Cell Injury

After the intervention of interest, cell plates were incubated for 5 min at room temperature with 0.4% trypan blue dye (Sigma Chemical, St. Louis, MO) dissolved in normal saline and were assessed for injury under an inverted light microscope (Nikon Canada Instrument, Mississauga, Ontario) at ×200 magnification. Injured cells were unable to exclude the high-molecular-weight dye and stained blue (42). Counts were read from five standard locations on each plate (center and 3, 6, 9, and 12 o'clock) and averaged. All counts were performed by a single blinded observer.

Biochemical Measurements

Adenosine assay. Ischemic supernatants were flash frozen in liquid nitrogen, lyophilized, and reconstituted immediately before adenosine assay using step-gradient high-performance liquid chromatography (20, 59). The resultant values were expressed as an adenosine concentration against a known adenosine standard.

PKC immunofluorescence microscopy. The method used for fluorescent antibody staining of myocytes is a modification of a technique we described previously (33). Myocytes were fixed in 100% methanol for 20 min after completion of one of four interventions: *1*) negative control (30 min of stabilization in perfusion PBS); *2*) positive control (20 min of incubation with 1 μmol/l PMA followed by 20 min of non-PMA-containing reperfusion); *3*) 20 min of incubation with 50 μmol/l adenosine followed by 20 min of non-adenosine-containing reperfusion; and *4*) preconditioning (20 min of ischemia followed by 20 min of reperfusion). The cells were then washed three times in PBS and stained with a 1:40 dilution of rabbit anti-human anti-protein kinase $C_{Concensus}$ immunoglobulin G antibody (catalog no. 539550, Calbiochem) at 37°C for 45 min. This antibody is known to bind to a unique peptide sequence (amino acids 543–550) in the catalytic site of rat, rabbit, bovine, and human PKC (Calbiochem). The antibody was then removed with three 15-min washings of PBS in a gentle shaker at room temperature. The cells were then stained with a 1:20 dilution of fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G antibody (ICN Biochemicals, Mississauga, Ontario) at 37°C for 45 min. The plates were washed three times as before except that to remove the effect of nonspecific staining, washing was continued until the fluorescence of a separate plate stained only with the second fluorescent antibody was barely detectable. The plates were viewed and photographed under ultraviolet light using an epifluorescent microscope with a blue filter (Nikon Canada Instrument) at ×200 magnification.

Measurement of PKC activity. PKC activity was measured by in situ phosphorylation of a PKC-specific peptide substrate, using a modification of a method described by Heasley and Johnson (16, 17). Myocytes were seeded onto 96-well flat-bottomed microtiter culture plates as described above. Myocytes were subjected to one of four interventions: *1*) negative control (30 min of stabilization in perfusion PBS); *2*) positive control (20 min of incubation with 1 μmol/l PMA followed by 20 min of non-PMA-containing reperfusion); *3*) 20 min of incubation with 50 μmol/l adenosine followed by 20 min of non-adenosine-containing reperfusion; and *4*) preconditioning (20 min of ischemia followed by 20 min of reperfusion). After this, the overlying supernatants were discarded and the cells were immediately exposed to 40 μl of buffered reaction solution containing 50 μg/ml digitonin, 137 mmol/l NaCl, 4.4 mmol/l KCl, 10 mmol/l $MgCl_2$, 0.3 mmol/l Na_2HPO_4, 0.4 mmol/l K_2HPO_4, 25 mmol/l β-glycerophosphate, 1 mmol/l $CaCl_2$, 20 mmol/l *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, 100 μmol/l [γ-^{32}P]ATP (5,000 cpm/pmol, Amersham Life Science, Oakville, Ontario), and 100 μmol/l of PKC-specific substrate (VRKRTLRRL) at 37°C and pH 7.20. This nine-amino acid peptide, shown to be phosphorylated by PKC independent of the action of adenosine 3′,5′-cyclic monophosphate- and guanosine 3′,5′-cyclic monophosphate-dependent kinases, S6 kinase, and Ca^{2+}/calmodulin-dependent kinase (16, 17), was synthesized with an automated solid-phase peptide synthesizer. Purity >95% and quantitation of the peptide were assessed by amino acid analysis. Except where indicated, all preceding reagents were obtained from Sigma Chemical.

The reaction was allowed to proceed for 10 min at 30°C before termination by the addition of 50 μl of ice-cold 25% (wt/vol) trichloroacetic acid. The phosphorylation rate of this reaction under the conditions specified was shown in similar systems to be constant over 30 min (17). Aliquots (45 μl) of the acidified reaction mixtures were spotted onto 2-cm-diameter phosphocellulose filters (P81, Gibco) and washed batchwise in three changes (500 ml each) of 75 mmol/l phosphoric acid with a final wash in 500 ml of 75 mmol/l Na_2HPO_4 (pH 7.50). The PKC substrate, due to its basicity, was retained on the filter paper at neutral pH. Total phosphorylation was determined by scintillation counting of the washed filter papers. Background phosphorylation (obtained by running the above reaction without peptide) was subtracted from the total to yield specific peptide phosphorylation. These values were then divided by the reaction time of 10 min and standardized for cell protein measured using the method of Lowry et al. (40).

Statistical Analysis

Statistical analysis was performed using the Statistical Analysis Systems program (SAS Institute, Cary, NC). Analysis of variance was used to identify significant differences between control and treatment groups. When found, these differences were specified with Duncan's multiple-range *t*-test. Statistical significance was assumed at the $P < 0.05$ level.

RESULTS

Supernatant and Adenosine Studies

Figure 1 shows the results of the supernatant preconditioning study. Addition of preconditioning supernatant to nonpreconditioned cells conferred protection from subsequent prolonged ischemia and reperfusion (ischemia 41.9 ± 4.0, preconditioning 23.4 ± 1.8, supernatant 29.0 ± 2.3% blue-staining cells; preconditioning and supernatant, $P < 0.05$ from ischemia). However, this effect was abolished if SPT was present in the supernatant [38.8 ± 2.1% blue-staining cells, P = not significant (NS) from ischemia but $P < 0.05$ from preconditioning and supernatant].

Figure 2 shows the results of preconditioning with variable volumes of anoxic perfusate. The results show

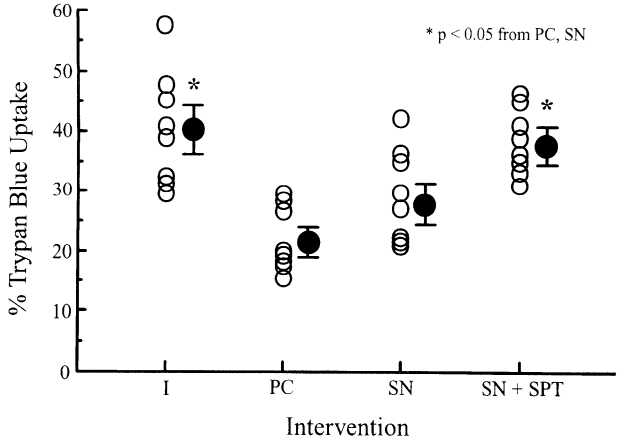

Fig. 1. Effect of "ischemic" supernatant (SN) pretreatment on cellular damage (expressed as trypan blue uptake) after prolonged simulated ischemia and reperfusion (I). Ischemic SN pretreatment induced cellular protection similar to preconditioning (PC). Addition of 100 μmol/l 8-(*p*-sulfophenyl)theophylline (SPT) to the supernatant abolished protection (SN + SPT). ○, counts on separate culture plates (n = 8 per group); ●, means ± SE.

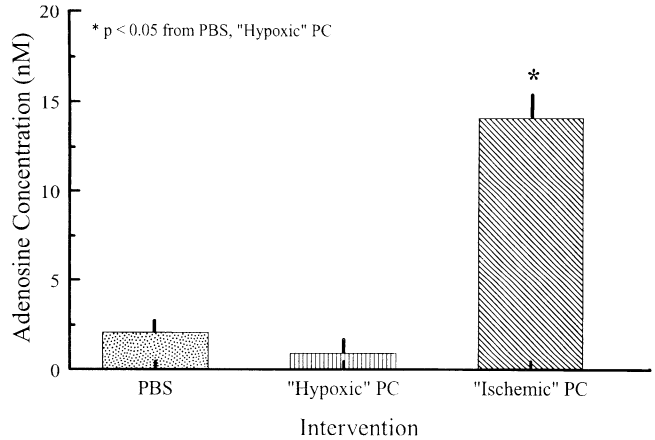

Fig. 3. SN adenosine (Ado) analysis. Ischemic PC SN (n = 7) was found to contain greater Ado concentration than hypoxic PC SN (n = 6) or SN from cells stabilized for 30 min in perfusion phosphate-buffered saline (PBS, n = 6). Values are expressed as means ± SE.

that, in contrast to low-volume ischemic preconditioning (19.5 ± 1.5% blue-staining cells), exposure to a high anoxic volume (simulating hypoxia) did not protect the myocytes from subsequent ischemia and reperfusion (41.6 ± 1.4, P = NS from ischemia 44.8 ± 3.9% blue-staining cells but $P < 0.05$ from ischemic preconditioning). Furthermore, high-performance liquid chromatography analysis (Fig. 3) revealed substantially greater adenosine present in the ischemic supernatant (14.1 ± 3.4 nM) than either the control (PBS, 2.4 ± 0.2 nM) or hypoxic (0.98 ± 0.10 nM) supernatants ($P < 0.05$).

In Fig. 4, separate plates were incubated with different adenosine concentrations for 20 min with 20 min of reperfusion before prolonged ischemia and reperfusion. The results show a U-shaped protective dose response [maximum (0 μmol/l adenosine) 45.7 ± 6.2% blue-staining cells; minimum (50 μmol/l adenosine) 27.9 ± 1.5% blue-staining cells], with the greatest protection observed with 10 and 50 μmol/l ($P < 0.05$).

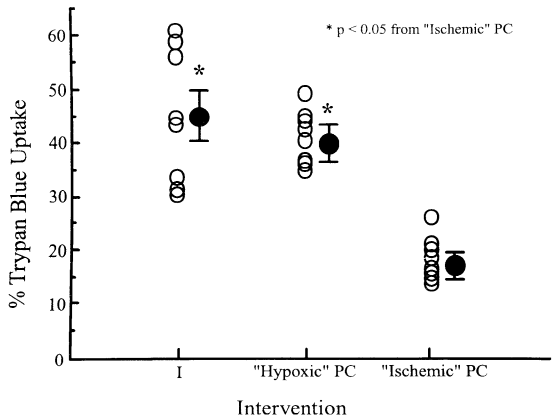

Fig. 2. Cell injury after ischemic (low anoxic volume) or "hypoxic" (high anoxic volume) PC before prolonged I. Ischemic PC but not hypoxic PC reduced cellular trypan blue uptake. ○, counts on separate culture plates (n = 8 per group); ●, means ± SE.

Figure 5 shows the results of the adenosine agonist and antagonist study. Incubation with 100 μmol/l PIA for 20 min with 20 min of reperfusion before prolonged ischemia and reperfusion significantly reduced the percentage of trypan blue uptake in a similar fashion to preconditioning (preconditioning 26.4 ± 2.1, PIA 31.8 ± 2.3%, both $P < 0.05$ from ischemia 42.1 ± 3.6%). Incubation with 100 μmol/l SPT for 30 min before preconditioning and during its ischemic component both abolished the protective effect of preconditioning and increased the percentage of trypan blue uptake relative to control ischemia and reperfusion (51.1 ± 4.1%, $P < 0.05$ from ischemia).

PKC Studies

Figure 6 shows the results of PKC fluorescent antibody staining. After stabilization in PBS, fluorescence was uniform throughout the cytoplasm (Fig. 6*A*). Preconditioning (Fig. 6*B*) and adenosine preincubation

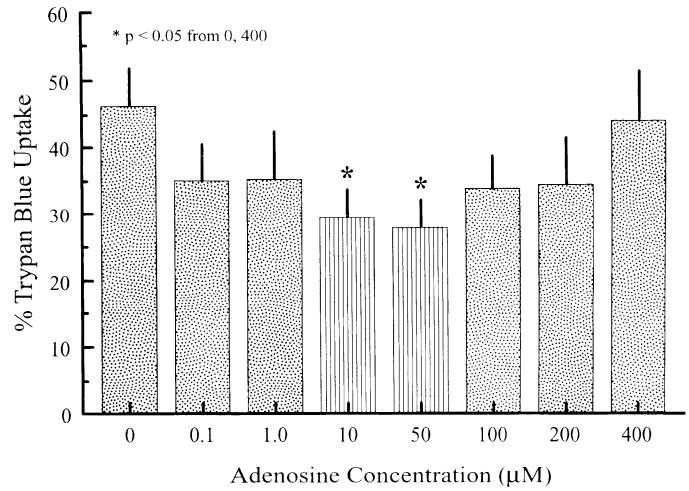

Fig. 4. Ado preincubation dose response against cellular damage before prolonged I. Results show a U-shaped response in trypan blue uptake to different Ado pretreatment concentrations. Protection was obtained with a concentration of 10 or 50 μmol/l. Values are expressed as means ± SE; n = 8 per group.

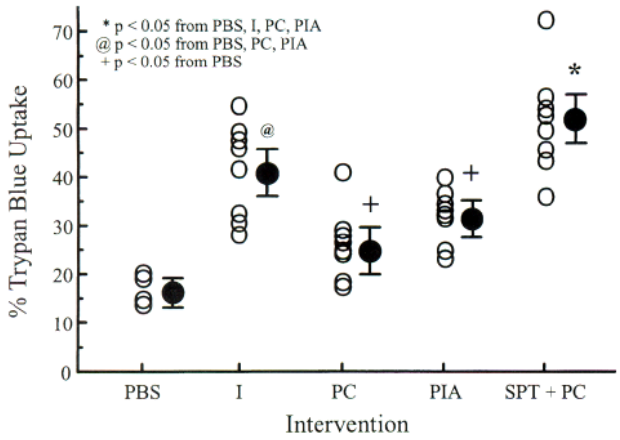

Fig. 5. Effect of Ado-receptor stimulation and inhibition on cellular damage after prolonged I. Pretreatment with Ado-receptor agonist $R(-)$-N^6-(2-phenylisopropyl)adenosine (PIA, 100 μmol/l) reduced cellular trypan blue uptake similar to PC but greater than control 30-min stabilization in PBS. Addition of 100 μmol/l SPT before and during PC ischemia (SPT + PC) abolished protection. ○, counts on separate culture plates [n = 8 per group except PBS (n = 4)]; ●, means ± SE.

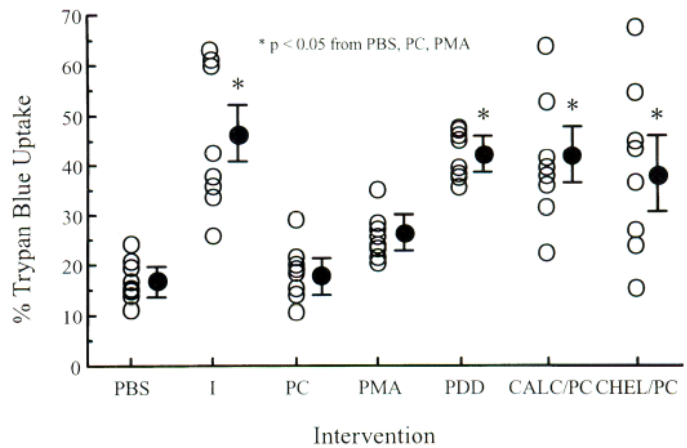

Fig. 7. Effect of PKC stimulation and inhibition on cellular damage after prolonged I. Pretreatment with phorbol ester agonist PMA (1 μmol/l) reduced cellular trypan blue uptake similar to PC and PBS stabilization. Pretreatment with inactive phorbol ester 4α-phorbol 12,13-didecanoate (PDD, 1 μmol/l) was not protective. Addition of PKC antagonist calphostin C (Calc/PC, 200 nmol/l) or chelerythrine (Chel/PC, 1 μmol/l), prevented PC. ○, counts on separate culture plates (n = 8 per group); ●, means ± SE.

(Fig. 6*D*) induced redistribution of fluorescence to the cell membrane and perinuclear area. This appearance was similar to that seen in cells preincubated with PMA (Fig. 6*C*).

In Fig. 7, the effects of PKC agonists and antagonists on preconditioning are shown. The results show that preincubation with PMA (25.4 ± 2.2% trypan blue uptake) but not PDD (43.5 ± 2.3%) resulted in protec-

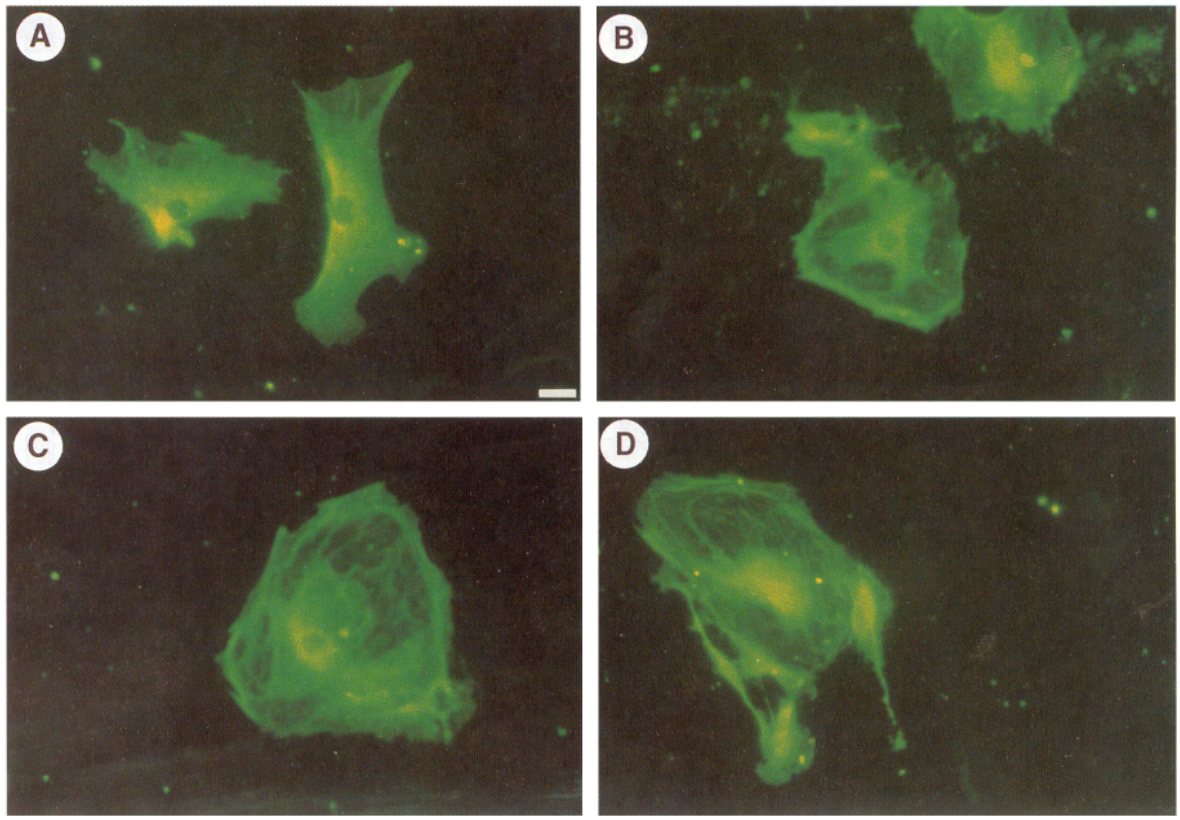

Fig. 6. Assessment of protein kinase C (PKC) translocation using specific fluorescein isothiocyanate-labeled fluorescent antibodies. Stabilization in PBS for 30 min showed uniform cytoplasmic staining for PKC (*A*). In contrast, PC (*B*), pretreatment with phorbol ester agonist 4β-phorbol 12-myristate 13-acetate (PMA, 1 μmol/l; *C*), or pretreatment with 50 μmol/l Ado (*D*) induced redistribution of antibody to sarcolemmal and perinuclear membranes compatible with translocation of PKC. Photomicrographs were shot at ×200 magnification. Scale bar, 10 μm.

tion from ischemia and reperfusion similar to preconditioning (18.5 ± 1.7%; preconditioning and PMA, $P <$ 0.05 from ischemia: (46.2 ± 5.4%). Furthermore, preconditioning could not be achieved when brief ischemia was applied in the presence of either calphostin C (40.6 ± 3.6% trypan blue uptake) or chelerythrine (37.7 ± 3.7%) (both P = NS from ischemia). Although both agents statistically abolished preconditioning, calphostin C seemed to provide more reliable inhibition. Both PMA preincubation and preconditioning resulted in an increase (Fig. 8) in PKC phosphorylation rate (PMA 252 ± 6.7, prcconditioning 227 ± 6.3, and PBS 80 ± 5.2 pmol peptide phosphorylated·mg cell protein^{-1}·min^{-1}; PMA and preconditioning $P < 0.05$ from PBS). When cells were preconditioned in the presence of calphostin C, PKC phosphorylation was reduced to below control PBS stabilization values (47 ± 3.6 pmol peptide phosphorylated·mg cell protein^{-1}·min^{-1}, $P < 0.05$ from PBS). The cellular protein content in each well averaged (mean ± SE) 10.6 ± 0.7 μg, with no significant differences between groups.

The interaction between adenosine and calphostin C is shown in Fig. 9. The results demonstrate that the protective effect of 20 min of preincubation in 50 μmol/l adenosine with 20 min of reperfusion [adenosine preincubation 22.1 ± 3.6% trypan blue uptake, P = NS from preconditioning (19.6 ± 2.2%) but $P < 0.05$ from ischemia (45.5 ± 4.8%)] is removed in the presence of calphostin C (42.6 ± 2.0%, $P < 0.05$ from preconditioning and adenosine preincubation but P = NS from ischemia). Similarly, adenosine preincubation increased PKC phosphorylation rate (Fig. 10) in a similar fashion to PMA (PMA 156 ± 5.4, adenosine 148 ± 5.7, and PBS 62 ± 3.2 pmol peptide phosphorylated·mg cell protein^{-1}·min^{-1}; PMA and adenosine $P < 0.05$ from PBS). However, with calphostin C present, this effect was lost (48 ± 2.1 pmol peptide phosphorylated·mg cell protein^{-1}·min^{-1}, $P < 0.05$ from PBS). The cellular protein content in each well averaged 11.0 ± 0.4 μg with no significant differences between groups.

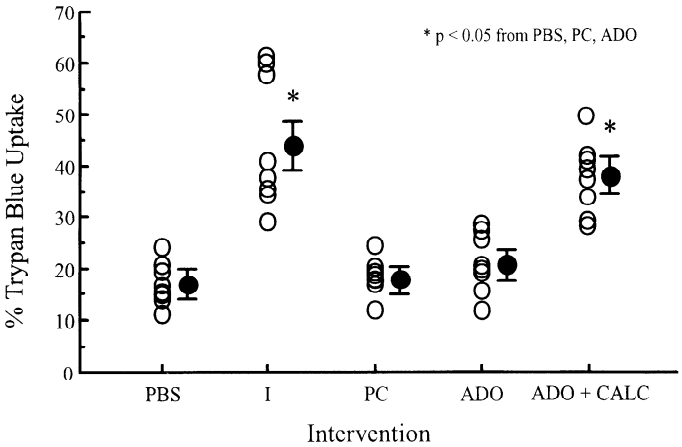

Fig. 9. Effect of Ado and PKC inhibition on cellular damage after prolonged I. Pretreatment with 50 μmol/l Ado reduced cellular trypan blue uptake similar to PC and PBS stabilization (PBS). Pretreatment with PKC antagonist Calc (200 nmol/l) before and during Ado preincubation (Ado + Calc) prevented protection. ○, counts on separate culture plates (n = 8 per group); ●, means ± SE.

DISCUSSION

The detrimental effects of myocardial ischemia may be blunted by antecedent exposure to brief episodes of ischemia and reperfusion. This phenomenon, termed "ischemic preconditioning," results in a delay in the onset of irreversible damage (47). Although our present knowledge of preconditioning comes primarily from animal studies, recent reports suggest that the human myocardium may be preconditioned (23, 29). In contrast to the large volume of data available on preconditioning the whole heart, fewer reports assess preconditioning in heart tissue preparations or cell cultures

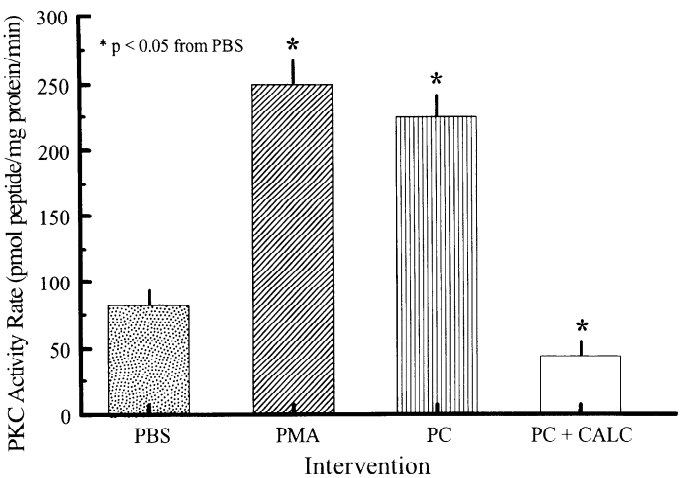

Fig. 8. Effect of PC and PKC activation or inhibition on in situ cellular phosphorylation rates of PKC-specific peptide. Pretreatment with phorbol ester agonist PMA (1 μmol/l) or PC significantly increased peptide phosphorylation rate compared with PBS stabilization. Addition of PKC antagonist Calc (200 nmol/l) before and during preconditioning ischemia (PC + Calc) prevented increase in peptide phosphorylation rate. Values are expressed as means ± SE; n = 10 per group.

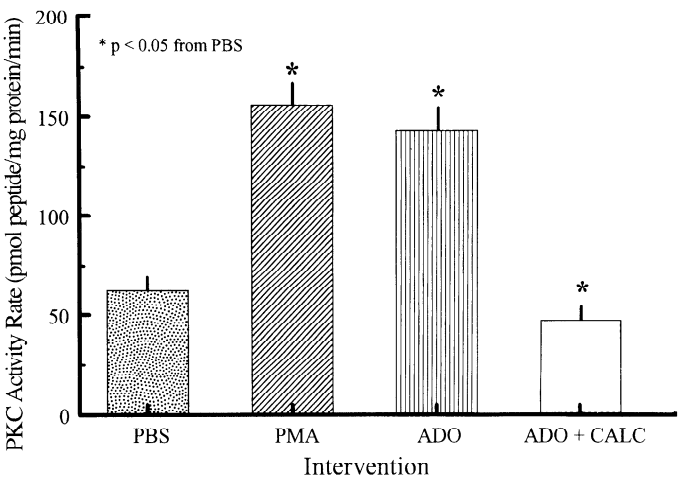

Fig. 10. Effect of Ado pretreatment and PKC activation or inhibition on in situ cellular phosphorlyation rates of PKC-specific peptide. Pretreatment with phorbol ester agonist PMA (1 μmol/l) or 50 μmol/l Ado significantly increased peptide phosphorylation rate compared with PBS stabilization. Addition of PKC antagonist Calc (200 nmol/l) before and during Ado preincubation (Ado + Calc) prevented an increase in peptide phosphorylation rate. Values are expressed as means ± SE; n = 10 per group.

(2–4, 14, 62). With the development of increasing interest in the signal transduction pathways involved in preconditioning, these latter preparations facilitate assessment of promising mechanisms.

We developed a unique model of preconditioning with simulated ischemia in human pediatric myocyte cultures. We reported previously that in these cells a single preconditioning cycle of 20 min of simulated ischemia and 20 min of simulated reperfusion optimized the reduction in cell death, increase in cellular survival, and reduction in both lactate production and adenine nucleotide degradation in response to 90 min of ischemia and 30 min of reperfusion (21). This model therefore behaved similarly to other published reports using intact hearts (47), with the exception that the duration of simulated ischemia required to induce preconditioning and to cause significant cell death seemed to exceed the time required in whole heart models. This observation is consistent with the results from other cell culture studies that demonstrate that longer periods of simulated ischemia are required to produce cell death (14, 60). In addition, we previously showed (21) that although 10 min of ischemia and 10 min of reperfusion had a preconditioning effect in our cells, the 20-min ischemia + 20-min reperfusion stimulus was more effective. This longer ischemic time requirement for maximal preconditioning may reflect the use of a single-cycle preconditioning stimulus in this model.

Supernatant and Adenosine Studies

Ischemic preconditioning may be mediated by adenosine released from cardiomyocytes in response to brief ischemia resulting in activation of adenosine receptors (38). Because our cells are preconditioned with low volumes of anoxic solution, this model permitted collection of this solution (the supernatant) after preconditioning and testing of this solution for transference of protection to other cells. Furthermore, the supernatant could be analyzed for potential mediators.

Evidence in the literature suggests the existence of a humoral mediator of preconditioning (52). The present study shows that a protective mediator is released into supernatant over myocytes in response to brief ischemia. Our hypothesis that this mediator is adenosine is supported by the abolishment of the protective capacity of ischemic supernatant when the adenosine-receptor blocker SPT was added and also by detection of adenosine in the ischemic supernatant.

Also supporting the adenosine hypothesis was the finding that a high volume of anoxic supernatant (hypoxia, which would dilute any released protective mediators) did not protect cells from subsequent prolonged ischemia and reperfusion. The concentration of adenosine recovered from the hypoxic supernatant (0.98 ± 0.10 nmol/l) is below the published dissociation constant (K_d) for the adult myocardial adenosine A_1 receptor (1.5–3.0 nmol/l; Ref. 49). In contrast, the ischemic preconditioning supernatant adenosine concentration (14.1 ± 3.4 nmol/l) greatly exceeds the reported K_d for the A_1 receptor. Some controversy exists as to whether adenosine induces preconditioning through activation of cardiac A_1 receptors (38) or the more recently implicated A_3 receptors (3, 37). K_d for the A_3 receptor is currently unknown (3), so at present it is unclear whether the adenosine concentration in the ischemic supernatant is sufficient for activation of this receptor.

This study showed that the adenosine-receptor agonist PIA exerted a preconditioning-mimetic effect on the myocytes, whereas SPT prevented the induction of preconditioning. Although these results support involvement of adenosine receptors in human myocyte preconditioning, neither of these agents is selective enough to allow distinction between the adenosine A_1 or A_3 receptor subtypes (3).

In our study, hypoxia did not precondition the myocytes; however, hypoxic perfusion has been used in other models to successfully precondition the heart (28, 55, 62). In multicellular preparations, adenosine may accumulate between cells before it is washed out. This "adenosine gradient," created by the presence of many cell layers, could result in retention of sufficient amounts of adenosine to allow receptor activation. Our preparation, however, consists of a cellular monolayer with no capacity for interstitial adenosine accumulation once released by the cell. Hence, exposure to high-volume anoxia would not support receptor-adenosine binding and, therefore, prevent induction of protection.

Although administration of adenosine with subsequent reperfusion was protective against prolonged ischemia and reperfusion, we were surprised that the protective effect of adenosine was lost at concentrations $\geq$100 μmol/l, resulting in a U-shaped dose response. Armstrong and Ganote (4) observed a similar phenomenon where adenosine induced a biphasic response, protecting from ischemic damage at low levels but inhibiting protection at high levels in rabbit cardiomyocytes. This response may have been due to partial agonist activity by adenosine (65), activation of additional inhibitory receptor types, or direct stimulation of metabolic pathways with high concentrations of adenosine. This adenosine dose response may also have resulted from receptor downregulation (30) such as that seen with adrenergic receptors exposed to high catecholamine concentrations (13, 41). We found that an adenosine concentration of 14 nmol/l in preconditioning supernatant protected myocytes from prolonged ischemia and reperfusion, but when given exogenously, a concentration of 10 μmol/l (almost 1,000-fold higher) was required to achieve protection. The reason for this observation is unclear. Adenosine protection may have a bimodal profile, which may reflect modulation of one receptor subtype (58) or recruitment of different adenosine receptor types with different adenosine concentrations (34). Alternatively, perhaps the small amount of adenosine present in preconditioning supernatant is enhanced by other protective mediators released during ischemia that potentiate the effects of adenosine. These mediators may either activate other membrane receptors, potentiate the binding of adenosine to its receptors, or stimulate further endogenous adenosine

release into an unstirred layer overlying the myocytes in intimate proximity to the receptors. Adenosine receptor occupation must be present, because SPT abolishes the preconditioning effect. Further studies are required to uncover possible additional mediators in preconditioning supernatant.

PKC Studies

Although some refuting data exists (27, 56, 61), most of the recent studies showed that PKC activation is necessary for ischemic preconditioning to occur. In rabbits, Brooks and co-workers (8) demonstrated that phosphorylation of the PKC-specific, naturally occurring myristolated alanine-rich C-kinase substrate (MARCKS) occurs as early as 5 min into a prolonged episode of myocardial ischemia, but only in hearts that had first been preconditioned. Ytrehus et al. (66) demonstrated that a single 5-min infusion of 1-oleoyl-2-acetylglycerol or PMA followed by 10 min of washout reduced the infarct size after 30 min of regional myocardial ischemia by 58 and 77%, respectively. Administration of staurosporine or polymyxin B after preconditioning abolished protection (66). Similar results were obtained by Armstrong et al. (2, 4) in their model of isolated adult rabbit cardiomyocyte preconditioning. Liu and colleagues (39) showed that blockade of PKC translocation during ischemia with colchicine also prevented preconditioning. In rats, Speechly-Dick and co-workers (57) showed a reduction in infarct size similar to preconditioning with administration of diacylglycerol analog 1,2-dioctanoylglycerol before 45 min of prolonged regional ischemia. The presence of chelerythrine after preconditioning but before prolonged ischemia blocked the protective effect (57). Mitchell et al. (44) demonstrated membrane translocation of multiple PKC isoforms in rat hearts in response to brief ischemia using specific fluorescent antibodies.

PKC, first identified by Nishizuka in 1977, represents a family of enzymes whose actions appear crucial for signal transduction of many short- and long-term cellular responses. The characteristics of these kinases have been extensively reviewed (6, 19, 50, 51, 53).

At least 10 members of the PKC family have been identified. Each of these isoforms are serine and/or threonine kinases of similar structure and molecular weight (68,000–83,000). In adult myocardium, at least five PKC isozymes have been identified (6), of which the β- and ε-isoforms are most abundant (7, 26).

Hydrolysis of membrane inositol phospholipids, in particular phosphatidylinositol 4,5-bisphosphate [PtdIns(4,5)P_2], by phospholipase C results in the formation of inositol-1,4,5-trisphosphate [Ins(1,4,5)P_3], released to the cellular cytoplasm to induce mobilization of Ca^{2+} from internal stores, and 1,2-diacylglycerol (DAG), which remains in membranes and initiates the membrane translocation and activation of PKC. This translocation process, which may not always be synonymous with kinase activity, probably involves cytoskeletal structural elements such as microtubules (35, 39) (evident in the fluorescent micrographs shown in Fig. 6) and is associated with binding to receptors for activated C kinase (RACKS) present on cytoskeletal elements and the cell membrane (45, 48). This preferential binding to RACKS is thought to confer specificity of PKC isoforms for phosphorylation of different proteins.

The results of our study support the role of PKC in preconditioning, because we demonstrated that preincubation with the PKC agonist PMA induced a preconditioning-mimetic effect and blockade of PKC during preconditioning resulted in the loss of protection. We chose to test the PKC inhibitors calphostin C and chelerythrine because of their high specificity for PKC over other kinases such as protein kinase A and because of their independent mechanisms of action (18, 24). Preincubation with either of these inhibitors before preconditioning resulted in loss of protection. In addition, we demonstrated with fluorescent antibody stains that both PMA preincubation and preconditioning induce redistribution of fluorescence to membranes consistent with PKC translocation.

To study the importance of PKC in preconditioning, particular biological properties of PKC such as translocation have been assessed with immunofluorescence antibody staining (44) and with agents that interfere with cellular structural filaments that PKC may require for translocation (39). In addition, the technique of using agonists and antagonists to assess PKC function has been employed (57, 66). However, to date there is little published evidence to show that preconditioning actually modulates the kinase function of PKC. Brooks et al. (8) measured MARCKS phosphorylation in whole rabbit hearts to show that PKC phosphorylation was stimulated by preconditioning. Use of a cell culture model greatly simplified the assessment of PKC phosphorylation in response to preconditioning through assay of in situ phosphorylation of PKC-specific peptide substrates. Many of these artificially synthesized peptides, consisting of ~5–20 amino acids are commercially available and in use (1, 16, 17, 64). For this study, we chose to study the phosphorylation of a nine-amino acid peptide derived from the threonine phosphorylation site of human epidermal growth factor receptors (12). This peptide shows remarkable specificity for PKC independent of cyclic nucleotide-dependent, Ca^{2+}/calmodulin-dependent, or other kinases (16, 17). The results of this study indicate that PMA or preconditioning induces phosphorylation of this PKC-specific peptide. The reduction in peptide phosphorylation to below baseline values with the addition of calphostin C gives confidence that this increased phosphorylation was due to PKC and not other kinases. The advantages of this method pertain to the rapidity and facile methodology of the assay, the specificity of these peptides for PKC, and the ability to measure PKC phosphorylation immediately after the selected stimulus, not at a later date using a different agonist to activate PKC in an extracted cellular membrane fraction. This method has the inherent disadvantages of an inability to localize and differentiate between specific isoforms of PKC. However, in conjunction with the other two PKC assessments used, we believe that these studies provide strong evidence that PKC kinase activity after mem-

brane translocation is required for transduction of the signal(s) of human myocyte preconditioning.

Previous work showed that activation of adenosine A_1 receptors in various tissues stimulates phospholipase C, the membrane enzyme responsible for conversion of PtdIns(4,5)P_2 to Ins(1,4,5)P_3 and DAG (54, 58, 63). In a guinea pig left atrial and papillary muscle preparation, Kohl and colleagues (25) observed that administration of PIA increased Ins(1,4,5)P_3 production with a concomitant fall in PtdIns(4,5)P_2. These data suggest that adenosine-receptor stimulation induces PKC activation. Our studies support this hypothesis, because we demonstrated that adenosine preincubation induces redistribution of PKC antibody staining patterns to cellular membranes in a manner compatible with PKC activation. In addition, the protective effect of adenosine against cellular damage was abolished in cells stabilized with calphostin C. Finally, adenosine preincubation caused increased phosphorylation of PKC-specific peptide that was negated in the presence of calphostin C. Because of the lack of specificity of the antibodies used, we cannot comment at present on which isoforms of PKC are activated by preconditioning or whether these differ from the isoforms activated by adenosine. More studies are required to specify these changes in greater detail.

Study Limitations

The use of cell culture systems for studies of preconditioning has certain limitations. For example, our myocyte culture system undergoes some cellular dedifferentiation and loss of sarcomeres with increasing time of culture (31), which does not appear to interfere with the capacity for these cells to be preconditioned. These changes are similar to those described by Moses and Claycomb (46) for adult rat cardiomyocytes, and they render these cells quiescent. However, we have recently demonstrated that the cardiac cell culture antioxidant profiles (43), ratio of CK to CK-MB, and membrane phospholipid and fatty acid composition (32) are similar to these same parameters in human myocardial tissue. With these cultures we reported differences in water-soluble antioxidant specificity against free radical injury (43) and identified oxygen-responsive genetic sequences that regulate glutathione peroxidase expression in response to varying oxygen tensions (10). Furthermore, we demonstrated the usefulness of these cells for studies of cellular damage and metabolism after simulated ischemia (21, 59). Hence, these cells possess many similarities to primary human cardiomyocytes but also have some important differences. Although our results may be clinically relevant because this work was performed on cell cultures obtained from human patients at the time of cardiac surgery, caution should be employed when translating these cell culture results to clinically relevant strategies for cardioprotection. Studies in beating whole hearts are required to test this information under more physiological conditions.

We have chosen to describe our model of low-volume anoxia as ischemia because of the significant acid and lactate production associated with prolonged exposure to the conditions specified (21, 59). However, it is unclear as to how closely this model approximates ischemic conditions in the intact myocardium. Furthermore, multiple cycles of preconditioning could not be assessed with this model because the repeated cell plate washings associated with multiple cycles of ischemia and reperfusion incur significant cellular damage.

In summary, the present study shows that adenosine, released from ischemic human pediatric myocytes, exerts an endogenously derived form of protection through binding to sarcolemmal adenosine receptors and activation of PKC. How adenosine-induced activation of PKC induces preconditioning is currently unknown. Possible effector mechanisms include activation of channels such as the ATP-sensitive K^+ channel (36) and modulation of the function of enzymes such as ectosolic 5′ nucleotidase (22). Further studies are required to elucidate the protein effectors of preconditioning, information that may hopefully be used to improve the treatment of human cardiovascular disease.

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