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Research Article

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Ischemic Preconditioning Stimulates Sodium and Proton Transport in Isolated Rat Hearts

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Abstract

One or more brief periods of ischemia, termed preconditioning, dramatically limits infarct size and reduces intracellular acidosis during subsequent ischemia, potentially via enhanced sarcolemmal proton efflux mechanisms. To test the hypothesis that preconditioning increases the functional activity of sodium-dependent proton efflux pathways, isolated rat hearts were subjected to 30 min of global ischemia with or without preconditioning. Intracellular sodium (Na_i) was assessed using ^{23}Na magnetic resonance spectroscopy, and the activity of the Na-H exchanger and Na-K-2Cl cotransporter was measured by transiently exposing the hearts to an acid load (NH_4Cl washout). Creatine kinase release was reduced by greater than 60% in the preconditioned hearts ($P < 0.05$) and was associated with improved functional recovery on reperfusion. Preconditioning increased Na_i by 6.24±2.04 U, resulting in a significantly higher level of Na_i before ischemia than in the control hearts. Na_i increased significantly at the onset of ischemia (8.48±1.21 vs. 2.57±0.81 U, preconditioned vs. control hearts; $P < 0.01$). Preconditioning did not reduce Na_i accumulation during ischemia, but the decline in Na_i during the first 5 min of reperfusion was significantly greater in the preconditioned than in the control hearts (13.48±1.73 vs. 2.54±0.41 U; $P < 0.001$). Exposure of preconditioned hearts to ethylisopropylamiloride or bumetanide in the last reperfusion period limited in the increase in Na_i during ischemia and reduced the beneficial effects of preconditioning. After the NH_4Cl prepulse, preconditioned hearts acidified significantly more than control hearts and had significantly more rapid recovery of pH (preconditioned, $\Delta pH = 0.35 \pm 0.04$ U over 5 min; control, $\Delta pH = 0.15 \pm 0.02$ U over 5 min). This rapid pH recovery was not affected by inhibition of the Na-K-2Cl cotransporter but was abolished by inhibition of the Na-H exchanger. These results demonstrate that preconditioning alters the kinetics of Na_i accumulation during global ischemia as well as proton transport after NH_4Cl washout. These observations are consistent with stimulation of the Na-K-2Cl cotransporter and Na-H exchanger by preconditioning. (*J. Clin. Invest.* **1995. 96:1464–1472.)**

Key words: preconditioning • ischemia • magnetic resonance spectroscopy • Na-H exchanger • Na-K-2Cl cotransporter

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Introduction

Preconditioning is a phenomenon in which one or more brief periods of ischemia protect the myocardium during a subsequent period of ischemia, with the degree of protection classically defined as limitation of infarct size (1–3). Preconditioning has been demonstrated in a variety of species, but there are no clearly defined and accepted mechanisms to explain the endogenous cardioprotection of preconditioning. Several theories have been proposed to explain the possible mechanism(s) by which ischemic preconditioning reduces infarct size, including preservation of myocardial high energy stores during ischemia (4), adenosine receptor stimulation (5), activation of ATP-sensitive K^+ channels (6), and translocation and activation of protein kinase C (7, 8).

Ischemic preconditioning has been shown, by this group as well as by others, to limit acidosis during global ischemia (4, 9). Limitation of acidosis could be due to either *(a)* reduced proton production or *(b)* enhanced proton efflux. Reduced proton production would reduce the stimulus for increasing sodium entry via the Na-H exchanger and potentially result in lower levels of intracellular sodium (Na_i) during ischemia. Enhanced proton efflux, which may occur because of stimulation of the Na-H exchanger, could, conversely, result in an *increase* in Na_i during ischemia. Stimulation of the Na-H exchanger by preconditioning would also alter the response of the heart to acidification resulting from an acid load in the absence of ischemia.

This study tested the hypothesis that ischemic preconditioning stimulates sodium-dependent proton efflux pathways (such as the Na-H exchanger) in the heart, resulting in increased proton efflux and reduced acidosis during subsequent ischemia. This hypothesis was tested using ^{23}Na and ^{31}P nuclear magnetic resonance (NMR)[1] spectroscopy in preconditioned and control hearts to measure *(a)* the response of intracellular Na^+ in perfused rat hearts during 30 min of global ischemia, *(b)* the effect on intracellular Na^+ of inhibiting the Na-H exchanger and Na-K-2Cl cotransporter in preconditioned hearts, and *(c)* the kinetics of proton extrusion in the absence of ischemia.

1. *Abbreviations used in this paper:* CK, creatine kinase; EIPA, ethylisopropylamiloride; gdw, grams dry weight; LVDP, left ventricular developed pressure; NMR, nuclear magnetic resonance; ppm, parts per million.

Methods

General methods

All experiments were performed with the approval of the University of California, Davis, Animal Research Committee.

Isolated heart model

Experiments were performed using an isovolumic isolated perfused rat heart preparation. Male Sprague-Dawley rats (~ 350 g) were pretreated with heparin (1,000 U intraperitoneally [IP]), followed by sodium pentobarbital (65 mg/kg IP). After deep anesthesia was achieved as determined by the absence of a foot reflex, the heart was rapidly excised and placed into iced saline. The arrested heart was perfused in a retrograde manner through the aorta within 2 min. The left ventricular developed pressure (LVDP) was determined using a latex balloon in the left ventricle, with high pressure tubing connected to a pressure transducer. Perfusion pressure (PP) was also monitored using high pressure tubing off the perfusion line. Hemodynamic measurements were recorded on a four-channel recorder (Windowgraf; Gould Inc., Valley View, OH). The heart was perfused using an accurate roller pump at a flow rate of 12.5 ml/min (Rainin Instrument Co., Woburn, MA). The perfusate consisted of (in millimoles per liter) NaCl, 118; KCl, 4; $CaCl_2$, 1.2; $MgCl_2$, 1; $NaHCO_3$, 25; with the substrate being 11 mM glucose. The perfusion apparatus was tightly temperature controlled, with heated baths used for the perfusate and for the water jacketing around the perfusion tubing to maintain heart temperature at 37±0.5°C under all conditions. Oxygenation of the perfusate was provided by a pediatric hollow fiber oxygenator (Capiax II; Terumo Corp., Tokyo, Japan) immediately proximal to the heart. This preparation has routinely been stable for 3–4 h (10).

Creatine kinase

Creatine kinase (CK) was measured from timed 5-min collections of the effluent for 60 min of reperfusion after the ischemic period. This extended period of collection was employed because preliminary experiments showed that, in control hearts, CK release was maximal after ~ 30 min of reperfusion and was stabilized within 60 min. Each 5-min collection was analyzed in duplicate using established spectrophotometric methods (11). Total integrated CK activity over the reperfusion period was calculated for each heart and corrected for dry weight of the heart. CK release was expressed in IU/g dry weight (gdw). As previously shown (12), the integral of the CK release is a representative measure of ischemic injury and infarction.

NMR spectroscopy

Phosphorus-31. All NMR spectroscopy was performed on a vertical bore spectrometer (AMX 400; Bruker Instruments Inc., Billerica MA) using a switchable 20-mm probe tuned for ^{31}P or ^{23}Na. ^{31}P NMR spectroscopy was performed using 228 acquisitions of a 60° pulse and a 1.6-s interpulse delay, with spectra processed using an exponential multiplication of 20 Hz and Fourier transformed. Intracellular pH was calculated from the chemical shift between the inorganic phosphate (P_i) and phosphocreatine (PCr) resonance using a titration curve established in this facility (13).

Sodium-23. Intracellular sodium content, Na_i, was determined using the shift reagent (7 mM Dy $(TTHA)^{3-}$) added to the standard perfusate throughout the entire experimental period. ^{23}Na spectra were acquired using the broad-band probe tuned to 105.85 MHz. 1,000 free induction decays were signal averaged over 5 min using 90° pulses with a ±4,000-Hz sweep width. ^{23}Na spectra were processed on the Bruker spectrometer. After baseline correction, the extracellular resonance was inverted, shifted, and subtracted from the original spectrum to obtain the intracellular resonance. The area of the intracellular resonance was determined without constraining the line fit to either a Lorentzian or Gaussian shape. These operations were performed in duplicate for each spectrum and the values averaged. The data acquired were normalized to an assumed baseline value of 10 at −45 min and are expressed as normalized area units. To rule out any change in relaxation times during the protocol, half-height linewidths of the intracellular sodium resonance were calculated at three time points during the protocols. The chemical shifts between the extracellular and intracellular resonances were determined before, during, and after ischemia.

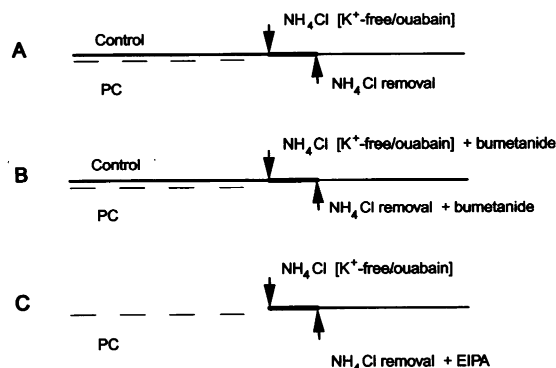

Figure 1. Protocol III. Schematic of the protocols measuring the pH response to an acid load (NH_4Cl exposure for 10 min and washout) in preconditioned and control hearts under the following conditions: *(A)* K^+-free perfusate with ouabain, *(B)* K^+-free perfusate with ouabain and bumetanide (10 μM) at the time of NH_4Cl exposure and removal, and *(C)* preconditioned hearts in which EIPA (10 μM) was administered during NH_4CL removal. Four hearts were studied in each group. Preconditioning consisted of four 5-min periods of total global ischemia with 5-min reperfusion periods (total 40 min). Control perfusion consisted of 40 min of perfusion at normal flow.

Statistical methods

Data were analyzed using INSTAT (GraphPad, San Diego, CA) software operating on an IBM-compatible personal computer. Differences at different time points within groups were assessed using ANOVA for repeated measures, with a subsequent Dunnett multiple comparisons test if the P value for ANOVA was significant. Differences between groups at a given time point were assessed using the Mann-Whitney nonparametric test. Reproducibility of the intracellular sodium measurements was evaluated using linear regression of duplicate measurements. A P value of less than 0.05 was used to reject the null hypothesis. All data are expressed as the mean±SEM.

Protocols

The protocols consisted of ischemia protocols (I and II) employing measurement of Na_i, cardiac function, and CK release and a nonischemia protocol (III) in which the changes in pH in response to an acid load were measured. Protocol I measured functional recovery, creatine kinase release, and intracellular sodium kinetics in control hearts and preconditioned hearts. Protocol II measured functional recovery, CK release, and intracellular sodium kinetics in preconditioned hearts in which either ethylisopropylamiloride (EIPA, 3 μM, an inhibitor of the Na-H exchanger) or bumetanide (5 μM, an inhibitor of the Na-K-2Cl cotransporter) was added to the perfusate during the last reperfusion period before 30 min of global ischemia. These hearts were reperfused after 30 min with standard perfusate containing neither EIPA nor bumetanide. Protocol III (shown in Fig. 1 and described in detail below) measured the response to an acid load under three conditions: *(A)* NH_4Cl prepulse; *(B)* NH_4Cl prepulse with inhibition of the Na-K-2Cl cotransporter with bumetanide; and *(C)* NH_4Cl prepulse with inhibition of the Na-H exchanger during pH recovery.

Functional recovery and CK release

To determine whether use of the shift reagent had any effects on functional recovery or infarct size, hearts with and without ischemic preconditioning were studied both with and without $DyTTHA^{3-}$ in the perfusate ($n = 6$ in each group). $DyTTHA^{3-}$ was added to the perfusate after an initial stabilization period. After 40 min of perfusion, control hearts were subject to 30 min of global ischemia followed by 60 min of reperfusion. Preconditioned hearts were subject to four 5-min periods of no-flow ischemia, with each period of ischemia followed by 5 min of reperfusion. As in the control hearts, the preconditioned hearts were then subject to 30 min of global ischemia followed by 60 min of reperfusion.

Response of intracellular sodium to ischemia and reperfusion

Control ($n = 8$) and preconditioned (PC, $n = 6$) hearts were studied using ^{23}Na NMR. As in the functional measurements, hearts in each group were subjected to 30 min of global ischemia followed by 60 min of reperfusion after 40 min of either control perfusion or four 5-min preconditioning episodes all in the presence of $DyTTHA^{3-}$. Preconditioned hearts in which EIPA ($n = 5$) or bumetanide ($n = 5$) was added to the hearts during the last reperfusion period before global ischemia were also studied using ^{23}Na NMR.

Response to an acid load

To test the hypothesis that preconditioning increases proton efflux, pH recovery was measured in 20 control and preconditioned hearts ($n = 4$ in each condition) after exposure to an acid load in the three protocols described earlier. In each protocol, hearts were exposed to 20 mM NH_4Cl for 10 min in a K^+-free buffer that contained 100 μM ouabain to inhibit Na transport via Na,K-ATPase. After this 10-min exposure to NH_4Cl, hearts were perfused for 30 min with K^+-free/ouabain buffer not containing NH_4Cl. This washout phase decreased pH as the NH_4^+ that had previously entered then dissociated into $NH_3 + H^+$ (14). The ability of the cell to extrude protons was then reflected in the subsequent increase in pH. The first protocol *(A)* did not contain any membrane transport inhibitors during the NH_4Cl exposure or washout other than the K^+-free/ouabain buffer. Because preconditioning could affect the Na-K-2Cl cotransporter, which is a potential mechanism for NH_4 transport into the cell (15), the second protocol *(B)* included 10 μM bumetanide during NH_4Cl exposure and removal to inhibit the Na-K-2C1 cotransporter. The third protocol *(C)* employed 10 μM EIPA during the NH_4Cl washout phase to determine the role of the Na-H exchanger in preconditioned hearts.

Results

Function

Use of the shift reagent $Dy(TTHA)^{3-}$ during ^{23}Na NMR experiments did not alter cardiac function in either the control or preconditioned hearts. LVDP was not different in any group under baseline conditions before global ischemia (84±8 mmHg in the control hearts, 76±7 mmHg in the control hearts with 7 mM $Dy(TTHA)^{3-}$, 82±8 mmHg in the preconditioned hearts, 79±7 mmHg in the preconditioned hearts with 7 mM $Dy(TTHA)^{3-}$) and fell to 0 during the first 5 min of global ischemia in all hearts. Similarly, heart rates were identical in all the groups during baseline conditions, and ischemia caused a rapid cessation of cardiac rhythm. Reperfusion of the control hearts resulted in limited functional recovery. LVDP and rhythmic activity did not recover in three hearts, with the average developed pressure only 12±5 mmHg after 60 min of reperfusion in the remaining hearts ($P < 0.01$ vs. PC). All the preconditioned hearts exhibited significant functional recovery, with LVDP recovering to 48±7 mmHg after 60 min of reperfusion. This degree of functional recovery was similar to that observed in our earlier studies (9).

Preconditioned hearts treated with EIPA in the last reperfusion period before ischemia had significant diminution of functional recovery compared with preconditioned hearts. Left ventricular developed pressure was 79±8 mmHg before ischemia and 27±7 mmHg ($P < 0.05$ vs. PC) at the end of reperfusion. Similarly, treatment with bumetanide reduced developed pressure from 78±6 mmHg before ischemia to 16±6 mmHg ($P < 0.01$) at the end of reperfusion.

Ischemic injury

CK release, a measure of myocardial injury and infarction, was significantly reduced after 30 min of global ischemia in the preconditioned hearts and was not affected by DyTTHA (CK release: 1186±117 IU/gdw in control hearts, 372±63 in the preconditioned hearts, and 414±54 in the preconditioned hearts with 7 mM $Dy(TTHA)^{3-}$); $P < 0.01$ preconditioned vs control hearts). These reductions in ischemic injury following preconditioning are similar to those observed in our earlier studies (9). Treatment with EIPA or bumetanide before ischemia resulted in CK release that was similar to that in control hearts and significantly greater than that in preconditioned hearts (EIPA, 1257±244 IU/gdw; bumetanide, 1522±236 IU/gdw; $P < 0.05$ vs preconditioned hearts).

Intracellular $[Na^+]$ in preconditioned hearts

Fig. 2 displays the ^{23}Na NMR spectra of intracellular and extracellular sodium in preconditioned and control hearts, while Fig. 3 illustrates the changes in Na_i in control and preconditioned hearts. The intracellular resonance appeared as a shoulder on the large extracellular resonance under baseline conditions, but became more conspicuous during the 30-min global ischemia period. Because accurate and reproducible measurement of the intracellular sodium resonance was critical in these experiments, we examined *(a)* the changes in T2 relaxation times and, hence, linewidths of the intracellular sodium resonance during the experiments, *(b)* the chemical shifts between the intra- and extracellular resonances before, during, and after ischemia; and *(c)* the reproducibility of the two separate analyses of the intracellular sodium resonance areas. The half-height linewidths did not change during the experiments. The linewidths in the preconditioned hearts were baseline (−45 min), 83±3 Hz; immediately before global ischemia (0 min), 84±4 Hz; and at the end of global ischemia (30 min), 86±4 Hz. Similarly, the linewidths in the control hearts were baseline (−45 min), 86±4 Hz; immediately before global ischemia (0 min), 86±4 Hz; and at the end of global ischemia (30 min), 84±4 Hz. The changes in chemical shift differences from immediately before global ischemia (0 min) to the end of global ischemia (30 min) were not different between the groups (control, 0.07±0.05; PC, 0.01±0.03 ppm); similarly the changes in chemical shift differences from the end of global ischemia and 5 min after reperfusion were not different (control, 0.01±0.002 ppm; PC, 0.07±0.03 ppm). The correlation coefficient for separate analyses of the raw intracellular sodium resonance areas was 0.95.

As observed in Fig. 3, the four preconditioning episodes progressively increased Na_i from its baseline value. Although the increases in Na_i from the baseline value were not significant by ANOVA, these changes resulted in Na_i that was greater in

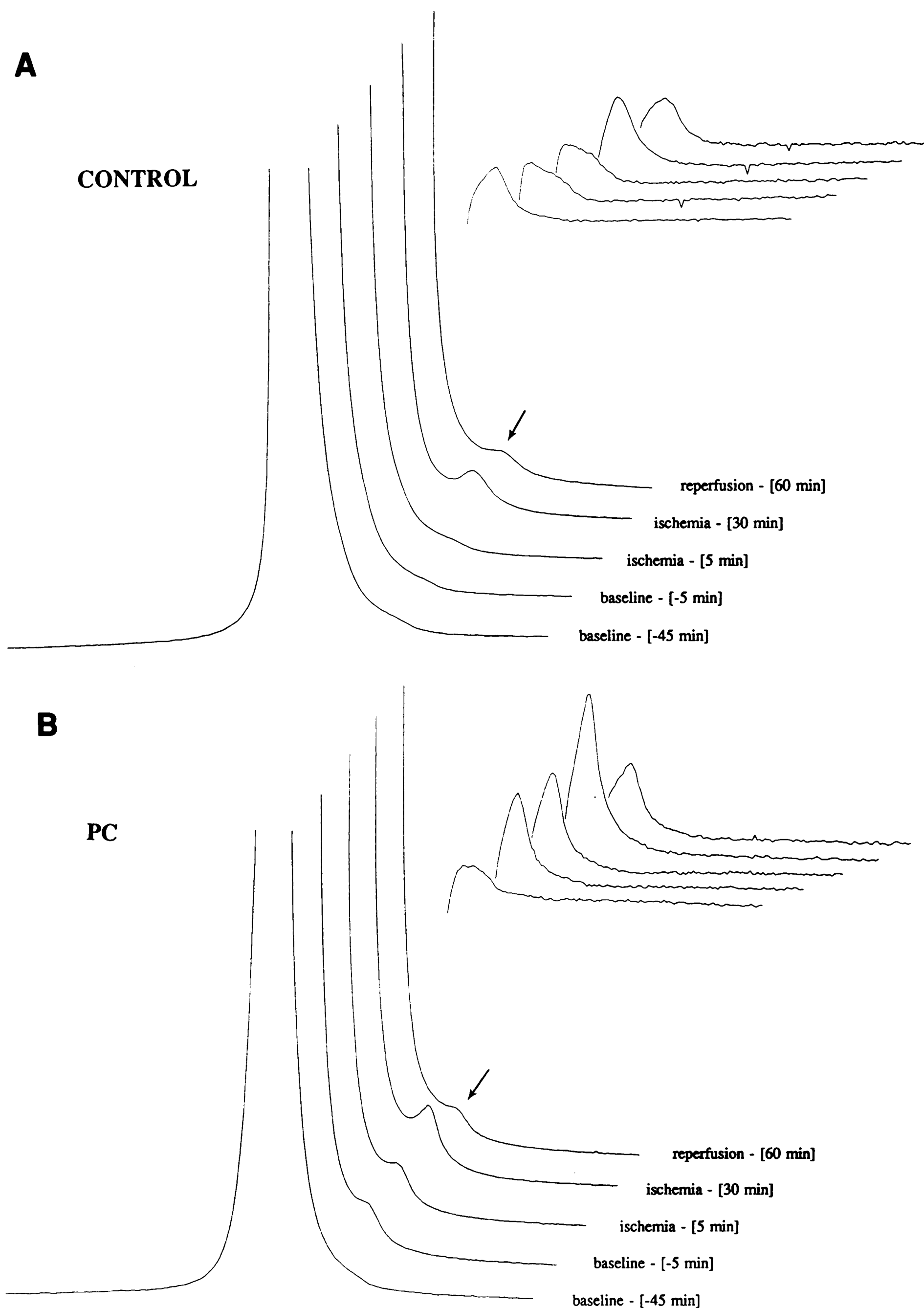

Figure 2. Representative ^{23}Na NMR spectra of isolated rat hearts perfused with 7 mM $Dy(TTHA)^{3-}$ in modified oxygenated Krebs-Hensleit buffer obtained from *(A)* control or *(B)* preconditioned (PC) hearts at the time points shown. The spectra demonstrate the large extracellular sodium resonance (shifted downfield by the shift reagent) and the small intracellular sodium resonances *(arrow)* under these conditions. Inset in each figure are the intracellular resonances derived by subtracting the extracellular resonances. Although the chemical shift of the resonances in these hearts is slightly different, it is apparent that the intracellular sodium resonance is increased by the preconditioning episodes (baseline, −5 min) and is further increased during ischemia. In contrast, the intracellular sodium resonance in the control hearts does not increase under baseline perfusion conditions.

the preconditioned than in the control hearts 5 min before the 30-min ischemia period ($P < 0.05$). As seen in Fig. 4, there was a rapid and significant increase in Na_i during the first 5 min of the ischemia period in the preconditioned hearts but not in the control hearts, with intracellular Na_i increasing 8.48±1.21 U in the preconditioned hearts compared with 2.57±0.81 U in the control hearts ($P < 0.01$). The subsequent changes in Na_i during ischemia were similar in the preconditioned and control hearts, yielding similar end-ischemia values before reperfusion. Intracellular sodium decreased by 13.48±1.73 U, or 30% of its final ischemia value, in the preconditioned hearts during the first 5 min of reperfusion, compared with a decrease of 2.54±1.24 U, or 6% of its final ischemia value, in the control hearts ($P < 0.001$). Thus, the response of Na_i during ischemia in the preconditioned hearts was characterized by an accelerated increase in Na_i at the onset of ischemia and accelerated decrease on reperfusion.

As in the untreated preconditioned hearts, the preconditioned hearts treated with either EIPA or bumetanide during the last reperfusion period before 30 min of global ischemia demonstrated gradual increases in Na_i during the preconditioning episodes (8.48±0.48 and 11.94±.9.92 U, respectively; Fig. 5), resulting in values of Na_i that were significantly higher than those of the control hearts before global ischemia ($P < 0.05$). The increase in Na_i during 30 min of global ischemia in the EIPA-treated hearts (7.50±0.94 U) was significantly re-

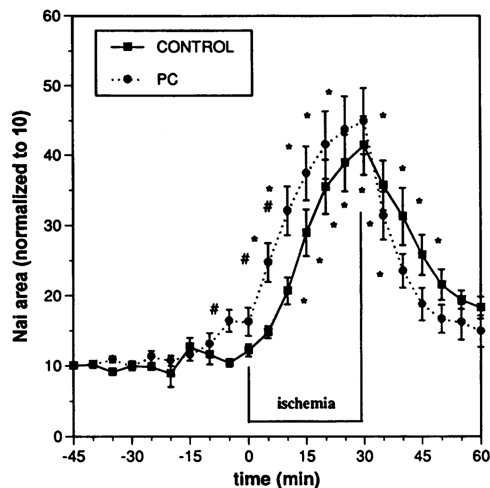

Figure 3. Changes in intracellular sodium resonance area (Na_i) as a function of time in control and preconditioned (PC) hearts, with the initial intracellular resonance at −45 min in each heart normalized to 10. $^{\#}P < 0.05$ vs. control hearts; $*P < 0.05$ vs. baseline measurement for each group.

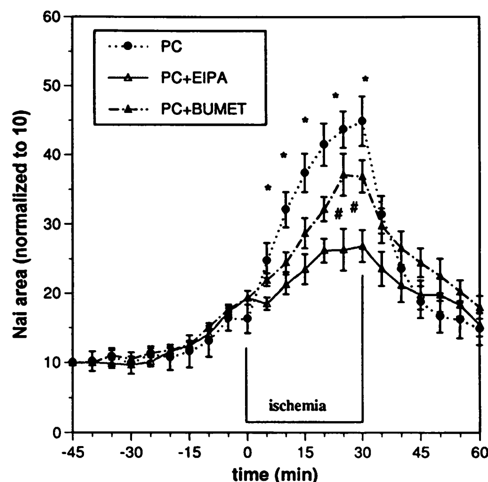

Figure 5. Changes in intracellular sodium (Na_i) resonance area as a function of time in preconditioned hearts exposed to either ethylisopropylamiloride (*PC + EIPA*, △) or bumetanide (*PC + BUMET*, ▲) during the last reperfusion period prior to global ischemia. The data from the preconditioned hearts (*PC*, ●) are from Fig. 3. $*P < 0.05$ PC hearts vs. PC + EIPA; $^{\#}P < 0.05$ PC + BUMET vs. PC + EIPA.

duced compared with that in either control or preconditioned hearts, while the increase in Na_i was not reduced by bumetanide (17.56±1.53 U). The end-ischemia value of Na_i in the bumetanide-treated hearts was significantly higher than that in the EIPA-treated hearts ($P < 0.05$). The decrease in Na_i during the first 5 min of reperfusion in the EIPA- and bumetanide-treated hearts was similar to that of control hearts and significantly less than that of preconditioned hearts. All groups had similar values of Na_i at the end of reperfusion.

Response to an acid load (NH_4Cl prepulse)

The response of control and preconditioned hearts to an acid load is shown in Fig. 6. Each 5-min preconditioning episode significantly reduced intracellular pH (range, 6.38±0.02 [first ischemia period] to 6.46±0.01 [last ischemia period]), with recovery to normal pH with each reperfusion episode (data not

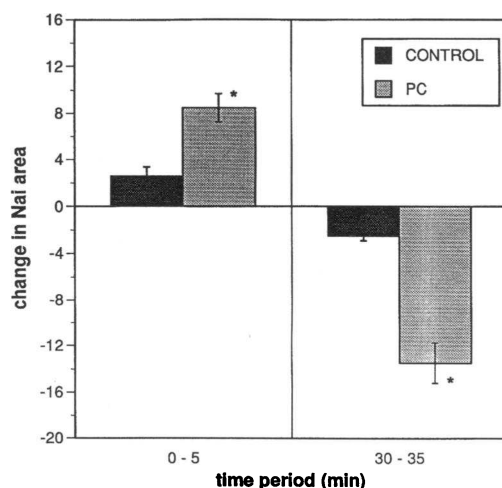

Figure 4. Changes in normalized intracellular sodium (Na_i) resonance area during the onset of ischemia (0–5 min) and the onset of reperfusion (30–35 min) in control and preconditioned (PC) hearts. Areas derived from data shown in Fig. 3. The preconditioned hearts had significantly greater changes in Na_i than did the control hearts during these time periods. $*P < 0.05$ preconditioned vs. control hearts.

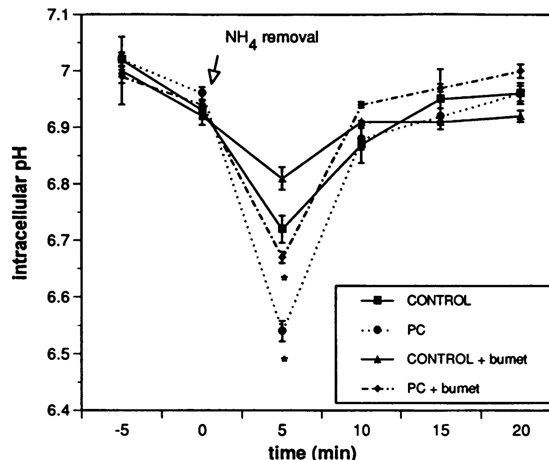

Figure 6. Changes in intracellular pH as a function of time in control and preconditioned hearts without (control, ■ and preconditioned (*PC*), ●) and with (control, ▲ and preconditioned, ◆) bumetanide added to the perfusate during NH_4Cl exposure and washout. The hearts were exposed to NH_4Cl for 10 min followed by washout in K^+-free buffer containing ouabain as described in Fig. 1. The data points during NH_4Cl exposure (time −5 and 0 min) and during NH_4Cl washout (time 5–20 min) are shown here. NH_4Cl was removed from the perfusate at the end of the acquisition period marked "NH_4 removal." Bumetanide eliminated the greater acidification of the preconditioned hearts, suggesting that greater acidification was due to increased activity of the Na-K-2Cl cotransporter. Rapid pH recovery was maintained in the preconditioned hearts *despite* the presence of bumetanide and a nadir pH equal to that of control hearts, suggesting that this recovery was independent of the cotransporter. $*P < 0.05$ PC and PC + bumetanide vs. control and control + bumetanide hearts, respectively.

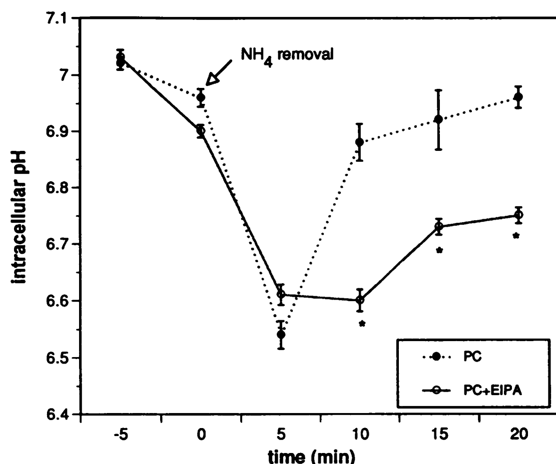

Figure 7. Changes in pH in preconditioned hearts (PC) during and after NH_4Cl exposure, with addition of EIPA at the time of NH_4Cl removal (○). Conditions were identical to experiments shown in Fig. 6. Data in the preconditioned hearts without EIPA (●) are from Fig. 6. $^*P < 0.05$ vs. preconditioned hearts without EIPA.

shown). The pH during the exposure to NH_4Cl was identical between the preconditioned and control hearts groups (7.02±0.02 after 10 min). Preconditioned hearts exhibited greater acidification and a more rapid recovery of pH after exposure and removal of NH_4Cl. The nadir pH was observed in the spectrum acquired 5 min after removal of NH_4Cl and equalled 6.72±0.02 in the control hearts compared with 6.54±0.02 in the preconditioned hearts ($P < 0.05$). The preconditioned hearts had a rapid pH recovery during the washout phase ($\Delta pH = 0.35 \pm 0.04$ over 5 min), while the control hearts had a significantly slower pH recovery ($\Delta pH = 0.15 \pm 0.02$). Both groups of hearts achieved the same final pH at the end of the measurement period (6.96±0.02).

Response to an acid load (NH_4Cl prepulse) with inhibition of the Na-K-2Cl cotransporter. Because the increased acidification observed in the preconditioned hearts could be a result of increased entry of NH_4^+ via the Na-K-2Cl cotransporter (NH_4^+ can substitute for K^+ on the cotransporter) (15), further experiments were performed in the presence of 10 μM bumetanide (a potent inhibitor of the Na^+-K^+-$2Cl^-$ cotransporter) (16, 17). The results, shown in Fig. 6, demonstrate that bumetanide significantly limited intracellular acidification in both the preconditioned and control hearts although, as in the prior experiments, the preconditioned hearts acidified more than the control hearts and alkalinized more rapidly ($\Delta pH = 0.28 \pm 0.02$ vs. $\Delta pH = 0.10 \pm 0.03$ over 5 min, preconditioned vs. control, respectively; $P < 0.001$). The lower nadir pH in the preconditioned hearts in the absence of bumetanide suggests greater NH_4^+ entry into the heart via the Na-K-2Cl cotransporter, and therefore that preconditioning stimulated inward flux of the cotransporter.

Despite these differences in nadir pH in the presence or absence of bumetanide, the *rate* of pH recovery after an ammonium prepulse in the preconditioned hearts was similar in the presence and absence of bumetanide ($\Delta pH = 0.28 \pm 0.02$ in the presence of bumetanide, $\Delta pH = 0.35 \pm 0.04$ in the absence of bumetanide over 5 min; Fig. 6), suggesting that the enhanced pH recovery of the preconditioned hearts after an acid load did not involve the Na-K-2Cl cotransporter.

Response to an acid load (NH_4Cl prepulse) with inhibition of the Na^+-H^+ exchanger. To determine the role of the NaH exchanger in pH recovery after an acid load, experiments similar to those above were performed in which the Na-H exchanger was inhibited by 10 μM EIPA during the washout phase after an ammonium prepulse (18). Fig. 7 demonstrates that the presence of EIPA retarded pH recovery in preconditioned hearts. The preconditioned hearts without EIPA had a rapid recovery to normal pH during the washout phase ($\Delta pH = 0.35 \pm 0.04$ in the first 15 min), while preconditioned hearts with EIPA had no pH recovery for the first 5 min, and subsequently had limited pH recovery. This response resulted in significantly lower pH values than in the absence of EIPA. Comparison of these differences in pH recovery with and without EIPA suggests that all of the pH recovery in the first 5-min period after the pH nadir in the preconditioned hearts occurred via the Na-H exchanger.

Discussion

This study has demonstrated that ischemic preconditioning resulted in a rapid increase in Na_i at the onset of global ischemia and a rapid decrease on reperfusion, consistent with stimulation of mechanisms that enhance sodium transport across the sarcolemma under these conditions. The response of preconditioned hearts to an acid load under conditions in which the Na-K-ATPase and Na-K-2Cl cotransporter were inhibited are consistent with preconditioning stimulating the Na-K-2Cl cotransporter (resulting in increased acidosis following a NH_4Cl prepulse) and the Na-H exchanger (resulting in more rapid recovery from acidosis). In support of a role for stimulation of these sodium transport mechanisms in the protective effect of preconditioning, inhibition of these changes in sodium transport blunted the protective effects of preconditioning on functional recovery and on ischemic injury.

Na^+_i accumulation in preconditioning. Few studies have measured intracellular sodium during ischemia in preconditioned hearts (19, 20). In adult rat hearts, Steenbergen et al. (19) used the sodium shift reagent thulium-DOTP^{5-} in combination with the calcium chelator 5F-BAPTA in a protocol similar to the one employed in the current study. They found no significant differences in Na_i between control and preconditioned hearts. The current observations of a rapid increase in Na_i at the onset of ischemia, no reduction in Na_i at the end of ischemia, and a rapid decrease in Na_i on reperfusion were not seen by Steenbergen et al. (19). The failure to demonstrate these kinetic changes in Na^+_i in their study may have been due to *(a)* loading of perfused hearts with the calcium chelator 5F-BAPTA, resulting in low developed pressure throughout the experiment; *(b)* the presence of at least 20 mM NH_4 as a counter-cation with their shift reagent (based on the preparation methodology employed); and *(c)* baseline ischemia preparations, signified by low pre-ischemia PCr/ATP ratios and progressive reductions in pH with the preconditioning episodes before global ischemia.

Na transport mechanisms. The observed changes in Na_i, and particularly the rapid increase in Na_i at the onset of ischemia in the preconditioned hearts, are consistent with stimulation of transport mechanisms that increase sodium entry into the cell. These mechanisms include the Na-K-2Cl cotransporter and the

Na^+-H^+ exchanger, with stimulation of either potentially resulting in reduced intracellular acidosis during ischemia. Regulation of the Na-K-2Cl cotransporter is multifactorial, with evidence that the cotransporter is regulated by changes in cell volume (15) and cyclic-AMP–dependent (21–23) and non-cAMP–dependent protein phosphorylation (24) as well as ionic concentration gradients. Increased inward cotransporter flux *during ischemia* after preconditioning could be beneficial in protecting the heart through its functional coupling with the Cl/HCO_3 exchanger (25). In this paradigm, chloride transported into the cell by the Na-K-2Cl cotransporter may exit via Cl/HCO_3 exchange, thus increasing intracellular HCO_3 and limiting acidosis.

The primary factors regulating the Na-H exchanger include the intracellular proton concentration and the phosphorylation state (26, 27). Under baseline conditions, the exchanger contributes to the extracellular/intracellular proton gradient with net outward transport of protons (28). Under conditions of ischemia in which protons are generated and intracellular pH decreases, the exchanger is stimulated to increase extrusion of protons, although with an obligatory increase in inward transport of sodium ions (29). The prior observations of reduced acidification during ischemia (9) and the current observations of an increased rate of intracellular sodium accumulation following preconditioning are consistent with stimulation of either the cotransporter or exchanger by preconditioning. These findings are similar to those observed by Bak and Ingwall (30) in hyperthyroid rat hearts in which increased activity of the Na-H exchanger resulted in higher pH during global ischemia, and may also explain the excellent recovery of function in hypertrophied hearts from hyperthyroid rats after global ischemia (31).

Reduction in Na_i on reperfusion. Despite similar levels of intracellular sodium during ischemia in the preconditioned hearts, there was a significantly more rapid reduction in Na_i on reperfusion, presumably reflecting increased sodium efflux. These findings are concordant with the rapid reduction in Na_i observed by Tani and Neely (32) in hearts pretreated by 10 min of anoxia before global ischemia (a likely ''preconditioning'' stimulus). Although previous studies have demonstrated that the cotransporter is directed into the cell under control conditions (33), arguments concerning thermodynamics and previous studies (34, 35) show that the cotransporter may be directed out of the cell during ischemia and reperfusion. Therefore, one potential mechanism that could increase sodium efflux under conditions of high intracellular sodium and chloride at the end of ischemia (36) is outward flux through the Na-K-2Cl cotransporter. A significant role for this mechanism is supported by the acidification of preconditioned hearts after exposure to NH_4Cl (as discussed below) and the deleterious effect that bumetanide (a Na-K-2Cl cotransporter inhibitor) had on inhibiting sodium efflux during reperfusion when Na,K-ATPase was inhibited (35).

Response to an acid load in preconditioning: acidification. The pH response to an acid load using ammonium prepulse experiments is an established measure to differentiate the possible mechanisms involved in proton extrusion and, as used in this study, provides insight into the effect of preconditioning on the Na-K-2Cl cotransporter and Na-H exchanger. Under the conditions of the present experiments (K^+-free perfusate containing ouabain), acidification on NH_4^+ removal was likely to be the result of entry of NH_4^+ by the K^+ channels or Na-K-2Cl cotransporter. Because the greater reduction in pH after removal of NH_4Cl in the preconditioned hearts was eliminated by bumetanide, increased acidification in the preconditioned hearts was likely due to stimulation of the Na-K-2Cl cotransporter. There are several mechanisms by which Na-K-2Cl cotransporter activity could be altered. Opening of ATP-sensitive potassium channels (K_{ATP} channels), known to be involved in several models of preconditioning (37), could increase extracellular K^+ and increase inward cotransporter flux (34). Alternatively, because protein kinase activation has been implicated in preconditioning (8), direct phosphorylation of the cotransporter could similarly increase inward flux.

Response to an acid load in preconditioning: pH recovery. The experiments measuring the effect of preconditioning after an acid load also support a significant effect of preconditioning on the activity of the Na-H exchanger. Potential mechanisms that ''effectively'' extrude protons from the myocyte include, in addition to the Na-H exchanger, the Cl^--HCO_3^- exchanger and the CO_2-lactate cotransporter, although involvement of the Na^+-dependent HCO_3 transporter is also possible (14). The more rapid rate of intracellular pH recovery from an acid load in the preconditioned hearts compared with control hearts demonstrates increased proton extrusion, while the substantial effect of EIPA in limiting pH recovery is clear evidence for a significant role of the Na-H exchanger on pH recovery in preconditioned hearts. These data demonstrating an important role for the Na-H exchanger in the recovery of pH after an acid load are consistent with data from Lieberman (28), in which stimulation of the Na-H exchanger enhanced pH recovery following an acid perturbation.

Potential mechanisms of Na-H exchanger activation in preconditioning. Although these data show that kinetics of Na_i accumulation are increased by preconditioning, the mechanism(s) of this increase can only be postulated. Recent data have shown that protein kinase C is activated and translocated during preconditioning, and that protein-kinase-C–dependent phosphorylation is necessary for the protective effects observed in preconditioned hearts (7, 8). It was shown in those studies that administering a protein kinase C inhibitor just before the sustained ischemia abolished the protective effect of preconditioning, while activation of protein kinase C by phorbol ester pretreatment mimicked preconditioning. Wallert and Frohlich (38) have also shown that the cardiac Na-H exchanger is stimulated by activation of protein kinase C, as well as by α-adrenergic stimulation, and that stimulation of the Na-H exchanger restored intracellular pH to normal values within minutes after an acid load. These studies therefore suggest a mechanism by which the Na-H exchanger is activated by preconditioning; namely, that activation and translocation of protein kinase C by preconditioning before ischemia stimulates the Na-H exchanger, subsequently limiting acidification during ischemia.

In addition to activation of protein kinase C, other mechanisms for enhanced activity of the Na-H exchanger may be related to the repetitive, transient acidosis elicited by the preconditioning episodes. The intracellular acidification seen with each preconditioning episode (pH approximately 6.45) is sufficient to transiently increase proton efflux via the Na-H exchanger (29), and it is possible that repetitive stimulation of the exchanger could increase the number of active sites or copies of the exchanger in the cell membrane.

Inhibition of the Na-H exchanger and/or Na-K-2Cl cotrans-

porter. The current experiments show that pharmacologic interventions that inhibit the Na-H exchanger or Na-K-2Cl cotransporter after preconditioning (but before global ischemia) blunt the protective effects of preconditioning on functional recovery and, in the case of EIPA, on ischemic injury. These results, while supporting the protective effect of stimulation of proton and sodium transport observed in preconditioned hearts, raise issues regarding the protective role of inhibition of either the Na-H exchanger or Na-K-2Cl cotransporter during ischemia. Because inhibition of the Na-H exchanger or Na-K-2Cl cotransporter before ischemia in the *absence* of preconditioning has been shown to limit the increase in Na_i and be protective (39, 40), the lack of significant protection by EIPA or bumetanide in the current experiments suggests that there may be beneficial effects of sodium transport stimulation by preconditioning that are subsequently counteracted by the inhibitory effects of these agents.

Limitations. Whereas the current findings strongly support the hypothesis that ischemic preconditioning increases intracellular sodium concentration before ischemia and enhances proton efflux during ischemia, these observations must be interpreted within the limitations of the experimental design. First, the isolated heart model may not represent the in situ preconditioning effects observed in regional ischemia (1). However, the beneficial effects of preconditioning on myocardial function and ischemic injury parallel those in other models, as do the effects on high energy phosphates and pH (4). Thus, these similarities suggest that this model is representative of preconditioning. Second, while we have primarily addressed alterations in the Na-H exchanger and Na-K-2Cl cotransporter, other mechanisms limiting proton production (9, 41) or enhancing effective proton efflux (such as the Cl/HCO_3 exchanger) may be altered by preconditioning, although the ammonium prepulse experiments with EIPA are consistent with previous results suggesting that a primary mechanism of proton efflux immediately after an acid load is Na-H exchange. Third, these data do not define the involvement of other cardioprotective mechanisms in preconditioning, such as ATP-sensitive K^+ channels or adenosine receptor stimulation (5, 37).

The technique of employing a shift reagent to measure intracellular sodium has inherent limitations. The low level of intracellular sodium under baseline conditions makes resolution and quantification of the intracellular resonance difficult and subject to greater error. However, the rise in Na_i during ischemia improved resolution of the intracellular sodium resonance and made its measurement more reliable. While changes in the volume of the extracellular compartment that occur during ischemia and reperfusion may have changed the relative concentration of the shift reagent in the extracellular space, resulting in a change in chemical shift, the absence of significant changes in these chemical shifts suggests that concentration changes of the shift reagent in the extracellular space were minimal. Furthermore, because the cell membrane is impermeable to the shift reagent (19), changes in extracellular concentration should not have altered the measurement of Na_i. Finally, changes in lineshape of the intracellular resonances were observed and may have been due to spectral processing techniques or inherent changes in the intracellular sodium environment. The integration algorithms were therefore chosen without assumption of either a Lorentzian or Gaussian lineshape and should represent accurately the resonance intensity. However, potentially different intrinsic changes in the intracellular and extracellular compartments between the groups cannot be excluded as a factor in the measurement of Na_i.

Conclusion. Ischemic preconditioning results in a rapid increase in Na_i at the onset of global ischemia and a rapid decrease on reperfusion, consistent with activation of mechanisms that enhance sodium transport across the sarcolemma under these conditions. Greater acidification of preconditioned hearts in response to a NH_4Cl prepulse is consistent with increased inward NH_4^+ flux via the Na-K-2Cl cotransporter flux after preconditioning, whereas more rapid recovery from acidosis, which is inhibited by EIPA, indicates that proton efflux in preconditioned hearts is increased primarily by stimulation of Na-H exchange. In combination with data showing reduced intracellular acidification during ischemia, these data support the postulate that a significant beneficial effect of ischemic preconditioning results from activation of the Na-H exchanger and/or Na-K-2Cl cotransporter before ischemia.

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