### Title

Age-related differences in Na+-dependent Ca2+ accumulation in rabbit hearts exposed to hypoxia and acidification




### Journal

American Journal of Physiology - Cell Physiology, 284(5)

### ISSN

0363-6143



### Authors

Anderson, SE

Liu, H

Ho, HS

et al.



### Publication Date

2003-05-01

### DOI

10.1152/ajpcell.00148.2002

# Age-related differences in $Na^+$-dependent $Ca^{2+}$ accumulation in rabbit hearts exposed to hypoxia and acidification

**S. E. Anderson, H. Liu, H. S. Ho, E. J. Lewis and P. M. Cala**

*Am J Physiol Cell Physiol* 284:C1123-C1132, 2003. First published 8 January 2003;
doi:10.1152/ajpcell.00148.2002



***AJP - Cell Physiology*** is dedicated to innovative approaches to the study of cell and molecular physiology. It is published 12 times a year (monthly) by the American Physiological Society, 9650 Rockville Pike, Bethesda MD 20814-3991.  ISSN: 0363-6143, ESSN: 1522-1563. Visit our website at http://www.the-aps.org/.

*Am J Physiol Cell Physiol* 284: C1123–C1132, 2003.
First published January 8, 2002; 10.1152/ajpcell.00148.2002.

# Age-related differences in $Na^+$-dependent $Ca^{2+}$ accumulation in rabbit hearts exposed to hypoxia and acidification

**S. E. Anderson, H. Liu, H. S. Ho, E. J. Lewis, and P. M. Cala**
*Department of Human Physiology, University of California, Davis, California 95616-8644*

Submitted 3 April 2002; accepted in final form 23 December 2002

**Anderson, S. E., H. Liu, H. S. Ho, E. J. Lewis, and P. M. Cala.** Age-related differences in $Na^+$-dependent $Ca^{2+}$ accumulation in rabbit hearts exposed to hypoxia and acidification. *Am J Physiol Cell Physiol* 284: C1123–C1132, 2003. First published January 8, 2002; 10.1152/ajpcell.00148.2002.—In this study, we test the hypothesis that in newborn hearts (as in adults) hypoxia and acidification stimulate increased $Na^+$ uptake, in part via pH-regulatory $Na^+/H^+$ exchange. Resulting increases in intracellular $Na^+$ ($Na_i$) alter the force driving the $Na^+/Ca^{2+}$ exchanger and lead to increased intracellular $Ca^{2+}$. NMR spectroscopy measured $Na_i$ and cytosolic $Ca^{2+}$ concentration ($[Ca^{2+}]_i$) and pH ($pH_i$) in isolated, Langendorff-perfused 4- to 7-day-old rabbit hearts. After $Na^+/K^+$ ATPase inhibition, hypoxic hearts gained $Na^+$, whereas normoxic controls did not [19 ± 3.4 to 139 ± 14.6 vs. 22 ± 1.9 to 22 ± 2.5 (SE) meq/kg dry wt, respectively]. In normoxic hearts acidified using the $NH_4Cl$ prepulse, $pH_i$ fell rapidly and recovered, whereas $Na_i$ rose from 31 ± 18.2 to 117.7 ± 20.5 meq/kg dry wt. Both protocols caused increases in $[Ca]_i$; however, $[Ca]_i$ increased less in newborn hearts than in adults ($P < 0.05$). Increases in $Na_i$ and $[Ca]_i$ were inhibited by the $Na^+/H^+$ exchange inhibitor methylisobutylamiloride (MIA, 40 μM; $P < 0.05$), as well as by increasing perfusate osmolarity (+30 mosM) immediately before and during hypoxia ($P < 0.05$). The data support the hypothesis that in newborn hearts, like adults, increases in $Na_i$ and $[Ca]_i$ during hypoxia and after normoxic acidification are in large part the result of increased uptake via $Na^+/H^+$ and $Na^+/Ca^{2+}$ exchange, respectively. However, for similar hypoxia and acidification protocols, this increase in $[Ca]_i$ is less in newborn than adult hearts.

newborn heart; intracellular $Na^+$, $Ca^{2+}$, and pH

MANY STUDIES HAVE SHOWN the newborn heart is less susceptible to hypoxia-induced dysfunction and damage than the adult. For example, mechanical function, high energy phosphates, and enzyme release are altered less by hypoxia in the newborn heart than the adult (23, 24, 39). However, no unifying hypothesis for the mechanisms of hypoxic injury, much less its age-related variations, has been accepted. We and others have reported results obtained from studies of adult and newborn hearts consistent with the general hypothesis that hypoxia/ischemia stimulates pH-regulatory $Na^+/H^+$ exchange, which increases net $Na^+$ uptake and thereby leads to reduction of the transmembrane $Na^+$ gradient and, consequently, increases $Ca^{2+}$ uptake via $Na^+/Ca^{2+}$ exchange (2, 4, 30, 46, 47). This hypothesis states that increased $Na^+$ uptake is the first step toward hypoxic/ischemic injury in that it gives rise to increased intracellular $Na^+$ ($Na_i$), $Ca^{2+}$ ($Ca_i$), and ATP consumption (30).

If our hypothesis is correct, age-related differences in response to myocardial hypoxia are likely to be the result of age-related differences in $Na^+$-dependent $Ca^{2+}$ accumulation. Given the scenario described above, this could arise from differences in proton production and/or ion transport through the $Na^+/H^+$ and/or $Na^+/Ca^{2+}$ exchangers. Here, we report the results of testing the general hypothesis in newborn hearts and compare the results with those from the adult. Newborn hearts were exposed to hypoxia or $NH_4Cl$ washout (9) to stimulate pH-regulatory $Na^+/H^+$ exchange under hypoxic and normoxic conditions, respectively. $Na_i$, $Ca_i$, and intracellular pH ($pH_i$), as well as high-energy phosphates, were measured using NMR. To our knowledge, this is the first report including measurement of all three ions in intact newborn hearts during hypoxia and after normoxic acidification.

## METHODS

*General.* The methods used were modified from those previously reported (1, 4, 22). New Zealand White rabbits (newborn, 4–7 days; adult, 11–14 wk) were anesthetized with pentobarbital sodium (35–65 mg/kg) and heparinized (1,000 USP units/kg). Hearts were removed and perfused at a constant rate (9–10 ml/min for newborns; 27–29 ml/min for adults) at 23–25°C. Control perfusate contained (in mM): 133 NaCl, 4.75 KCl, 1.25 $MgCl_2$, 1.82 $CaCl_2$, 25 $NaHCO_3$, (or 20 HEPES, 8 NaOH), and 11.1 dextrose. $^{23}Na$, $^{19}F$, and $^{31}P$ NMR were used to measure $Na_i$, $Ca_i$, $pH_i$, and high-energy phosphates, respectively. To measure $Na_i$, 15 mM dysprosium triethylenetetraminehexaacetic acid (DyTTHA) was substituted iso-osmotically for NaCl in the perfusate, and $Ca^{2+}$ was added to reach a perfusate concentration of 1.8–2 mM as measured by $Ca^{2+}$ electrode. To measure $Ca_i$, hearts were loaded during the control interval (30–40 min) with perfusate containing the acetoxymethyl ester of 5-fluoro-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (FBAPTA) at 2.5 μM for newborns or 5 μM for adults (26). FBAPTA was then washed out of the extracellular space with control solution for 15 min before measurement of $Ca_i$. Per-

Address for reprint requests and other correspondence: S. E. Anderson, Dept. of Human Physiology, Univ. of California, One Shields Ave., Davis, California 95616-8644 (E-mail: seanderson@ucdavis.edu).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

  

fusates were titrated to pH 7.35–7.45 and equilibrated with 95% $O_2$-5% $CO_2$ or 95% $N_2$-5% $CO_2$ (100% $O_2$ or 100% $N_2$ with HEPES) for normoxic and hypoxic conditions, respectively. The latter provided a $Po_2$ at the aorta of 20 ± 1 torr during hypoxic perfusion. To discriminate between changes in dissipative $Na^+$ uptake and active $Na^+$ extrusion, $Na^+$ efflux via $Na^+/K^+$-ATPase was inhibited by removal of KCl from the perfusate (osmotic substitution with sucrose) (4, 45).

Because the precipitating event for the observed responses is hypothesized to be a decrease in $pH_i$, we also used normoxic acidification to test the hypothesis. Normoxic acidification was achieved using the $NH_4Cl$ prepulse (9), which consisted of *1*) 10–15 min of control perfusion, *2*) 40 min of perfusion with perfusate to which 20 mM $NH_4Cl$ was added, *3*) 5 min of perfusion with $K^+$-free perfusate to which 20 mM $NH_4Cl$ was added, *4*) 30 min of $K^+$-free perfusion without $NH_4Cl$, and *5*) 30–40 min of perfusion with normal $K^+$ control perfusate.

To identify the pathway responsible for changes in $Na^+$ uptake, methylisobutylamiloride (MIA, 40μM), a known inhibitor of pH-regulatory $Na^+/H^+$ exchange (27), was added during hypoxia or $NH_4Cl$ washout. Hypertonic perfusion has also been shown to diminish $Na^+$ accumulation during hypoxia (22). To test for this effect in newborn hearts, another set of experiments was conducted in which 30 mosM of sucrose was added to all perfusates, beginning with the $K^+$-free portions of the hypoxic and $NH_4Cl$ washout protocols.

After perfusions were complete, hearts were weighed wet and dried to constant weight (at least 48 h) at 65°C to determine dry weight.

*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 1,000, 1,500, and 148 excitation pulses (90°, 45°, and 60°) using 2K-, 2K-, and 4K-word data files and ±4,000-, ±5,000-, and ±4,000-Hz sweep widths, respectively. For all nuclei, data files were collected over 5-min intervals. To improve the signal-to-noise ratio for $^{19}F$ measurement of $Ca_i$, two 5-min $^{19}F$ files were added together. Data are represented in time as corresponding to the midpoint of the appropriate 5- or 10-min acquisition interval. Please note also that lines connecting data points are not meant to imply that the measured variable follows a linear path from point to point.

$Na_i$ content (in meq/kg dry wt) was calculated from the calibrated area under the unshifted peak of the $^{23}Na$ spectra after subtracting the extracellular peak (4, 31). $[Ca]_i$ in nanomoles per liter of cell water was calculated as the product of the ratio of the areas of the $Ca^{2+}$-bound and $Ca^{2+}$-free peaks in the FBAPTA spectrum and the 500 nM $Ca^{2+}$-FBAPTA dissociation constant (26). Using calibrated areas for newborn and adult $^{19}F$ spectra and assuming cytosolic volume is 2.5 l/kg dry wt (2), the total FBAPTA in the cytosol (bound + free) was calculated as 27.5 ± 8.0 μM in newborn hearts and 69.5 ± 13.1 μM in adult hearts. The $pH_i$ was determined from the chemical shift of the inorganic phosphate ($P_i$) resonance [with reference to control phosphocreatine (PCr)] calibrated at 25°C (1). High-energy phosphates are reported as a percentage of baseline peak intensity (30).

*Statistics.* Results are reported as means ± SE unless otherwise indicated. Two-factor analysis of variance (ANOVA) with repeated measures on one factor (time) was used to test for differences among treatment and age groups. The Tukey multiple comparison test was used to identify significant differences between treatments and age groups when differences among groups were significant (17). The Tukey test was used to compare both full data sets, as well as data for specific time points, and significant differences for the latter are indicated by asterisks in the figures. For all comparisons, differences were considered significant at $P < 0.05$.

*Validation of $Na_i$ measurement.* It has been suggested that the method employed to measure $Na_i$ using DyTTHA as a shift reagent is inferior to the more recently developed method using thulium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) (TmDOTP) (11). To address this issue, two series of experiments were performed using the hypoxia protocol. One used 4 mM TmDOTP, and the other used 15 mM DyTTHA to shift the extracellular $Na^+$ resonance. In Fig. 1, the data indicated by open squares depict $Na_i$ during $K^+$-free hypoxia measured using DyTTHA and analyzed by reversing the spectra and subtracting the extracellular $Na^+$ peak (4, 31). (This method was used for all $Na_i$ measurements in this study except those described immediately following for Fig. 1.) The data depicted by the closed circles and closed squares were acquired from another four hearts exposed to the same hypoxic conditions but using TmDOTP to shift the extracellular $Na^+$ resonance. The data depicted by the closed squares were analyzed using the reverse and subtract method (4, 31), whereas the data depicted by the closed circles were analyzed using NMR1 software (New Methods Research, Syracuse, NY) to deconvolute the $Na^+$ spectra. The sample size required to "prove" that these three sets of data are not different prohibits statistically testing that hypothesis. Nevertheless, it is apparent that neither the method used to alter the extracellular $Na^+$ resonance frequency (DyTTHA or TmDOTP) nor the method of analysis has a significant effect on the measured change in $Na_i$ (reverse and subtract vs. NMR1; $P = 0.967$ by ANOVA for the two methods of analyses of TmDOTP data). Thus we find no benefit in using TmDOTP. Given the decrease in arterial blood pressure caused by TmDOTP in vivo (8), we

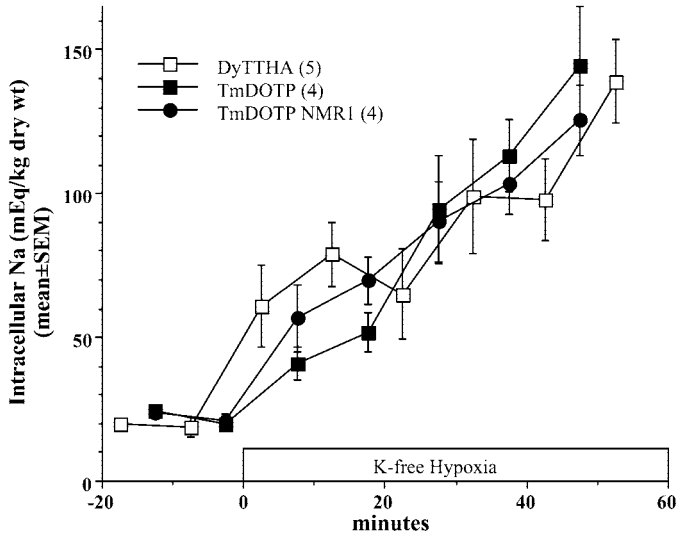


Fig. 1. Comparison of hypoxia-induced $Na^+$ uptake in newborn hearts measured with 2 different shift reagents: dysprosium triethylenetetraminehexaacetic acid (DyTTHA) and thulium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) (TmDOTP). Intracellular $Na^+$ is plotted vs. minutes of $K^+$-free hypoxic perfusion using DyTTHA (□) or TmDOTP (■) to shift the extracellular $Na^+$ peak. Data depicted by closed and open squares were analyzed by subtracting the extracellular $Na^+$ peak from the spectrum. For comparison, the TmDOTP spectra were also analyzed using NMR1 deconvolution (●). Please see text for explanation. In all figures, number of experiments are given in parentheses.

prefer using DyTTHA, which has no measurable effect on blood pressure in vivo (7).

## RESULTS

*Hypoxia stimulates $Na^+$ uptake.* Our hypothesis predicts that during hypoxia, decreased $pH_i$ will stimulate $Na^+/H^+$ exchange (functioning in a pH-regulatory mode), resulting in increased $Na^+$ uptake (4, 12). To measure $Na^+$ uptake, $Na^+$ efflux via the $Na^+/K^+$ pump must be quantified. To achieve this, we measured $Na_i$ accumulation under conditions in which the $Na^+$ pump was allowed to function (normal $K^+$ perfusion) and in which $Na^+$ efflux via the $Na^+$ pump was inhibited by $K^+$-free perfusion (4, 45). Figure 2 shows the results of experiments comparing $Na_i$ in newborn hearts during normoxic and hypoxic $K^+$-free perfusion. After 52.5 min of $K^+$-free perfusion, $Na_i$ had increased from 19 ± 3.4 to 139 ± 14.6 meq/kg dry wt during hypoxia but did not change measurably under normoxic conditions (from 22 ± 1.9 to 22 ± 2.5 meq/kg dry wt). Because these experiments were conducted while $Na^+$ efflux was inhibited by $K^+$-free perfusion, the data unequivocally demonstrate that hypoxia stimulates an increase in $Na^+$ uptake. (Please see DISCUSSION for further explanation.)

Figure 3 shows a comparison of $Na_i$ in newborn and adult hearts during hypoxic $K^+$-free perfusion, as well as the effect of the $Na^+/H^+$ inhibitor MIA (40 μM) (27) on $Na_i$ in newborn hearts for the same perfusion protocol. Although an increase in $Na_i$ occurs under hypoxic conditions in both adults and neonates, there is no measurable difference between age groups. Additionally, $Na^+$ uptake is inhibited in both adult and newborn hearts when the $Na^+/H^+$ exchange inhibitor MIA is added to the hypoxic perfusate (adult data not shown).

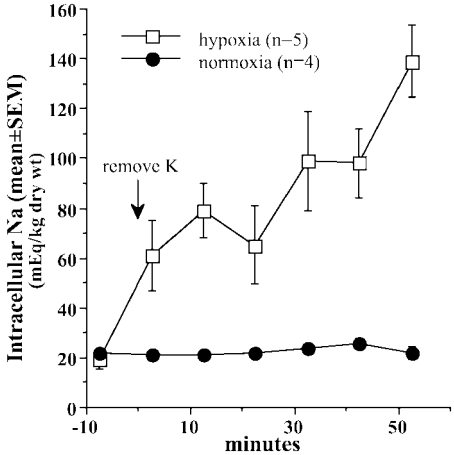


Fig. 2. Isolated newborn heart intracellular $Na^+$ (means ± SE) during hypoxic (□) and normoxic (●) $K^+$-free perfusion. Mean intracellular $Na^+$ (meq/kg dry wt) is plotted vs. minutes. (In all figures, signal-averaged NMR data are represented by points plotted at the time corresponding to the midpoint of the interval over which the data were acquired.) After 52.5 min of hypoxic perfusion, mean intracellular $Na^+$ is more than 6 times greater than after normoxic perfusion.

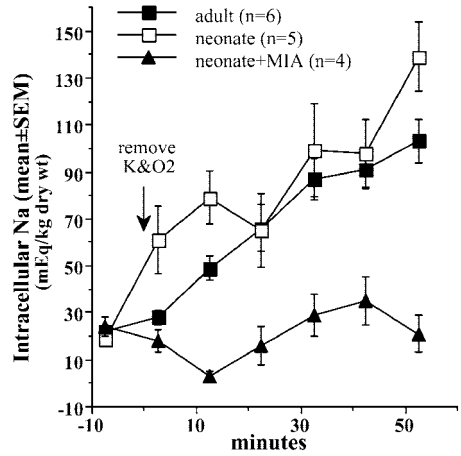


Fig. 3. Hypoxic $Na^+$ uptake is not measurably different in perfused newborn (□) and adult (■) hearts, and in newborns, like adults, uptake is inhibited by the $Na^+/H^+$ exchange inhibitor methylisobutylamiloride (40 μM MIA; ▲). Mean intracellular $Na^+$ (meq/kg dry wt) is plotted vs. minutes.

*Decreased $pH_i$ stimulates $Na^+$ uptake and proton efflux.* $Na_i$ was also measured after decreasing $pH_i$ under normoxic conditions. Figure 4*A* shows that in newborn hearts $NH_4Cl$ (20 mM) washout results in rapid acidification ($pH_i$ falls from 7.23 ± 0.12 to 6.55 ± 0.24), which is followed by regulation of pH back to 7.25 ± 0.14 within 20 min. Figure 4*B* shows that while $pH_i$ is being regulated in the newborn heart, $Na^+$ uptake is increased ($Na_i$ increases from 31 ± 18.2 to 117.7 ± 20.5 meq/kg dry), with $Na_i$ reaching a plateau as $pH_i$ returns to the control level. Furthermore, addition of the $Na^+/H^+$ exchange inhibitor MIA (40 μM) to the perfusate used to washout $NH_4Cl$ prevents both pH regulation and $Na^+$ uptake (data not shown). Finally, Fig. 4, *A* and *B*, shows that after $NH_4$ washout, for a given proton load, there is no measurable difference between age groups with regard to excursions in $pH_i$ and $Na_i$.

*Hypoxia and normoxic acidification stimulate increases in $[Ca]_i$.* Figure 5*A* shows changes in $[Ca]_i$ in newborn and adult hearts exposed to $K^+$-free hypoxic perfusion. In addition, these data illustrate that increases in $[Ca]_i$ in newborn hearts during $K^+$-free hypoxic perfusion are inhibited by the $Na^+/H^+$ exchange inhibitor MIA (40 μM). Finally, Fig. 5*A* also provides evidence that during hypoxia, increases in $[Ca]_i$ are less in newborn than adult hearts ($P < 0.05$ by ANOVA).

Changes in $[Ca]_i$ were also measured after acidifying the hearts under normoxic conditions. The data in Fig. 5*B* demonstrate that exposure to normoxic acidification (20 mM $NH_4Cl$ prepulse) increases $[Ca]_i$ in newborn hearts and that for the similar acidification conditions (see Fig. 4*A*), $[Ca]_i$ increases less in newborn hearts than adults ($P < 0.05$ by ANOVA).

*The effects of hypertonic perfusion on intracellular $Na^+$ and $Ca^{2+}$.* To further test the general hypothesis, and in particular to test the assertion that pH-regulatory $Na^+/H^+$ exchange is the pathway responsible for

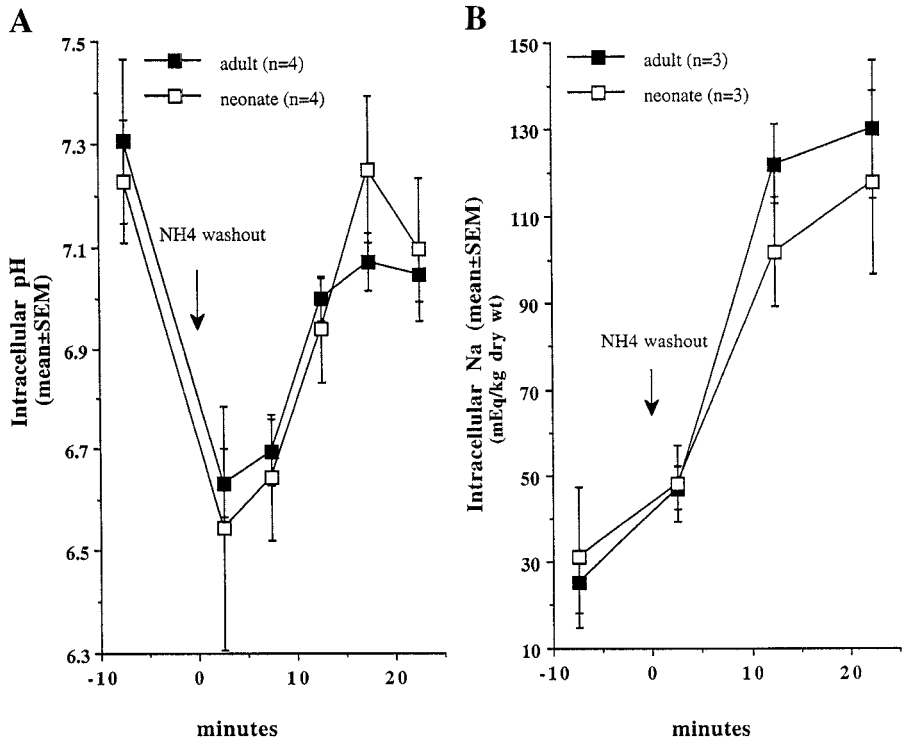


Fig. 4. Intracellular acidification (20 mM $NH_4Cl$ washout) under normoxic conditions stimulates pH regulation and $Na^+$ uptake. Mean intracellular pH (*A*) and intracellular $Na^+$ (meq/kg dry wt) (*B*) are plotted vs. minutes. Changes in intracellular pH and $Na^+$ after $NH_4Cl$ washout are not measurably different in adult (■) and newborn (□) hearts. $Na^+$ uptake appears to plateau as intracellular pH returns to control, supporting the hypothesis that uptake is via pH-regulatory $Na^+/H^+$ exchange.

increased $Na^+$ uptake during hypoxia, hypertonic perfusion was used to inhibit hypoxia- and acidification-induced $Na^+$ uptake (12, 13, 22). (Please see DISCUSSION for further explanation of this hypothesis.)

Figure 6, *A* and *B*, show the effect of hyperosmotic perfusion on $Na_i$ and $[Ca]_i$, respectively, in neonate hearts exposed to hypoxia. (Neonate data from Figs. 2 and 5*A* are included for comparison.) compared with isotonic, hypertonic perfusion significantly decreased $Na_i$ during hypoxia and reoxygenation ($P < 0.05$). Similarly, hypertonic perfusion diminished increases in $[Ca]_i$ during hypoxia ($P < 0.05$). Note also that the recovery of $Na_i$ and $[Ca]_i$ toward control when $K^+$ and $O_2$ are replaced provides further evidence for the dependence of $[Ca]_i$ on $Na_i$.

*Inhibition of $Na^+$ accumulation preserves high-energy phosphates and diminishes changes in coronary resistance during hypoxia.* Figure 7 demonstrates the

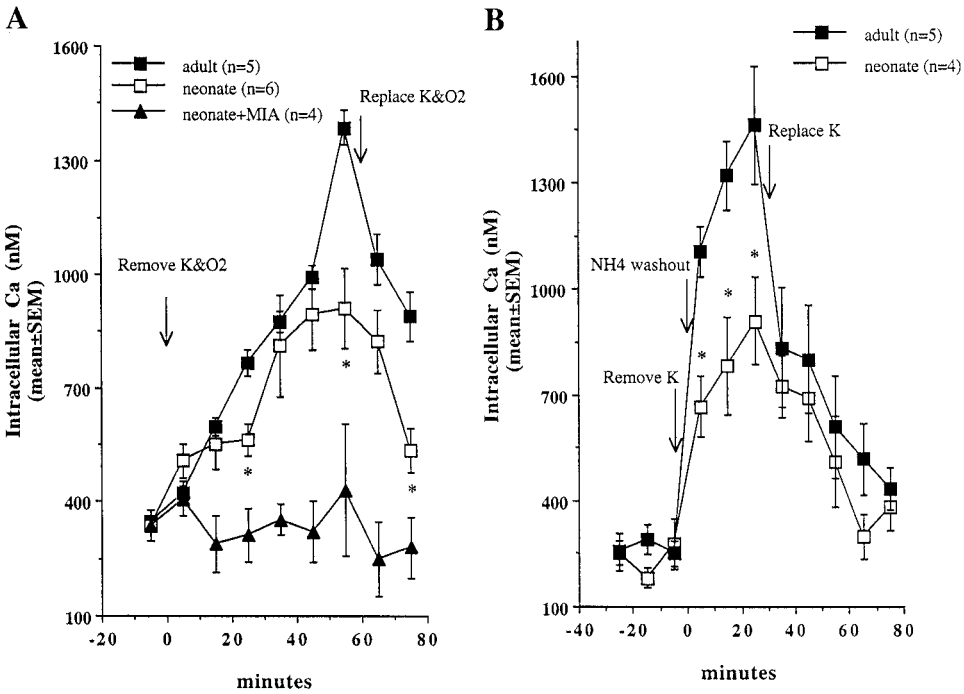


Fig. 5. Intracellular $Ca^{2+}$ ($[Ca]_i$) increases more in adult (■) than newborn (□) hearts during hypoxia (*A*) and after normoxic acidification (*B*) (20 mM $NH_4Cl$ washout). Additionally, in newborn (like adult) hearts, increases in $[Ca]_i$ during hypoxia are inhibited by the $Na^+/H^+$ exchange inhibitor MIA (40 μM; ▲). Mean $[Ca]_i$ (nM) is plotted vs. minutes (*$P < 0.05$ between age groups).

effects of MIA and hypertonic perfusion on $pH_i$ and high-energy phosphates during hypoxia. Significant differences for individual time points are indicated by the symbols described in Fig. 7 ($P < 0.05$), but the most salient features are summarized as follows. Figure 7*A* shows that when $Na^+$ accumulation is inhibited by MIA (see Fig. 3), $pH_i$ decreases more during hypoxia than without MIA ($P < 0.05$). The general hypothesis predicts that inhibiting increases in $Na_i$ and $[Ca]_i$ during hypoxia (Figs. 3, 5*A*, and 6, *A* and *B*), would decrease ATP consumption. This hypothesis is supported by the results depicted in Fig. 7, *B–D*, where MIA and hypertonic perfusion are shown to limit depletion of ATP and PCr and limit accumulation of $P_i$ ($P < 0.05$ in each case).

In addition to the effects on $Na^+$, $H^+$, $Ca^{2+}$, and high-energy phosphates discussed above, MIA and hypertonic inhibition of $Na^+$ accumulation both limit increases in perfusion pressure otherwise observed during $K^+$-free hypoxic perfusion. That is, in newborn hearts under constant flow conditions during 60 min of $K^+$-free hypoxic perfusion, perfusion pressure increases 89% in isotonic controls, 23% in hearts treated with 40 μM MIA, and 40% in hearts treated with 30 mosM hypertonic perfusion (from 44.9 ± 3.1 to 84.9 ± 7.1 mmHg, 42.0 ± 4.0 to 51.7 ± 1.8 mmHg and 44.8 ± 1.6 to 62.5 ± 7.0 mmHg, respectively). These data thus demonstrate that inhibiting $Na^+$, and thereby $[Ca_i]$, accumulation diminishes hypoxia-induced increases in coronary resistance, the latter indicating decreased hypoxia/ischemia-induced myocardial damage (28).

Finally, to better characterize the pathway responsible for the observed changes in $Na_i$ and $pH_i$, we measured $pH_i$ during the $NH_4Cl$ washout protocol with and without 40 μM MIA. The results are shown in Fig. 8, which demonstrates that MIA prevents $pH_i$ regulation under these conditions. (Again, please see DISCUSSION for further interpretation of this result.)

## DISCUSSION

*The $H^+$, $Na^+$, $Ca^{2+}$ paradigm: hypoxia and the control of $pH_i$.* In newborn rabbit hearts, as in adults, hypoxia stimulates an increase in cell $Na^+$ uptake, which is accompanied by an increase in $[Ca]_i$ (Figs. 2 and 5*A*). Similarly, after normoxic acidification, $Na_i$ increases with a time course similar to that of $pH_i$ and net $Na^+$ uptake ceases when $pH_i$ has returned to its control value (~7.25) (Fig. 4). Increased $Na^+$ uptake after normoxic acidification is also accompanied by an increase in $[Ca]_i$ (Fig. 5*B*). The $Na^+/H^+$ exchange inhibitor MIA inhibits increases in $[Na]_i$ and $[Ca]_i$ during hypoxia (Figs. 3 and 5*A*). MIA also limits $Na^+$ uptake (data not shown) and $pH_i$ recovery after normoxic acidification (Fig. 8). Inhibition of $Na^+$ accumulation by hypertonic perfusion (Fig. 6*A*) is also associated with a reduction in the $Ca_i$ accumulation during hypoxia (Fig. 6*B*). Parallel increases in $Na_i$ and $[Ca]_i$ during hypoxia and after normoxic acidification are reversed when $Na^+/K^+$ ATPase function is restored and $Na_i$ is allowed to return toward control, providing further evidence that the changes in $[Ca]_i$ are secondary to changes in $Na_i$ (Figs. 5 and 6). Thus, as previously demonstrated in the adult heart, these results are all consistent with the hypothesis that myocardial hypoxic/ischemic injury is in part a result of intracellular proton accumulation, which stimulates pH-regulatory ($[H]_i$-activated) $Na^+/H^+$ exchange, which increases net $Na^+$ uptake, reduces the transmembrane $Na^+$ gradient, and thereby increases $Ca^{2+}$ uptake via $Na^+/Ca^{2+}$ exchange (4, 12, 22, 30, 35, 36, 40, 47, 48).

*$Na^+$ and $Ca^{2+}$ accumulation in the adult and neonate heart.* Our results demonstrate that under both hypoxic and acidotic conditions, the increase in $[Ca]_i$ is less in the newborn heart than in the adult (Fig. 5, *A* and *B*) . Given the accepted notion that cell injury is a result of increases in $[Ca]_i$ (46), this is consistent with the well-documented finding that the newborn heart is resistant to hypoxic injury (23, 24, 39). The mechanism responsible for this age-related difference in $Ca_i$ accumulation, however, remains to be investigated. One simple explanation is that differences in $Ca^{2+}$ are the result of differences in $[Na]_i$ and its effect on $Na^+/Ca^{2+}$ exchange. On the basis of $Na^+$ uptake per dry weight, our data show no significant difference between age groups. Thus our $Na_i$ data appear to be inconsistent

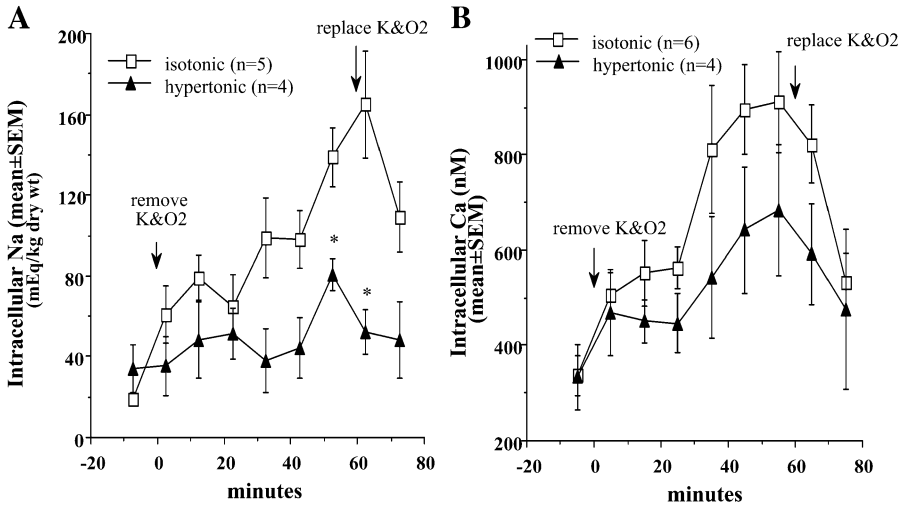


Fig. 6. In newborn hearts, $Na^+$ uptake and $Ca^{2+}$ accumulation during hypoxic $K^+$-free perfusion are inhibited by hypertonic solution ($P < 0.05$ by ANOVA for repeated measures). Mean intracellular $Na^+$ (meq/kg dry wt) (*A*) and $[Ca]_i$ (nM) (*B*) are plotted vs. minutes (*$P < 0.05$ for individual time intervals). Hypertonic perfusion is achieved by addition of 30 mosM of sucrose to the perfusate 5 min before and during hypoxic perfusion.

with a previous report by Seguchi et al. (44) showing that the $Na^+/H^+$ exchange rate is higher in newborn hearts than adults during hypoxic respiratory acidosis. Seguchi et al., however, drew their conclusions from the amiloride sensitivity of changes in developed tension and $^{22}Na$ uptake in sarcolemmal vesicles via unidentified pathways. On the other hand, our results are consistent with a more recent report from Nakanishi et al. (37), which concludes, based on ethylisopropylamiloride sensitivity of pH changes, that there is no difference between $Na^+/H^+$ exchange rates in newborn and adult hearts after $NH_4Cl$ washout.

The age-related differences we find in $Ca_i$ accumulation might at first be considered inconsistent with previous reports that $Na^+/Ca^{2+}$ exchange activity of newborn myocardial sarcolemma is greater than that of adult in rabbit hearts (5, 50) and not different than that of adult in dog hearts (21). However, for a number of reasons, results from sarcolemmal preparations cannot be directly compared with those acquired from the intact perfused organ. First, transport activity in vesicles is unlikely to be modulated by transduction pathways, which function under more physiological conditions in intact cells. Therefore, the number and activity of transporters in vesicles isolated from cells may be largely irrelevant to flux that occurs in the hypoxic organ. Second, the activity of the $Na^+/Ca^{2+}$ exchanger in vesicles is commonly determined in media which have high $Na^+$ (100–140 mM). This is much greater than physiological $[Na]_i$ and likely to be saturating for the transporter. That being the case, meaningful comparisons with cells whose $[Na]_i$ may be near or below the $K_{1/2}$ for $Na^+/Ca^{2+}$ exchange (41) may not be possible. A recent study using the whole cell patch-clamp method demonstrated that $Na^+/Ca^{2+}$ exchange current in cardiac myocytes isolated from newborn rabbit hearts is greater than that measured from cells isolated from adult rabbit hearts (6). Although it was not explicitly stated, it is assumed that these studies were conducted under normoxic conditions, making it difficult to compare the results with our findings during hypoxia and after acidification. Finally, at least one study of $Na^+$ transport proteins ($Na^+/K^+$ pumps) has demonstrated that increased numbers of transporters may be associated with a decrease in net transport function (42). Thus, even though the capacity of the $Na^+/Ca^{2+}$ exchanger in newborn myocardium may exceed that of adults under resting or saturating conditions, our data do not support the interpretation that there is greater net $Ca^{2+}$ transport via $Na^+/Ca^{2+}$ exchange in intact newborn rabbit hypoxic or acidotic myocardium than in adult. On the contrary, our data are consistent with decreased net $Ca^{2+}$ transport via $Na^+/Ca^{2+}$ exchange in newborn relative to the adult under the conditions tested. Contributions of other $Ca^{2+}$ transport pathways, however, remain to be investigated.

It will be noted that FBAPTA, as a $Ca^{2+}$ buffer, is likely to create artifacts in our $[Ca]_i$ measurements. Nevertheless, when the artifacts and limitations of FBAPTA are compared with other techniques (32), we conclude that $^{19}F$ NMR remains the best method for measuring mean $[Ca]_i$ in the intact organ. That is, other methods for measuring $[Ca]_i$ (as opposed to $Ca_i$ content) are limited to use in isolated cells or cells on the surface of a tissue that are accessible using microelectrode or optical techniques. We argue that we may reasonably draw conclusions from our data for the following reasons. Even though FBAPTA will have a large capacity for buffering $Ca_i$ in our experiments, its primary effect will be to slow or diminish changes in $[Ca]_i$ away from its $K_d$ (500 nM). More specifically, because the response time of FBAPTA to changes in $[Ca]_i$ is conservatively estimated to be on the order of 20 ms (32) and we report time-averaged values for $[Ca]_i$ acquired over 10-min intervals, the $[Ca]_i$ values we report are inaccurate to the extent that the mean value of $[Ca]_i$ is biased toward the $K_d$. This means that under control conditions, our estimates of $[Ca]_i$ may be somewhat high, but as the mean value of $[Ca]_i$ rises past 500 nM, our measurements provide an underestimate. Furthermore, because the buffer capacity of FBAPTA decreases as $[Ca]_i$ moves away from 500 nM, changes in $[Ca]_i$ at low and, especially, high $[Ca]_i$ will be measured with less artifact due to FBAPTA buffering of $Ca_i$. In addition, because the effects of hypoxia and normoxic acidification on $Na_i$ and $[Ca]_i$ are measured during $K^+$-free perfusion (cells are depolarized and asystolic), the effects of FBAPTA on myocardial contractility are minimized. Finally, and most importantly, because our conclusions are based on statistical assessment of differences in $[Ca]_i$ between groups and treatments that develop over time from the same baseline, our conclusions do not depend on the effects of FBAPTA on $[Ca]_i$ (25). In other words, with regard to $[Ca]_i$, our conclusions are based only on differences between measured values of $[Ca]_i$ and not on the actual values of $[Ca]_i$.

*Effects of hypertonicity on pH-regulatory $Na^+/H^+$ exchange.* Hypertonic perfusion has previously been shown to inhibit pH-regulatory $Na^+/H^+$ exchange in *Amphiuma* red blood cells (12, 13) and to limit hypoxia-induced increases in $Na_i$ and $[Ca]_i$ in adult rabbit hearts (22). The results presented in this study similarly show that in the newborn heart hypertonic perfusion initiated before, and continued during, hypoxia decreases $Na^+$ accumulation and $[Ca]_i$ compared with isotonic perfusion. The relative decrease in $Na^+$ uptake is similar to that previously reported for adult hearts (22) and consistent with the hypothesis that hypertonic solutions decrease the response of $Na^+/H^+$ exchange to decreased intracellular pH. Although the mechanism responsible for the decrease in $Na^+$ accumulation under hypertonic conditions remains obscure, the effect of hypertonic perfusion on $[Ca]_i$ is consistent with the hypothesis that hypoxia-induced increases in $[Ca]_i$ are $Na^+$ dependent in the newborn heart.

Figure 7*A*, however, suggests a corollary or alternative explanation for the effect of hypertonic perfusion on $Na^+$ uptake during hypoxia. In this case, the initiation of hypertonic perfusion 5 min before beginning hypoxic perfusion is shown to increase $pH_i$ compared

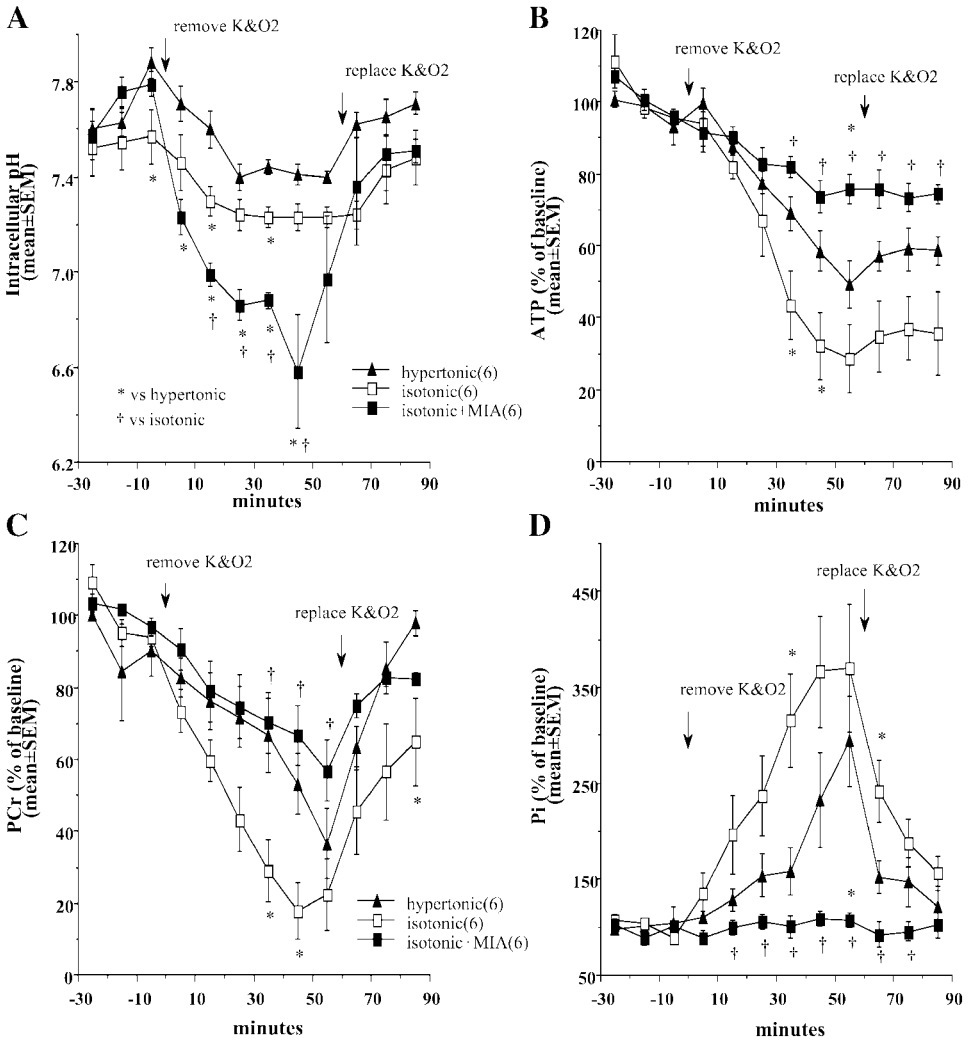


Fig. 7. The $Na^+/H^+$ exchange inhibitor MIA (40 μM) increases, and 30 mM hypertonic perfusion decreases, the change in intracellular pH ($pH_i$), otherwise observed during hypoxic perfusion. Both interventions decrease $Na^+$ uptake and $[Ca]_i$ during hypoxia and likewise decrease associated changes in high-energy phosphates. $pH_i$ and high-energy phosphates [ATP, phosphocreatine (PCr), and inorganic phosphate ($P_i$) as a percentage of baseline] are plotted vs. minutes with (■) and without (□) MIA and after hypertonic perfusion (▲). Inhibition of intracellular $Na^+$ accumulation apparently diminishes energy consumption during hypoxia (*$P < 0.05$ vs. hypertonic; †$P < 0.05$ vs. isotonic).

with isotonic perfusion. Because our hypothesis states that increased $[H]_i$ provides the stimulus for $Na^+/H^+$ exchange during hypoxia, the data shown in the top curve of Fig. 7*A* suggest that hypertonic perfusion would decrease the stimulus for $Na^+/H^+$ exchange during hypoxia. This interpretation is also consistent with Fig. 6, *A* and *B*. That is, if hypertonic perfusion increases $pH_i$ secondary to a volume regulatory response (13, 22), the relatively higher $pH_i$ during hypoxia (Fig. 7*A*) will attenuate $H_i$-induced $Na^+/H^+$ exchange and result in less $Na^+$ and, therefore, $Ca^{2+}$ uptake.

*Energy cost of stimulating pH-regulatory $Na^+/H^+$ exchange.* The results summarized for perfusion pressure and in Fig. 7 are consistent with previous reports that inhibition of pH-regulatory $Na^+/H^+$ exchange preserves high-energy phosphates and function in myocardium and cardiac myocytes exposed to hypoxia/ischemia (4, 30, 35, 36, 40). As such, they also support the hypothesis that increased $Na^+$ and, therefore, $Ca^{2+}$ uptake resulting from increased pH-regulatory $Na^+/H^+$ exchange are central to hypoxic/ischemic injury (30, 46). More specifically, the data presented for perfusion pressure and in Fig. 7 suggest that both pharmacological as well as hypertonic inhibition of $Na^+$ accumulation protect the myocardium from the changes otherwise observed during hypoxia. Further insight into the metabolic cost of increasing $Na^+$ uptake can be gained from experiments conducted when $Na_i$ was measured during hypoxia with normal $K^+$ in the perfusate (data not shown). Again, in newborn rabbit hearts, when $Na^+$-$K^+$-ATPase was not inhibited by $K^+$-free perfusion, there was no measurable change in $Na_i$ after 52.5 min of hypoxic perfusion (from 21 ± 16 to 27 ± 18 meq/kg dry weight; $n = 3$). ($Na^+$ uptake during hypoxic perfusion with normal $K^+$ is essentially the same as that shown in Fig. 3 for hypoxic $K^+$-free perfusion with MIA.) In comparison, when $Na^+$-$K^+$-ATPase was inhibited by $K^+$-free perfusion during hypoxia (Figs. 2 and 3), $Na_i$ rose from 19 ± 3.4 to 139 ± 14.6 meq/kg dry wt. Here, the difference between $Na_i$ measured with normal $K^+$ and $K^+$-free perfusates represents the amount of $Na^+$ leaving the cells due to $Na^+$-$K^+$-ATPase activity during hypoxia. In other words, in order for $Na_i$ to remain unchanged during normal $K^+$ hypoxic perfusion, the $Na^+$ extrusion ($Na^+$-$K^+$ pump) rate must actually increase to match the increase in uptake shown in Figure 2. Thus the

data demonstrate that, contrary to historical opinion, increases in $Na_i$ observed during myocardial hypoxia are not the result of decreased $Na^+/K^+$ pump rate but, instead, as observed during ischemia (2, 30), are the result of a relatively larger increase in uptake rate compared with a measurable but smaller increase in $Na^+/K^+$ pump rate. As a result, the hypoxia-induced increase in $Na^+$ uptake will increase the rate of ATP consumption by $Na^+$-$K^+$-ATPase and likely increase the rate and magnitude of ATP depletion. However, because most of the experiments reported in this study were conducted using $K^+$-free perfusion to inhibit the $Na^+$-$K^+$-ATPase, the observed effects of limiting $Na^+$ uptake on ATP depletion are more likely to stem from limiting $Na^+$- and $Ca^{2+}$-dependent ATP consumption other than $Na^+$-$K^+$-ATPase. Inhibition of $Na^+$ uptake may also diminish effects of $Na_i$ and $Ca_i$ accumulation on mitochondria (19, 29), which could limit ATP depletion by marginally increasing ATP production.

To reiterate, our comparisons of $Na^+$ uptake during normoxic and hypoxic conditions have been completed under conditions of $K^+$-free perfusion to measure $Na^+$ uptake directly in the absence of $Na^+$ efflux via the $Na^+$-$K^+$ pump. Furthermore, the perfusions were performed at ~23°C in order decrease the rate of change in $Na_i$ and $[Ca]_i$ and thereby increase the sensitivity of the NMR measurements (increased number of acquisitions per unit change in $Na_i$ and $[Ca]_i$), while at the same time limiting irreversible changes in cell membrane ion transport. These procedures undoubtedly decreased the heart's consumption of energy for contraction, but they have allowed us to assess the relative rates of $Na^+$ uptake and efflux via Na+-$K^+$-ATPase under normoxic and hypoxic conditions. Our previous studies of adult hearts demonstrated that $K^+$-free perfusion completely inhibits $Na^+$ efflux via the $Na^+$-$K^+$ pump (4). Similar studies using ouabain and $K^+$-free superfusion with chick heart cells to assess the role of $Na^+$ uptake in cell swelling have led the authors to conclusions similar to ours, that $Ca^{2+}$ uptake after $Na^+$-$K^+$ pump inhibition is via $Na^+/Ca^{2+}$ exchange and that "swelling during ischemic injury may not result from $Na^+/K^+$ pump failure alone" (45). It could be further argued that because our studies were conducted under asystolic conditions, metabolism (including proton production) associated with muscle contraction is minimized and, therefore, the changes in high-energy phosphates that occur in response to MIA and hypertonic perfusion reflect changes in metabolism resulting from changes in $Na^+$ uptake in the absence of changes in contractility. We have considered this to be a reasonable compromise between minimizing uncontrolled variables and using a physiologically relevant model to the extent that the changes in $Na_i$ and $[Ca]_i$ that we measure are reversible (and therefore mediated by cell membranes that maintain near normal $Na^+$ and $Ca^{2+}$ transport function) and predictably consistent with those reported for other preparations at a variety of temperatures, including body temperature (4, 25, 26, 36, 40, 47, 48). It will also be noted that during 55-min normoxic $K^+$-free perfusion, $Na_i$ appeared to be unchanged in newborn hearts, whereas we have previously shown in adult hearts that $Na_i$ increases nominally by 120% under the same conditions (4). The difference between these groups is, however, not significant ($P = 0.981$ by ANOVA), reflecting the difficulty of measuring small changes in $Na_i$ using notoriously insensitive NMR.

It will be further noted that we have used 40 μM MIA to inhibit $Na^+/H^+$ exchange in these studies. This dosage was chosen because it was the lowest dose at which we were unable to measure $Na^+$ uptake during 1 h of hypoxic $K^+$-free perfusion. That is, the criteria for inhibition of $Na^+/H^+$ exchange is based on $Na^+$ uptake measured under physiological extracellular $Na^+$ conditions (33) rather than changes in $pH_i$ or $Na^+$ flux measured under less than physiological $Na^+$ conditions (15, 18, 37, 48). Controversy remains concerning the effect of amiloride and its analogs on $Na^+$, and $Na^+$-dependent, transport in cardiac myocytes. Although the lack of specificity of the amiloride analogs is well documented (43), the potency of analogs such as MIA and ethylisopropylamiloride (EIPA) for $Na^+/H^+$ exchange inhibition is well accepted (34). Thus, at the concentration used in this study, the major effect of MIA is likely to be inhibition of $Na^+/H^+$ exchange. We cannot, however, rule out an effect of MIA on noninactivating $Na^+$ channels and $Na^+/Ca^{2+}$ exchange, which have also been implicated under the conditions of this study. For example, based on the fact that EIPA inhibits veratridine-induced hypercontracture, Haigney et al. (20) concluded that EIPA inhibits noninactivating $Na^+$ channels, and similar conclusions were reached by others using a whole cell patch-clamp technique (14). On the other hand, Frelin et al. (15) reported that in chick cardiac cells, $Na^+/Ca^{2+}$ exchange is not affected by the most active inhibitors of $Na^+/H^+$ exchange (including MIA) at concentrations <1 mM, whereas Gar-

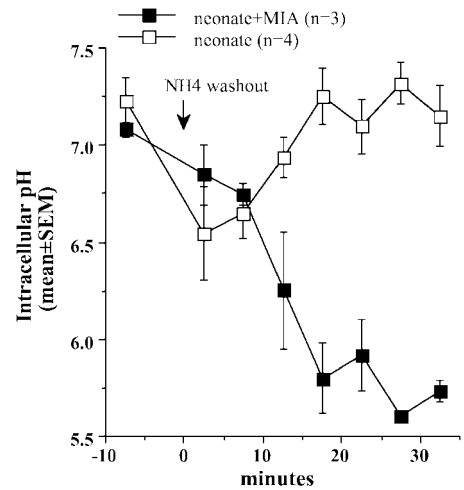


Fig. 8. $Na^+/H^+$ inhibition prevents pH regulation after 20 mM $NH_4Cl$ washout. $pH_i$ is plotted vs. minutes with (■) and without (□) 40 μM MIA. During the first 10 min after $NH_4Cl$ washout, MIA has no measurable effect on $pH_i$, allowing calculation of buffer capacity as $\beta = \{[NH_4]_o \times 10 \exp(pH_o - pH_i)\}/\Delta pH_i$. Please see text for explanation.

cia et al. (16) reported that, in porcine cardiac sarcolemmal vesicles, these same amiloride analogs competitively inhibit binding of L-type $Ca^{2+}$ channel inhibitors and, by inference, will themselves inhibit L-type $Ca^{2+}$ channels. Although we have not determined the specificity of MIA in newborn myocardium, our results remain consistent with the interpretation that the response we measure is the result of inhibiting $Na^+$-dependent $Ca^{2+}$ accumulation.

*Mechanisms of newborn resistance to hypoxic cell injury.* Although the mechanisms responsible for the newborn heart's apparent resistance to hypoxia (23, 24, 39) and acidosis (38) remain unclear, our data can be used to address at least two current explanations. First, it has been hypothesized that the proton load or the total number of protons added to the intracellular solution during hypoxia or acidosis is less in the newborn than the adult heart. This could be the result of less proton production and/or greater proton buffering in the newborn heart. Second, the newborn heart may have $Na^+$-independent pH-regulatory transport systems that are not active in the adult, e.g., there may be age-related differences in $Cl^-/HCO_3^-$ exchange (37). Either one, or the combination, of these scenarios would result in less stimulation of pH-regulatory $Na^+/H^+$ exchange and, therefore, less $Na^+$-dependent $Ca^{2+}$ uptake.

The results of the $NH_4$ prepulse experiment most directly address these questions because $H^+$ delivery or initial proton load will be the same for both age groups. That is, from the Henderson-Hasselbalch equation, we calculate that when $pH_i$, $pH_o$, and $[NH_4]_o$ are the same in both age groups before $NH_4$ washout, $[NH_4]_i$ is the same and, therefore, intracellular [H] added by $NH_4$ washout will be the same. Figure 4 shows no measurable difference between age groups in the initial acidification after $NH_4Cl$ washout. If no pH-regulatory transport has occurred during this interval, the data would indicate that there is no measurable age-related difference in the heart cell's "intrinsic" or fixed buffer capacity. However, this is only true if there are no active pH-regulatory processes functioning (10). Figure 8 addresses this issue, illustrating that the $Na^+/H^+$ exchange inhibitor MIA has no measurable effect on $pH_i$ during the first 10 min of $NH_4$ washout [$NH_4$ washout should be complete in $<2$ min (3)]. Thereafter, $pH_i$ continues to fall, presumably the result of continued proton production, while $Na^+/H^+$ exchange remains inhibited. Previous experiments (data not shown) similarly demonstrated the same response to $Na^+/H^+$ inhibition in the adult heart (4). The buffer capacity calculated from the data shown in Fig. 4 ($\beta = \Delta[NH_4]_i/\Delta pH_i = [NH_4]_i/\Delta pH_i = \{[NH_4]_o \times 10 \exp(pH_o - pH_i)\}/\Delta pH_i$, where o and i refer to extra- and intracellular compartments, respectively, $[NH_4]_o$ is the concentration of $NH_4$ in the perfusate used before washout, and $\Delta pH_i$ is the change in $pH_i$ measured during the first 10 min after washout) is 36.7 meq/l pH unit for adult hearts and 43.16 meq/l pH unit for newborn hearts. These values are not significantly different, similar to those previously measured in guinea pig papillary muscle, isolated ferret hearts, and sheep Purkinje fibers (10, 48, 49), and somewhat higher than isolated myocytes (10). Thus our results do not support reports that the "intrinsic" buffer capacity of newborn hearts is greater than that of adults (44). Our results showing that the $Na^+/H^+$ exchange inhibitor MIA completely inhibits pH regulation in nominally $HCO_3^-$-free, HEPES-buffered perfusate are consistent with previous reports from adult and newborn hearts (18, 37) that suggest that $Na^+/H^+$ exchange mediates a major portion of pH recovery after normoxic acidification. We have not, however, tested whether $Cl^-/HCO_3^-$ exchange may serve to perform a greater portion of newborn heart pH regulation than in adults (37) under $HCO_3^-$-buffered conditions.

*Conclusions.* The data presented here are consistent with the hypothesis that newborn hearts, like adult, respond to hypoxia and normoxic acidification with an increase in pH-regulatory $Na^+/H^+$ exchange, which leads to increased $Na^+$ uptake, collapse of the transmembrane $Na^+$ gradient, and, consequently, increased uptake and accumulation of $Ca^{2+}$ via $Na^+/Ca^{2+}$ exchange. The data also demonstrate that under similar conditions of hypoxia and acidification, while age-related differences in $Na^+$ uptake are not significant, $[Ca]_i$ is significantly less in newborn than adult hearts.

This work was done during the tenure of a research fellowship from the American Heart Association, California Affiliate, and was supported by the University of California Medical Center Hibbard Williams Fund and by National Heart, Lung, and Blood Institute Grants HL-21179 and HL-56681. NMR spectrometer expense was funded in part by National Institutes of Health Grant RR-02511 and National Science Foundation Grant PCM-8417289.

## REFERENCES

1. **Anderson SE, Carr LJ, Schierling TD, and Kost GJ.** Are age-related differences in response to myocardial ischemia and cardioplegia pH dependent? *Biol Neonate* 65: 25–35, 1994.
2. **Anderson SE, Dickinson CZ, Liu H, and Cala PM.** Effects of Na-K-2Cl cotransport inhibition on myocardial Na and Ca during ischemia and reperfusion. *Am J Physiol Cell Physiol* 270: C608–C618, 1996.
3. **Anderson SE and Johnson JA.** Tissue-fluid pressure measured in perfused rabbit hearts during osmotic transients. *Am J Physiol Heart Circ Physiol* 252: H1127–H1137, 1987.
4. **Anderson SE, Murphy E, Steenbergen C, London RE, and Cala PM.** Na/H exchange in myocardium: effects of hypoxia and acidification on Na and Ca. *Am J Physiol Cell Physiol* 259: C940–C948, 1990.
5. **Artman M.** Sarcolemmal $Na^+$-$Ca^{2+}$ exchange activity and exchanger immunoreactivity in developing rabbit hearts. *Am J Physiol Heart Circ Physiol* 263: H1506–H1513, 1992.
6. **Artman M, Ichikawa H, Avkiran M, and Coetzee WA.** $Na^+/Ca^{2+}$ exchange current density in cardiac myocytes from rabbits and guinea pigs during postnatal development. *Am J Physiol Heart Circ Physiol* 268: H1714–H1722, 1995.
7. **Balschi JA, Bittl JA, Springer CS Jr, and Ingwall JS.** $^{31}P$ and $^{23}Na$ NMR spectroscopy of normal and ischemic rat skeletal muscle. Use of a shift reagent in vivo. *NMR Biomed* 3: 47–58, 1990.
8. **Bansal N, Germann MJ, Seshan V, Shires GT 3rd, Malloy CR, and Sherry AD.** Thulium 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetrakis(methylene phosphonate) as a $^{23}Na$ shift reagent for the in vivo rat liver. *Biochemistry* 32: 5638–5643, 1993.
9. **Boron WF and De Weer PD.** Intracellular pH transients in squid giant axons caused by $CO_2$, $NH_3$, and metabolic inhibitors. *J Gen Physiol* 67: 91–112, 1976.

10. **Bountra C, Powell T, and Vaughan-Jones RD.** Comparison of intracellular pH transients in single ventricular myocytes and isolated ventricular muscle of guinea-pig. *J Physiol* 424: 343–365, 1990.
11. **Buster DC, Castro MM, Geraldes CF, Malloy CR, Sherry AD, and Siemers TC.** Tm(DOTP)5-: a $^{23}Na^+$ shift agent for perfused rat hearts. *Magn Reson Med* 15: 25–32, 1990.
12. **Cala PM, Maldonado H, and Anderson SE.** Cell volume and pH regulation by the *Amphiuma* red blood cell: a model for hypoxia-induced cell injury. *Comp Biochem Physiol* 102A: 603–608, 1992.
13. **Cala PM and Maldonado HM.** pH regulatory Na/H exchange by *Amphiuma* red blood cells. *J Gen Physiol* 103: 1035–1054, 1994.
14. **Chattou S, Coulombe A, Diacono J, Le Grand B, John G, and Feuvray D.** Slowly inactivating component of sodium current in ventricular myocytes is decreased by diabetes and partially inhibited by known $Na^+$-$H^+$ exchange blockers. *J Mol Cell Cardiol* 32: 1181–1192, 2000.
15. **Frelin C, Vigne P, Barbry P, and Lazdunski M.** Molecular properties of amiloride action and of its $Na^+$ transporting targets. *Kidney Int* 32: 785–793, 1987.
16. **Garcia ML, King VF, Shevell JL, Slaughter RS, Suarez-Kurtz G, Winquist RJ, and Kaczorowski GJ.** Amiloride analogs inhibit L-type calcium channels and display calcium entry blocker activity. *J Biol Chem* 265: 3763–3771, 1990.
17. **Glantz SA and Slinker BK.** *Primer of Applied Regression and Analysis of Variance*. New York: McGraw-Hill, 2001.
18. **Grace AA, Kirschenlohr HL, Metcalfe JC, Smith GA, Weissberg PL, Cragoe EJ Jr, and Vandenberg JI.** Regulation of intracellular pH in the perfused heart by external $HCO_3^-$ and $Na^+$-$H^+$ exchange. *Am J Physiol Heart Circ Physiol* 265: H289–H298, 1993.
19. **Griffiths EJ.** Reversal of mitochondrial Na/Ca exchange during metabolic inhibition in rat cardiomyocytes. *FEBS Lett* 453: 400–404, 1999.
20. **Haigney MC, Lakatta EG, Stern MD, and Silverman HS.** Sodium channel blockade reduces hypoxic sodium loading and sodium-dependent calcium loading. *Circulation* 90: 391–399, 1994.
21. **Hanson GL, Schilling WP, and Michael LH.** Sodium-potassium pump and sodium-calcium exchange in adult and neonatal canine cardiac sarcolemma. *Am J Physiol Heart Circ Physiol* 264: H320–H326, 1993.
22. **Ho HS, Liu H, Cala PM, and Anderson SE.** Hypertonic perfusion inhibits intracellular Na and Ca accumulation in hypoxic myocardium. *Am J Physiol Cell Physiol* 278: C953–C964, 2000.
23. **Jarmakani JM, Nagatomo T, Nakazawa M, and Langer GA.** Effect of hypoxia on myocardial high-energy phosphates in the neonatal mammalian heart. *Am J Physiol Heart Circ Physiol* 235: H475–H481, 1978.
24. **Jarmakani JM, Nakazawa M, Nagatomo T, and Langer GA.** Effect of hypoxia on mechanical function in the neonatal mammalian heart. *Am J Physiol Heart Circ Physiol* 235: H469–H474, 1978.
25. **Kirschenlohr HL, Grace AA, Clarke SD, Shachar-Hill Y, Metcalfe JC, Morris PG, and Smith GA.** Calcium measurements with a new high-affinity n.m.r. indicator in the isolated perfused heart. *Biochem J* 293: 407–411, 1993.
26. **Kirschenlohr HL, Metcalfe JC, Morris PG, Rodrigo GC, and Smith GA.** $Ca^{2+}$ transient, $Mg^{2+}$, and pH measurements in the cardiac cycle by $^{19}F$ NMR. *Proc Natl Acad Sci USA* 85: 9017–9021, 1988.
27. **Kleyman TR and Cragoe EJ Jr.** Amiloride and its analogs as tools in the study of ion transport. *J Membr Biol* 105: 1–21, 1988.
28. **Kolocassides KG, Galinanes M, and Hearse DJ.** Ischemic preconditioning, cardioplegia or both? *J Mol Cell Cardiol* 26: 1411–1414, 1994.
29. **Korge P and Langer GA.** Mitochondrial $Ca^{2+}$ uptake, efflux, and sarcolemmal damage in $Ca^{2+}$-overloaded cultured rat cardiomyocytes. *Am J Physiol Heart Circ Physiol* 274: H2085–H2093, 1998.
30. **Liu H, Cala PM, and Anderson SE.** Ethylisopropylamiloride diminishes changes in intracellular Na, Ca, and pH in ischemic newborn myocardium. *J Mol Cell Cardiol* 29: 2077–2086, 1997.
31. **Liu H, Cala PM, and Anderson SE.** Ischemic preconditioning: effects on pH, Na and Ca in newborn rabbit hearts during ischemia/reperfusion. *J Mol Cell Cardiol* 30: 685–697, 1998.
32. **Marban E, Kitakaze M, Chacko VP, and Pike MM.** $Ca^{2+}$ transients in perfused hearts revealed by gated $^{19}F$ NMR spectroscopy. *Circ Res* 63: 673–678, 1988.
33. **McLean LA, Zia S, Gorin FA, and Cala PM.** Cloning and expression of the $Na^+/H^+$ exchanger from *Amphiuma* RBCs: resemblance to mammalian NHE1. *Am J Physiol Cell Physiol* 276: C1025–C1037, 1999.
34. **Meng H, Maddaford TG, and Pierce GN.** Effect of amiloride and selected analogs on postischemic recovery of cardiac contractile function. *Am J Physiol Heart Circ Physiol* 264: H1831–H1835, 1993.
35. **Meng H and Pierce GN.** Protective effects of 5-(*N*,*N*-dimethyl)amiloride on ischemia-reperfusion injury in hearts. *Am J Physiol Heart Circ Physiol* 258: H1615–H1619, 1990.
36. **Myers ML, Mathur S, Li G, and Karmazyn M.** Sodium-hydrogen exchange inhibitors improve postischaemic recovery of function in the perfused rabbit heart. *Cardiovasc Res* 29: 209–214, 1995.
37. **Nakanishi T, Gu H, Seguchi M, Cragoe EJ Jr, and Momma K.** $HCO_3^-$-dependent intracellular pH regulation in the premature myocardium. *Circ Res* 71: 1314–1323, 1992.
38. **Nakanishi T, Seguchi M, Tsuchiya T, Yasukouchi S, and Takao A.** Effect of acidosis on intracellular pH and calcium concentration in the newborn and adult rabbit myocardium. *Circ Res* 67: 111–123, 1990.
39. **Nakanishi T, Young HH, Shimizu T, Nishioka K, and Jarmakani JM.** The relationship between myocardial enzyme release and $Ca^{2+}$ uptake during hypoxia and reoxygenation in the newborn and adult heart. *J Mol Cell Cardiol* 16: 519–532, 1984.
40. **Nishida M, Borzak S, Kraemer B, Navas JP, Kelly RP, Smith TW, and Marsh JD.** Role of cation gradients in hypercontracture of myocytes during simulated ischemia and reperfusion. *Am J Physiol Heart Circ Physiol* 264: H1896–H1906, 1993.
41. **Philipson KD.** Sodium-calcium exchange in plasma membrane vesicles. *Annu Rev Physiol* 47: 561–571, 1985.
42. **Pickar JG, Carlsen RC, Atrakchi A, and Gray SD.** Increased $Na^+$-$K^+$ pump number and decreased pump activity in soleus muscles in SHR. *Am J Physiol Cell Physiol* 267: C836–C844, 1994.
43. **Pierce GN, Cole WC, Liu K, Massaeli H, Maddaford TG, Chen YJ, McPherson CD, Jain S, and Sontag D.** Modulation of cardiac performance by amiloride and several selected derivatives of amiloride. *J Pharmacol Exp Ther* 265: 1280–1291, 1993.
44. **Seguchi M and Jarmakani JM.** Effect of respiratory acidosis on hypoxic newborn myocardium. *J Mol Cell Cardiol* 21: 927–934, 1989.
45. **Smith TW, Rasmusson RL, Lobaugh LA, and Lieberman M.** $Na^+/K^+$ pump inhibition induces cell shrinkage in cultured chick cardiac myocytes. *Basic Res Cardiol* 88: 411–420, 1993.
46. **Steenbergen C, Fralix TA, and Murphy E.** Role of increased cytosolic free calcium concentration in myocardial ischemic injury. *Basic Res Cardiol* 88: 456–470, 1993.
47. **Tani M and Neely JR.** Role of intracellular $Na^+$ and $Ca^{2+}$ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. *Circ Res* 65: 1045–1056, 1989.
48. **Vandenberg JI, Metcalfe JC, and Grace AA.** Mechanisms of $pH_i$ recovery after global ischemia in the perfused heart. *Circ Res* 72: 993–1003, 1993.
49. **Vaughan-Jones RD and Wu M.** pH dependence of intrinsic $H^+$ buffering power in the sheep cardiac Purkinje fibre. *J Physiol* 425: 429–448, 1990.
50. **Vetter R, Kemsies C, and Schulze W.** Sarcolemmal $Na^+$-$Ca^{2+}$ exchange and sarcoplasmic reticulum $Ca^{2+}$ uptake in several cardiac preparations. *Biomed Biochim Acta* 46: S375–S381, 1987.