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Authors

Liu, H

Cala, PM

Anderson, SE

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Ethylisopropylamiloride Diminishes Changes in Intracellular Na, Ca and pH in Ischemic Newborn Myocardium

H. Liu, P. M. Cala and S. E. Anderson

Department of Human Physiology, University of California, Davis, CA, 95616-8644, USA

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H. Liu, P. M. Cala and S. E. Anderson. Ethylisopropylamiloride Diminishes Changes in Intracellular Na, Ca and pH in Ischemic Newborn Myocardium. *Journal of Molecular and Cellular Cardiology* (1997) **29**, 2077–2086. Numerous studies suggest that in adult hearts myocardial ischemic injury is in part the result of proton stimulation of Na/H exchange which increases intracellular Na (Na_i) and thus leads to increases in intracellular Ca concentration ($[Ca]_i$) due to changes in Na/Ca exchange flux. Corollary to the hypothesis, inhibition of Na/H exchange diminishes Na and Ca accumulation and improves heart function after ischemia. To test this hypothesis and its corollary in newborn hearts, NMR spectroscopy was used to measure intracellular pH (pH_i), Na_i, $[Ca]_i$, and high energy phosphates in isolated, 4–7-day-old rabbit hearts, Langendorff-perfused with Krebs–Henseleit solution at pH 7.4 ± 0.5 equilibrated with 95% O_2/5% CO_2 at 36 ± 1°C. Control hearts were perfused for 30 min before initiating 40 min of global ischemia followed by 40 min of reperfusion. In a second group of hearts ethylisopropylamiloride (EIPA–10 μM) was added to the perfusate 20 min before global ischemia to inhibit Na/H exchange. After 15 min ischemia, pH_i in EIPA-treated hearts (6.41 ± 0.04) was higher than that of the control hearts (6.20 ± 0.08; $P<0.05$). EIPA also limited the increase in Na_i and $[Ca]_i$ during ischemia and improved Na_i and $[Ca]_i$ recovery during reperfusion ($P<0.05$). Na_i (mEq/kg dry weight) rose from 18.1 ± 3.2 to 110.6 ± 14.0 and recovered to 53.3 ± 12.3 in the control group. The corresponding Na_i values for EIPA-treated hearts were 16.2 ± 2.4, 39.6 ± 9.6 and 12.6 ± 3.5, respectively. In control hearts $[Ca]_i$ (nM/l) rose from 332 ± 42 to 1157 ± 89 and recovered to 842 ± 55, whereas in EIPA-treated hearts the values were 255 ± 32, 616 ± 69 and 298 ± 34, respectively. EIPA also preserved cellular ATP during ischemia and reperfusion and diminished inorganic phosphate during reperfusion ($P<0.05$). Finally, EIPA treatment improved recovery of left ventricular developed pressure (68.2 ± 8.9 *v* 16.2 ± 3.6% of control) and limited myocardial injury as indicated by decreased total creatine kinase release during reperfusion (348 ± 132 *v* 2432 ± 639 IU/g dry weight). Thus, as in adults, the results from newborn hearts are consistent with the hypothesis.

Key Words: H; Na; Ca; Ischemia; Na/H exchange; EIPA; Newborn hearts.

Introduction

In the last decade it has become clear that many pathophysiological processes in cardiac ischemia and reperfusion are associated with derangement of cellular ion homeostasis (Scholz and Albus, 1993; Fliegel and Dyck, 1995; Myers *et al.*, 1995). The interdependent effects of decreased intracellular pH (pH_i), increased intracellular Na (Na_i), and Ca overload seem to play key roles in the impairment of ischemic and reperfused tissue and possibly cause arrhythmias (Dennis *et al.*, 1991). In adult hearts, myocardial ischemic/hypoxic damage is associated with increased Ca influx (Nayler, 1987) and, therefore, increased intracellular Ca concentration ($[Ca]_i$) (Lee *et al.*, 1988), which has been identified as a causal factor in reperfusion injury (Steenbergen *et al.*, 1993). The Na dependence of myocyte Ca uptake (Philipson *et al.*, 1982; Kim *et al.*, 1987) implicated Na/Ca exchange as a major effector in Ca_i dependent reperfusion/reoxygenation injury (Grinwald and Brosnaham, 1987; Hearse and Tosaki, 1988). Re-

Please address all correspondence to: Steve Anderson, Department of Human Physiology, University of California, Davis, 95616-8644, USA.

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cently, we and others have reported results consistent with the general hypothesis that the sequence of events leading to myocardial ischemic/hypoxic cell damage is: (1) increased anaerobic metabolism; (2) decreased pH_i; (3) proton stimulation of Na/H exchange; (4) increased Na_i; (5) decreased efflux and ultimately Ca influx via Na/Ca exchange; (6) increased $[Ca]_i$; (7) a cascade of Ca-dependent events leading to cell damage (Tani and Neely, 1989; Wallert and Frohlich, 1989; Anderson *et al.*, 1990; Steenbergen *et al.*, 1990; Pike *et al.*, 1993). Consistent with this hypothesis, in adult hearts, inhibition of Na/H exchange has been shown to diminish intracellular Na and Ca accumulation as well as decrease myocardial injury during ischemia and reperfusion (Murphy *et al.*, 1991; Myers *et al.*, 1995; Scholz and Albus, 1995).

Differences between adult and newborn heart responses to ischemia and hypoxia, as well as age-related treatments for prevention of associated myocardial injury remain controversial. Some investigators indicate that the newborn is more susceptible to ischemic myocardial injury (Wittnich *et al.*, 1987; Kempsford and Hearse, 1990; Fujiwara *et al.*, 1991), whereas others suggest the newborn is less susceptible to ischemic myocardial injury (Pridjian *et al.*, 1987; Yano *et al.*, 1987). This is in the context of the incidence of open heart surgery in newborns increasing (Pollock, 1994), while morbidity and mortality rates for open heart surgery in newborns are unacceptably high. Thus, it remains important to gain a better understanding of the newborn myocardium's response to ischemia. In particular, the hypothesis outlined above has not been tested in the newborn heart, nor have the effects of Na/H exchange inhibition been measured in ischemic newborn hearts. The results of testing the hypothesis demonstrate that in newborn myocardium, treatment with ethylisopropylamiloride (EIPA—10 μM) decreases ischemia induced Na_i and Ca_i accumulation. Furthermore, EIPA treatment improves recovery of left ventricular developed pressure and diminishes release of creatine kinase during reperfusion. A portion of these results have been previously reported in abstract form (Liu *et al.*, 1996).

Materials and Methods

General

The methods used were modified from those previously described (Anderson *et al.*, 1990, 1994a; Carr *et al.*, 1992). New Zealand white rabbits (4–7 days old) were anesthetized with sodium pentobarbital (65 mg/kg) and heparinized (1000 USP units/kg). Hearts were removed and perfused at a constant rate (9–10 ml/min) at 36 ± 1°C. Perfusion pressure was measured continuously by a fluid filled cannula connecting the aortic cannula to a pressure transducer. Left ventricular pressure was measured by a fluid filled cannula inserted into the left ventricle and connected to a pressure transducer. Left ventricular developed pressure (LVDP) was measured as the difference between end-diastolic pressure and end-systolic pressure. Ischemia started at time 0 ($t = 0$ min), lasted for 40 min, and was followed by 40 min of reperfusion. Control perfusate contained (mmol/l): 133 NaCl; 4.75 KCl; 1.25 $MgCl_2$; 1.82 $CaCl_2$; 25 $NaHCO_3$; 11.1 dextrose. Perfusates were equilibrated with 95% O_2/5% CO_2 which provided a pH of 7.35–7.45. Ethylisopropylamiloride (EIPA—10 μM) was added to the perfusate 20 min before ischemia in the EIPA-treated group (Pike *et al.*, 1993), but was not included in the reperfusion solution. ^{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 during the control interval (30–40 min) with perfusate containing the acetoxymethyl ester of 5F-1,2-bis(2-aminophenoxy)ethane-N,N,N′,N′-tetraacetic 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 $[Ca]_i$. Total creatine kinase was measured during 40 min reperfusion and used as an indicator of myocardial ischemic injury and infarction (Ramasamy *et al.*, 1995). After perfusions were complete hearts were weighed wet and dried to constant weight (at least 48 h) at 65°C to determine dry weight. The mean wet and dry heart weights were 0.54 ± 0.015 and 0.08 ± 0.002 g, respectively ($n = 35$).

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 gen-

erated 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 graphed at the midpoint of the appropriate 5 or 10 min acquisition interval.

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, standard Bruker AMX400 software was used to reverse each spectrum along the frequency axis. Then each original spectrum was subtracted from its own reversed spectrum after precisely shifting the reversed spectrum so that the original and reversed extracellular peaks overlapped. The extracellular peaks were thus subtracted out of the difference spectrum and the Na_i spectral area was measured 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 ratio of the areas of the Ca-bound and Ca-free peaks in the FBAPTA spectrum (Kirschenlohr *et al.*, 1988). 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.*, 1994a). High energy phosphates are reported as percent of control peak height where the control for each heart is the mean value for data acquired during baseline perfusion prior to ischemia without any treatment. For technical reasons, data for each of the three nuclei of interest must be acquired on separate hearts. Representative ^{23}Na, ^{19}F, and ^{31}P spectra are shown in Figures 1, 2, and 3, respectively.

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 treatments. When differences between treatments were significant, the unpaired *t*-test was used to determine the times at which differences between treatments occurred. Please note that the latter comparisons were not made across time, only across treatments for a particular time. Thus multiple comparison statistics were not required. For all comparisons differences were considered significant when $P<0.05$.

Results

Na_i increases during ischemia and EIPA diminishes ischemia-induced Na_i accumulation

The hypothesis predicts that during ischemia, anaerobic metabolism increases H production which decreases pH_i and, thus, stimulates Na/H exchange to increase Na_i (Cala, 1986; Anderson *et al.*, 1990). The data shown in Figure 4 demonstrate that 40 min ischemia stimulates a large increase in Na_i neonatal hearts from 18.1 ± 3.2 to 110.6 ± 14.0 (mEq/kg dry weight). The data also demonstrate that when EIPA (10 μM) is added to the perfusate 20 min prior to ischemia, the ischemia-induced increase in Na_i is diminished by 75% at the end of ischemia. That is, Na_i only rises to 39.6 ± 9.6 in EIPA-treated hearts at the end of ischemia ($P<0.05$ compared to control). Na_i is also less in EIPA-treated hearts at the end of reperfusion (12.6 ± 3.5) than in control hearts (53.3 ± 12.3).

pH_i decreases during ischemia and EIPA diminishes ischemia-induced proton accumulation

Intracellular pH was measured during ischemia ± 10 μM EIPA in order to assess the effect of Na/H exchange inhibition on myocardial proton accumulation during ischemia. Figure 5 demonstrates that pH_i in EIPA-treated hearts is significantly higher than that of control hearts (ANOVA; $P=0.011$). In particular, after 15 min of ischemia pH_i in EIPA-treated hearts (6.41 ± 0.04) is higher than that of control hearts (6.20 ± 0.08) ($P=0.029$). Additionally, pH_i in the EIPA-treated group recovered to a significantly higher value than the control group after 35 min of reperfusion ($P=0.0001$). EIPA had no significant effect on pH_i prior to ischemia.

$[Ca]_i$ increases during ischemia and EIPA diminishes ischemia-induced Ca_i accumulation

The general hypothesis predicts that inhibition of Na/H exchange will decrease Na accumulation which will in turn increase Ca efflux and/or decrease Ca influx via Na/Ca exchange to diminish ischemia-induced increases in $[Ca]_i$. As predicted, Figure 6 demonstrates $[Ca]_i$ rose less during ischemia and recovered better during reperfusion in EIPA-treated

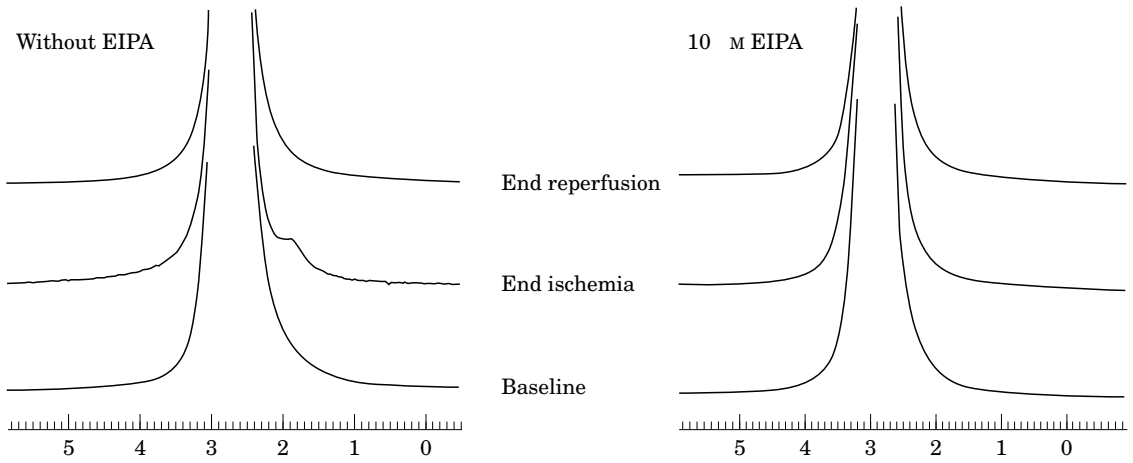

Figure 1 Representative 23Na spectra with and without treatment with 10 μM EIPA on the right and left respectively. The spectra show that EIPA prevents the increase in intracellular Na resonance (upfield shoulder) which is otherwise observed at the end of ischemia.

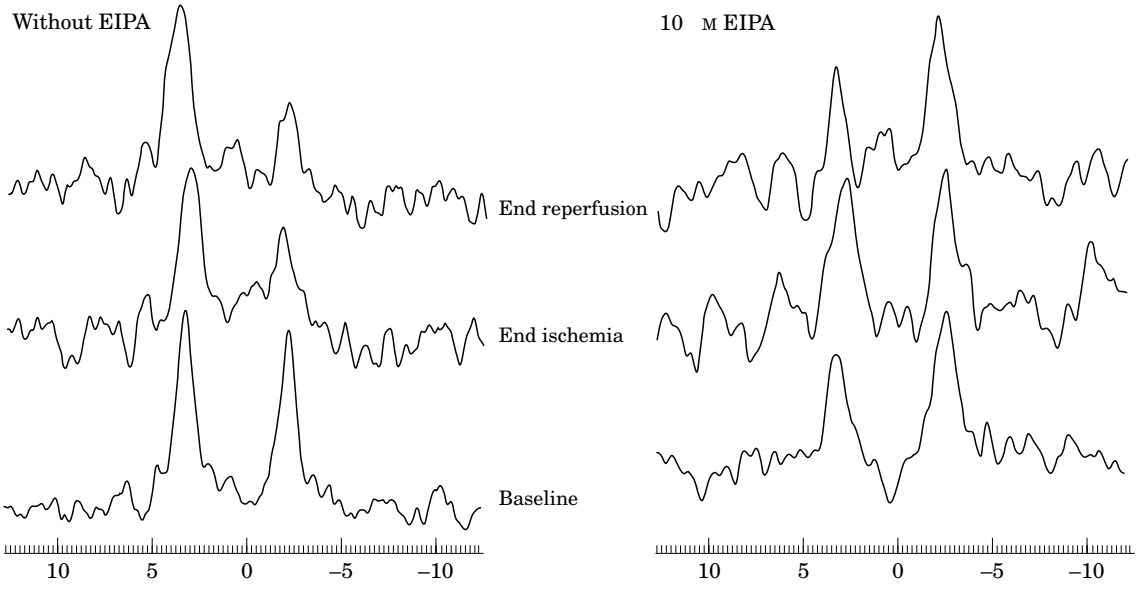

Figure 2 Representative 19F spectra with and without treatment with 10 μM EIPA on the right and left respectively. The spectra show that EIPA limits the increase in the ratio of the areas of the Ca-bound to Ca-free FBAPTA peaks (downfield and upfield, respectively) which is indicative of the increase in intracellular [Ca] observed at the end of ischemia and reperfusion. Please see Materials and Methods for calculation of intracellular [Ca].

hearts ($P<0.05$). In the control group $[Ca]_i$ (nM/l) rose from 332 ± 42 to 1157 ± 89 during ischemia and recovered to 842 ± 55. On the other hand, in the EIPA-treated group the $[Ca]_i$ rose from 255 ± 32 to 616 ± 69 during ischemia and recovered to 298 ± 34.

EIPA preserves myocardial high energy phosphates during ischemia and reperfusion

Numerous studies consistent with our hypothesis have demonstrated that in adult hearts Na/H exchange inhibitors decrease ATP depletion during

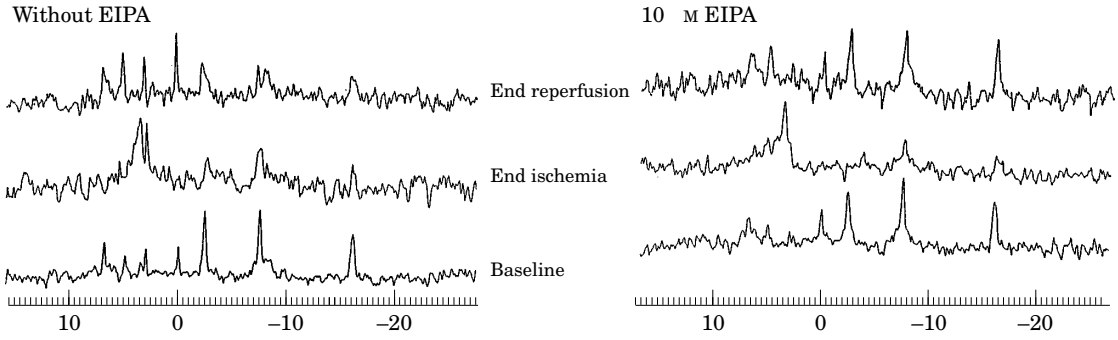

Figure 3 Representative 31P spectra with and without treatment with 10 μM EIPA on the right and left respectively. PCr resonance is set at 0 p.p.m. in the baseline spectra; γ, α and β ATP phosphates resonate upfield, Pi resonates downfield. The spectra most clearly show that EIPA improves the recovery of ATP during reperfusion after ischemia. Please see Materials and Methods for further description of high energy phosphate and pH_i analysis.

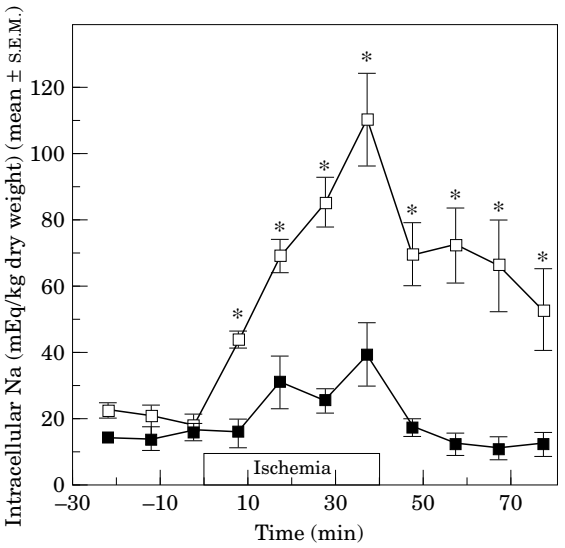

Figure 4 In newborn rabbit hearts, the Na/H exchange inhibitor EIPA (10 μM) decreases accumulation of intracellular Na during ischemia and reperfusion. Intracellular Na content (mEq/kg dry weight) is plotted *v* time before, during, and after ischemia with EIPA (closed squares) and without EIPA (open squares). *P<0.05; number of experiments is given in parentheses.

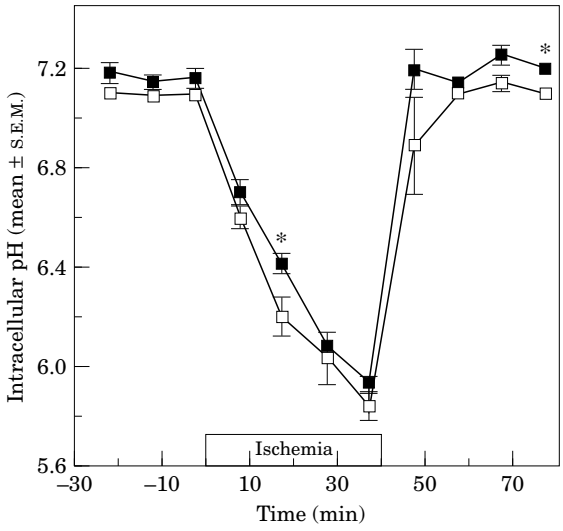

Figure 5 In newborn rabbit hearts, the Na/H exchange inhibitor EIPA (10 μM) decreases intracellular H concentration during ischemia and reperfusion. Intracellular pH is plotted *v* time before, during, and after ischemia with EIPA (closed squares) and without EIPA (open squares).*P<0.05; number of experiments is given in parentheses.

hypoxia/ischemia and improve functional recovery after ischemia (Tani and Neely, 1989; Anderson *et al.*, 1990; Pike *et al.*, 1993; Myers *et al.*, 1995). As shown in Figure 7, during 40 min of ischemia myocardial ATP declined to 28.4 ± 3.5% of preischemic baseline in EIPA-treated hearts and to 19.0 ± 3.4% of baseline in control hearts. [Although differences in ATP between groups ± EIPA were not significant at the end of ischemia, they were different after 15 and 25 min of ischemia (P<0.05)]. During reperfusion, ATP recovered to 36.0 ± 5.2% of baseline in control hearts and 49.3 ± 4.0% in the EIPA hearts (P<0.05). On the other hand, there was no significant effect of EIPA on PCr before, during, or after ischemia (data not shown). In both groups, PCr fell rapidly at the onset of ischemia to 20–25% of control at the end of ischemia. During reperfusion, PCr recovered rapidly and attained 80–90% of control by the end of reperfusion. Finally, in EIPA-treated hearts Pi increased to 283.4 ± 22.3% during ischemia and recovered to 60.7 ± 5.2% at the end of reperfusion, whereas the respective values for control hearts were 300.3 ± 48.9% and 111.4 ± 5.8% (P<0.05 during reperfusion; data not shown).

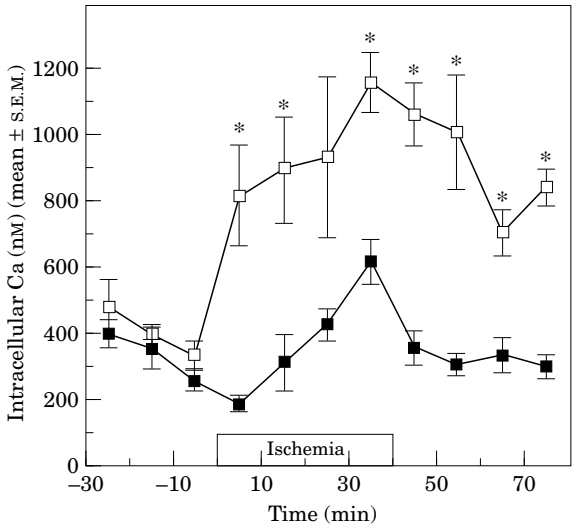

Figure 6 In newborn rabbit hearts, the Na/H exchange inhibitor EIPA (10 μM) decreases intracellular Ca accumulation during ischemia and reperfusion. Intracellular Ca concentration (nM) is plotted *v* time before, during, and after ischemia with EIPA (closed squares) and without EIPA (open squares). **P*<0.05; number of experiments is given in parentheses.

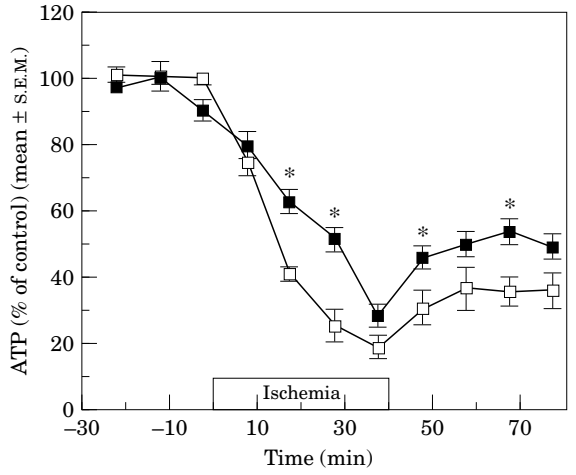

Figure 7 In newborn rabbit hearts, the Na/H exchange inhibitor EIPA (10 μM) decreases ATP depletion during ischemia and reperfusion. Myocardial ATP (% of control) is plotted *v* time before, during, and after ischemia with EIPA (closed squares) and without EIPA (open squares). **P*<0.05; number of experiments is given in parentheses.

EIPA decreases creatine kinase release and improves left ventricular functional recovery during reperfusion

The hypothesis predicts that inhibition of Na/H exchange will prevent myocardial damage by limiting the increase in $[Ca]_i$. In order to assess myocardial damage LVDP was measured before, during and after ischemia ± EIPA and the results are shown in Figure 8. Prior to ischemia LVDP dropped to 62.1 ± 8.9% of baseline after 20 min of perfusion with 10 μM EIPA, but during reperfusion without EIPA, LVDP recovered better in EIPA-treated hearts that in control hearts (68.2 ± 8.9 *v* 16.2 ± 3.6%; *P*<0.05). In order to further assess myocardial injury, total CK release during reperfusion was measured. During 40 min reperfusion, CK release was significantly lower in EIPA-treated hearts than in control hearts (348 ± 132 *v* 2432 ± 639 IU/g dry weight; *P*<0.05).

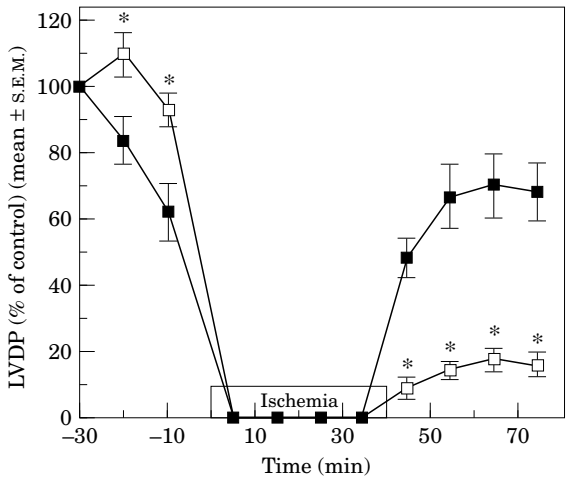

Figure 8 In newborn rabbit hearts, the Na/H exchange inhibitor EIPA (10 μM) improves LVDP recovery during reperfusion. LVDP (% of control) is plotted *v* time before, during, and after ischemia with EIPA (closed squares) and without EIPA (open squares). **P*<0.05; number of experiments is given in parentheses,

Discussion

We and others have demonstrated that hypoxia and ischemia cause increases in intracellular [H], [Na] and [Ca] in adult hearts (Tani and Neely, 1989; Anderson *et al.*, 1990; Steenbergen *et al.*, 1990; Pike *et al.*, 1993) and that hypoxia induces increases in intracellular [H], [Na] and [Ca] in newborn hearts (Liu *et al.*, 1992). Again, these data are consistent with the general hypothesis that hypoxic/ischemic cell injury is the result of the following chain of events: (1) hypoxia/ischemia increases anaerobic metabolism and thereby decreases pH_i; (2) pH-regulatory Na/H exchange is stimulated to increase Na_i; (3) increased $[Na]_i$ decreases the driving force for Ca efflux and/or increases the driving force for Ca uptake via Na/Ca exchange; (5) increased $[Ca]_i$ stimulates Ca-dependent processes causing cell injury. Thus, we

would predict that any intervention which inhibited any step in this process (but preferably earlier steps) would diminish cell injury. Although numerous studies have demonstrated that Na/H exchange inhibition is effective in protecting against ischemic damage in adult hearts (Pike *et al.*, 1993; Scholz and Albus, 1993) and in isolated rat neonatal myocytes (Harper *et al.*, 1993), in the context of the general hypothesis the effects of Na/H exchange inhibition in intact newborn hearts have not yet been studied.

These studies provide new information about the relationships between Na, H, and Ca in newborn ischemic hearts as well as demonstrate that on a qualitative basis, in terms of these ions and Na uptake inhibition, the newborn heart is similar to the adult. Given a plethora of evidence for age-related differences in response to myocardial ischemia (Wittnich *et al.*, 1987; Yano *et al.*, 1987; Carr *et al.*, 1992; Anderson *et al.*, 1994a), and more specifically, differences in accumulation and transport of Na, H, and Ca during and after acidification, hypoxia, and ischemia (Nakanishi *et al.*, 1984; Uemura *et al.*, 1985; Nakanishi *et al.*, 1990; Anderson *et al.*, 1994a), this could not be taken for granted. For instance, 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) and that during hypoxic respiratory acidosis the Na/H exchange rate is greater in newborn rabbit hearts than in adults (Seguchi and Jarmakani, 1989). On the other hand, in isolated rabbit cardiac myocytes, a later study reported no developmental change in the Na/H exchange activity, but instead HCO_3/Cl exchange was found to be more active in premature myocardium (Nakanishi *et al.*, 1992). In addition, we have previously reported that under hypoxic conditions Na/Ca exchange is more active in newborn heart than in adult (Anderson *et al.*, 1994b). Thus, the working hypothesis used in this study could not be accepted for newborn hearts a priori.

EIPA decreases changes in pH_i during ischemia

It has been reported that after inhibition of Na/H exchange, pH_i is lower than control in adult hearts during ischemia as well as after simulated "ischemia" in myocytes isolated from neonatal rat hearts (Harper *et al.*, 1993). In contrast, in adult hearts it has also been reported that there is no significant effect of Na/H exchange inhibition on pH_i during ischemia (Murphy *et al.*, 1991; Pike *et al.*, 1993). The present study of newborn rabbit hearts demonstrates that compared to control, 10 μM EIPA causes a significant increase in pH_i during ischemia and reperfusion. Although it remains unclear how EIPA alters pH_i, one explanation is as follows; during ischemia, decreased pH_i activates Na/H exchange. H loss via Na/H exchange is obligatorily coupled to Na uptake which may stimulate Na-K ATPase (Anderson *et al.*, 1996) and increase ATP consumption. Increasing the rate of ATP hydrolysis would increase the rate of net H production (Dennis *et al.*, 1991). Thus, EIPA inhibition of Na accumulation during ischemia may decrease H production and thereby accumulation, even though proton efflux via Na/H exchange is diminished.

EIPA decreases changes in Na_i and $[Ca]_i$ during ischemia

As predicted by the hypothesis, this study demonstrates that, as in adult hearts, in newborn hearts myocardial ischemia increases Na_i (largely via Na/H exchange) which in turn results in an increase in $[Ca]_i$ (Steenbergen *et al.*, 1987; Tani and Neely, 1989; Pike *et al.*, 1993; Anderson *et al.*, 1996). The hypothesis further predicts that increases in $[Na]_i$ and $[Ca]_i$ during ischemia will be decreased by inhibition of Na/H exchange. Our results demonstrate that EIPA (10 μM) diminishes the increase in Na_i otherwise observed in newborn rabbit hearts during ischemia by nearly 75%. This may be compared to Na_i accumulation in adult rat hearts exposed to 20 min of normothermic ischemia where $\approx$83% inhibition was achieved by treatment with 2.7 μM EIPA (Pike *et al.*, 1993), and essentially 100% inhibition achieved by treatment with 1 mM amiloride (Murphy *et al.*, 1991).

As described above, accumulation of intracellular Na during myocardial ischemia is thought to play a critical role in irreversible injury because of its effect on intracellular Ca as mediated by Na/Ca exchange (Steenbergen *et al.*, 1987; Tani and Neely, 1989; Scholz and Albus, 1993; Fliegel and Dyck, 1995). That is, if the stoichiometry of Na/Ca exchange is 3/1 and the membrane potential remains at its resting level, when $[Na]_i$ increases by a factor of two to three, the Na/Ca exchanger will reach equilibrium and therefore no longer extrude Ca. If $[Na]_i$ increases further and/or if the membrane potential becomes more positive, flux through the Na/Ca exchange will reverse direction and it will mediate net Ca uptake. [Normally, the Na/Ca exchanger acts as the major Ca efflux pathway in order to maintain myocardial Ca homeostasis (Bers,

1991).] The data for the control group are consistent with this scenario.

On the other hand, when Na uptake was inhibited by EIPA, the aforementioned decrease in Na accumulation was accompanied by a decrease of more than 56% in cytosolic Ca accumulation during ischemia. This may be compared with an 84% decrease in ischemia-induced cytosolic Ca accumulation in adult rat hearts after Na uptake was inhibited by 1 mM amiloride (Murphy *et al.*, 1991). It will be noted, however, that in EIPA-treated newborn hearts Na_i is only increased above preischemic baseline by a factor of 2.4 at the end of ischemia. All things remaining equal, this is not high enough to allow the Na/Ca exchanger to mediate net Ca uptake. Nevertheless, $[Ca]_i$ increases during ischemia after treatment with EIPA (Fig. 6—albeit the increase is less than without EIPA). There are a number of possible explanations for the increase in $[Ca]_i$. First, if the membrane potential rose to -55 mV Na/Ca exchange could mediate net Ca uptake when Na_i reached the observed 39.6 mEq/kg dry weight (assuming 2.5 kg cell water/kg dry weight). And, if the average membrane potential is more positive than -55 mV, the Na/Ca exchanger could mediate net Ca uptake at even lower Na_i. Another explanation for the increase in $[Ca]_i$ is that decreased pH_i has been shown to inhibit the Na/Ca exchanger (Philipson *et al.*, 1982).

Although the fact that EIPA limits increases in $[Ca]_i$ during ischemia (Fig. 6) supports the hypothesis that ischemia-induced increases in $[Ca]_i$ are the result of increased Na uptake via Na/H exchange, it is possible that EIPA affects Na transport pathways other than Na/H exchange. For instance, EIPA has been reported to inhibit the Na/Ca exchanger directly (Kleyman and Cragoe, 1988). There are, however, at least two pieces of evidence suggesting this effect is not large in our experiments. First, the K_i for inhibition of Na/Ca exchange by EIPA is 129 μM (Kleyman and Cragoe, 1988): more than 10-fold greater than the concentration of EIPA (10 μM) used in this study. Second, since the Na/Ca exchanger acts as the primary pathway for Ca efflux under baseline conditions (Bers, 1991), if it were significantly inhibited by EIPA one would expect to see increases in $[Ca]_i$ during perfusion with solution containing EIPA prior to ischemia: this was not observed. EIPA has also been reported to inhibit Na channels and Na-dependent HCO_3 transport (Haigney *et al.*, 1994; Khandoudi *et al.*, 1996). With respect to the latter, however, low tissue $[HCO_3]$ during ischemia would minimize any effect of EIPA on Na-dependent HCO_3 transport. We cannot, however, rule out the possibility that a portion of the EIPA-dependent decrease in ischemia-induced Na_i accumulation is due to EIPA inhibition of Na channels.

Regardless of non-specific effects of EIPA on Na transporters including Na/Ca exchange, however, the data remain consistent with the hypothesis that Na/Ca exchange is the pathway responsible for changes in $[Ca]_i$ during ischemia. Thus, the results of this study are consistent with our previous results as well as those of other investigators (Anderson *et al.*, 1990; Murphy *et al.*, 1991; Liu *et al.*, 1992), which support the hypothesis that Na/H exchange inhibition reduces Na_i accumulation during ischemia and, thereby, diminishes the change in the force driving Na/Ca exchange, thus limiting Ca uptake and intracellular Ca accumulation.

EIPA preserves high energy phosphates during ischemia and improves function during reperfusion

Na/H exchange inhibition has been reported to reduce depletion of myocardial ATP during ischemia and reperfusion in adult hearts (Pike *et al.*, 1993). In this study, after exposure to EIPA, ATP is significantly preserved during ischemia and reperfusion (Fig. 7) and Pi accumulation is significantly decreased during reperfusion. We postulate that by limiting ischemia induced increases in Na_i and $[Ca]_i$, Na/H exchange inhibition could diminish changes in ATP and Pi by decreasing Na- and Ca-dependent energy consuming processes (e.g. Na/K ATPase and Ca-ATPase activity) (Scholz and Albus, 1995).

Again, increases in $[Na]_i$ and $[Ca]_i$ have been implicated in development of post-ischemic myocardial dysfunction (Steenbergen *et al.*, 1993). Our results from newborn hearts (Fig. 8) are consistent with those from adult hearts in which Na/H exchange inhibition improved recovery of myocardial function (Tani and Neely, 1989; Dennis *et al.*, 1991; Meng *et al.*, 1993; Pike *et al.*, 1993). Specifically, this study demonstrates that inhibition of Na uptake (10 μM EIPA) during 40 min ischemia improved recovery of LVDP in newborn rabbit hearts from 16–68% of control after 40 min reperfusion. This may be compared with adult rabbit hearts, where inhibition of Na/H exchange (200 μM amiloride) during 60 min of ischemia improved recovery of left ventricular force from 43–72% of control after 40 min reperfusion (Myers *et al.*, 1995), or with adult rat hearts where perfusion with 2.7 μM EIPA prior to 21 min of ischemia improved recovery

of LVDP from 36–104% of control after 30 min reperfusion (Pike *et al.*, 1993).

Finally, this study is also consistent with results from adult hearts in which EIPA treatment during reperfusion was shown to decrease release of myocardial creatine kinase. Our results show that pretreatment with 10 μM EIPA decreases total CK release by more than 85%, as compared with a decrease of 73% in adult rat right ventricles reperfused with 1 μM EIPA after 60 min ischemia (Meng *et al.*, 1993).

Conclusions

The data presented here are consistent with the hypothesis that newborn hearts, like adult hearts, respond to ischemia with an increase in Na uptake in part via Na/H exchange which leads to increased accumulation of Ca due to alterations in the force driving Na/Ca exchange. Ca_i overload is associated with myocardial cell damage as evidenced by creatine kinase release and loss of myocardial contractility. Further consistent with the hypothesis, EIPA decreases accumulation of intracellular Na and [Ca]. Finally, decreases in intracellular Na and Ca accumulation are associated with decreased CK release and improved recovery of heart function during reperfusion.

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