Preconditioning in Rabbit Cardiomyocytes

Role of pH, Vacuolar Proton ATPase, and Apoptosis

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Abstract

Ischemic preconditioning signals through protein kinase C (PKC) to protect against myocardial infarction. This protection is characterized by diminished intracellular acidification. Acidification is also a feature of apoptosis, and several agents act to prevent apoptosis by preventing acidification through activation of ion channels and pumps to promote cytoplasmic alkalinization. We characterized metabolic inhibition, recovery, and preconditioning through a PKC-dependent pathway in cardiomyocytes isolated from adult rabbit hearts. Preconditioning reduced loss of viability assessed by morphology and reduced DNA nicking. Blockade of the vacuolar proton ATPase (VPATPase) prevented the effect of preconditioning to reduce metabolic inhibition-induced acidosis, loss of viability, and DNA nicking. The beneficial effect of Na^+/H^+ exchange inhibition, which is thought to be effective through reduced intracellular Na^+ and Ca^{++}, was also abrogated by VPATPase blockade, suggesting that acidification even in the absence of Na^+/H^+ exchange may lead to cell death. We conclude that a target of PKC in mediating preconditioning is activation of the VPATPase with resultant attenuation of intracellular acidification during metabolic inhibition. Inhibition of the "death protease," interleukin-1-beta converting enzyme or related enzymes, also protected against the injury that followed metabolic inhibition. This observation, coupled with the detection of DNA nicking in cells subjected to metabolic inhibition, suggests that apoptotic cell death may be preventable in this model of ischemia/reperfusion injury. (*J. Clin. Invest.* 1996. 97:2391–2398.) Key words: ischemia • reperfusion • bafilomycin

Introduction

Ischemic preconditioning, which protects against myocardial infarction, is defined experimentally as a reduction in ultimate tissue death when a test ischemic episode is preceded by a brief, nonlethal period of ischemia and reperfusion (1). It is one of the most potent ways to protect against ischemia reperfusion injury, and it appears to be active in all experimental models and in humans (2, 3). The exact mechanisms of protection are unknown; however, the signal transduction pathway involves A1 or A3 adenosine receptor activation in most species (4, 5) and can be mimicked by cholinergic and alpha adrenergic receptor stimulation in some species (6–8). Signal transduction proceeds through a protein kinase C (PKC)[1]-dependent pathway that results in translocation of PKC to the membrane where it phosphorylates as yet undefined targets (9). One consistent observation in virtually every model of myocardial preconditioning for lethal ischemic injury is a diminution in the fall of intracellular pH (1, 10–14). Mechanisms of improved pH homeostasis include reduced proton production from anaerobic glycolysis and increased proton export through pumps or ion exchange channels. The delayed or diminished fall in pH during ischemia might be unimportant for cell survival, or could be protective per se (14). An example of how preserving intracellular pH is protective for the apoptotic cell death pathway is found in hematopoietic stem cells where granulocyte-macrophage colony stimulating factor (GM-CSF) delays apoptosis by cytoplasmic alkalinization through activation of the Na^+/H^+ exchanger and in neutrophils where granulocyte colony stimulating factor (G-CSF) delays apoptosis by activation of the vacuolar proton ATPase (VPATPase) (15–17). The VPATPase is known to be activated by PKC (15, 18, 19), suggesting it could be activated during preconditioning.

Until recently, only necrosis was recognized as a cell death pathway in cardiomyocytes. Apoptosis and necrosis are distinct mechanisms of cell death; however, both have been implicated in myocardial ischemia/reperfusion injury. Apoptosis is the morphologic description of several processes involved in programmed cell death. We recently found that myocardial ischemia and reperfusion in intact rabbit myocardium induced apoptosis in cardiomyocytes (20), and Tanaka et al. found that hypoxia induces apoptosis in neonatal rat cardiomyocytes (21).

We considered the hypothesis that preconditioning might involve activation of ion pumps to reduce acidosis, and thus be protective by preventing myocyte cell death by apoptosis. While reduced H^+ production in preconditioned ischemic

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1. *Abbreviations used in this paper:* BAF, bafilomycin A_1; EIPA, (5-*N*-ethyl-*N*-isopropyl) amiloride; ICE, IL-1-beta converting enzyme; MI, metabolic inhibition; NHE-1, Na^+/H^+ exchanger; PKC, protein kinase C; R, recovery; VPATPase, vacuolar proton ATPase.

muscle is also a likely and tenable hypothesis, the basis for the work we now report comes from consideration of mechanisms to export H^+ from the cell.

Extruding H^+ by activation of the Na^+/H^+ exchanger during reperfusion is thought to be detrimental because this mechanism increases intracellular Na^+, which in turn increases Ca^{++} through Na^+/Ca^{++} exchange (22–26). Calcium overload can lead to necrosis, perhaps through hypercontraction and sarcolemma disruption (25, 27) or activation of other detrimental processes (28, 29). Inhibition of Na^+/H^+ exchange during ischemia and reperfusion reduces arrhythmias (30), Ca^{++} overload (23, 26), hypercontraction (22, 25, 31), and cell death (25, 32). The blockade of the Na^+/H^+ exchanger (NHE-1) is effective only when applied during ischemia; blockade only during reperfusion has little effect. The protective effect is thought to be mediated by inhibiting Na^+ entry, thus limiting Ca^{++} entry by Na^+/Ca^{++} exchange (23, 25, 26). A mechanism of preconditioning whereby activation of NHE-1 would reduce acid accumulation and protect myocytes seems unlikely because (*a*) enhanced Na^+ and Ca^{++} accumulation would be detrimental and (*b*) inhibition of this channel has little effect on pH during ischemia (33–38).

Based on our findings in neutrophils (15), we considered another potential mechanism for reduction of H^+ accumulation: activation of the VPATPase. We used an isolated myocyte system to simulate ischemia through metabolic inhibition with cyanide and 2-deoxyglucose. This model system was found to exhibit preconditioning through a PKC-dependent pathway. Our results identify the VPATPase as a potential target of protein kinase C in mediating preconditioning and suggest that pH regulation is a critical feature of preconditioning in our model system.

Methods

Cell isolation and culture. All studies were approved by the Veterans Affairs Medical Center Animal Committee, an AAALAC accredited institution. Rabbits of either sex, 2–2.5 kg, were tranquilized with acepromazine, xylazine and ketamine, anticoagulated with 1,000 U heparin, then killed with 100 mg pentobarbital i.v., and the heart was rapidly excised. The heart was attached to a perfusion apparatus via the aorta. Calcium-free buffer was infused for 5 min at 60 mmHg constant pressure and 37°C containing the following: 118 mM NaCl, 2.6 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 25 mM $NaHCO_3$, 11.1 mM glucose. Perfusion was switched to a collagenase buffer containing the same electrolytes as above plus 4.7 μM $CaCl_2$ and collagenase, Worthington type II, 110 U/ml. After 30 min, the left ventricle was trimmed free of other tissue, minced, and strained through cell dissociation mesh size 50 (Sigma Chemical Co., St. Louis, MO). The isolated myocytes were then enriched for viable cells by layering on a Ficoll cushion (SG 1.077) and centrifuging for 4 min at 250 *g*. Cells recovered from the interface consist of 75–90% rod-shaped viable myocytes. The cells were allowed to rest in DME with 5% FBS, streptomycin, penicillin, and Fungizone (culture medium) for 30 min before use in experimental protocols. Cells were transferred to culture medium in 15 ml conical tubes for experimental protocols. The percentage of viable cells remained stable when cultured for 1–3 d in culture medium in a 5% CO_2 incubator at 37°C. After 3 d, cell viability began to decline.

Viable cells. In all subsequent analyses, a viable myocyte is defined as a rod-shaped, striated cell. Hypercontracted myocytes (reduced length to width ratios) were also considered viable but generally represented $< 2\%$ of the total population. Rounded or irregular shaped cells with loss of striations were considered nonviable. In preliminary experiments comparison with other measures of viability, including tetrazolium salt (MTT) reduction and trypan blue exclusion indicated that all rod-shaped cells met these criteria for viability. Depending on conditions, as many as half of the cells which were rounded also reduced MTT. Rod-shaped morphology is therefore the strictest criteria but may tend to underestimate viable cells. The observer was blinded to conditions, and interobserver variation was $< 10\%$.

Scoring of viable cells. After either 4 h or overnight recovery, cells were fixed in 4% formalin and placed on microscope slides. Approximately 150 cells were counted for determination of viability by the rod-shaped criteria by an observer blinded to conditions. Interobserver variability was $< 10\%$. Results are presented as percent viable relative to control cells.

Nick translation assay. In situ nick translation assay was performed to estimate the number of cells undergoing DNA fragmentation. Formalin-fixed cells (4% for 15 min) were washed with PBS twice and stored in cold 70% EtOH. After resuspension in PBS, cells were exposed to proteinase K (20 μg/ml; Gibco BRL, Gaithersburg, MD) for 15 min at room temperature, washed, and suspended in terminal deoxynucleotidyl transferase (TdT) reaction buffer (200 mM K Cacodylate, 25 mM Tris, 0.25 mg/ml BSA., 2.5 mM $CaCl_2$, at pH 6.6) for 1 h at 37°C with terminal deoxynucleotide transferase 0.3 U/μl. The reaction was initiated with 4 μM biotin-dATP (Gibco BRL). The TdT was omitted for negative controls. Positive controls were pretreated with DNase I, 10 U/ml for 10 min at 37°C. The assay was developed with alkaline phosphatase–conjugated streptavidin (Dako Corp., Santa Barbara, CA, 1:100 dilution, 30 min, room temp.). Slides were washed with PBS followed by 0.1 M Tris (pH 9.5) and incubated with substrate (SK 5400; Vector Laboratories Inc., Burlingame, CA) for 30 min.

Metabolic inhibition protocol. Metabolic inhibition (MI) was induced by placing the cells in the following buffer: 106 mM NaCl, 4.4 mM KCl, 1.0 mM $MgCl_2$, 38 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 20 mM 2-deoxyglucose, 1.0 mM NaCN, at pH 6.6. In preliminary experiments, we established that a 30-min exposure caused a reproducible injury that resulted in a reduction of viability of $\sim$ 30–40% when scored 4 h later and that preconditioning was very effective in improving viability.

Preconditioning protocol. Preconditioning in isolated myocytes was accomplished by a 2-min period of metabolic inhibition followed by washout of the MI buffer by 2 solution changes in culture medium and 5 min rest.

Experimental protocols. For technical reasons no more than ~ 15 conditions could be tested in a single experiment. In each experiment at least one culture medium control, one control for the intervention being performed in the absence of MI, and when appropriate, controls for DMSO were performed. At most, two experiments could be carried out on cells from one heart, one on the day of harvest and one on the following day, when cell viability did not change appreciably overnight. In each experimental group, the number of experiments indicates the number of hearts used. For all experiments, cells were transferred to culture medium and maintained at 37°C in a 5% CO_2 atmosphere. In all experiments, preconditioning was performed for 2 min, followed by 5 min recovery, metabolic inhibition for 30 min, followed by recovery for 4 h. For detection of DNA fragmentation by nick translation assay, and amiloride inhibition studies, recovery was extended to 16–20 h (overnight). The percentage of viable cells does not change after an additional 20-h recovery (Fig. 1), but the nick translation assay signal is stronger. The following interventions were used to assess their effect on preconditioning and/or MI; (*a*) PMA, 100 nM pretreatment, and continued exposure during MI/recovery; (*b*) bafilomycin A_1 (50 nM) (a macrolide antibiotic specific VPATPase inhibitor (39–41); (*c*) chelerythrine, 2 μM, (protein kinase C inhibitor); (*d*) DMSO, 1:500 (0.2%) in culture medium as a control for bafilomycin A_1 (*e*) amiloride 10 μM, (an inhibitor of the sodium/hydrogen exchanger); (*f*) (5-*N*-ethyl-*N*-isopropyl) amiloride (EIPA) (10 μM, (a more potent NHE-1 inhibitor); (*g*) Z-VAD-fmk (fluoro-methylketone) (Kamiya Biomedical,

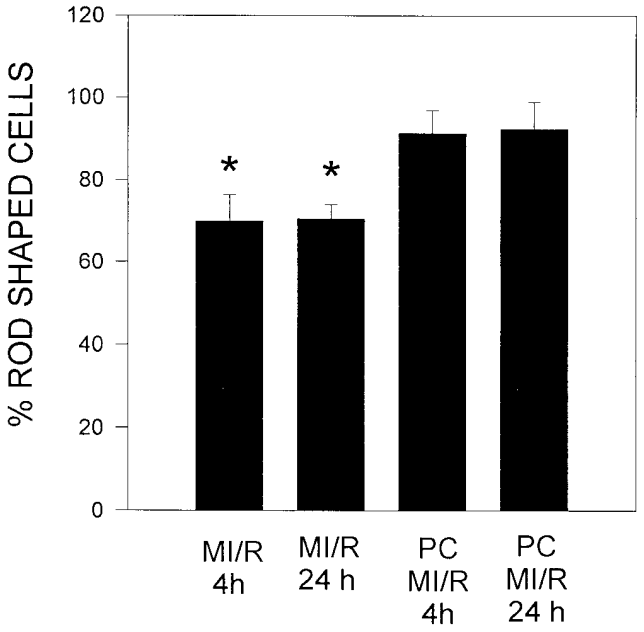

Figure 1. Viability of isolated myocytes 4 and 24 h after metabolic inhibition (*MI*) and recovery (*R*). Myocytes were subjected to metabolic inhibition for 30 or 2 min preconditioning (*PC*) before MI, then returned to culture medium for 4–24 h of recovery. Viability is presented as percent rod-shaped cells (relative to untreated control), mean±SD ($n = 7$; $^*P < 0.05$ for MI versus PC).

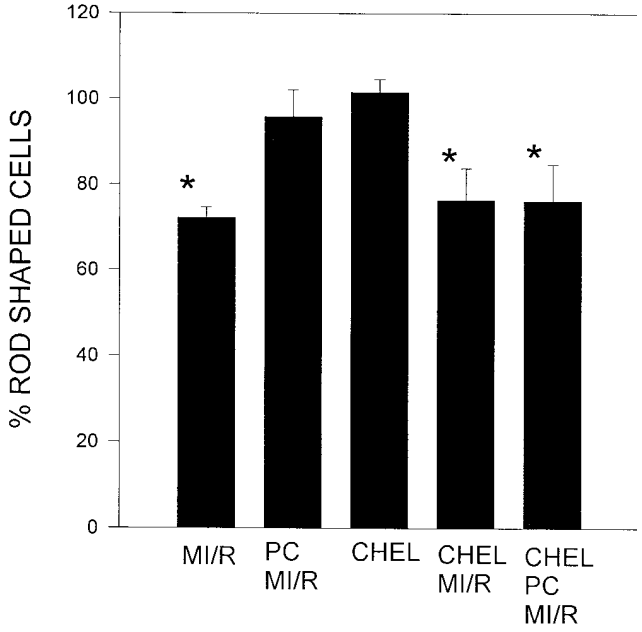

Figure 3. Effect of chelerythrine (*CHEL*) on preconditioning. Where indicated, myocytes were pretreated with CHEL (2 μM), then subjected to preconditioning (*PC*) and/or metabolic inhibition (*MI*) followed by 4-h recovery (*R*). Viability is relative to untreated controls, mean±SD. ($n = 4$; $^*P < 0.05$, PC-MI/R versus CHEL-PC-MI/R, and PC-MI/R versus MI/R or CHEL-MI/R.)

Thousand Oaks, CA), 100 μM, (a tetrapeptide inhibitor of the interleukin-1-beta converting enzyme [ICE] family of cysteine proteases) (42).

Myocyte pH measurement. Myocytes were isolated as described. Cells cultured 24 h after isolation were studied to measure intracellular pH during MI, preconditioning MI, and bafilomycin A_1/preconditioning/MI with essentially the same protocol as above, except that Hepes-based buffers (rather than bicarbonate) were used and bafilomycin A_1 was 500 nM. Intracellular pH measurements were per-

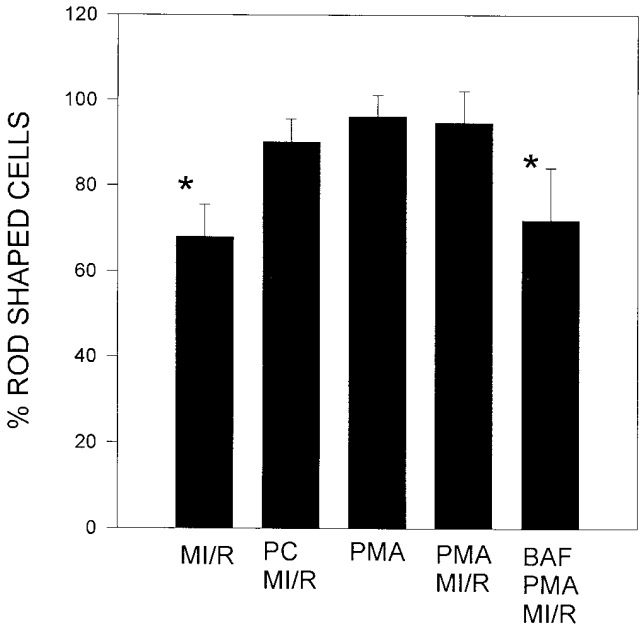

Figure 2. Effect of bafilomycin A_1 (BAF) on preconditioning mediated by phorbol esters. Myocytes were incubated with or without BAF (50 nM) 5 min before PMA (100 nM), then subjected to metabolic inhibition (30 min) and recovery (4 h). Viability is relative to untreated controls, mean±SD. ($n = 10$; $^*P < 0.05$, PMA-MI/R versus BAF-PMA-MI/R, and MI/R versus PC-MI/R or PMA-MI/R.)

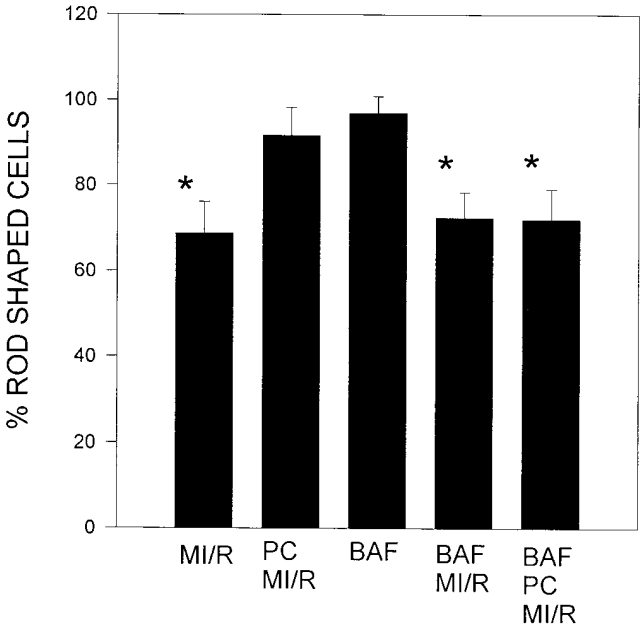

Figure 4. Effect of bafilomycin A_1 on preconditioning. Where indicated, cells were pretreated with BAF (50 nM), then subjected to preconditioning and/or metabolic inhibition (30 min) and recovery (4 h). Viability is relative to untreated controls, mean±SD. ($n = 10$; $^*P <$ 0.05 PC-MI/R versus BAF-PC-MI/R or BAF-MI/R or MI/R.)

formed on cells which had been loaded for at least 30 min with 20 μM BCECF-AM (Molecular Probes Inc., Eugene, OR) using dual excitation (440, 495 nm) ratiometric image analysis (MCID; Imaging Research Inc., St. Catherines, CN) on a fluorescence microscope. Measurements were made once every minute beginning 5 min before MI and finishing 5 min after the addition of recovery buffer.

Statistical analysis. All results for cell viability are expressed as percentage of the simultaneous appropriate control±SD to account for minor day to day variability (e.g., for the effect of bafilomycin A_1 on preconditioning, the bafilomycin A_1 preconditioning/MI results are expressed as percentage of bafilomycin A_1/culture medium control). Unpaired comparisons across experimental groups were not performed. Comparison within experimental groups for viability or nick translation assay was by ANOVA with post hoc Tukey test. Isolated myocyte pH data was tested by repeated measures ANOVA and post hoc Duncan multiple range test.

Results

Experimental model of preconditioning. We wished to establish conditions in which we could simulate ischemia/reperfusion and preconditioning. 30 min of MI followed by 4 h of recovery (R) resulted in loss of viability down to 70±6% of control characterized by rounded morphology. DNA fragmentation at 24 h of recovery after MI was detected by nick translation assay in 28±8% of cells. Recovery for 24 h did not alter the viability or the effect of preconditioning (Fig. 1). Consistent with earlier work in intact rabbit hearts, changes in nuclear morphology could not be detected at the light microscope level. Preconditioning preserved viability after MI (91±5%), an effect also observed with pretreatment with the PKC activator PMA (95±7%) (Fig. 2). PMA pretreatment alone or pretreatment and continued PMA during MI were equally protective (data not shown). Thus, preconditioning and phorbol ester treatment protected cells from metabolic inhibition. Chelerythrine, 2 mM, an inhibitor of PKC, completely blocked the protective effect of preconditioning (Fig. 3). These results parallel those reported by others in the intact heart and suggest that the signal transduction pathway mediating preconditioning involves PKC. To examine the role of the VPATPase in preconditioning, we used the potent and specific inhibitor bafilomycin A_1.

Bafilomycin A_1, alone in culture medium did not affect cell viability (97±4%) relative to control, and bafilomycin A_1 did not worsen viability after metabolic inhibition (72±6%) (Fig. 4). In this series of experiments, preconditioning retained cell viability at 90±5%. Furthermore, Bafilomycin A_1 pretreatment blocked the effect of preconditioning (71±7%), as well as the protective effect of PMA (72±11%) (Fig. 2). These findings suggest that the protective effect of preconditioning, whether induced by brief metabolic inhibition or by direct activation of PKC, requires activity of the VPATPase.

To evaluate the contribution of Na^+/H^+ exchange to myocyte viability after MI, we used amiloride and the more specific analogue EIPA. Both amiloride and EIPA preserved myocyte viability after metabolic inhibition (Fig. 5). Bafilomycin A_1 abrogated the protective effect of blockade of the Na^+/H^+ exchange, suggesting that inactivation of both mechanisms of proton extrusion has adverse effects on cell viability after MI.

Intracellular pH changes before and during metabolic inhibition. We loaded cardiomyocytes with 10 μM BCECF and used ratiometric fluorescence microscopy to monitor intracellular pH changes during metabolic inhibition, preconditioning, and preconditioning with bafilomycin A_1. We found that the resting pH of cardiomyocytes measured in a Hepes-based buffer is ~ 7.2 and that within 5 min of initiation of metabolic inhibition, the intracellular pH decreases to ~ 6.9 (Fig. 6). Myocyte pH decreased by 0.44 pH units from 7.28±0.21 to

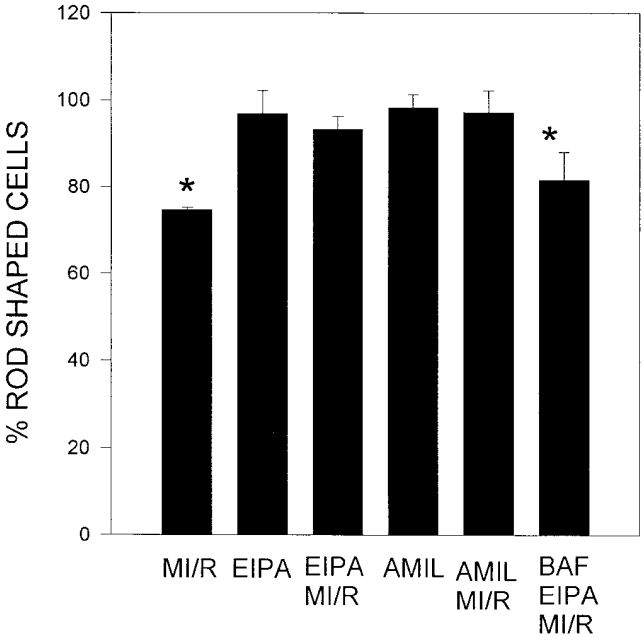

Figure 5. Effect of amiloride (*AMIL*) and EIPA on viability after metabolic inhibition and recovery (*MI/R*). Cardiomyocytes were treated with AMIL (10 μM) or EIPA (10 μM) throughout MI and 4-h recovery. Where noted, bafilomycin (*BAF*, 50 nM) was also included. Viability is relative to untreated controls, mean±SD. ($n = 4$ *$P <$ 0.05 EIPA-MI/R or AMIL-MI/R versus MI/R or BAF-EIPA-MI/R.)

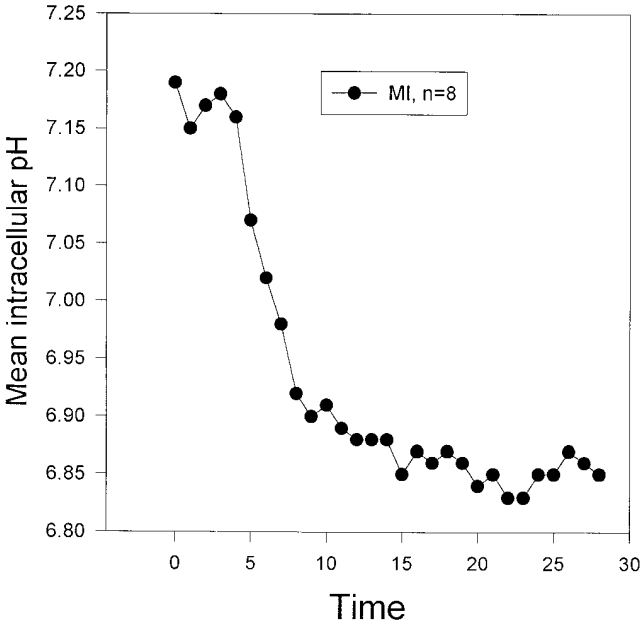

Figure 6. Time course of pH change during metabolic inhibition in isolated myocytes. Cells were loaded with BCECF-AM in Hepes-based buffer, then monitored for pH by dual excitation fluorescence microscopy before and during metabolic inhibition (see Methods). The buffer was changed to one containing 2-deoxyglucose and NaCN at 4 min.

6.84±0.09 at 20 min of MI. Preconditioning (2 min of metabolic inhibition, 15 min of recovery) attenuated the extent of acidification to 0.2 pH units, resulting in a drop from 7.20±0.13 to 7.00±0.13 over the same time interval. To examine the effect of bafilomycin A_1 on intracellular pH of preconditioned cells, cardiomyocytes were incubated for 5 min with bafilomycin A_1 (500 nM), then subjected to preconditioning, recovery, and MI. Bafilomycin A_1 was included throughout. The mean intracellular pH during MI fell 0.63 pH units from 7.26±0.15 to 6.63±0.09 (Fig. 7). Thus, bafilomycin A_1 negated the effect of preconditioning on intracellular pH.

Evidence for apoptosis: nick translation assay/Z-VAD. Incubation of cells for 24 h in culture medium resulted in 7.6±3.6% of nuclei with DNA nicking. Metabolic inhibition and 24-h recovery caused nicking in 28.8±7.5%. Preconditioning reduced nuclear DNA damage to 17.7±8.6% of cells ($P < 0.05$ versus MI/R) but did not prevent it completely ($P < 0.05$ versus control). The reduction in DNA nicking by preconditioning was seen in both round and rod-shaped cells (Fig. 8). To examine further the possibility that the cell death observed with MI/R was apoptosis, we tested the requirement for ICE protease activity. Myocytes were preincubated for 45 min in 10 or 100 μM Z-VAD-fmk. Metabolic inhibition resulted in 79±3% viability, preconditioning improved viability to 97±8%, pretreatment with Z-VAD-fmk 100 μM improved viability after MI/R to 95±8% and Z-VAD-fmk 10 μM improved viability to 88±1%. To the extent that ICE family protease activation is a specific requirement of apoptosis, these findings suggest that apoptotic cell death occurs in cardiomyocytes subjected to MI/R.

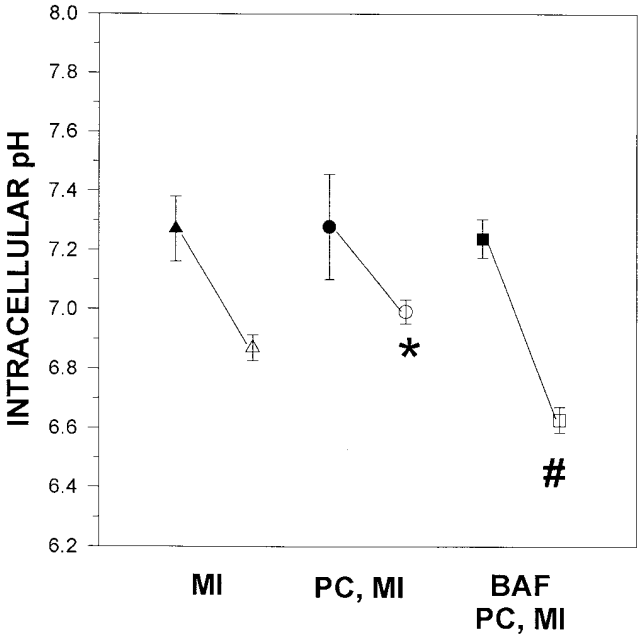

Figure 7. Effect of interventions on myocyte pH_i before and after 20 min of metabolic inhibition (*MI*). Cells underwent no pretreatment (△), preconditioning (○), or pretreatment with bafilomycin A_1 (500 nM) before preconditioning (□), then pH was monitored during metabolic inhibition. Solid symbols represent pH values before MI, and open symbols represent pH values after 20 min of MI. ($n = 9$ for MI, 10 for PC-MI, and 13 for BAF-PC-MI; pH values before MI were not different; at 20-min MI: $^*P < 0.05$ PC-MI versus MI; $^\#P < 0.05$ BAF-PC-MI versus MI or PC-MI).

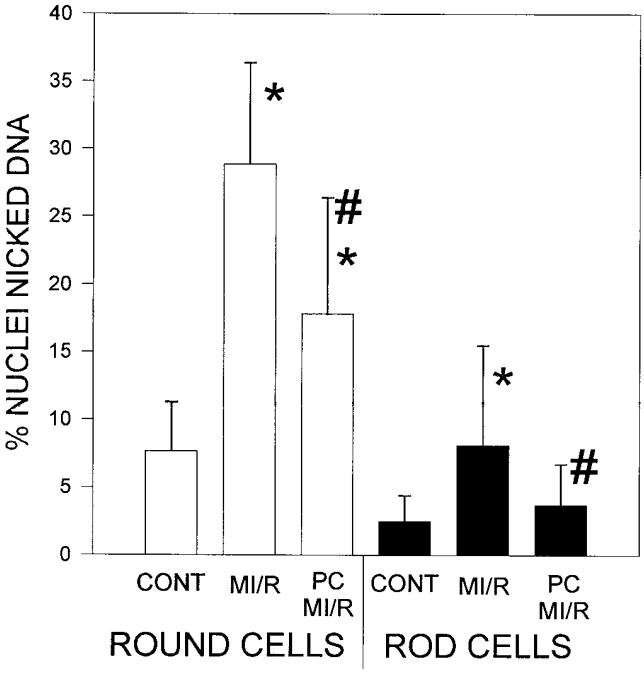

Figure 8. Presence of nicked DNA in nuclei of myocytes subjected to metabolic inhibition. DNA fragmentation in cardiomyocytes was detected by terminal transferase 16–24 h after recovery from metabolic inhibition (30 min). Where indicated, cells were subjected to preconditioning (2 min) before metabolic inhibition. The percentage (mean±SD) of cells with positively labeled nuclei (indicating DNA fragmentation) are shown for both rounded-up cells (*open bars*) and rod-shaped cells (*solid bars*). ($n = 5$; $^*P < 0.05$ versus control (*CONT*); $^\#P < 0.05$ versus MI/R.)

Discussion

Our main findings are: (*a*) the VPATPase is activated by preconditioning, (*b*) VPATPase contributes to preservation of myocyte pH during metabolic inhibition, (*c*) preconditioning may act through PKC to activate the VPATPase, and (*d*) MI/R causes preventable cell death that may be apoptotic, based on reduced DNA nicking after preconditioning and reduced cell death by the Z-VAD-fmk inhibitor of ICE.

Myocardial ischemia and reperfusion is accompanied by profound ionic alterations, including acidification, accumulation of sodium, and elevation of cytosolic calcium. During the acute phase of ischemia, acidification occurs as a result of anaerobic metabolism. One consistently identified feature of preconditioning is the attenuation of intracellular acidification during ischemia (or in isolated myocytes, during metabolic inhibition). Since our studies (15, 43) and those of others (16, 17, 44–47) have indicated that acidification is a general feature of apoptotic cell death, and since preconditioning is known to minimize the drop in pH observed during ischemia or metabolic inhibition (1, 10–14), we reasoned that preconditioning might directly affect mechanisms of pH homeostasis. Intracellular pH is largely maintained through activity of the sodium/hydrogen exchanger, the chloride/bicarbonate exchanger, and under certain conditions, the VPATPase. We have previously shown that G-CSF–mediated protection from apoptosis in neutrophils depends on activity of the VPATPase, and it has been shown (18, 19) that phorbol esters rapidly activate the

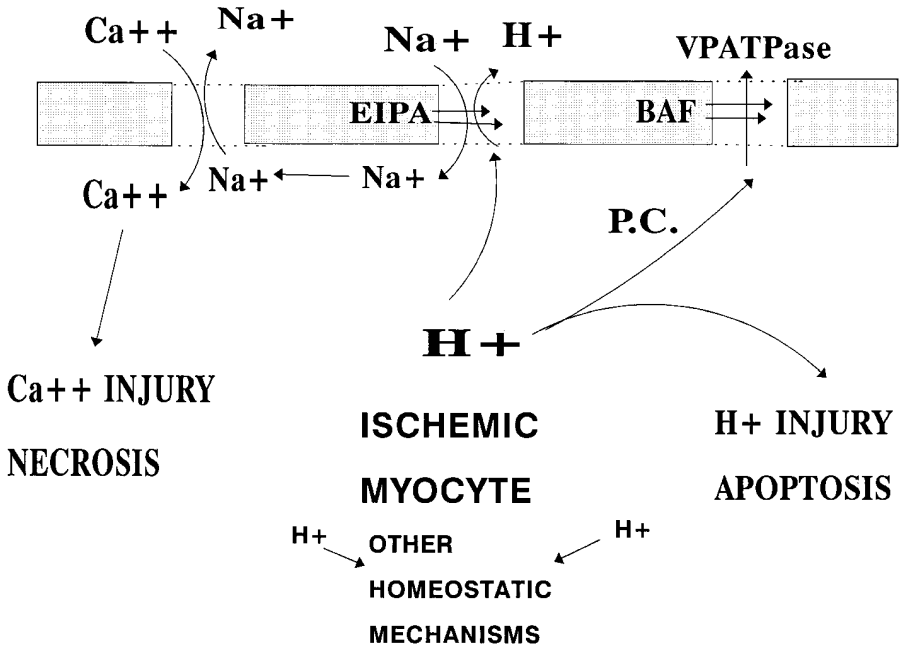

Figure 9. Proposed interaction of Na^+/H^+ exchange and VPATPase in two cell death pathways, apoptosis and necrosis. Inhibition of the Na^+/H^+ exchanger prevents sodium accumulation and secondary calcium influx which would lead to calcium injury and necrosis. Preconditioning activates the VPATPase to extrude protons, thereby minimizing sodium influx. Extrusion of protons prevents apoptosis. EIPA inhibits the Na^+/H^+ exchanger, and bafilomycin A_1 (*BAF*) inhibits the VPATPase.

VPATPase. We therefore considered the possibility that preconditioning might be mediated through activity of the proton pump. To test this, we made use of bafilomycin A_1, which is a potent and specific inhibitor of the VPATPase (39–41, 48, 49).

Bafilomycin A_1 does not inhibit the Na^+/H^+ exchanger, the K^+/H^+ exchange of *Manduca sexta* or other ATPases (50–53). The VPATPase is associated with lysosomal vacuoles and the plasma membrane (54–56). Bafilomycin A_1 prevented the protective effect of preconditioning on myocyte viability (Fig. 4) and increased the degree of acidification during MI (Fig. 7). These data indicate that the VPATPase is activated by preconditioning and contributes to improved viability by attenuating the degree of acidosis. In addition, inhibition of the VPATPase with bafilomycin A_1 abolishes the protective effect conferred by phorbol ester treatment, suggesting that activity of the VPATPase is the most important target of protein kinase C in this model system of preconditioning.

It is possible that inhibition by bafilomycin A_1 of proton sequestration in acidic organelles would exacerbate injury by metabolic inhibition, regardless of preconditioning. This consideration is based on the observation that protons leak out of acidic organelles during ATP depletion (40) with consequent worsening of acidosis. Several observations argue against this, however: (*a*) bafilomycin A_1 pretreatment has no effect on viability of control cells; (*b*) bafilomycin A_1 does not worsen the effect of metabolic inhibition, suggesting that it is not an additive injury; (*c*) bafilomycin A_1 pretreatment has no effect on the resting pH of the cells; and (*d*) bafilomycin A_1 added 5 min after preconditioning (rather than before) does not interfere with the cell viability protection afforded by preconditioning (data not shown). PKC activation of the VPATPase may render it resistant to subsequent inhibition by bafilomycin A_1. These findings suggest that bafilomycin A_1 pretreatment specifically interferes with preconditioning. Since bafilomycin A_1 is a specific inhibitor of the VPATPase, these findings indicate that preconditioning requires activity of the vacuolar proton pump to prevent cytoplasmic acidification during subsequent metabolic inhibition.

A number of investigators have shown that inhibition of the sodium/hydrogen exchanger is protective against metabolic inhibition (22–26). Since both VPATPase and NHE-1 function to extrude protons and preserve intracellular pH, why is inhibition of NHE-1 protective, while inhibition of VPATPase is deleterious? One possible explanation may relate to the nonspecificity of the amiloride derivatives, which also inhibit diamine oxidase (57), *src* family tyrosine kinases (58), and cytotoxicity mediated by tumor necrosis factor (59). Alternatively, the protective effect of inhibition of NHE-1 may relate to secondary effects on ion homeostasis (Fig. 9). During ischemia, sodium will accumulate intracellularly due to activity of the NHE-1 and inactivity of the Na^+/K^+-ATPase (due to energy depletion). Sodium homeostasis is maintained through activity of the Na^+/Ca^{++}-exchanger, leading to calcium overload (25, 26, 28, 29). This process has been offered to explain the so-called "pH paradox", (25) in which external acidosis is protective during reperfusion, possibly by limiting calcium influx (26, 60). This was previously suggested by the finding that acidosis was protective by preventing calcium-dependent injury on the thick ascending limb of Henle (61). If, however, during preconditioning, the vacuolar proton pump is responsible for proton extrusion (at least until ATP stores are more severely depleted), then less sodium (and consequently less calcium) will accumulate. Consistent with this mechanism, blockade of Na^+/H^+ exchange during ischemia does not result in any change in the intracellular pH (23, 25, 29, 37), indicating that the VPATPase and other mechanisms are able to compensate for the increased H^+ load. When myocardium is preconditioned, the protective effect of Na^+/H^+ blockade is significantly enhanced as would be expected from the proposed interaction in Fig. 9 (32). When both Na^+/H^+ exchange and VPATPase are blocked, the beneficial effect on cell survival of NHE-1 blockade is lost. In contrast to these studies where extracellular acidosis is beneficial for survival, intracellular aci-

dosis reduces stunning after nonlethal injury by undefined mechanisms (62).

The deleterious effects of elevated intracellular calcium are well documented and include activation of phospholipases, proteases, endonucleases, and disruption of mitochondrial function and cytoskeletal architecture (28, 29). However, the abrupt rise in pH precipitated by reperfusion appears to be injurious, independent of calcium influx in studies of neonatal cardiomyocytes subjected to 3 h of metabolic inhibition (25). Bond et al. have suggested that acidosis attenuates the activity of proteases and other degradative enzymes which have pH optima above 7.0. Calcium release from mitochondria via the mitochondrial permeability transition pore would also be attenuated in settings in which the matrix pH drops below 7.0 (63, 64). In contrast, there is evidence that acidosis induces delayed cell death (65), and we have shown that prevention or attenuation of acidosis protects cells from apoptosis (15, 43). This may relate to the fact that there are a number of enzymes implicated in the process of apoptosis that have pH optima well below 7.0 including the acid endonuclease (66, 67), acidic sphingomyelinase (68), tissue transglutaminase (69), and gelsolin (70). Thus the short-term protection observed by Bond et al. might prevent immediate cell death (necrosis?) but may not ameliorate delayed cell death (apoptosis?).

Our results by no means exclude other mechanisms of preconditioning. For example, reduction in anaerobic glycolysis is recognized as an effect of preconditioning which can reduce H^+ production. After longer periods of MI or during in vivo ischemia, other mechanisms of cell injury counteracted by preconditioning may well be active that play only a minor role in our model system.

Apoptosis occurs during myocardial ischemia and reperfusion (20) and during hypoxia/reoxygenation (21), and must be considered along with necrosis. Although these two forms of cell death involve different pathophysiologic mechanisms, both may proceed in a single cell. Our finding of reduced DNA nicking when preconditioning precedes metabolic inhibition suggests that apoptosis was reduced. However, the reduced DNA damage was present in both round and rod-shaped cells. Rod-shaped cells would be considered viable using the cell shape criteria established and validated by tetrazolium staining, these cells probably go on to late cell death. Round cells suggest loss of membrane integrity and necrotic cell death. In our studies of in vivo ischemia and reperfusion in the rabbit heart, we also found that the nick translation assay can be positive for DNA damage in cells that appear by other criteria to have undergone necrosis. The finding that Z-VAD-fmk reduced cell death after MI/R helps to confirm that apoptosis contributes to injury after MI/R. Z-VAD-fmk is a potent inhibitor of ICE and related proteases critically involved in apoptosis (42). Thus, one may not be able to distinctly differentiate between necrosis and apoptosis during ischemia and reperfusion because both cell death pathways may be involved (Fig. 9). However, protection by preconditioning indicates that regardless of nomenclature, cell death consequent to MI/R is preventable, and that this is critically dependent on ion homeostasis.

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