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Ischemic Preconditioning: Effects on pH, Na and Ca in Newborn Rabbit Hearts During Ischemia/Reperfusion

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H. Liu, P. M. Cala and S. E. Anderson. Ischemic Preconditioning: Effects on pH, Na and Ca in Newborn Rabbit Hearts During Ischemia/Reperfusion. *Journal of Molecular and Cellular Cardiology* (1998) **30**, 685–697. In adult hearts, ischemic preconditioning (PC) has been shown to decrease ischemia-induced changes in intracellular pH (pH_i) and [Ca] ($[Ca]_i$) and decrease associated injury. These results are consistent with the interpretation that PC decreases the stimulus for Na uptake via Na/H exchange, thereby decreasing intracellular Na (Na_i) accumulation, and thus decreasing the change in force driving Na/Ca exchange, which otherwise contributes to ischemia-induced increases in $[Ca]_i$. Given documented age-related differences in myocardial responses to ischemia, we tested the hypothesis that in newborn hearts, PC will diminish intracellular [H], Na_i, and $[Ca]_i$ during ischemia/reperfusion. NMR was used to measure pH_i, Na_i, $[Ca]_i$, ATP, and PCr in isolated newborn (4–7 days) rabbit hearts Langendorff-perfused with Krebs–Henseleit solution equilibrated with 95% O_2/5% CO_2 at 36 ± 1°C. Control hearts were perfused 30 min before initiating 40 min global ischemia followed by 40 min reperfusion. PC hearts were treated the same except four 5-min intervals of ischemia each followed by 10 min of perfusion which preceded global ischemia. At end ischemia, pH_i was higher in PC than control hearts (6.31 ± 0.03 *v* 5.83 ± 0.05; $P<0.05$). Similarly, PC diminished Na_i-accumulation during ischemia and reperfusion ($P<0.05$). Control Na_i rose from 16.2 ± 2.6 to 108.8 ± 10.3 (mEq/kg dry weight) and recovered to 55.2 ± 10.1 and the corresponding values for PC hearts were 25.6 ± 6.2, 70.0 ± 7.9 and 21.9 ± 5.2. PC also improved $[Ca]_i$ recovery during reperfusion ($P<0.05$). Control $[Ca]_i$ rose from 418 ± 43 to 1100 ± 78 (nM/l) and recovered to 773 ± 63, whereas in PC hearts the values were 382 ± 40, 852 ± 136 and 371 ± 45, respectively. In addition, PC decreased coronary resistance during reperfusion ($P<0.05$) as reflected by lower perfusion pressures under constant flow conditions (65.9 ± 1.5 *v* 56.1 ± 4.1 mmHg at end of reperfusion). Finally, PC improved recovery of left-ventricular developed pressure (LVDP—43.8 ± 12.0 *v* 17.2 ± 3.0% of control; $P<0.05$) and diminished CK release (607 ± 245 *v* 2432 ± 639 IU/g dry weight; $P<0.05$) during reperfusion. The results are consistent with the hypothesis.

Key Words: H; Na; Ca; Ischemic preconditioning; Na/H exchange; Newborn.

Introduction

Ischemic preconditioning (PC—alternating short intervals of ischemia and perfusion) has been referred to as "state of the art myocardial protection" (Lawson and Downey, 1993). The mechanism(s) by which preconditioning decrease(s) ischemic heart damage has been the subject of considerable interest and several theories have been proposed. For instance, release of adenosine during PC is associated with protection during subsequent episodes of ischemia (Liu *et al.*, 1991). It has also been proposed that the effects of PC are mediated by ATP-sensitive K channels (Yao and Gross, 1994), altered carbohydrate metabolism (Murry *et al.*, 1990), decreased free oxygen radicals (Das *et al.*, 1992),

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altered fatty acid metabolism (Simkhovich *et al.*, 1993), and, more recently, the activation of protein kinase C (Ytrehus *et al.*, 1994). Thus, the mechanism of PC remains controversial (Li and Kloner, 1993; Steenbergen *et al.*, 1993a; Thornton *et al.*, 1993). While PC has been shown to be effective in protection against ischemic damage in adult hearts of dogs, pigs, rats, rabbits, and humans (Li *et al.*, 1990; Schott *et al.*, 1990; Cohen *et al.*, 1991; Li *et al.*, 1992; Yellon *et al.*, 1993), the effect of PC in newborn hearts still needs to be studied.

In the last decade, it has become clear that most pathophysiological processes in cardiac ischemia and reperfusion are associated with derangement of cellular ion homeostasis (Allen *et al.*, 1993). In adult hearts, myocardial ischemic/hypoxic damage is associated with increased intracellular calcium (Ca_i) or Ca influx (Naylor, 1987), and increased Ca_i has been identified as a causal factor in reperfusion injury (Allen *et al.*, 1993; Pierce and Czubryt, 1995). The Na-dependence of myocyte Ca uptake (Kim *et al.*, 1987) implicates Na/Ca exchange as a major effector in Ca_i-dependent reperfusion/reoxygenation injury. In this context, we and others have reported results consistent with the "general hypothesis" that one important sequence of events leading to myocardial ischemic/hypoxic cell damage is: (1) increased anaerobic metabolism; (2) decreased intracellular pH (pH_i); (3) H_i stimulation of pH-regulatory Na/H exchange; (4) increased intracellular Na (Na_i); (5) decreased and/or reversed Na/Ca exchange; (6) increased $[Ca]_i$; (7) a cascade of Ca-dependent events leading to cell damage (Anderson *et al.*, 1990; Pike *et al.*, 1993; Steenbergen *et al.*, 1993b). Even though the mechanism(s) by which preconditioning decreases ischemic heart damage remains controversial, if this general hypothesis is correct, PC must alter one or more of the events in the sequence outlined above. Results consistent with this point of view have been presented for the adult heart (Steenbergen *et al.*, 1993a). That is, PC has been shown to diminish the increases in H_i and $[Ca]_i$ otherwise observed during prolonged ischemia. Although this study did not show a significant effect of PC on Na_i, the trend was consistent with the interpretation that the changes in Ca_i are Na-dependent. On the other hand, a more recent study using a similar model demonstrated that PC initially increased Na_i accumulation during ischemia, but it too concluded that PC had no significant effect on Na_i after 30 min of ischemia (Ramasamy *et al.*, 1995). Thus, the effect of PC on Na uptake and accumulation in adult hearts during ischemia remains unclear and will require further investigation.

While it is well understood that newborn and adult hearts are different both histologically and biochemically, the differences between adult and newborn heart responses to ischemia and hypoxia, as well as in appropriate treatments for prevention of associated myocardial injury, also remain controversial (Nishioka and Jarmakani, 1982; Parrish *et al.*, 1987; Kempsford, 1989). More specifically, relatively little is known about age-related differences in proton, Na, and Ca accumulation and transport during ischemia. For example, in a model similar to that used in this study, ATP fell more in adult hearts than newborns during ischemia, whereas percent decreases in PCr during ischemia were not significantly different (Carr *et al.*, 1992). Although it has been argued that the major source of proton production arises from glycolysis (Allen and Orchard, 1987), because the balance between ATP and PCr hydrolysis also influences net proton production (Dennis *et al.*, 1991), differences in PCr and ATP depletion may be implicated in age-related differences in proton accumulation during ischemia and reperfusion. Indeed, an aforementioned study demonstrated that although there were no significant differences in proton accumulation after 30 min of ischemia, pH_i was higher after 30 min of reperfusion in newborns than in adults (Carr *et al.*, 1992).

With respect to Na/H and Na/Ca exchange in particular, there have also been conflicting reports of age-related differences. For instance, during hypoxic respiratory acidosis, the Na/H exchange rate was less in adults than in newborn rabbit hearts (Seguchi and Jarmakani, 1989), whereas in isolated rabbit cardiac myocytes, a subsequent study reported no age-related differences in the Na/H exchange activity, but HCO_3/Cl exchange was more active in premature myocardium (Nakanishi *et al.*, 1992). Additionally, while it has been reported that there is approximately 2.5 times more Na/Ca exchanger protein in fetal and newborn rabbit sarcolemma than in adult preparations (Artman, 1992), no age-related differences in Na/Ca exchange activity were found in canine cardiac sarcolemmal preparations (Hanson *et al.*, 1993). Finally, it has recently been reported that there may be a reciprocal change in Na/K pump and Na/Ca exchange in rat myocardium between 8–15 days, with Na/Ca exchanger abundance decreasing with age (Magyar *et al.*, 1995).

The effects of PC on ion distribution in newborn hearts, however, have not been previously reported and, based on the studies cited above, one could not confidently predict that newborn hearts would respond to PC as do adults. We therefore used NMR

to test the hypothesis that in newborn hearts PC will diminish intracellular [H], Na_i, and $[Ca]_i$ during ischemia/reperfusion. Our results support this hypothesis and further demonstrate that during reperfusion, PC-dependent limitation of $[Ca]_i$ is associated with diminished coronary resistance and creatine kinase release and improved recovery of left-ventricular developed pressure (LVDP). Thus, the results are further consistent with the "general hypothesis" outlined above, which provides a framework for understanding the mechanisms responsible for changes in the distribution of these ions and associated myocardial injury. A portion of these results has been presented previously in abstract form (Liu *et al.*, 1994).

Materials and Methods

General

The methods used were modified from those previously reported (Anderson *et al.*, 1990, 1994). New Zealand white rabbits (4–7 days old) were anesthetized with sodium pentobarbital (35–65 mg/kg) and heparinized (1000 USP units/kg). Hearts were removed and the aorta was cannulated and perfused at a constant rate (9–10 ml/min) at 36 ± 1°C. Control perfusate contained (mmol/l) 133 NaCl, 4.75 KCl, 1.25 $MgCl_2$, 1.82 $CaCl_2$, 25 $NaHCO_3$, 11.1 dextrose and was equilibrated with 95% O_2/5% CO_2, which provided a pH of 7.35–7.45. In order to measure left-ventricular developed pressure (LVDP—end systolic minus end diastolic pressure), a fluid filled balloon was placed in the left ventricle and secured by means of a ligature immediately proximal to the mitral valve. Perfusion pressure and left-ventricular pressure were monitored using a Gould RS2000 oscillographic recorder. The control ischemia protocol consisted of 30 min perfusion, followed by 40 min ischemia, followed by 40 min reperfusion. In the preconditioning (PC), protocol hearts were perfused for 10 min, followed by four episodes of 5 min of ischemia, followed by 10 min reperfusion each. After preconditioning, hearts were treated the same as in the control ischemia protocol: 40 min ischemia and 40 min reperfusion. Initiation of 40 min of ischemia was designated $t = 0$ min for all hearts. ^{23}Na, ^{19}F, and ^{31}P NMR were used to measure Na_i, $[Ca]_i$, and pH_i and high-energy phosphates, respectively. In order to measure Na_i, 7.5 mM dysprosium triethylenetetraminehexaacetic acid (DyTTHA) was substituted iso-osmotically for NaCl in the perfusate and Ca was added to reach a perfusate concentration of 1.8–2 mM, as measured by Ca electrode. In order to measure $[Ca]_i$, hearts were perfused for 30–40 min prior to the control interval with perfusate containing the acetoxymethyl ester of 5F-1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetra-acetic acid (FBAPTA) at 2.5 μM (Anderson *et al.*, 1990). FBAPTA was then washed out of the extracellular space with control solution for 15 min before measurement of $[Ca]_i$. After perfusions had been completed, hearts used to measure ^{23}Na were weighed wet and dried to constant weight (at least 48 h) at 65°C to determine dry weight. Their wet and dry weights were 0.555 ± 0.020 and 0.085 ± 0.003 g, respectively ($n = 14$).

NMR spectroscopy

^{23}Na and ^{31}P experiments were conducted using a Bruker AMX400 spectrometer and ^{19}F experiments were conducted using a GE Omega 300 horizontal bore system. ^{23}Na, ^{19}F, and ^{31}P spectra were generated from the summed free induction decays of 1000, 1500, and 148 excitation pulses (90°, 45° and 60°) using 2K, 2K, and 4K word data files and ± 4000-, ± 5000-, and ± 4000-Hz sweep widths, respectively. For all nuclei, data files were collected over 5-min intervals. In order to improve signal-to-noise for ^{19}F measurement of $[Ca]_i$, two 5-min ^{19}F files were added together. Because the NMR signal intensity reflects the time average for the interval over which data are collected, data are represented in time as corresponding to the midpoint of the appropriate 5- or 10-min acquisition interval. For technical reasons, spectra for each of the three nuclei were acquired from separate hearts. Representative ^{23}Na, ^{19}F, and ^{31}P spectra are shown in Figures 1–3, respectively.

Na_i in mEq/kg dry weight was calculated from the calibrated area under the unshifted peak of the ^{23}Na spectra, as previously described (Anderson *et al.*, 1990). Briefly, each spectrum was reversed along the frequency axis using standard Bruker AMX400 software. Then, after precisely shifting the reversed spectrum so that the original and reversed extracellular peaks overlapped, each original spectrum was subtracted from its own reversed spectrum. Thus, the extracellular peaks were subtracted out of the difference spectrum. Finally, the Na_i spectral area was determined by integrating over the remaining positive peak. $[Ca]_i$ in nmol/l cell water was calculated as the product of the 500 nM Ca-FBAPTA dissociation constant and the

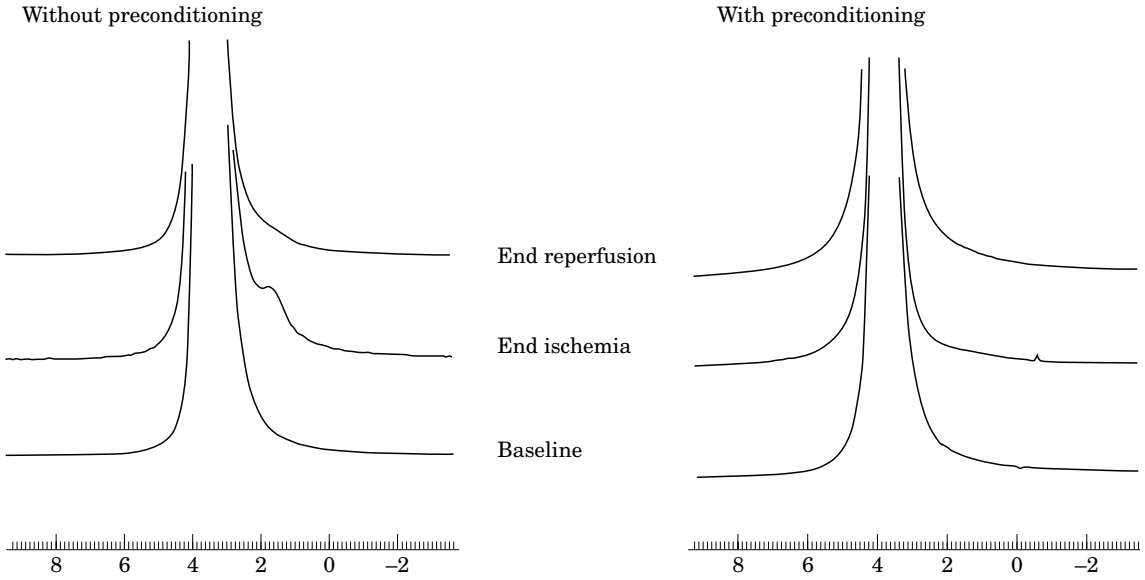

Figure 1 Representative 23Na spectra with and without preconditioning (PC) on the right and left respectively. The spectra show that PC prevents the increase in intracellular Na resonance (upfield shoulder) which is otherwise observed at the end of ischemia (compare middle spectra).

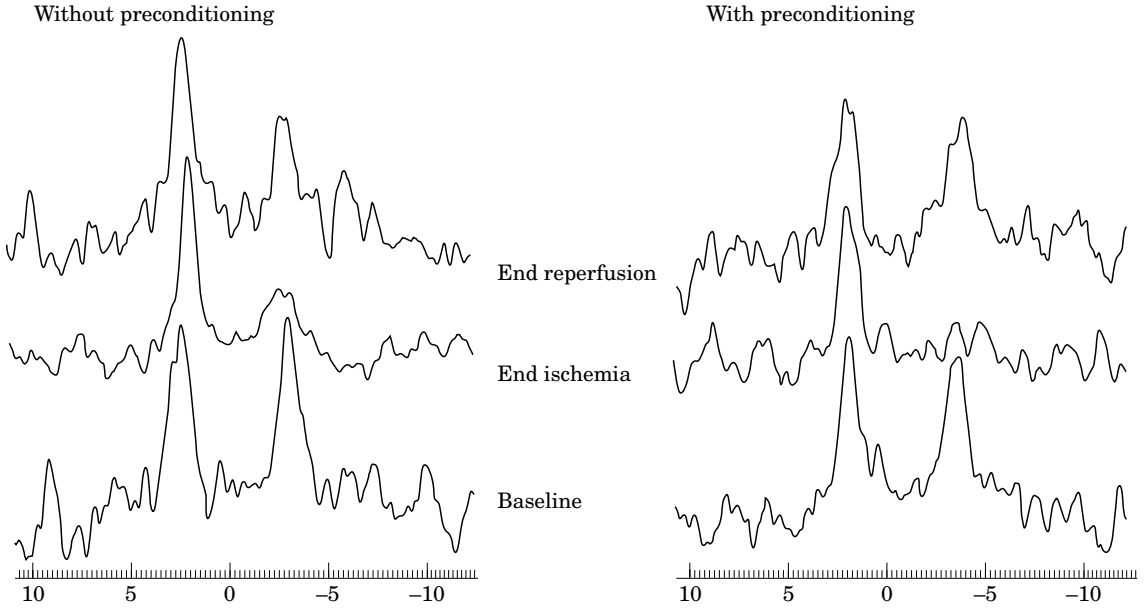

Figure 2 Representative 19F spectra with and without preconditioning (PC) on the right and left respectively. The spectra show that PC improves the recovery of the ratio of the areas of the Ca-bound to Ca-free FBAPTA peaks (downfield and upfield, respectively), which is indicative of improved recovery of intracellular [Ca] during reperfusion. Please see Materials and Methods for calculation of intracellular [Ca].

ratio of the areas of the Ca-bound (downfield) and Ca-free (upfield) peaks in the FBAPTA spectrum (Anderson *et al.*, 1990). Intracellular pH was determined from the chemical shift of the inorganic phosphate (Pi) resonance [with reference to control phosphocreatine (PCr)] calibrated at 37°C (Anderson *et al.*, 1994). High-energy phosphates are reported as percent of control peak intensity (Anderson *et al.*, 1990).

In order to assess ischemic injury, total creatine kinase (CK) released during reperfusion was measured from timed collections of effluent perfusate for

Figure 3 Representative 31P spectra with and without preconditioning (PC) on the right and left respectively. PCr resonance is set at 0 p.p.m. in the baseline spectra. From PCr, major upfield peaks arise from γ, α and β phosphates of ATP and downfield peaks arise from phosphodiesters, Pi, and phosphomonoesters, respectively. Closer inspection reveals that PC limits the change in Pi chemical shift during ischemia indicating decreased proton accumulation. Please see Materials and Methods for further description of high energy phosphate and pH_i analysis.

40 min of reperfusion. CK in IU/g dry weight was measured spectrophotometrically, as previously described (Ramasamy *et al.*, 1995).

Unless otherwise stated, results are reported as mean $\pm$ S.E.M. Analysis of variance for repeated measures was used to test for differences between control and PC treatments. When differences between treatments were found, the unpaired *t*-test was used to determine the times at which differences between treatments occurred. Please note that the *t*-test was used only across treatments after the effect of PC had been demonstrated by ANOVA, and only for a particular time interval, thus obviating the need for multiple comparison tests. Differences were considered significant when $P<0.05$.

Results

Intracellular proton accumulation

Intracellular pH (pH_i) was measured during ischemia $\pm$ PC as one variable required to assess the effect of PC on Na/H exchange during and after ischemia. The data shown in Figure 4, demonstrate that at the end of 40 min sustained ischemia pH_i in preconditioned hearts is higher than that of control hearts (6.31 ± 0.03 *v* 5.83 ± 0.05; $P<0.05$). The pH_i was not measurably different after 40 min of reperfusion (7.12 ± 0.01 *v* 7.11 ± 0.02). We also found pH_i at the end of preconditioning (prior to ischemia, $t = -2.5$ min) is higher than without preconditioning ($P<0.05$).

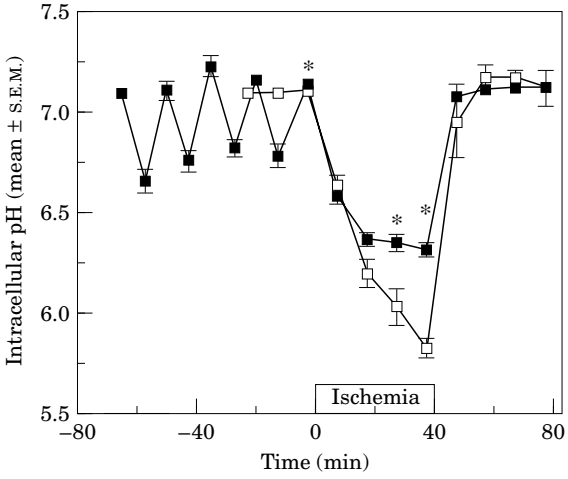

Figure 4 In newborn rabbit hearts, ischemic preconditioning decreases intracellular [H] prior to and during ischemia. Intracellular pH is plotted *v* time before, during and after ischemia with (closed squares) and without (open squares) ischemic preconditioning (PC). *$P<0.05$; ischemia ($n=8$), isch + PC ($n=7$) (n = number of experiments). The large oscillations in intracellular pH prior to prolonged ischemia are due to PC.

Intracellular Na accumulation

The data shown in Figure 5 demonstrate that, compared to the control group and consistent with the hypotheses, PC diminished Na_i during ischemia/reperfusion. During 40 min of ischemia, Na_i (mEq/kg dry weight) rose from 16.2 ± 2.6 to 108.8 ± 10.3 and recovered to 55.2 ± 10.1 after 40 min of reperfusion in the control group, whereas in the PC group, Na_i rose from 25.6 ± 6.2 to 70.7 ± 7.9 and

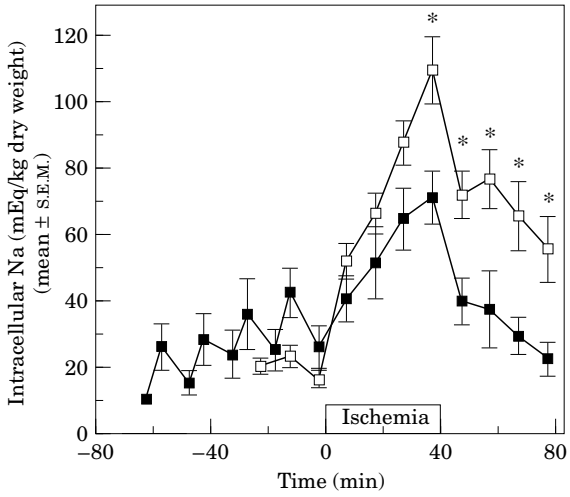

Figure 5 In newborn rabbit hearts, ischemic preconditioning decreases intracellular Na during ischemia and reperfusion. Intracellular Na (mEq/kg dry weight) is plotted *v* time before, during, and after ischemia with (closed squares) and without (open squares) ischemic preconditioning (PC). **P*<0.05; ischemia ($n=8$), isch+PC ($n=6$) (n=number of experiments).

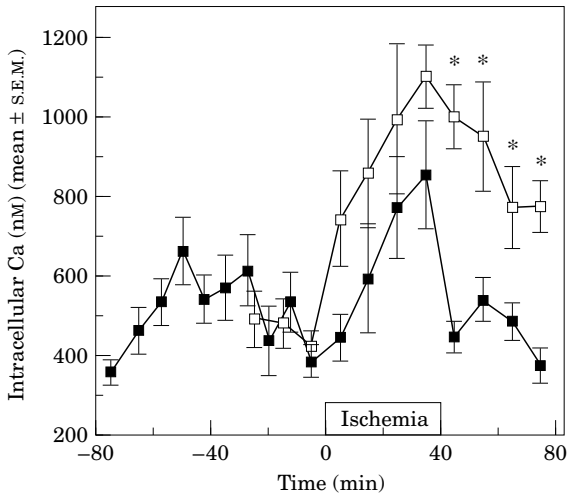

Figure 6 In newborn rabbit hearts, ischemic preconditioning improves recovery of intracellular [Ca] during reperfusion. Intracellular [Ca] (nM) is plotted *v* time before, during, and after ischemia with (closed squares) and without (open squares) ischemic preconditioning (PC). **P*<0.05; ischemia ($n=8$), isch+PC ($n=7$) (n= number of experiments).

recovered to 21.9 ± 5.2. Thus, Na_i rose less during ischemia and recovered to a lower value during reperfusion after PC ($P<0.05$).

Intracellular [Ca] changes

The results shown in Figure 6 demonstrate that, also consistent with the hypotheses, for data

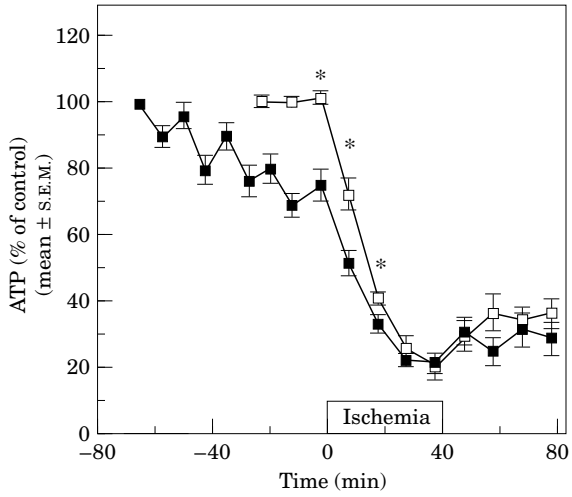

Figure 7 In newborn rabbit hearts, ischemic preconditioning significantly decreases ATP prior to and at the beginning of prolonged ischemia but has no significant effect on ATP during subsequent ischemia and reperfusion. Myocardial ATP (% of control) is plotted *v* time before, during, and after ischemia with (closed squares) and without (open squares) ischemic preconditioning (PC). **P*<0.05; ischemia ($n=8$), isch+PC ($n=7$) (n=number of experiments).

acquired between $t=-10$ and $t=80$, $[Ca]_i$ was less in the PC-treated hearts than in the control ($P=0.0055$ by ANOVA). In the control group, $[Ca]_i$ rose from 418 ± 43 to 1100 ± 78 (nM/l) during 40 min of ischemia, and recovered to 773 ± 63 after 40 min reperfusion, whereas in the PC group, $[Ca]_i$ rose from 382 ± 40 to 852 ± 136 nM/l during ischemia, and recovered to 371 ± 45 during reperfusion. The *t*-test demonstrated that while $[Ca]_i$ was only nominally less during ischemia after PC ($P=0.053$ at $t=5$ min), it was significantly less throughout reperfusion in the PC-treated hearts ($P<0.05$).

Myocardial high-energy phosphate metabolism

One simple prediction based on the preceding results is that PC-diminished increases in Na_i and $[Ca]_i$ would result in diminished ATP consumption by active Na and Ca transport processes, and therefore PC would diminish high energy phosphate depletion during ischemia/reperfusion. Figures 7 and 8 summarize the results of experiments conducted to test this prediction in our neonatal rabbit model. During preconditioning, ATP declined from 100% to 74.6 ± 4.8% of pre-ischemic baseline. Thus, ATP in the PC group was significantly less than that of control group prior to, and for the first 20 min

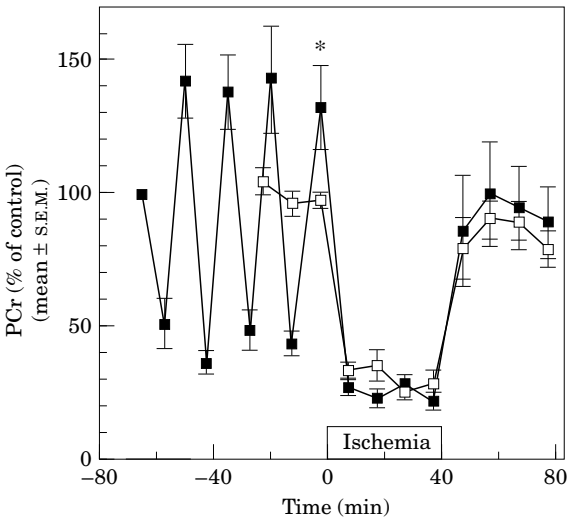

Figure 8 In newborn rabbit hearts, preconditioning increases phosphocreatine (PCr) prior to prolonged ischemia. Myocardial PCr (% of control) is plotted *v* time before, during, and after ischemia with (closed squares) and without (open squares) ischemic preconditioning (PC). *$P<0.05$; ischemia ($n=8$), isch + PC ($n=7$) ($n=$ number of experiments).

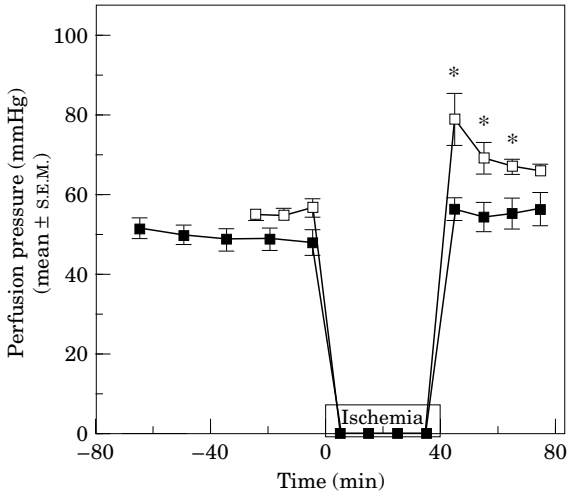

Figure 9 In newborn rabbit hearts, ischemic preconditioning prevents the increase in coronary resistance (measured by increased perfusion pressure at constant flow) otherwise observed during reperfusion. Perfusion pressure (mmHg) is plotted *v* time before, during, and after ischemia with (closed squares) and without (open squares) ischemic preconditioning (PC). *$P<0.05$; ischemia ($n=4$), isch + PC ($n=4$) ($n=$number of experiments).

of prolonged ischemia ($P<0.05$), but PC had no significant effect on ATP at the end of ischemia and during reperfusion. On the other hand, PCr increased from 100% to 132 ± 16% of the baseline value after preconditioning ($P<0.05$). Thereafter, PC had no significant effect on PCr, with PCr falling to approximately 20% and recovering to approximately 85% of control in both groups. PC also had no significant effect on inorganic phosphate accumulation before, during, or after prolonged ischemia (data not shown).

Functional recovery

The results summarized in Figure 9 demonstrate that PC diminishes the increase in coronary resistance otherwise observed during reperfusion ($P<0.05$), as measured by relatively decreased perfusion pressure under constant flow conditions. At the end of reperfusion, perfusion pressure was 65.9 ± 1.5 (mmHg) in control hearts and 56.1 ± 4.1 in PC-treated hearts. As shown in Figure 10, PC also improved recovery of LVDP ($P<0.05$). At the end of reperfusion, LVDP was 17.2 ± 3.0% of baseline in control hearts and 43.8 ± 12.0% in PC-treated hearts. Finally, as depicted in Figure 11, PC decreased total CK released during reperfusion from 2432 ± 639 IU/g dry weight to 607 ± 245 ($P<0.05$).

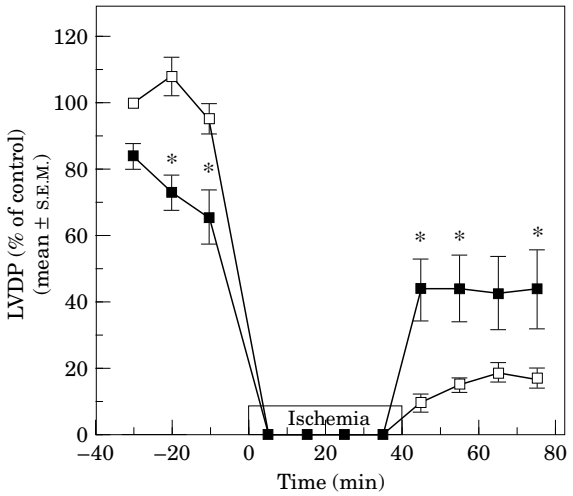

Figure 10 In newborn rabbit hearts, ischemic preconditioning improves recovery of left-ventricular developed pressure (LVDP) during reperfusion. LVDP (% of control) is plotted *v* time before, during, and after ischemia with (closed squares) and without (open squares) ischemic preconditioning (PC). *$P<0.05$; ischemia ($n=5$), isch + PC ($n=4$) ($n=$number of experiments).

Discussion

Numerous studies have demonstrated that ischemic preconditioning is effective in protection against ischemic damage in adult hearts of a variety of species, including humans (Li *et al.*, 1990; Schott

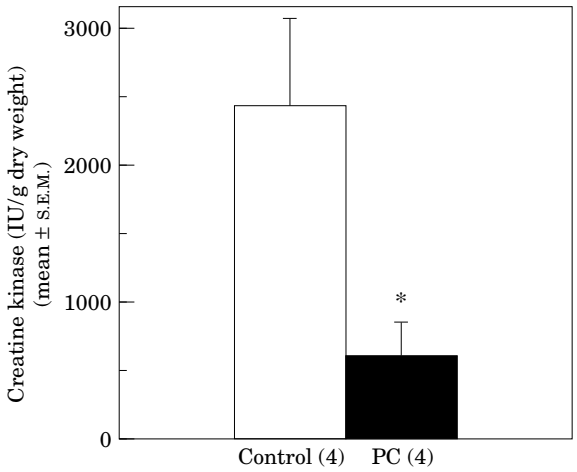

Figure 11 In newborn rabbit hearts, ischemic preconditioning decreases release of creatine kinase (CK) during reperfusion. Total CK released during reperfusion (IU/g dry weight) is plotted *v* treatment: with (closed bar) and without (open bar) ischemic preconditioning (PC). *$P<0.05$; number of experiments is given in parentheses.

et al., 1990; Cohen *et al.*, 1991; Li *et al.*, 1992; Yellon *et al.*, 1993). The results reported in this study, however, are the first to assess the effect of PC on newborn hearts and, more specifically, to test the hypothesis that PC will diminish intracellular [H], Na_i, and $[Ca]_i$ during ischemia/reperfusion in neonates.

We and others have demonstrated that hypoxia and ischemia cause increases in intracellular [H], [Na] and [Ca] in both newborn and adult hearts (Anderson *et al.*, 1990, 1996; Liu *et al.*, 1992, 1994,1997; Pike *et al.*, 1993; Steenbergen *et al.*, 1993a), consistent with the interpretation that hypoxia/ischemia-induced increases in Na_i and $[Ca]_i$ are the result of increased Na-uptake, in part via Na/H exchange, which results in Ca_i accumulation mediated by changes in Na/Ca exchange. Thus, we would predict that any process which inhibited any "link" in this chain (but preferably earlier steps) would diminish cell injury. Data addressing this prediction, however, have been conflicting in adult animals. That is, one set of results from rat hearts are consistent with the interpretation that as a result of limiting intracellular proton accumulation PC limits Ca_i accumulation (Steenbergen *et al.*, 1993a). On the other hand, a more recent study in rat hearts showed a similar PC-dependent limitation of proton accumulation was associated with an increase in Na-accumulation-rate early during ischemia (Ramasamy, 1995). The results of the current experiments, as well as relevant age-related differences in ion transport and PC-sensitive ischemia-induced changes in pH_i, Na_i, and $[Ca]_i$ are discussed in the respective subsections below.

Ischemic preconditioning diminishes intracellular H accumulation

While it remains unclear what causes the changes in pH_i after PC, the data are consistent with the interpretation that during ischemia after PC, decreases in pH_i are diminished compared to control ischemia. That is, as shown in adult hearts (Steenbergen *et al.*, 1993a; de Albuquerque *et al.*, 1994) in newborn rabbit hearts, after PC pH_i decreases more slowly during the later portion of ischemia, and thus pH_i is higher at the end of 40 min ischemia than in non-preconditioned hearts (Fig. 4). In this study, we also observed that pH_i is higher ($P<0.05$) in preconditioned hearts (7.14 ± 0.01) than in non-preconditioned hearts (7.10 ± 0.01) prior to prolonged ischemia. We subscribe to the postulate that the cell regulates its pH_i in part by H_i activation of pH-regulatory Na/H exchange (Lazdunski *et al.*, 1985; Piwnica-Worms *et al.*, 1986; Pierce and Czubryt, 1995). Thus, higher pH_i prior to and later during prolonged ischemia would tend to diminish Na/H exchange stimulation.

In adult dog hearts, it has been reported that early depletion of ATP and accumulation of glycolytic products occurs less in preconditioned hearts during ischemia. This suggests that PC reduces the rates of glycolysis and ATP-hydrolysis during prolonged ischemia (Murry *et al.*, 1990). Both of these effects would result in less proton production (Dennis *et al.*, 1991). In contrast, we are unable to show significant differences in the levels of ATP and creatine phosphate between preconditioned and non-preconditioned newborn hearts at the end of 40 min of ischemia. However, as previously suggested (Asimakis *et al.*, 1992), the PC-dependent increase in PCr prior to prolonged ischemia (Fig. 8) may play a role in limiting the subsequent fall in pH_i. In addition, since the decrease in ATP levels during prolonged ischemia are significantly less after PC, the data are consistent with previous observations that ATP utilization is diminished after PC. Thus, we cannot rule out the possibility that PC causes a reduced rate of ATP consumption and/or glycolysis and thereby decreases the fall in pH_i during ischemia.

Ischemic preconditioning decreases Na_i during ischemia/reperfusion

We and others have demonstrated in adult hearts that myocardial ischemia stimulates Na uptake (in part via Na/H exchange), which in turn results in an increase in $[Na]_i$ and $[Ca]_i$ (Pike *et al.*, 1993; Steenbergen *et al.*, 1993b; Anderson *et al.*, 1996; Liu *et al.*, 1997). In adult rat hearts, Na_i rises in both preconditioned and non-preconditioned hearts during ischemia (Steenbergen *et al.*, 1993a; Ramasamy *et al.*, 1995). Although the Na_i in non-preconditioned hearts appeared to be higher than that of preconditioned hearts in the earlier study, there were no significant differences. The more recent study, however, reported that PC significantly increased Na_i at the beginning of ischemia, but again no significant differences were found at the end of ischemia. In contrast, our results demonstrate that PC significantly diminishes the increase in Na_i otherwise observed in newborn rabbit hearts during ischemia (Fig. 5). Whether the differences between these studies are the result of species- or age-related differences remains unclear. Nevertheless, all of these results are qualitatively consistent with previous studies (Piwnica-Worms *et al.*, 1986), which support the interpretation that increased H_i stimulates Na/H exchange, and therefore increases net Na influx during ischemia. That is, pH_i falls during ischemia and Na uptake and Na_i increase. Furthermore, in this study, newborn hearts in the PC group have less proton accumulation and less Na_i accumulation.

We cannot, however, rule out the possibility that decreased Na_i accumulation is the result of PC-dependent increases in Na efflux or decreases in Na uptake via Na transport pathways other than Na/H exchange. For example, in adult rabbit hearts, we have previously demonstrated that hypoxia and ischemia initially increase Na efflux via Na/K ATPase (Anderson *et al.*, 1990, 1996). (But this increase is less than the increase in net uptake.) Others have reported similar results after stimulating Na-uptake by acidifying chick cardiac myocytes (Lazdunski *et al.*, 1985; Piwnica-Worms *et al.*, 1986). However, previous studies in adults, as well as the newborn data, discussed in the previous section, do not support the idea that ATP utilization (by Na/K ATPase) is even further increased by PC to diminish Na_i.

Particularly with respect to ischemia-induced Na accumulation, a number of studies provide data which suggest that changes in Na transport are more complicated than described by the general hypothesis. These include studies which demonstrate that amiloride inhibition of Na_i accumulation during ischemia is incomplete (Tani and Neely, 1989; Anderson *et al.*, 1996), and thus suggest that a portion of Na uptake is via pathways other than Na/H exchange. This postulate is consistent with more recent studies which suggest that hypoxia/ischemia increases Na uptake via Na channels (Silverman and Stern, 1994). On the other hand, Na_i does not increase during 15 min of simulated ischemia in isolated papillary muscle (Vanheel *et al.*, 1990), consistent with studies from isolated chick cardiac myocytes, which conclude, based on effects of decreasing pH_o, that Na/H exchange may be inhibited during ischemia (Lazdunski *et al.*, 1985). Thus, further studies will have to be conducted to unambiguously determine whether Na transport via channels or other pathways is altered by PC.

Ischemic preconditioning decreases $[Ca]_i$ during reperfusion

Although the mechanism(s) responsible for Na_i-accumulation cannot be unequivocally identified, the results of this study remain consistent with the interpretation that increases in intracellular Na decrease the force driving Ca out of the cardiac myocyte via Na/Ca exchange. This would stop and/or reverse flux through the exchanger, and thus decrease net Ca-efflux and cause an increase in $[Ca]_i$ during ischemia. Normally, the Na/Ca exchanger transports 3 Na into the cell for each Ca out, and thus acts as the major Ca efflux pathway in order to maintain myocardial Ca homeostasis (Bers, 1991). Assuming the average membrane potential does not change, if the $[Na]_i$ increases to two to three times its normal concentration, the cell membrane Na/Ca exchanger will reach equilibrium, and may reverse its direction such that Na moves out of the cell and Ca moves into the cell. (During ischemia, when the cell is depolarized and changes in intra- and extracellular Na are in opposite directions, even smaller increases in $[Na]_i$ will result in a change in driving force to favor Ca entering the cell via Na/Ca exchange.) Thus, not only do the control hearts in this study provide evidence consistent with the general hypothesis for newborn hearts, but as one could also predict from the general hypothesis, significantly lower Na_i in the PC group is associated with significantly lower $[Ca]_i$ during reperfusion (Figs 5 and 6).

On the other hand, it will also be noted that during ischemia significantly lower Na_i in the PC group is not associated with significantly lower

$[Ca]_i$. That is, while ANOVA demonstrates that $[Ca]_i$ in the two groups are different after PC, when individual time intervals are compared, significant differences in $[Ca]_i$ occur later (only during reperfusion) than do differences in Na_i. (Although $P=0.053$ at $t=5$ min for $[Ca]_i$.) This might lead one to question whether the effects of PC on $[Ca]_i$ are confined to the reperfusion interval. The answer to this question is beyond the scope of this investigation, but amongst the many possible explanations for the "lag" in significant $[Ca]_i$ differences, one simple explanation consistent with the data is described below.

Again, we must reiterate that the hypothesis tested in this study does not address Na and Ca transport via pathways other than Na/H and Na/Ca exchange nor the possibility that PC alters flux via Na/Ca exchange in ways other than those based upon the thermodynamic changes associated with alterations in the Na gradient. One way to address these possibilities is to assess whether the data are consistent with the Na/Ca exchanger being the dominant Ca transport pathway. If it is the dominant pathway, one would expect it to reach equilibrium (given enough time). Using the measured values for Na_i and $[Ca]_i$ (Figs 5 and 6) and assumptions described previously (Steenbergen *et al.*, 1993a; Anderson *et al.*, 1996), one can calculate that on the average (over the 10-min intervals of the NMR measurements) the force driving the exchanger is not at equilibrium in either group except early during ischemia (when it changes direction) and during most of reperfusion in the control group. In particular, during the last 35 min of ischemia, the force driving the exchanger is directed so as to promote Ca entry in both groups and the force is larger in magnitude in the control group. One way this could occur is if the rate of net flux via the Na/Ca exchanger were insufficient to allow the exchanger to reach equilibrium, e.g. if the exchanger were inhibited during ischemia. Indeed, it has been demonstrated in vesicular and giant patch preparations that Na/Ca exchange activity is diminished by decreases in pH and ATP (Bers, 1991). With respect to the effects of ATP, however, recent studies demonstrate that in intact cardiac myocytes, ATP must fall below 10% of control before Na/Ca exchange is affected (Haworth and Goknur, 1996), whereas ATP does not fall below 20% of control in this study. Furthermore, PC has no significant effect on ATP (Fig. 7) during reperfusion, when the most significant effects of PC on $[Ca]_i$ (Fig. 6) are observed. Thus, it appears that the effects of PC on $[Ca]_i$ are not referable to changes in ATP. On the other hand, given the forces driving the exchanger, proton-inhibition of Na/Ca exchange would limit Ca-uptake in both groups during ischemia. Additionally, the lower pH_i in the control group would likely inhibit Na/Ca exchange more, and thereby limit Ca accumulation more than in the PC group. This could in part explain the lack of significant difference in $[Ca]_i$ during ischemia (i.e. differences in Na_i will not cause Na/Ca-exchange-dependent differences in $[Ca]_i$, to the extent that Na/Ca exchange is inhibited by low pH). Furthermore, upon reperfusion, this limitation upon Na/Ca exchange would rapidly be released as pH recovers. Again, this is consistent with the data from the control group, which relax to the point where the exchanger is near equilibrium (as predicted if Na/Ca exchange is the dominant Ca transport process). On the other hand, hearts in the PC group recover better, back to a point similar to that prior to ischemia, where Na_i is low enough so the force driving the exchanger would promote Ca efflux. Thus, although the hypothesis remains consistent with the data, the additional postulate of excess proton inhibition of Na/Ca exchange could be added to explain why (1) $[Ca]_i$ does not increase more in both groups during ischemia (and the exchanger does not reach equilibrium), as well as (2) $[Ca]_i$ is not statistically different in the two groups during that interval.

Ischemic preconditioning and myocardial high energy phosphate metabolism

Although the above-described abnormalities in ion homeostasis are vitally important, changes in availability of high energy phosphates are also likely to play an important role in irreversible myocyte injury during ischemia and reperfusion. In adult dog hearts, PC has been reported to reduce myocardial ATP content by approximately 30% (Murry *et al.*, 1990). In the present study, the ATP content in newborn hearts was reduced by approximately 25% by the end of preconditioning (Fig. 7). While these results are similar, during ischemia following PC, ATP was not preserved in newborn hearts as observed in adult hearts (Murry *et al.*, 1990).

Also, as previously reported in adult rat hearts (Asimakis *et al.*, 1992), in newborn rabbit hearts PCr content was increased above control levels at the end of preconditioning (Fig. 8), but the PCr content then fell to the same level in both preconditioned and non-preconditioned hearts after 40 min ischemia, and there was no significant difference between groups in PCr recovery.

We previously reported that myocardial high-energy phosphates are significantly preserved in this model when intracellular Na and Ca accumulation are limited by Na/H-exchange-inhibition with amiloride analogues during hypoxia and ischemia (Liu *et al.*, 1992, 1997). Thus, the lack of effect of PC on high energy phosphates during ischemia/reperfusion (even though PC significantly decreases Na_i and $[Ca]_i$) suggests that in the newborn, PC is less effective than pharmacological inhibition of Na uptake in reducing high-energy phosphate consumption.

Ischemic preconditioning and preservation of function

In *in vivo* studies of adult hearts, ischemic preconditioning has been shown to significantly decrease myocardial infarct size, and enhance recovery of contractile function of the ischemic regions during reperfusion (Cohen *et al.*, 1991; Li *et al.*, 1992; Piacentini *et al.*, 1993). Similar responses have also been observed in isolated heart preparations (Cave *et al.*, 1993; Steenbergen *et al.*, 1993a; Ramasamy *et al.*, 1995). Numerous studies have demonstrated an increase in $[Ca]_i$ during ischemia and $[Ca]_i$ may remain increased for some time during reperfusion (Nayler, 1987; Pike *et al.*, 1993; Steenbergen *et al.*, 1993b; Pierce and Czubryt, 1995; Anderson *et al.*, 1996). This has led a number of investigators to conclude that the magnitude of increase in $[Ca]_i$ is a powerful predictor of ischemia-induced injury (as measured by infarct size and recovery of myocardial function), and therefore suggest a cause–effect relationship (Allen *et al*, 1993; Steenbergen *et al.*, 1993b). As previously shown in adult hearts (Steenbergen *et al.*, 1993a), our results demonstrate that compared to control, PC significantly decreases $[Ca]_i$ during reperfusion. Thus, the relative decrease in $[Ca]_i$ observed in our study would lead us to predict the observed improvements in functional recovery discussed below.

Increased coronary resistance is a well-documented response associated with ischemia-induced injury and diminished functional recovery (Hearse *et al.*, 1993). In adult hearts, PC has been shown to diminish this response (Kolocassides *et al.*, 1994). The mechanism of this effect remains unclear, although evidence has been presented that it is in part due to an effect of PC on coronary endothelium (Richard *et al.*, 1994). Our observation that PC diminishes the increase in coronary resistance which otherwise occurs during reperfusion (Fig. 9) further supports the postulate that PC decreases ischemia-induced injury in newborn hearts and, more specifically, is also consistent with previous results from adult rat hearts, which suggest that PC limits the no-reflow phenomenon (Asimakis *et al.*, 1992).

Finally, PC significantly improved recovery of LVDP (Fig. 10) and diminished CK release (Fig. 11) during reperfusion. In hearts that were not preconditioned, greater CK release, increased coronary resistance, and poor recovery of LVDP are associated with incomplete recovery of $[Ca]_i$ during reperfusion. Again, these results are consistent with previous studies of adult hearts (Allen *et al.*, 1993; Steenbergen *et al.*, 1993b) and are likely to be indicative of irreversible injury which may be limited by PC-dependent relative decreases in $[Ca]_i$.

Conclusions

We conclude that the data support the hypothesis that in newborn hearts PC will diminish intracellular [H], Na_i, and $[Ca]_i$ during ischemia/reperfusion. The results are further consistent with the general hypothesis that (as in adult hearts) ischemia-induced proton accumulation stimulates Na/H exchange, which leads to increased Na-uptake, collapse of the transmembrane Na gradient and, consequently, increased uptake and accumulation of Ca via Na/Ca exchange. This Ca_i overload is associated with loss of myocardial contractility, increased coronary resistance and myocardial infarct (as assessed by CK release), during reperfusion. Conversely, in newborn hearts, the observed PC-dependent limitation of increases in $[H]_i$ and Na_i during ischemia and the improved recovery of Na_i and $[Ca]_i$ during reperfusion are associated with improved recovery of LVDP, reduced coronary resistance, and decreased CK release during reperfusion.

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References

Allen DG, Orchard CH, 1987. Myocardial contractile function during ischemia and hypoxia. *Circ Res* **60**: 153–168.

Allen DG, Cairns SP, Turvey SE, Lee JA, 1993. Intracellular calcium and myocardial function during ischemia. *Adv Exp Med Biol* **346**: 19–29.

Anderson SE, Murphy E, Steenbergen C, London RE, Cala PM, 1990. Na/H exchange in myocardium: effects of hypoxia and acidification on Na and Ca. *Am J Physiol* **259** (*Cell Physiol* **28**): C940–C948.

Anderson SE, Carr LJ, Schierling TD, Kost GJ, 1994. Are age-related differences in response to myocardial ischemia and cardioplegia pH dependent? *Biol Neonate* **65**: 25–35.

Anderson SE, Dickinson CZ, Liu H, Cala PM, 1996. Effects of Na–K–2Cl cotransport inhibition on myocardial Na and Ca during ischemia and reperfusion. *Am J Physiol* **270** (*Cell Physiol* **39**): C608–C618.

Artman M, 1992. Sarcolemmal Na^{+}–Ca^{2+} exchange activity and exchanger immunoreactivity in developing rabbit hearts. *Am J Physiol* **263** (*Heart Circ Physiol* **32**): H1506–H1513.

Asimakis GK, Inners-McBride K, Medellin G, Conti VR, 1992. Ischemic preconditioning attenuates acidosis and postischemic dysfunction in isolated rat heart. *Am J Physiol* **263** (*Heart Circ Physiol* **32**): H887–H894.

Bers DM, 1991. *Excitation–contraction coupling and cardiac contractile force.* Dordrecht: Kluwer.

Carr LJ, VanderWerf QM, Anderson SE, Kost GJ, 1992. Age-related response of rabbit heart to normothermic ischemia: a 31P-MRS study. *Am J Physiol* **262** (*Heart Circ Physiol* **31**): H391–H398.

Cave AC, Collis CS, Downey JM, Hearse DJ, 1993. Improved functional recovery by ischaemic preconditioning is not mediated by adenosine in the globally ischaemic isolated rat heart. *Cardiovasc Res* **27**: 663–668.

Cohen MV, Liu GS, Downey JM, 1991. Preconditioning causes improved wall motion as well as smaller infarcts after transient coronary occlusion in rabbits. *Circulation* **84**: 341–349.

Das DK, Prasad MR, Lu D, Jones RM, 1992. Preconditioning of heart by repeated stunning. Adaptive modification of antioxidative defense system. *Cell Mol Biol* **38**: 739–749.

de Albuquerque C, Gerstenblith G, Weiss RG, 1994. Importance of metabolic inhibition and cellular pH in mediating preconditioning contractile and metabolic effects in rat hearts. *Circ Res* **74**: 139–150.

Dennis SC, Gevers W, Opie LH, 1991. Protons in ischemia: where do they come from; where do they go to? *J Moll Cell Cardiol* **23**: 1077–1086.

Hanson GL, Schilling WP, Michael LH, 1993. Sodium–potassium pump and sodium–calcium exchange in adult and neonatal canine cardiac sarcolemma. *Am J Physiol* **264** (*Heart Circ Physiol* **33**): H320–H326.

Haworth RA, Goknur AB, 1996. Regulation of sodium–calcium exchange in intact myocytes by ATP and calcium. *Ann NY Acad Sci* **779**: 464–479.

Hearse DJ, Maxwell L, Saldanha C, Gavin JB, 1993. The myocardial vasculature during ischemia and reperfusion: a target for injury and protection. *J Mol Cell Cardiol* **25**: 759–800.

Kempsford RD, 1989. Adenine nucleotide preservation and post-ischemic recover in the immature myocardium. *J Mol Cell Cardiol* **21** (Suppl. IV): S12.

Kim D, Okada A, Smith TW, 1987. Control of cytosolic calcium activity during low sodium exposure in cultured chick heart cells. *Circ Res* **61**: 29–41.

Kolocassides KG, Galinanes M, Hearse DJ, 1994. Ischemic preconditioning, cardioplegia or both? *J Mol Cell Cardiol* **26**: 1411–1414.

Lawson CS, Downey JM, 1993. Preconditioning: state of the art myocardial protection. *Cardiovasc Res* **27**: 542–550.

Lazdunski M, Frelin C, Vigne P, 1985. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J Mol Cell Cardiol* **17**: 1029–1042.

Li GC, Vasquez JA, Gallagher KP, Lucchesi BR, 1990. Myocardial protection with preconditioning. *Circulation* **82**: 609–619.

Li Y, Kloner RA, 1993. The cardioprotective effects of ischemic 'preconditioning' are not mediated by adenosine receptors in rat hearts. *Circulation* **87**: 1642–1648.

Li YW, Whittaker P, Kloner RA, 1992. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am Heart J* **123**: 346–353.

Liu GS, Thornton J, Winkle DMV, Stanley AW, Olsson RA, Downey JM, 1991. Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. *Circulation* **84**: 350–356.

Liu H, Anderson SE, Cala PM, 1992. Effects of pH-regulatory Na/H exchange inhibition in hypoxic newborn rabbit myocardium (Abstract). *Circulation* **86**: I–28.

Liu H, Anderson SE, Cala PM, 1994. Preconditioning decreases changes in intracellular pH, Na, and Ca in newborn rabbit hearts during ischemia (Abstract). *FASEB J* **8**: A855.

Liu H, Cala PM, Anderson SE, 1997. Ethylisopropylamiloride diminishes changes in intracellular Na, Ca, and pH in ischemic newborn myocardium. *J Mol Cell Cardiol* **29**: 2077–2086.

Magyar CE, Wang J, Azuma KK, McDonough AA, 1995. Reciprocal regulation of cardiac Na–K–ATPase and Na/Ca exchanger: hypertension, thyroid hormone, development. *Am J Physiol* **269** (*Cell Physiol* **38**): C675–C682.

Murry CE, Richard VJ, Reimer KA, Jennings RB, 1990. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* **66**: 913–931.

Nakanishi T, Gu H, Seguchi M, Cargoe EJ Jr, Momma K, 1992. $HCO_3^{(-)}$-dependent intracellular pH regulation in the premature myocardium. *Circ Res* **71**: 1314–1323.

Nayler WG, 1987. Calcium antagonists and the ischemic myocardium. *Int J Cardiol* **15**: 267–285.

Nishioka K, Jarmakani JM, 1982. Effect of ischemia on mechanical function and high-energy phosphates in rabbit myocardium. *Am J Physiol* **242** (*Heart Circ Physiol* **11**): H1077–H1083.

Parrish MD, Payne A, Fixler DE, 1987. Global myocardial ischemia in the newborn, juvenile, and adult

isolated isovolumic rabbit heart. Age-related differences in systolic function, diastolic stiffness, coronary resistance, myocardial oxygen consumption, and extracellular pH. *Circ Res* **61**: 609–615.

Piacentini L, Wainwright CL, Parratt JR, 1993. The antiarrhythmic effect of ischaemic preconditioning in isolated rat heart involves a pertussis toxin sensitive mechanism. *Cardiovasc Res* **27**: 674–680.

Pierce GN, Czubryt MP, 1995. The contribution of ionic imbalance to ischemia/reperfusion-induced injury. *J Mol Cell Cardiol* **27**: 53–63.

Pike MM, Luo CS, Clark MD, Kirk KA, Kitakaze M, Madden MC, Cragoe EJ Jr, Pohost GM, 1993. NMR measurements of Na^+ and cellular energy in ischemic rat heart: role of Na^+–H^+ exchange. *Am J Physiol* **265** (*Heart Circ Physiol* **34**): H2017–H2026.

Piwnica-Worms D, Jacob R, Shigeto N, Horres CR, Lieberman M, 1986. Na/H exchange in cultured chick heart cells: secondary stimulation of electrogenic transport during recovery from intracellular acidosis. *J Mol Cell Cardiol* **18**: 1109–1116.

Ramasamy R, Liu H, Anderson S, Lundmark J, Schaefer S, 1995. Ischemic preconditioning stimulates sodium and proton transport in isolated rat hearts. *J Clin Invest* **96**: 1464–1472.

Richard V, Kaeffer N, Tron C, Thuillez C, 1994. Ischemic preconditioning protects against coronary endothelial dysfunction induced by ischemia and reperfusion. *Circulation* **89**: 1254–1261.

Schott RJ, Rohmann S, Braun ER, Schaper W, 1990. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* **66**: 1133–1142.

Seguchi M, Jarmakani JM, 1989. Effect of respiratory acidosis on hypoxic newborn myocardium. *J Mol Cell Cardiol* **21**: 927–834.

Silverman HS, Stern MD, 1994. Ionic basis of ischaemic cardiac injury: insights from cellular studies. *Cardiovasc Res* **28**: 581–597.

Simkhovich BZ, Hale SL, Ovize M, Przyklenk K, Kloner RA, 1993. Ischemic preconditioning and long-chain acyl carnitine in the canine heart. *Coron Artery Dis* **4**: 387–392.

Steenbergen C, Perlman ME, London RE, Murphy E, 1993a. Mechanism of preconditioning. *Circ Res* **72**: 112–125.

Steenbergen C, Fralix TA, Murphy E, 1993b. Role of increased cytosolic free calcium concentration in myocardial ischemic injury. *Basic Res Cardiol* **88**: 456–470.

Tani M, Neely JR, 1989. Role of intracellular Na^+ and Ca^{2+} overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. *Circ Res* **65**: 1045–1056.

Thornton JD, Thornton CS, Downey JM, 1993. Effect of adenosine receptor blockade: preventing protective preconditioning depends on time of initiation. *Am J Physiol* **265** (*Heart Circ Physiol*): H504–H508.

Vanheel B, de Hemptinne A, Leusen I, 1990. Acidification and intracellular sodium ion activity during myocardial ischemia. *Am J Physiol* **259** (*Cell Physiol* **28**): C169–C179.

Tao Z, Gross GJ, 1994. A comparison of adenosine-induced cardioprotection and ischemic preconditioning in dogs. Efficacy, time course, and role of KATP channels. *Circulation* **89**: 1229–1236.

Yellon DM, Alkhulaifi AM, Pugsley WB, 1993. Preconditioning the human myocardium. *Lancet* **342**: 276–277.

Ytrehus K, Liu Y, Downey J, 1994. Preconditioning protects ischemic rabbit heart by protein kinase C activation. *Am J Physiol* **266** (*Heart Circ Physiol* **35**): H1145–H1152.