Mechanisms of pH preservation during global ischemia in preconditioned rat heart: roles for PKC and NHE

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Rehring, Thomas F., Joseph I. Shapiro, Brian S. Cain, Daniel R. Meldrum, Joseph C. Cleveland, Alden H. Harken, and Anirban Banerjee. Mechanisms of pH preservation during global ischemia in preconditioned rat heart: roles for PKC and NHE. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H805–H813, 1998.—Ischemic preconditioning (PC) attenuates cardiac acidosis during global ischemia. This adaptation to ischemia is detectable before other better known indexes of PC are manifested. Clarification of the endogenous mechanisms may provide insights into how protein kinase C (PKC) signaling might be linked to altered intracellular biochemistry.31P NMR studies of isolated, buffer-perfused rat heart were performed to determine whether functionally cardioprotective PC by cyclic ischemia (CI) and α1-adrenergic stimuli [phenylephrine (PE)] attenuated acidosis during ischemia and, if so, whether this 1) involves a PKC-dependent pathway and is due to 2) decreased glycolytic proton production, 3) an increase in proton buffering, or 4) proton extrusion. At the end of 20 min of global ischemia, both CI-PC (pH: 6.68 ± 0.14) and PE-PC (pH: 6.90 ± 0.13) attenuated end-ischemic acidosis (control pH: 6.54 ± 0.1). PKC blockade with chelerythrine (Chel) and HOE-694 eliminated the attenuation of ischemic acidosis by PC stimuli (end-ischemic pH: CI + Chel, 6.43 ± 0.06; PE + Chel, 6.17 ± 0.17). End-ischemic lactate accumulation was decreased in CI-PC hearts (7.54 ± 0.5 vs. control, 14.61 ± 2.1 µmol/g wet wt) but not in those preconditioned through the α1-adrenergic receptor (12.25 ± 0.9 µmol/g wet wt). Physiologically relevant buffers were not increased in the preconditioned groups. Blockade of the Na+/H+ exchanger [NHE; with 5-(N-ethyl-N-isopropyl)amiloride (EIPA) or HOE-694] eliminated the attenuation of ischemic acidosis seen with PC stimuli (pH: CI + EIPA, 6.5 ± 0.1; PE + EIPA, 6.46 ± 0.2; PE + HOE-694, 6.26 ± 0.15; not significantly different from control). We conclude that CI and α1-adrenergic PC stimuli attenuate ischemic acidosis, and this may involve the cardiac amiloride-sensitive NHE. The signaling pathways of both these stimuli appear to involve PKC.

Although PKC can phosphorylate numerous myocardial proteins, its role in the regulation of ischemic physiology is unclear.

An attractive mechanism proposed for ischemic injury implicates acidosis as a trigger (26, 27). In this view, unbalanced H+ production in the ischemic myocardium stimulates Na+/H+ exchange, adding to Na+ influx. Increased intracellular Na+ concentration ([Na+]i) load can subsequently cause an increase in cytosolic free Ca2+ concentration ([Ca2+]i) by a variety of mechanisms, including both influx and endogenous release (22, 26, 27). Interventions that delay the rise in [Ca2+]i correspondingly delay the onset of myocardial necrosis.

An early and reproducible benefit of CI-PC that can be detected even before reperfusion is a reduction of intracellular acidosis during ischemia (1, 3, 25, 27, 35). On the basis of evidence of decreased glycolytic H+ production, Steenbergen et al. (27) proposed that by maintaining a higher intracellular pH (pHi) during ischemia, the Na+/H+ exchanger (NHE) would be less active. By maintaining lower [Na+]i until end ischemia, less Ca2+ should be exchanged into the cell. Therefore, the protected myocardium would exhibit diminished necrosis and improved mechanical function. Indeed, CI-PC prevents the deleterious increases in [Na+]i and [Ca2+]i that occur during ischemia (27, 31).

Although this interpretation fits well with the data obtained, decreased intracellular H+ concentration ([H+]i) during global ischemia is possible as a result not only of decreasing production (reflected by lactate) but also of increasing intracellular buffering or proton extrusion (9, 26).

Our group and others have shown that α1-adrenergic PC protects postischemic function in rat heart (4, 12, 30, 32). This mechanism involves PKC (12, 16, 30). α1-Adrenoceptor agonists such as phenylephrine (PE) produce intracellular alkalization, possibly via indirect stimulation of the NHE (22). We hypothesized that both CI or PE preconditioning stimuli would attenuate ischemic acidosis, perhaps by similar mechanisms mediated by PKC. Specifically, we determined whether 1) CI and α1-adrenergic PC stimuli attenuate ischemic acidosis, 2) both stimuli limit ischemic acidosis via a PKC-dependent pathway, 3) CI and α1-adrenergic PC decrease ischemic proton production (as measured by lactate accumulation), 4) CI- and α1-adrenergic-induced limitation of ischemic acidosis involves buffering by producing increased P4, and 5) CI- and α1-adrenergic-induced limitation of ischemic acidosis is due to proton extrusion by the NHE.
METHODS

Materials. Male Sprague-Dawley rats (weight 300–350 g; Sasco, Omaha, NE) were fed a standard diet and quarantined in a quiet environment for 14 days before experimentation. The animal protocol was reviewed and approved by the Animal Care and Research Committee of the University of Colorado Health Sciences Center. PE was obtained from American Regent Laboratories (Shirley, NY). Chelerythrine chloride (Chel) was from LC Laboratories (Woburn, MA). 5-(N-ethyl-N-isopropyl) amiloride (EIPA) was obtained from Research Biochemicals International (Natick, MA). (3-Methylsulfonyl-4-piperidinobenzoyl)guanidinio methanesulfonate (HOE-694) was a generous gift from Dr. H. J. Lang (Hoechst-Roussel Pharmaceuticals, Somerville, NJ). All other reagents utilized were obtained from Sigma Chemical (St. Louis, MO).

Isolated rat heart ischemia-reperfusion. After appropriate anesthesia (pentobarbital sodium, 60 mg/kg ip) and anticoagulant (heparin, 500 U ip) were administered, hearts were excised and placed in a 4°C buffer solution. Within 45 s, hearts were retrogradely perfused in the nonworking, isovolumetric Langendorff mode (80 mmHg, 37°C) with nonrecirculated phosphate-free Krebs-Henseleit buffer solution (116 mM NaCl, 11 mM glucose, 4.0 mM KCl, 24 mM NaHCO3, 1.2 mM CaCl2, and 1.19 mM MgSO4) saturated with 92.5% O2-7.5% CO2 and achieving a pH of 7.4, a P O2 of at least 450 mmHg, and a PCO2 of 40 mmHg.

A water-filled latex balloon was inserted through the left atrium into the left ventricle and adjusted to a left ventricular end-diastolic pressure (LVEDP) of 8–10 mmHg during the initial equilibration period. This preload volume was not adjusted after initial equilibration. Left ventricular developed pressure was measured with an intraventricular balloon catheter attached to a computerized bridge amplifier/digitizer (MacLab 8, ADInstruments, Milford, MA) and continuously recorded on a Macintosh Quadra 800 minicomputer (Apple Computers, Cupertino, CA) in millimeters of Hg. A 1-mm apical ventriculostomy was created. Global ischemia was induced by stopping flow of buffer to the heart, and the heart was held at 35 ± 0.4°C by flowing thermostatically heated air around the perfusion chamber while the probe was within the bore of the magnet. The experiment protocol is outlined in Fig. 1.

All NMR studies were performed in a 7.05-tesla, 10-cm vertical bore cryomagnet equipped with an AM-300 spectrometer (Bruker Instruments, Billerica, MA). 31P NMR studies of isolated perfused hearts were performed using a constructed loop gap resonator tuned to 121.5 MHz as previously described (28). Free induction decays were collected using 3,000 transients obtained with a 10° pulse angle applied every 0.1 s using a sweep width of 10 kHz (2 K data arrays). These arrays were zero filled to 4 K before exponential multiplication with 10-Hz line broadening, Fourier transformation, and zero-order phase correction. Signal-to-noise ratio exceeded 50:1 on all spectra (Fig. 2). Relative peak areas were determined with the use of a Lorenzian line-fitting routine written in QuickBasic that optimized the fit on the basis of a least-squares method. The relative peak areas were corrected for partial saturation and chemical concentrations reported. pH was determined from the shift of P1 relative to phosphocreatine (PCr) (28). ATP content was determined by integrating the β-phosphate resonance of ATP and was expressed as a percentage of the baseline ATP concentration. PCr and P1 were determined also by measuring the area under the curves corresponding to their respective resonances.

For the determination of lactate and alanine, hearts were rapidly frozen in liquid N2 at the end of ischemia. The ventricles were powdered under liquid N2, weighed, and extracted with perchloric acid (6% wt/vol). The samples were neutralized with KOH to a pH of 7.0 and centrifuged at 12,000 g for 20 min to remove the precipitate. After rapid freezing and lyophilization, the samples were reconstituted in 2H2O and assayed for lactate with the use of a commercial 1H 5-mm probe employing a tip angle of 90°, 10-s relaxation delays, a sweep width of 4,000 Hz, and 8 K data arrays. The free induction decay was not apodized before fourier transfor-
imination and zero-order phase correction. Lactate and alanine were identified on the basis of chemical shift relative to trimethylsilylpropionic acid, quantified relative to this same standard with the use of integration software available on the AM-300 spectrometer console as previously reported, and expressed as micromoles per gram of wet weight (28).

**Experimental design.** A total of 67 animals were examined in this study. Eighteen rats were utilized to assess functional data, and this group was divided into three treatment groups (control, CI, and PE; n = 6 per group) (Fig. 1). Control hearts were perfused for 20 min and then subjected to normothermic global ischemia for 20 min, followed by 40 min of reperfusion (Fig. 1A). A three-way stopcock on the aortic cannula was turned to halt perfusion and initiate global ischemia. Normothermia was maintained by bathing the heart in 37.5°C perfusate during ischemia. Functional recovery of initial developed pressure was utilized as the primary outcome measurement. Preconditioning with CI was established with cessation of perfusate flow for four periods of 5 min, each separated by 5 min of perfusion, followed by 20 min of global ischemia and 40 min of reperfusion (Fig. 1B). A three-way stopcock on the aortic cannula was turned to halt perfusion and initiate global ischemia. Normothermia was maintained by bathing the heart in 37.5°C perfusate during ischemia. Functional recovery of initial developed pressure was utilized as the primary outcome measurement. Preconditioning with CI was established with cessation of perfusate flow for four periods of 5 min, each separated by 5 min of perfusion, followed by 20 min of global ischemia and 40 min of reperfusion (Fig. 1C).

The remainder of the animals were utilized for the determination of pH$_i$, high-energy phosphate levels, and metabolic data. The standard $^{31}$P NMR perfusion protocols for the three groups described were followed until end ischemia, when hearts were freeze-clamped and stored for assay. PKC blockade was established in the PE-treated group with the addition of Chel (0.4 µmol/min, n = 6) commencing 1 min before, throughout, and 1 min after the PE infusion as reported previously (16). In the CI group, Chel was administered during the four intermittent perfusion periods before sustained ischemia (0.4 µmol/min, n = 4). Inhibition of the NHE (26) was accomplished by administering EIPA (0.5 µmol/min) or HOE-694 (20 nmol/min) during the 5 min before the sustained ischemic insult (EIPA, n = 4; CI + EIPA, n = 6; PE + EIPA, n = 6; HOE-694 + PE, n = 7). Eighteen animals were utilized for determination of metabolic data and were divided into three treatment groups (control, CI, and PE, n = 6 per group).

Statistical analysis. Measured parameters are presented as means ± SE. Differences were assessed by using a repeated-factor ANOVA model (with one between factor comprising the treatment and one repeated, within factor).

Differences due to ischemic duration and interaction between factors (groups × time) were detected at P < 0.05 (SUPERANOVA, Abacus Concepts, Berkeley, CA). Selected linear contrasts were performed for group means (i.e., comparisons assessing treatment efficacy and blocker efficacy) with significance accepted at P < 0.001. Subsequently, the data were also reanalyzed using a one-factor ANOVA model (with less power but with the ability to perform Scheffé's post hoc test with significance preset at P < 0.05 (StatView 4.0.1, Abacus Concepts)). The significance detected between groups was similar with both statistical approaches.

**RESULTS**

PC stimuli. On the basis of previous experience with the isolated, perfused rat heart model (4, 16), we utilized 20 min of global, normothermic ischemia, followed by 40 min of reperfusion. We first reproduced the functional protection that we previously reported (4, 16) with α$_1$-adrenergic stimulation and verified improved postischemic function after PC with four cycles of ischemia (5 min of ischemia followed by 5 min of reperfusion) (18, 19, 27, 31). Although a transient decrement in function followed the CI stimulus, function rapidly returned to the equilibration baseline. No difference in preconditioning function was noted between groups. In untreated hearts, reperfusion after 20 min of ischemia resulted in a recovery of 50.2 ± 6.1% of preischemic baseline function. PE-treated hearts, however, recovered 75.3 ± 3.5% of initial function (P < 0.05), whereas hearts pretreated with CI achieved 86.2 ± 1.6% recovery (P < 0.05). Pretreatment with either CI or PE improved LVEDP during postischemic reperfusion. At end reperfusion, LVEDP in untreated hearts was 34.8 ± 2.0 mmHg, in contrast to 17.2 ± 0.7 mmHg (P < 0.05) and 22.8 ± 1.4 mmHg (P < 0.05) for CI- and PE-treated hearts, respectively.

Regulation of ischemic acidosis. Pretreatment of hearts with either CI (Fig. 3) or PE (Fig. 4) attenuated ischemic acidosis. pH$_i$ at the onset of ischemia was similar between groups. The mean pH$_i$ in untreated hearts at the end of ischemia was 6.54 ± 0.10. In contrast, the end-ischemic pH$_i$ fell to only 6.90 ± 0.13 (P < 0.05 vs. control) in PE-treated hearts and 6.86 ± 0.14 (P < 0.05 vs. control) in CI-PC hearts. Indeed, preconditioned pH$_i$ curves diverged from control rapidly and became different after the initial 5 min of ischemia, which is in agreement with the findings of others (27, 35).

We have previously (16) linked PE (at this dose) to PKC activation (4, 16). The attenuation of ischemic acidosis conferred with α$_1$-adrenergic stimuli disappeared when a PKC blocker was infused at the time of the α$_1$-adrenergic stimulus (Fig. 4). Similarly, Chel eliminated the protection against ischemic acidosis conferred by CI pretreatment [end-ischemic pH$_i$: 6.43 ± 0.06, P < 0.05 vs. CI alone, P = not significant (NS) vs. control] (Fig. 3). Chel infusion had no effect on the normoxic pH$_i$. However, during ischemia, the pH$_i$ in hearts treated with a combination of PE + Chel fell rapidly to values more acidic than in untreated control hearts (6.17 ± 0.17 by the end of the ischemic period, P < 0.05 vs. PE alone or control). This increased
Acidosis could be due to a nonspecific effect of the PKC blocker or, more likely, could be peculiar to the PE stimulus (because this difference was not significant vs. the drug alone or the Chel + CI hearts at the end of ischemia).

Effector mechanisms. The changes in tissue lactate and alanine after 20 min of ischemia are shown in Fig. 5. At the end of the ischemic period, untreated hearts produced 14.61 ± 2.09 µmol/g wet wt of lactate. CI-PC hearts generated significantly less lactate (7.54 ± 0.54 µmol/g wet wt, P < 0.05). However, pretreatment with an α₁-adrenergic stimulus did not reduce ischemic lactate accumulation (12.25 ± 0.91 µmol/g wet wt, P = NS). Alanine levels were small and did not differ between any group.

Increases in myocardial Pi from breakdown of high-energy phosphates are shown in Table 1. At the onset of the sustained ischemic insult, all groups revealed similar levels of Pi. Differences between CI and PE did not achieve statistical significance. End-ischemic amounts of Pi were similar between groups, with control hearts producing 25.5 ± 3.1 µmol/g, CI-treated hearts 21.0 ± 2.3 µmol/g, and PE-treated hearts 27.5 ± 2.5 µmol/g (P = NS).
Changes in 31P NMR metabolite spectra during global ischemia in control and conditioned hearts

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<th>10</th>
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<tr>
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Values are means ± SE and are expressed as percentages of baseline values. CI, cyclic ischemia; PE, phenylephrine; PCr, phosphocreatine.

*P < 0.05 vs. control at matched time; †P < 0.05 vs. CI at matched time.

**DISCUSSION**

In different mammalian species, PC protects hearts from postischemic cardiac injury. Although it appears that the proximal signaling pathways might involve PKC (4, 5, 11, 12, 16, 32), the distal effector mechanisms remain elusive. The roles of PKC in cardiac physiology and PC are also under investigation. The PKC family of kinases is itself regulated by combinations of factors and can phosphorylate a wide variety of protein targets in the cell (4, 5, 32). The variety of locations, regulation, and targets suggest that these kinases might comprehend a spectrum of effects.

One of the earliest differences that can be noted between control and PC hearts is the preservation of pH during ischemia (1, 25, 27, 35). In this study we examine the prevailing explanation for this observation. We analyze the intracellular H+ pool during global ischemia as a balance of competing metabolic production and elimination processes (Fig. 6). These mechanisms could be regulated by PKC and other PC signals (4, 10, 24). We hypothesize that analysis of this early adaptation against ischemic acidosis might further our general understanding of how PC and PKC regulate ischemic physiology.

In this discussion, we first dissect the role of decreased ischemic acidosis as an early effect of PC signals from the potential regulatory roles that pH and PC might play in affecting subsequent developments (ischemic and postischemic) in the rat heart. Next, we evaluate the role of three antiacidosis mechanisms and the role of PKC on NHE-driven H+ extrusion within the context of global ischemia. Finally, we consider the ramifications of pH extrusion by NHE and the clues pointing toward unsuspected PKC functions in PC.

Role of pH in PC. Steenbergen et al. (27) originally investigated ischemic acidosis as a potential mechanism of protection in rat heart preconditioned with cyclic ischemic episodes (CI-PC). Murry et al. (19) had previously shown that CI-PC dog hearts produced less lactate during regional ischemia. H+ efflux conducted by the NHE is believed to trigger Na+ entry into acidified myocytes (22, 26). There is substantial data...
implicating the subsequent rise in \([\text{Ca}^{2+}]\) in mediating ischemic injury, myocellular dysfunction, and death (22, 26). Therefore, Steenbergen et al. (27) suggested that decreased \([\text{H}^+]\), production from glycolysis might account for decreased \(\text{Na}^+\) entry via \(\text{Na}^+/\text{H}^+\) exchange and, consequently, might lessen \(\text{Ca}^{2+}\) overload. Measurements of tissue \(\text{Na}^+\) and \(\text{Ca}^{2+}\) in preconditioned hearts suggest that this is indeed the case (27, 30, 31). Therefore, the attenuation of ischemic acidosis, and hence \(\text{Ca}^{2+}\) overload, was perceived as an attractive mechanism to explain the postischemic protection induced by PC.

The proposal that attenuated acid production is not a perfect explanation of the ionic cascade leading to injury was recently provided by two of the original proponents of the hypothesis (2, 34). Asimakis (2) found that low glycogen levels during the diurnal cycle did not correlate with protection. Similarly, a collaboration that included Steenbergen and co-workers (34) reported that glycogen depletion (with glucagon) reduced lactate formation (a surrogate for ischemic \(\text{H}^+\) production) but not lethal ischemic injury. Schaefer et al. (25) have also reported that glycogen depletion (by hypoxic episodes), followed by a delay, decreases acidosis but does not relate infallibly to postischemic recovery. These studies emphasize that decreasing \(\text{H}^+\) production (by interventions other than CI-PC) is not intrinsically sufficient to confer protection. Using different approaches, we show that PC mechanisms might decrease ischemic acidosis by extrusion mechanisms that are independent of \(\text{H}^+\) production (Figs. 3–5).

Other studies offer further evidence to dissociate ischemic acidosis from postischemic developments. Several studies report that (in nonadapted hearts) the pH at end ischemia does not predict postischemic recovery (8, 13). Investigations in our laboratories (15) also indicate that certain PC stimuli protect postischemic function (after modest ischemia) despite severe ischemic acidosis. However, a closer analysis shows that only those stimuli that prevent ischemic acidosis protect tissue viability (in addition to function) (15). Furthermore, we have shown (15) that phorbol 12-myristate 13-acetate (PMA), which does not protect postischemic function, prevents ischemic acidosis and also protects tissue viability (15). Conversely, studies that pharmacologically inhibit postischemic protection and ischemic pH preservation [see Fig. 4 and our previous reports (4, 15, 16)] concurrently associate, but do not establish, causality between acidosis and postischemic developments. In another interesting study, Murphy et al. (17) showed that lipoxygenase inhibitors eliminated functional protection induced by CI-PC but not protection against acidosis. This could imply that PC engages separate mechanisms of protection against both acidosis and stunning (4, 15). Alternatively, distal lipoxygenase-related mechanisms might synergize together with preserved pH to diminish postischemic injury (4, 17). Therefore, without a better understanding of the mechanism of pH regulation induced by PC, the distal interactions within the host of ischemic and PC factors cannot be easily separated into causes (necessary vs. sufficient) and effects (protection against infarction, apoptosis, stunning, etc.).

Another approach presumes that ischemic acidosis itself may be a lesser problem compared with the consequences of NHE-driven \(\text{Na}^+\) entry. Indeed, pharmacological inhibition of NHE-1 (particularly during the active pH-restitution phase of early reperfusion) appears therapeutically useful (22, 26). This leads to the prospect that the endogenous adaptation mechanism of CI-PC and inhibition of NHE (particularly during reperfusion) might both provide similar coverage against \([\text{Na}^+]\)-driven ionic overload and other postischemic injury end points. However, these two protective strategies affect the development of ischemic acidosis (which precedes subsequent reperfusion events) in distinct ways. Typically, during ischemia (in nonconditioned hearts) extracellular acidification and ATP depletion quickly retard \(\text{H}^+\) efflux by the NHE (22, 26). Thus, in these hearts, NHE blockers do not significantly affect the development of ischemic acidosis (20). However, after protracted ischemia, NHE inhibitors may actually increase acidosis (14). Our data show that PC of rat hearts additionally prevents acidosis from the beginning of ischemia. (The significations of this adaptation are considered below.)

Taken together, these data indicate that if acidosis is relieved by NHE (notably during early reperfusion), then the ensuing \(\text{Na}^+\) influx can trigger subsequent ionic exchanges and injury. Interruptions of this cascade at any of the several intervening steps can decouple the usual detrimental association between ischemic acidosis and postischemic injury. Such a decoupling could be achieved by either pharmacological inhibitors or by signal-induced cellular adaptation. Conversely, although the attenuation of acidosis may be an useful component of some PC strategies, it is not sufficient. Indeed, certain protective maneuvers such as NHE
inhibition or hypothermia may operate independently of mechanisms that preserve pH during ischemia. Therefore, the regulation of pH during ischemia by PC stimuli is likely to be most useful for identifying PKC-regulated targets and other ancillary mechanisms that cooperate together to produce observable adaptations during and after ischemia.

Effect of PC on regulation of ischemic acidosis. Numerous studies have shown that ischemic PC attenuates acidosis during sustained ischemia. The best-studied case is the globally ischemic rat heart, but the effect has been reproduced in rabbit and pig hearts. In rats, Murphy and co-workers (7) found that CI-PC prevented ischemic acidosis and also protected postischemic function, both by PKC-dependent mechanisms. Furthermore, they found that direct PKC stimulation with diacylglycerol preserved ischemic acidosis and postischemic function, albeit weakly. Surprisingly, the prototypical PKC stimulant PMA, which is effective in protective postischemic viability in rabbits, did not improve either outcome. In agreement with the previous study (7), we have reported (15) that, in rats, the nonphysiological PKC stimulant PMA does not induce protection of postischemic function but does protect cell viability at higher doses.

In contrast, physiological signaling by either ischemic PC or α₁-adrenergic receptors apparently induces the antiacidotic adaptation through PKC mechanisms (Figs. 3 and 4). This suggests that phorbol esters and endogenous PKC stimuli engage different second messengers and activate PKC isoforms in distinct ways (4, 15). Although several studies (2, 8, 25, 34) have surmised that decreased production of H⁺ from the glycolytic pathway is unrelated to the protective adaptations induced by CI-PC, this is the first report to investigate the mechanisms for regulating ischemic acidosis in these preconditioned hearts. Figures 3 and 4 show that, quite independent of H⁺ production, H⁺ extrusion through the NHE is a prominent part of the adaptive mechanism responsible for regulating ischemic pH after CI- or α₁-adrenergic PC.

However, in rabbits, Bugge and Ytrehus (6) have suggested that NHE blockade provides protection that is additive to that provided by CI-PC. This suggests that, in rabbits, CI-PC-stimulated PKC isoform mechanisms are different from those in rats (32) and thus might not regulate ischemic pH through the NHE. Indeed, in isolated hypoxic rabbit cardiomyocytes, direct H⁺ extrusion by another mechanism (H⁺-ATPase) has been identified. These investigators found that, in these rabbit myocytes, simulated ischemic PC activated the H⁺-ATPase via PKC. This resulted in protection against apoptosis over the next 25 h, providing a rationale explaining why preservation of the pH can be important (11).

These studies of PC against ischemic acidosis emphasize that signaling may stimulate subsets of PKC isoform(s) to conduct specific changes (4, 15). The interaction of PKC isoforms with specific targets appears to include selected mechanisms for preserving pH during ischemia acting with other independent mechanisms regulating postischemic functional recovery and viability. The precise details of PKC-mediated adaptation may differ among species (4, 32).

Mechanism of PC against ischemic acidosis in rats. Until recently, the view that CI-PC preserved the ischemic pH by decreasing H⁺ production was virtually unquestioned. In an isolated system such as the globally ischemic heart, decreased intracellular acidosis is due to either decreased H⁺ production, increased extrusion, or augmented buffering capacity (9) (Fig. 6). Many investigators have offered different types of circumstantial evidence to support the first mechanism, decreased H⁺ production during ischemia (1, 3, 27, 35). Thus CI-PC rat hearts invariably have less glycolytic flux, as indicated by accumulated lactate. Moreover, glycogen depletion (by hypoxic periods similar to the CI-PC protocol) reduced lactate accumulation and caused postischemic protection (1, 3, 27, 35). However, as noted earlier, reduced ischemic glycogen and lactate contents per se appear insufficient to confer protection (2, 8, 25, 34).

Our data are the first to show that decreased lactate accumulation does not explain decreased acidosis after PC in globally ischemic rat hearts. While investigating the ability of the α₁-adrenergic pathway to mimic CI-PC (4, 16), we observed that, although both stimuli preserved pH during ischemia, only CI-PC decreased accumulated lactate (Fig. 5). Thus, for CI-PC hearts, decreased glycolysis might well be one mechanism contributing to preserving pH during ischemia (1, 3, 8, 27, 35). However, the α₁-adrenergic PC mechanism must have achieved cardiac antiacidosis by another process. We therefore proceeded to examine the alternative possibilities of extrusion and buffering. Upon inhibition of H⁺ extrusion by the cardiac NHE, α₁-adrenergic PC did not preserve pH any longer. This striking result was corroborated when two different NHE inhibitors (EIPA and HOE-694) also abolished the preservation of ischemic pH induced by CI-PC (Figs. 3 and 4). For both PC stimuli, the antiacidotic effect was abolished by PKC blockade. This suggests that, in the globally ischemic rat heart, PKC-dependent mechanisms are available for modulating H⁺ extrusion, thereby helping to preserve the pH during ischemia.

Proton buffering does not appear to be a major mechanism of preserving the ischemic pH in these preconditioned hearts. P_i [with an acidic dissociation constant (pK_a) of 6.9], bicarbonate, and histidyl residues of intracellular proteins comprise the primary ischemic myocardial intracellular buffers (9). At a pH >6.4, the buffering capacity of the ischemic heart depends mainly on P_i. The endogenous bicarbonate buffering system, with a pK_a of 6.1, is not an optimal intracellular buffer and is unavailable during extended global ischemia. Only at very low pH values do the high-capacity, low-pK_a (5.2) intrinsic protein buffers become significant (1, 9, 33). During ischemia, PCR is broken down to sustain cellular ATP, accumulating P_i as a by-product of this reaction (Table 1), which could increase intracellular buffering capacity. However, an increase in intracellular buffering capacity was not

ATTENUATED ISCHEMIC ACIDOSIS IN PRECONDITIONING
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evident in the preconditioned groups as measured by $P_i$ (Table 1). Indeed, despite less $P_i$ in CI-treated hearts, ischemic acidosis remains attenuated. These results are consistent with a recent study that also failed to find any difference in buffering capacity in CI-PC rat hearts (1).

The NHE functions as an electroneutral, gradient-driven, facilitated transport mediator across the lipid bilayer for $Na^+$ and $H^+$ (22, 26). Activation of the NHE can occur through either acidification of the cytosol, which allosterically alters its affinity for $H^+$, or by direct phosphorylation of the antiporter protein (10, 22, 26). The activity of the NHE is primarily driven by a transsarcolemmal proton gradient, and it is therefore incapacitated if the transmembrane gradients of pH and $Na^+$ collapse (i.e., high [Na$^+$], with or without extracellular acidification). The NHE is exquisitely sensitive to small changes in pH, yet is active over a relatively small range of pH. Thus, during global ischemia in nonconditioned hearts, NHE activity has been difficult to detect (20, 26). Conversely, during reperfusion, its role is more prominent (26). However, direct measurements of pH regulation by the NHE under perfused conditions are partially obscured by other mechanisms of $Na^+$-linked pH regulation (notably $Na^+$-HCO$_3^-$ transport) (26, 33). Therefore, Akirnin and co-workers (26) have shown (in nonconditioned rat hearts) that the role of $Na^+$/$H^+$ exchange is often difficult to demonstrate in the presence of bicarbonate buffer (26). In agreement with the literature, HOE-694 or EIPA did not alter the course of ischemic acidification in nonconditioned hearts, indicating that pH regulation through this mechanism is not normally significant during global ischemia in rat hearts. In contrast, in both CI- and PE-PC rat hearts (perfused with physiological bicarbonate-based Krebs-Henseleit buffer), the exchanger appears to be active during global ischemia (Figs. 3 and 4). Upon inhibition of the NHE, these PC stimuli can no longer attenuate the development of ischemic acidosis. Hence, the ischemic pH drops into the acidic range (pH 6.5), similar to control values. We infer that proton extrusion via the NHE may play a principal role in the antiacidotic effects of the PC stimuli tested.

The cardiac PKC mechanism that affects $H^+$ extrusion during global ischemia may not involve direct phosphorylation of the NHE. NHE-1, the predominant isoform in the heart (10, 29), is an 815-residue, 200-kDa membrane-spanning dimer with three consensus sites for glycosylation and several putative sites for phosphorylation. These sites do not appear to be PKC consensus sites (10). Therefore, the NHE may not be directly activated by PKC. One possibility is PKC phosphorylation of regulatory protein units that modify the NHE. Alternatively, PKC may activate mitogen-activated protein kinases or calmodulin-dependent protein kinase II, which have phosphorylation sites on the NHE-1 (10). Berk and co-workers (29) have proposed that ribosomal S6 kinase may be one such NHE-1 kinase that regulates the allosteric and pH set point. At the other extreme, it is equally possible that the NHE is not modified at all. Indeed, the PKC family impacts on a large range of proteins (other than kinases) that could help preserve the transmembrane driving gradients over longer ischemic periods and thereby permit NHE function to continue during ischemia.

We conclude that decreased $H^+$ production during ischemia is not sufficient to explain how PC preserves the ischemic pH. In contrast with normal rat hearts, the contribution of the NHE to ischemic pH regulation becomes significant after PC with either CI or the $\alpha_2$-adrenergic agonist PE. The PC stimuli achieve this physiological adaptation through PKC signaling, but the exact sites of regulation require further examination.

Although our data show that NHE activity occurs during ischemia in preconditioned hearts, neither CI-PC (27, 31) nor PE-PC (31) appears to create a corresponding intracellular $Na^+$ overload in these hearts. Steenbergen et al. (27) found that intracellular $Na^+$ levels were comparable to those in untreated controls, whereas intracellular $Ca^{2+}$ overload was attenuated (27). Similarly, Tosaki and co-workers (31) found that both CI-PC and PE-PC hearts avoided excess $Na^+$ as well as $Ca^{2+}$ accumulations. Our estimations of the ionic contents in CI-PC hearts at end ischemia [performed according to Tosaki et al. (30, 31)] are confirmatory (unpublished data). Ramasamy et al. (23) observed a small increase in intracellular $Na^+$ resonance area after CI-PC, but only during the first 10 min of ischemia (not thereafter). They found that CI-PC upregulated intracellular $Na^+$ efflux noticeably during reperfusion and suggested that both the NHE and the $Na^+$-$K^+$-$2Cl^-$ exchanger may be involved. Although some studies have suggested that $\alpha_2$-adrenergic receptors stimulate NHE activity, detailed investigations show that this does not appear to involve PKC (22). Therefore, it is possible that NHE activity is not upregulated per se but is simply drawn along kinetically because of $Na^+$ extrusion by ancillary processes.

We speculate that the PC stimulus sets up an adaptive mechanism for the ischemic myocardium to remove its intracellular $Na^+$ load without incurring the lethal increase in intracellular $Ca^{2+}$. Mechanisms that extrude intracellular $Na^+$ together with counteranions (24) such as bicarbonate (22), lactate (33), and phosphate (21) are present in heart and could be PKC targets (24). This could allow the heart to effectively export $H^+$ during ischemia, together with a dispensable counteranion, electroneutrally and without consuming ATP. Other effects might include other synergistic actions such as inhibition of $Na^+$/$Ca^{2+}$ exchange or glycolysis.

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