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Hypertonic perfusion inhibits intracellular Na and Ca accumulation in hypoxic myocardium

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Ho, Hung S., Hong Liu, Peter M. Cala, and Steven E. Anderson. Hypertonic perfusion inhibits intracellular Na and Ca accumulation in hypoxic myocardium. *Am J Physiol Cell Physiol* 278: C953–C964, 2000.—Much evidence supports the view that hypoxic/ischemic injury is largely due to increased intracellular Ca concentration ($[Ca]_i$) resulting from *1*) decreased intracellular pH (pH_i), *2*) stimulated Na/H exchange that increases Na uptake and thus intracellular Na (Na_i), and *3*) decreased Na gradient that decreases or reverses net Ca transport via Na/Ca exchange. The Na/H exchanger (NHE) is also stimulated by hypertonic solutions; however, hypertonic media may inhibit NHE's response to changes in pH_i (Cala PM and Maldonado HM. *J Gen Physiol* 103: 1035–1054, 1994). Thus we tested the hypothesis that hypertonic perfusion attenuates acid-induced increases in Na_i in myocardium and, thereby, decreases Ca_i accumulation during hypoxia. Rabbit hearts were Langendorff perfused with HEPES-buffered Krebs-Henseleit solution equilibrated with 100% O_2 or 100% N_2. Hypertonic perfusion began 5 min before hypoxia or normoxic acidification (NH_4Cl washout). Na_i, $[Ca]_i$, pH_i, and high-energy phosphates were measured by NMR. Control solutions were 295 mosM, and hypertonic solutions were adjusted to 305, 325, or 345 mosM by addition of NaCl or sucrose. During 60 min of hypoxia (295 mosM), Na_i rose from 22 ± 1 to 100 ± 10 meq/kg dry wt while $[Ca]_i$ rose from 347 ± 11 to 1,306 ± 89 nM. During hypertonic hypoxic perfusion (325 mosM), increases in Na_i and $[Ca]_i$ were reduced by 65 and 60%, respectively ($P < 0.05$). Hypertonic perfusion also diminished Na uptake after normoxic acidification by 87% ($P < 0.05$). The data are consistent with the hypothesis that mild hypertonic perfusion diminishes acid-induced Na accumulation and, thereby, decreases Na/Ca exchange-mediated Ca_i accumulation during hypoxia.

intracellular pH; myocardial hypoxia; nuclear magnetic resonance spectroscopy

ION HOMEOSTASIS IS TIGHTLY controlled in most cells and tissues under normal conditions, whereas it has been demonstrated that ion homeostasis is disturbed in a wide variety of pathological conditions, including hypoxia and ischemia. The traditional view of hypoxia/ischemia-induced disruption in cellular ion homeostasis has been based on the notion that ATP depletion leads to impaired function of ATP-dependent transport systems, resulting in dissipation of ion gradients, e.g., those dependent on Na-K-ATPase (39, 45). Recent studies provide evidence that disturbances in ion homeostasis may be among the earliest alterations in cell function and may ultimately lead to cell injury and death (44). In particular, during myocardial hypoxia and ischemia, disturbances in intracellular pH (pH_i), Na (Na_i), and Ca (Ca_i) occur before ATP is depleted (1, 5). The discovery of robust intracellular proton-induced Na/H exchange in myocardium (27, 38), combined with earlier evidence for Na-dependent Ca transport (26), led to the development and testing of a unifying hypothesis linking changes in pH_i, Na_i, and Ca_i during hypoxia and ischemia (5, 10, 44, 47). Thus we and others have presented data consistent with the general hypothesis that, in the heart, hypoxia/ischemia results in the following chain of events: *1*) anaerobic metabolism increases proton production and decreases pH_i; *2*) decreased pH_i stimulates Na/H exchange, which increases Na uptake; *3*) increased Na uptake increases Na_i concentration ($[Na]_i$); *4*) increased $[Na]_i$ initially decreases and may ultimately reverse the force that normally drives net Ca efflux via Na/Ca exchange; and *5*) decreased efflux or increased influx of Ca via Na/Ca exchange results in increased Ca_i concentration ($[Ca]_i$). Although recent studies suggest that other Na transport pathways may also contribute to Na_i accumulation after acidification and during hypoxia/ischemia (4, 14, 42), the relationship between Na_i and Ca_i accumulation is well described by the hypothesis regardless of the Na uptake pathway. Furthermore, most evidence is consistent with the postulate that intracellular acidification stimulates Na/H exchange and that a major portion of acidification- and/or hypoxia/ischemia-induced Na uptake is via Na/H exchange (4, 5, 14, 29, 32, 37, 41, 48).

On the other hand, in numerous cell types, including cardiac myocytes, the Na/H exchanger is also stimulated by exposure to hypertonic solutions (12, 54). Yet operation in one mode (volume or pH regulation) may preclude functional response to a second stimulus. Briefly, previous studies from this laboratory have demonstrated that when *Amphiuma* red blood cells (RBCs) are shrunk by exposure to hypertonic solutions, the Na/H exchanger is stimulated to cause net uptake of Na, along with osmotically obligated water, until normal cell volume is restored (8). In these same cells, when pH_i is decreased, the Na/H exchanger is stimulated to cause net Na uptake and proton efflux until normal pH_i is restored (12). However, these two modes of stimulating the Na/H exchanger, either exposure to

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increased osmolarity or decreased pH_i, appear to result in mutually exclusive responses (10–12). That is, after *Amphiuma* RBCs or cardiac myocytes are exposed to hypertonic media, the Na/H exchanger is capable of increasing pH_i to a level higher than would occur in response to decreased pH_i (12, 54); i.e., pH regulation is disrupted. Conversely, in *Amphiuma* RBCs, it has been demonstrated that when the Na/H exchanger is stimulated by lowering pH_i, it is capable of increasing cell volume far above the level that would normally occur in response to exposure to hypertonic media (12); i.e., volume regulation is disrupted. Thus, with respect to exposure to hypertonic solution and decreased pH_i, data from *Amphiuma* RBCs and cardiac myocytes suggest that the Na/H exchanger responds to the stimulus it receives first and "ignores" the other stimulus if it occurs later.

We therefore conducted the studies described here to further support the general hypothesis described above and to test the corollary or subhypothesis that modest increases in perfusate tonicity will limit Na uptake via the Na/H exchanger in response to decreased myocardial pH_i. The results of the experiments reported here demonstrate that moderately hypertonic perfusion decreases Na accumulation after normoxic acidification and during hypoxia, and this decrease in Na accumulation is associated with a smaller increase in $[Ca]_i$ than observed during hypoxia under isotonic conditions.

The impetus for this work arose in part from our awareness of, and involvement with, previous studies in which it was shown that infusion of hypertonic solution has beneficial effects in shock resuscitation (24). Not all these effects can be explained on the basis of fluid shifts alone. The results described here provide a new and rational explanation, based on alterations in ion transport, for a portion of the benefits of hypertonic resuscitation.

METHODS

General

The methods have been modified slightly from those previously reported (5). New Zealand White rabbits were anesthetized with pentobarbital sodium (65 mg/kg) and heparinized (1,000 U/kg). Hearts were removed and perfused at a constant rate of 27–29 ml/min at 22–25°C. Control perfusate contained (mmol/l) 133 NaCl, 4.75 KCl, 1.25 $MgCl_2$, 1.82 $CaCl_2$, 20 HEPES, 8 NaOH, and 11.1 dextrose. Perfusate osmolarity was measured ±2 mosM by a freezing-point osmometer; control was 295 mosM. Perfusates were titrated to pH 7.35–7.45 and equilibrated with 100% O_2 before and after hypoxia and with 100% N_2 during hypoxia. Under these conditions, the partial pressure of O_2 measured at the aorta was >550 Torr during normoxic perfusion and <20 Torr during hypoxic perfusion. To measure Na_i, 15 mM dysprosium triethylenetetramine hexaacetic acid (DyTTHA) was substituted for osmotic equivalents of NaCl in the perfusate, and Ca was added to reach a concentration of 1.8–2 mM as measured by Ca electrode (3). To measure $[Ca]_i$, hearts were loaded during the control interval (30–40 min) with perfusate containing 5 μM 5F-1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (FBAPTA)-AM (5, 22, 30). FBAPTA was then washed out of the extracellular space with control solution for 15 min before measurement of $[Ca]_i$. To discriminate between changes in Na uptake and Na efflux, Na efflux via Na-K-ATPase was inhibited by addition of 1 mM ouabain to, or removal of KCl from, the perfusate (43, 46) (osmotic substitution with sucrose) during the 60-min hypoxic interval in all hypoxic experiments except control. This allowed us to equate increases in Na_i with net Na uptake (4, 5), as opposed to other studies that report changes in Na_i without assessing Na uptake and Na efflux independently (17, 34, 37, 47, 53).

Normoxic acidification was achieved using the NH_4Cl prepulse technique (7), in which 20 mM NH_4Cl is added to the perfusate for 45 min and then washed out of the heart (5). After all perfusions were complete, hearts were weighed wet and dried for ≥48 h at 65°C to determine dry weight.

NMR Spectroscopy

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°) with 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, and technical limitations required that data from only one nuclear resonance frequency were acquired from each heart. To improve signal-to-noise ratio for ^{19}F measurement of $[Ca]_i$, the free induction decays from two contiguous 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.

Na_i (in meq/kg dry wt) was calculated from the calibrated area under the unshifted peak of the ^{23}Na spectrum after subtraction of the extracellular peak (3, 29). $[Ca]_i$ (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 (5, 21). Because the NMR techniques measure Na as an amount and Ca as a concentration, results are reported as such. The pH_i was determined from the chemical shift of the P_i resonance [with reference to control phosphocreatine (PCr)] calibrated at 25°C (5).

Unless otherwise stated, values are means ± SE, and ANOVA for repeated measures was used to test for differences among treatments. When differences among treatments were found, Tukey's test for multiple comparisons was used to determine the times at which differences between treatments occurred. Tukey's test was not used for comparisons across time, only across treatments for a particular time. For all comparisons, differences were considered significant when $P < 0.05$.

RESULTS

Na Uptake vs. Na Efflux During Hypoxia

To test the hypothesis that hypoxia stimulates Na uptake (and thus increases Na_i), hearts were exposed to hypoxia with and without the Na-K-ATPase inhibited by K-free perfusion. As shown in Fig. 1*A*, when the Na-K pump was allowed to function (normal K, open squares), Na_i increased from 22 ± 1 to 32 ± 4 meq/kg dry wt during 60 min of hypoxia. In contrast, when Na efflux via Na-K-ATPase was inhibited by K-free perfusion, the increase in Na_i was more than sevenfold greater (from 22 ± 1 to 100 ± 10 meq/kg dry wt) during hypoxia ($P < 0.05$, open vs. filled squares). Furthermore, when these data are taken together with those previously published (5), they demonstrate that al-

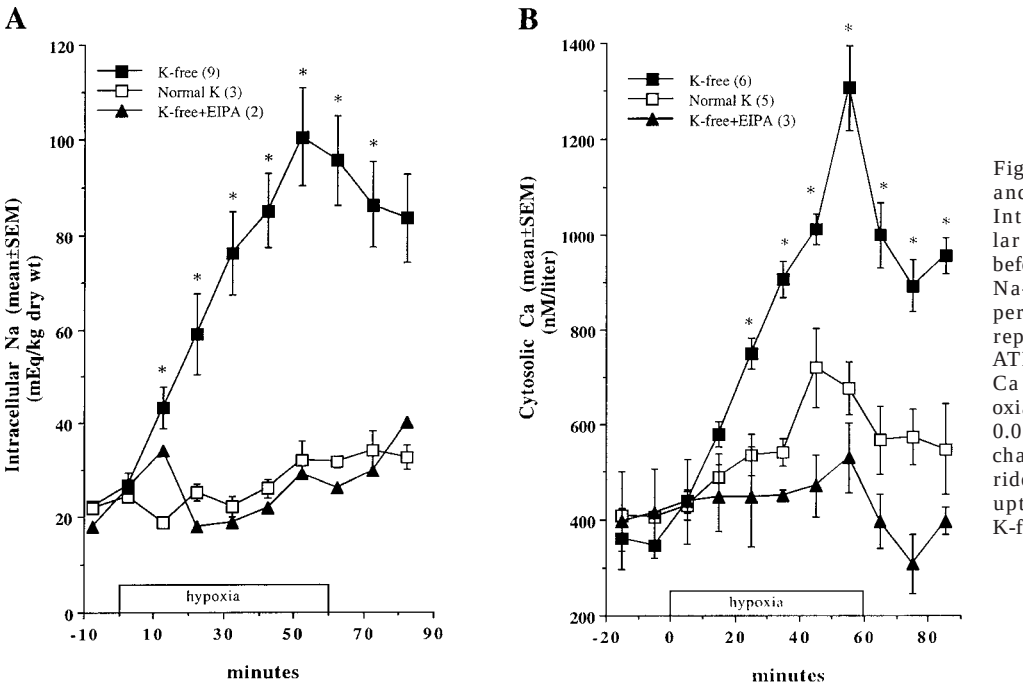

Fig. 1. Hypoxia stimulates Na uptake and Na-dependent Ca accumulation. Intracellular Na (Na_i, *A*) and intracellular Ca ($[Ca]_i$, *B*) are plotted vs. time before and during hypoxic perfusion. Na-K-ATPase inhibition with K-free perfusion began at *time 0* for protocols represented with filled symbols. Na-K-ATPase inhibition (■) increased Na and Ca accumulation compared with hypoxia with normal-K perfusion (□). *$P <$ 0.05 vs. normal-K perfusion. Na/H exchange inhibitor ethylisopropylamiloride (EIPA, 100 μM; ▲) inhibited Na uptake and Ca accumulation during K-free hypoxic perfusion.

though Na uptake and Na efflux via Na-K-ATPase are increased during hypoxia, uptake is increased more than efflux. In this case, the difference between the normal-K data (open squares) and the K-free data (filled squares) in Fig. 1*A* represents Na efflux via Na-K-ATPase. For data collected during the first 35 min of hypoxia, Na_i increases almost linearly at a rate of ~1.65 meq·min^{-1}·kg dry wt^{-1} (from 27 ± 3 to 76 ± 9 meq/kg dry wt) when Na-K-ATPase was inhibited by K-free perfusion, whereas there was no measurable change in Na_i with normal-K perfusion at 23°C (functional Na-K-ATPase). For the latter to be true, during the first 35 min of normal-K hypoxia the Na-K pump must be extruding Na_i at a rate equal to that of Na uptake (~1.65 meq·min^{-1}·kg dry wt^{-1}). In contrast, we previously showed (5) that, during 65 min of K-free normoxic perfusion, Na_i increases almost linearly by 28 meq/kg dry wt (0.43 meq·min^{-1}·kg dry wt^{-1}), whereas Na_i does not change over this interval during normoxic perfusion without Na-K-ATPase inhibition (data not shown). Therefore, under normoxic conditions, the Na-K-ATPase-mediated Na efflux rate is 0.43 meq·min^{-1}·kg dry wt^{-1} or ~26% of the rate of Na efflux (pump rate) calculated above for the first 35 min of hypoxic perfusion.

Figure 1*A* also shows the result of two experiments in which 100 μM ethylisopropylamiloride (EIPA; filled triangles) was added to the K-free perfusate 5 min before and during hypoxia. The results are consistent with previous reports that EIPA inhibits Na uptake during hypoxia/ischemia (5, 29).

Na Dependence of Ca_i Accumulation

Figure 1*B* shows how $[Ca]_i$ changes during hypoxia with and without Na-K-ATPase inhibition by K-free perfusion. During 60 min of hypoxia when the Na-K pump was allowed to function under normal-K conditions, $[Ca]_i$ rose from 407 ± 10 to 676 ± 55 nM; yet when the Na-K pump was inhibited by K-free hypoxic perfusion, $[Ca]_i$ rose from 347 ± 11 to 1,306 ± 89 nM. Thus, after 20 min of hypoxia and during reoxygenation, $[Ca]_i$ was significantly greater during the K-free (filled squares) than during the normal-K (open squares) protocol.

Figure 1*B* also shows the effect of 100 μM EIPA on Ca_i accumulation during hypoxia. As shown previously (5), EIPA inhibits Na uptake and, as predicted, Ca_i accumulation ($P < 0.05$). Figure 1 supports the hypothesis that increases in $[Ca]_i$ during hypoxia are dependent on increases in Na_i (presumably mediated by Na/Ca exchange).

Hypertonic Perfusion Decreases Na_i and Ca_i Accumulation During K-Free Hypoxic Perfusion

Again, previous studies by this laboratory and others have demonstrated that the Na/H exchanger's response to changes in pH_i is modified by exposure to hypertonic solutions (10–12, 54). To examine parallel behavior in the hypoxic heart, we added 10, 30, or 50 mmol/l of sucrose to the perfusate 5 min before hypoxic perfusion and throughout the remainder of the experiment. Figure 2 illustrates that hypoxia-induced changes in Na_i are altered in a time- and osmolarity-dependent fashion by the imposed increases in perfusate osmolarity. That is, during the interval between 10 and 15 min of hypoxic perfusion, Na_i was less for the 10 and 30 mosM hypertonic solutions than for the isotonic solution ($P <$ 0.05). However, only the 30 mosM hypertonic solution limits Na_i accumulation for the entire 60-min hypoxic interval ($P < 0.05$; see Fig. 3 for significant differences between isotonic and 30 mosM hypertonic experiments and see DISCUSSION for an explanation of the differences between responses to the 3 different tonicities).

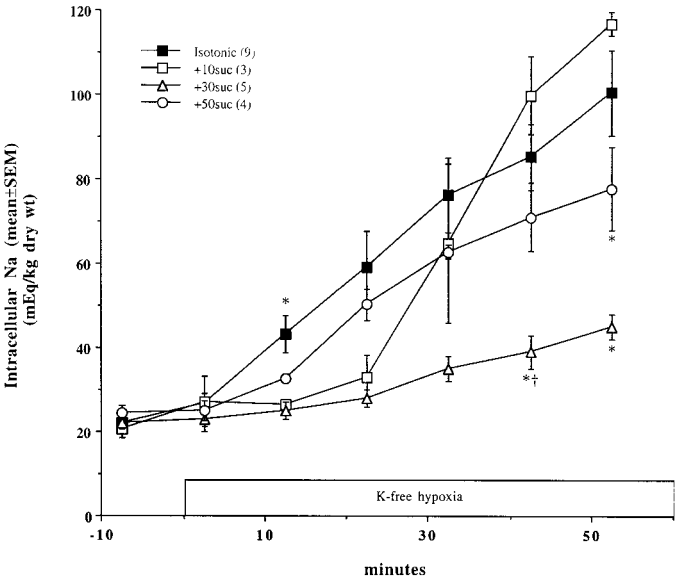

Fig. 2. Hypertonic perfusion inhibits Na uptake during hypoxia. Na_i is plotted vs. time before and during K-free hypoxic perfusion. When Na efflux via Na-K-ATPase is inhibited by K-free perfusion, changes in Na_i are equal to Na uptake. Isotonic perfusion data (■) are identical to Fig. 1. Compared with isotonic perfusion (■), perfusion with solution made hypertonic by addition of 10 (□) or 50 (○) mosM sucrose decreased Na uptake between 10 and 15 min, whereas addition of 30 mosM sucrose (△) decreased Na uptake between 10 and 55 min of hypoxia. Hypertonic perfusion began 5 min before K-free hypoxia; see Fig. 3 for +30 mosM effect and DISCUSSION for explanation of dose effect. *$P < 0.05$ vs. +10 mosM sucrose; †$P < 0.05$ vs. +50 mosM sucrose.

Figure 3 provides further evidence that, during hypoxia, *1*) increases in $[Ca]_i$ are Na dependent and *2*) increases in Na_i and $[Ca]_i$ are diminished by hypertonic perfusion. In particular, Fig. 3*A* demonstrates that perfusion with solutions made hyperosmotic (at −5 min) by addition of 30 mosM NaCl (filled triangles) or sucrose (open triangles) limits Na_i accumulation during hypoxia ($P < 0.05$), and there is no measurable difference between the two solutes. Thus the response is dependent on tonicity, not solute type or ionic strength. Figure 3*B* shows the changes in $[Ca]_i$ that accompany the changes in Na_i shown in Fig. 3*A*. Compared with the isotonic control (filled squares), $[Ca]_i$ is significantly decreased when perfusate osmolarity is increased by 30 mosM (triangles) 5 min before K-free hypoxia ($P < 0.05$). This is not only true for all data collected during hypoxia after 20 min but also for data acquired during the indicated intervals of recovery with normoxic, normal-K perfusion. Again the results are consistent with the hypothesis that exposure to mild hypertonic perfusion before and during hypoxia decreases Na_i accumulation and, therefore, limits increases in $[Ca]_i$ mediated by Na/Ca exchange.

In addition, Fig. 3 includes data from experiments that demonstrate that addition of 30 mosM sucrose after 30 min of hypoxia (open squares) has no measurable effect on Na_i and Ca_i accumulation. These data provide further support for the hypothesis that responses of the Na uptake pathway (presumably the Na/H exchanger) to volume or pH stimuli are mutually exclusive and that Ca_i accumulation is Na dependent.

Hypertonic Perfusion Decreases Na Accumulation After K-Free Normoxic Acidification

We have hypothesized that the sequence of events leading to increased Na_i and $[Ca]_i$ during hypoxia is the result of increased intracellular H concentration ($[H]_i$) stimulating pH-regulatory Na/H exchange and not a response to hypoxia per se (5). Circumstantial evidence supporting this view is given by our previous demonstration that, after normoxic acidification, pH recovery is

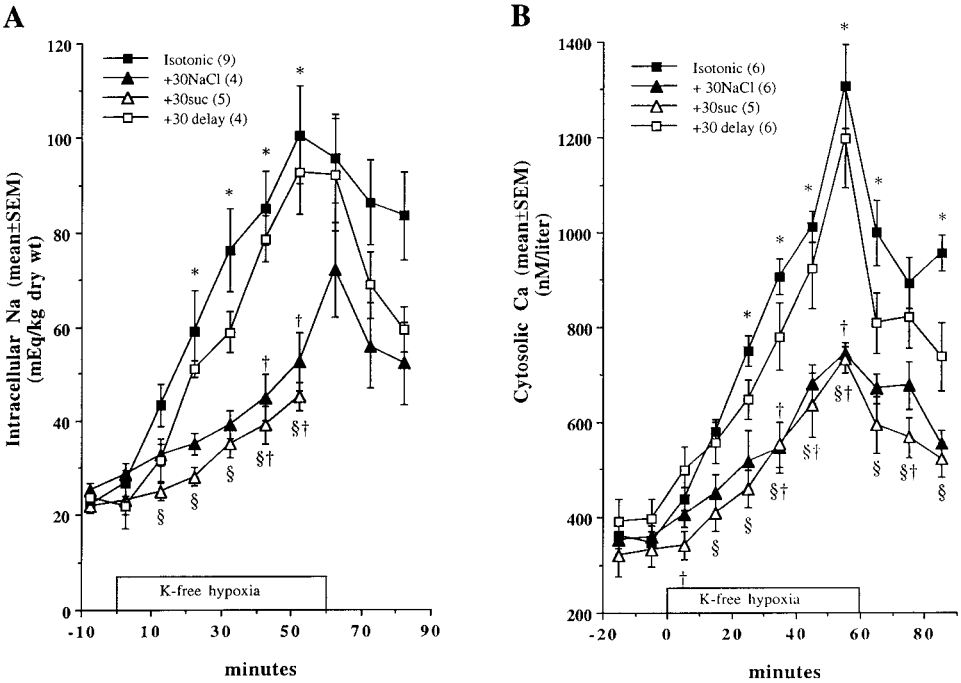

Fig. 3. Hypertonic solutions decrease Na uptake and Ca accumulation during hypoxic perfusion. Na_i (*A*) and $[Ca]_i$ (*B*) are plotted vs. time before and during hypoxic perfusion. Isotonic perfusion data are identical to Fig. 1. Addition of 30 mosM NaCl (▲) and sucrose (△) 5 min before K-free hypoxia are equally effective in preventing changes in Na_i and $[Ca]_i$. Addition of 30 mosM sucrose after 30 min of K-free hypoxia (□) has no measurable effect on Na_i or $[Ca]_i$. *$P < 0.05$ vs. +30 mosM NaCl; §$P < 0.05$ vs. isotonic; †$P < 0.05$ vs. +30 delay.

associated with increased Na uptake as well as increased $[Ca]_i$ and that both are similar in magnitude and sensitivity to EIPA to those observed after exposure to hypoxia (5). If our corollary hypothesis that increased osmolarity inhibits acid-induced Na/H exchange is correct, hypertonic perfusion before and during normoxic acidification should similarly inhibit Na uptake. The results shown in Fig. 4 are consistent with this hypothesis and demonstrate that increasing osmolarity, by addition of 30 mosM sucrose (open triangles) 5 min before and during normoxic acidification (NH_4Cl washout), decreases acid-induced Na uptake compared with isotonic perfusion (filled squares; $P < 0.05$).

Effect of Hypertonic Perfusion on Coronary Resistance During Hypoxia

Pharmacological inhibition of Na_i accumulation has previously been shown to diminish increases in coronary resistance during hypoxia (5). Our hypothesis that hypertonic perfusion limits pH-regulatory Na/H exchange led us to predict that hypertonic perfusion would similarly decrease coronary resistance during hypoxia. Figure 5 shows mean perfusion pressure (as percentage of baseline) plotted vs. time before and during K-free hypoxia with and without hypertonic perfusion. As discussed above, the responses to addition of 30 mosM NaCl (filled triangles) or sucrose (open triangles) are not measurably different in terms of Na uptake. Figure 5 shows that both treatments significantly diminished the increase in perfusion pressure otherwise observed during hypoxia (filled squares; $P < 0.05$). Thus, under the constant-flow conditions used in this study, the results demonstrate that hypertonic perfusion decreases coronary resistance during hypoxia. Given that increased coronary resistance is strongly associated with cell damage (18, 23), these data provide evidence that hypertonic perfusion limits hypoxia-induced myocardial injury.

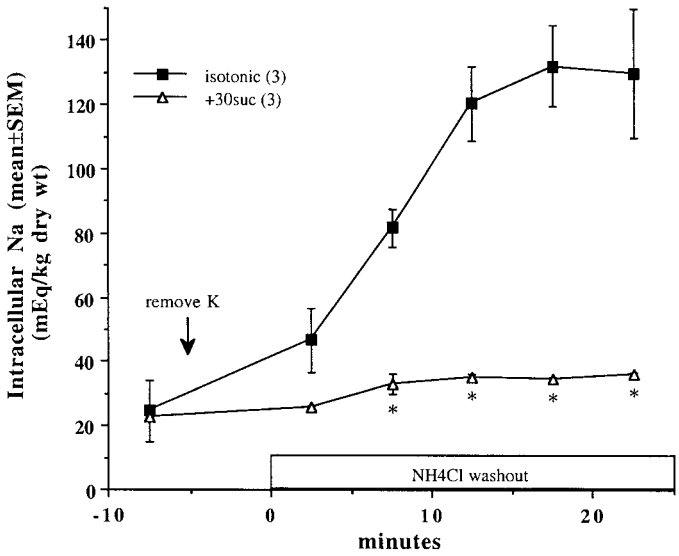

Fig. 4. Hypertonic perfusion decreases Na uptake after normoxic acidification. Na_i is plotted vs. time before and after normoxic acidification (NH_4Cl washout) with isotonic (■) and 30 mosM hypertonic (△) solutions. Hypertonic perfusion and Na-K-ATPase inhibition with K-free perfusion began 5 min before normoxic acidification. *$P < 0.05$.

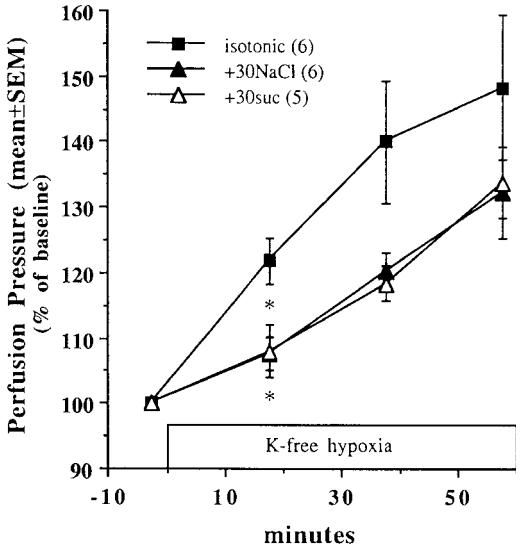

Fig. 5. Hypertonic perfusion inhibits increase in coronary resistance otherwise observed during hypoxia. Perfusion pressure as percentage of baseline during constant-flow perfusion is plotted vs. time before and during hypoxic perfusion with isotonic (■) and 30 mosM hypertonic solutions (▲, +NaCl; △, +sucrose). Hypertonic perfusion began 5 min before K-free hypoxia. *$P < 0.05$ vs. isotonic.

Effect of Hypertonic Perfusion on Hypoxic pH_i and Energy Stores

We previously demonstrated that amiloride inhibition of Na uptake during hypoxia diminished the depletion of high-energy phosphate in the form of PCr (5). Presumably, limiting Na uptake diminishes ATP consumption by Na-K-ATPase. To test the hypothesis that hypertonic inhibition of pH-regulatory Na/H exchange would have similar effects, we repeated the hypertonic experiments while measuring pH_i and high-energy phosphates. Figure 6 shows that increasing perfusate osmolarity by addition of 30 mosM NaCl decreased the fall in pH_i otherwise observed during hypoxia ($P < 0.05$). During hypertonic perfusion (filled triangles), pH_i was significantly greater than during isotonic perfusion (filled squares) 7.5, 12.5, 17.5, and 27.5 min after beginning hypoxia. On the other hand, hypertonic inhibition of Na uptake during hypoxia had no measurable effect on ATP or PCr (data not shown). Possible explanations for the relative increase in pH_i and lack of effect on high-energy phosphates are included in the DISCUSSION.

Hypertonic Perfusion Decreases Na_i Accumulation During Normal-K Hypoxic Perfusion

Most of the experiments described above were conducted using K-free perfusate to inhibit Na efflux via the Na-K-ATPase to assess the effect of hypertonic perfusion on Na uptake independent of efflux via the Na pump. However, it is now apparent that an Na-K-

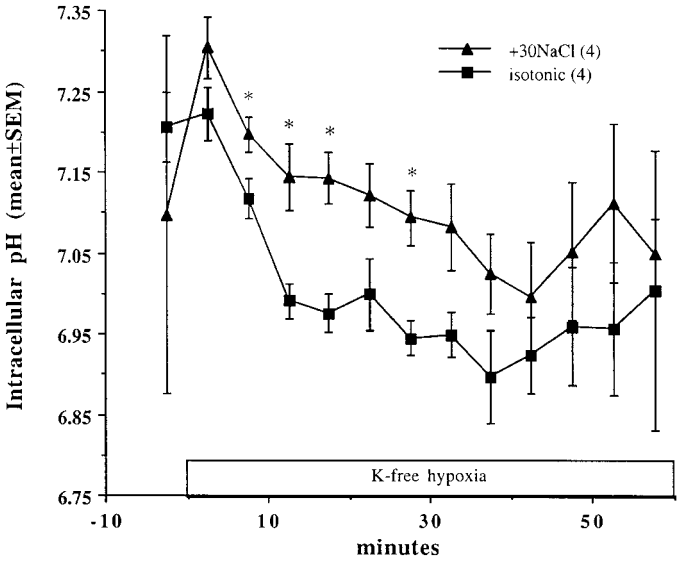

Fig. 6. Hypertonic perfusion decreases fall in intracellular pH during hypoxia. Intracellular pH is plotted vs. time before and during hypoxic perfusion with isotonic (■) and 30 mosM hypertonic (▲) solutions. Hypertonic perfusion began 5 min before K-free hypoxia. *$P < 0.05$.

2Cl cotransporter is present in myocardium and may be active under hypoxic/acidotic conditions (4). If the Na-K-2Cl cotransporter were active, under K-free conditions it would mediate net Na, Cl, and K efflux from the cells and could contribute to the relative decrease in Na accumulation observed in response to hypertonic perfusion. To test for this effect, we therefore repeated some of the experiments summarized in Fig. 2 but substituted 4.75 mM K + 1 mM ouabain for K-free perfusion to inhibit the Na-K pump without initially causing the driving force for the Na-K-2Cl cotransporter to be directed out of the cell. The results of these experiments are shown in Fig. 7 and demonstrate that normal-K hypertonic perfusion similarly decreases Na accumulation ($P < 0.05$) during hypoxia. That is, with normal-K + 1 mM ouabain perfusate, mean Na_i rises from 22 ± 1 to 80 ± 4 meq/kg dry wt in isotonic experiments but only from 23 ± 2 to 64 ± 3 meq/kg dry wt after addition of 30 mosM sucrose to the hypoxic perfusate. In this protocol, hypertonic perfusion significantly limited Na_i accumulation early during hypoxia ($P < 0.05$ at 7.5 and 17.5 min), whereas the force driving the Na-K-2Cl cotransporter is predicted to be directed into the cell (4). Thus the result is consistent with the hypothesis that hypertonic perfusion is limiting Na uptake (presumably in large part via Na/H exchange) and not stimulating Na efflux.

DISCUSSION

Hypoxia Stimulates Na Uptake More Than Na Efflux

It has been previously demonstrated that increases in cardiac myocyte $[Na]_i$ are stimulated by hypoxia (5, 10, 39), ischemia (4, 35, 37, 47), and normoxic acidification (5, 38, 54). The consistency of this response, as well as its sensitivity to Na/H exchange inhibitors, has led many investigators to conclude that these increases in $[Na]_i$ are mediated in large part by Na/H exchange functioning in a pH-regulatory capacity (4, 5, 27, 37, 38, 47). However, although increases in $[Na]_i$ in response to hypoxia, ischemia, and normoxic acidification have been demonstrated, it remains controversial (particularly in the cases of hypoxia and ischemia) to what extent the increases in $[Na]_i$ are due to increases in Na uptake as opposed to decreases in Na efflux.

With regard to the latter, increases in $[Na]_i$ under hypoxic/ischemic conditions have historically been attributed to decreased ATP production leading to a decrease in Na-K-ATPase activity and, therefore, a decrease in Na efflux (39, 45). However, in combination with our previous work (5), the data presented here (Fig. 1*A*) and data of others support the hypothesis that Na efflux via Na-K-ATPase is actually increased early during hypoxia (15). We recently reported a similar response to increased Na uptake during ischemia (4), and others have reported increased Na pump activity in heart cells after normoxic acidification (38) or exposure to hypotonic media (55).

It is important to reiterate the assertion that increases in $[Na]_i$ during hypoxia are a direct consequence of an increase in Na uptake. That is, changes in Na_i may be due, in general, to changes in Na uptake and/or efflux, and increases in Na_i only reflect increased uptake to the extent that efflux via the Na-K pump is unchanged. Having conducted our studies under conditions where Na efflux via the Na-K pump is near zero (K-free or plus 1 mM ouabain perfusion), we have met this criterion. Similarly, to evaluate the effects of inhibiting the hypothesized Na uptake pathway (with EIPA or hypertonic perfusion), Na efflux must be inhibited or quantified. Unlike previous reports in which Na efflux was not assessed (14, 47, 48, 53), the studies reported here have allowed us to

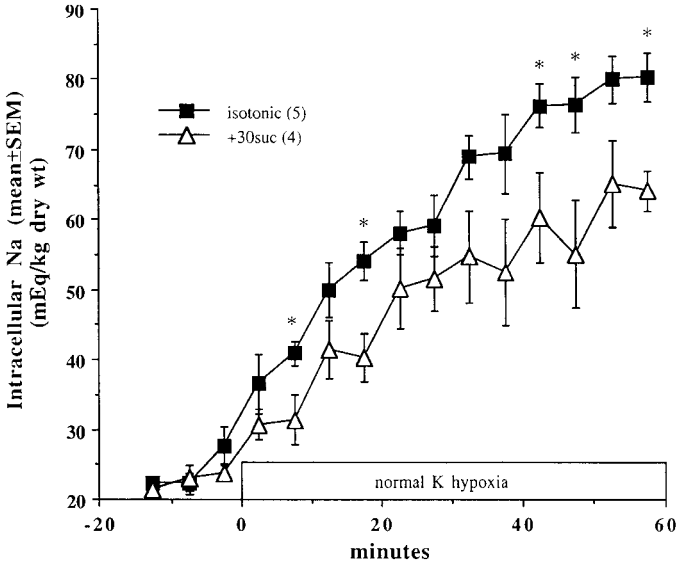

Fig. 7. Hypertonic perfusion inhibits Na uptake during hypoxia with normal-K perfusion. Na_i is plotted vs. time before and during normal-K hypoxic perfusion. Compared with isotonic perfusion (■), perfusion with solution made hypertonic by addition of 30 mosM sucrose (△) decreased Na accumulation at 7.5, 17.5, 42.5, 47.5, and 57.5 min of hypoxic perfusion. Hypertonic perfusion began 5 min before hypoxia. *$P < 0.05$ vs. hypertonic perfusion.

determine how each of the protocols alters Na uptake as well as the way in which $[Ca]_i$ responds to these changes.

Hypertonic Perfusion Inhibits Na Accumulation During Hypoxia

We and others have presented evidence that the increase in myocardial $[Na]_i$ during hypoxia and after normoxic acidification is largely due to increased Na/H exchange stimulated by decreased pH_i (5, 38, 41, 54). This laboratory has further presented evidence that in *Amphiuma* RBCs the Na/H exchanger can also be stimulated by decreases in cell volume. Recent studies in cardiac myocytes conclude that this is also true for heart muscle (54). At least in *Amphiuma* cells, however, the data suggest that the responses of the Na/H exchanger to decreased cell volume and pH may be mutually exclusive (12).

More specifically, when *Amphiuma* RBCs are shrunk in hypertonic media, Na/H exchange is stimulated to take up Na and osmotically obliged water to restore cell volume (8). However, as a consequence of obligated proton efflux, pH_i increases to levels much higher than those required to inactivate Na/H exchange stimulated by decreased pH_i. In contrast, when Na/H exchange is stimulated by acidification, Na and water uptake and proton efflux continue until normal pH_i is restored; yet, as a consequence, the cells swell to volumes much higher than those required to inactivate Na/H exchange stimulated by cell shrinkage. Briefly, the volume and pH dependence of the Na/H exchanger in *Amphiuma* RBCs are somehow prioritized and mutually exclusive (10–12), such that the pathway responds to the stimulus (volume or pH) presented first and is unresponsive to the other stimulus (volume or pH) if it is presented subsequently.

These results led us to question whether cardiac myocytes might respond similarly. That is, if hearts were first exposed to hypertonic perfusion, would the cardiac myocyte Na/H exchanger lose its normal response to decreases in pH_i that occur during hypoxia and after normoxic acidification? Figure 2 shows that solutions made hypertonic by addition of 10 or 30 mosM sucrose initially demonstrate decreased Na uptake during hypoxia, but only the 30 mosM hypertonic solution decreases Na_i accumulation after 20 min of hypoxia (Fig. 3). Possible reasons for the latter are discussed in *Perspectives*. Nevertheless, the data in Fig. 2 illustrate a response similar to that of pharmacological Na/H exchange inhibition (5, 38, 41, 54) and are thus consistent with the hypothesis that hypertonic perfusion also inhibits hypoxia-induced increases in Na/H exchange.

Role of Na-K-2Cl Cotransport in the Hypertonic Effect

As noted above, most of the data presented here were acquired using the K-free perfusion protocol to inhibit Na-K-ATPase (5) to determine the effects of hypertonic perfusion on Na accumulation in the absence of Na efflux via the Na pump. One potential drawback in using this protocol is that K-free perfusion will cause the force driving the Na-K-2Cl cotransporter to be directed out of the cell (4). To the extent that it is active, the Na-K-2Cl cotransporter will then act as an Na efflux pathway, and Na accumulation will underestimate Na uptake. To assess the importance of this effect, we repeated experiments shown in Fig. 2 but used normal-K perfusate (4.75 mM) + 1 mM ouabain instead of K-free perfusate to inhibit the Na-K-ATPase. The results demonstrate that, with normal-K perfusate, hypertonic perfusion limits Na_i accumulation during hypoxia (Fig. 7). These results are of further interest, because they show that hypertonic perfusion even limits Na_i accumulation early during hypoxia ($P < 0.05$ at 7.5 and 17.5 min) while the force driving the Na-K-2Cl cotransporter remains directed into the cell. That is, with the assumption that decreases in intracellular K concentration approximate increases in $[Na]_i$, as previously measured (5), and intracellular Cl concentration increases during hypoxia, as previously measured during ischemia (4, 25, 40), one can calculate the force driving the cotransporter (4). For the conditions of these experiments, the force driving the Na-K-2Cl cotransporter will not be directed out of the cell until Na_i rises above 50 meq/kg dry wt (after 20 min). Thus not only does mild hypertonic perfusion limit Na_i accumulation during hypoxia under K-free (Fig. 2) and normal-K (Fig. 7) conditions, but if the calculated driving force is correct, for normal-K conditions the significant hypertonic effect observed before 20 min could not be mediated by net efflux via the Na-K-2Cl cotransporter (i.e., the driving force is in the wrong direction). Again, this result is consistent with the hypothesis that, compared with isotonic perfusion, hypertonic perfusion diminishes Na uptake during hypoxia.

Hypertonic Perfusion Decreases Ca Accumulation During Hypoxia

The results shown in Fig. 3*B* provide evidence that hypertonic perfusion before hypoxia decreases Ca accumulation during and after hypoxia. As shown for Na_i, increasing the perfusate osmolarity by 30 mosM 5 min before hypoxia decreases Ca_i accumulation regardless of whether the added solute is sucrose or NaCl. Thus the data in Fig. 3 lend further support to the hypothesis that hypertonic perfusion (introduced before hypoxia) decreases Na uptake, which, through its effect on the force driving Na/Ca exchange, decreases Ca_i accumulation. The hypothesis that the responses of the Na/H exchanger to volume and pH disturbances are mutually exclusive further predicts that hypertonic perfusion will have no effect on hypoxia-induced Na uptake and Ca_i accumulation if exposure to hypertonic media occurs after exposure to hypoxia. The results shown in Fig. 3 are consistent with this prediction, because when hearts are exposed to hypertonic perfusate after 30 min of hypoxic perfusion (30 mosM sucrose added), Na_i and $[Ca]_i$ are greater than when exposed to hypertonic perfusate before hypoxia ($P < 0.05$) and not significantly different from isotonic hypoxic perfusion.

Hypertonic Perfusion, pH_i, High-Energy Phosphates, and Coronary Resistance

With respect to the effects of hypertonic perfusion on pH_i, we and others previously reported that inhibition of the Na/H exchanger by amiloride analogs decreases the ability of the heart to regulate pH after normoxic acidification (5, 14, 49), during hypoxia (28), and after ischemia (48). On this basis, one might predict that inhibition of pH-regulatory Na/H exchange by hypertonic perfusion would cause a greater decrease in pH_i during hypoxia. Instead, hypertonic perfusion diminishes the fall in pH_i otherwise observed during isotonic hypoxic perfusion (cf. filled triangles and squares in Fig. 6). Although this response requires further study, we suggest that, in part, it is due to the effect of hypertonic perfusion on Ca_i accumulation during hypoxia (cf. filled triangles and squares in Fig. 3*B*). The data in Figs. 6 and 3*B* demonstrate clearly that hypoxia induces increases in $[Ca]_i$ and $[H]_i$ (decreased pH_i) and that the +30 mosM hypertonic treatment decreases this response in $[Ca]_i$ and $[H]_i$. At least two scenarios consistent with this response have been reported. First, during hypoxia the Na-dependent increase in $[Ca]_i$ will tend to increase ATP consumption by such Ca-dependent processes as muscle contraction (51) and thus increase proton production (13). Second, increased $[Ca]_i$ will tend to increase $[H]_i$ by displacing protons from intracellular buffers (52). Thus, by inhibiting Na uptake and, therefore, Ca_i accumulation during hypoxia, hypertonic perfusion is likely to diminish proton production and release and, thereby, diminish the fall in pH_i.

Recent studies consistent with this interpretation demonstrate that inhibition of Na/H exchange does not decrease and may increase pH_i during ischemia (29, 37), whereas it consistently preserves or improves the recovery of the cell's high-energy phosphate stores during and after hypoxia/ischemia (5, 37) and improves recovery of function after ischemia (29, 32, 33, 47). In contrast, we find that inhibition of Na uptake by hypertonic perfusion during hypoxia has no measurable effect on high-energy phosphates (PCr and ATP; data not shown). Perhaps the simplest explanation for this difference is that amiloride analogs are more effective than hypertonic perfusion in inhibiting Na uptake.

Finally, our hypothesis that hypertonic perfusion inhibits hypoxia-induced Na/H exchange predicts that hypertonic perfusion will have the same effect on vascular resistance during hypoxia as pharmacological Na/H exchange inhibitors. The results of experiments summarized in Fig. 5 are consistent with the prediction as well as previous studies in which it was shown that inhibiting Na/H exchange diminishes hypoxia/ischemia-induced myocardial injury (29, 32, 41, 47, 53).

Limitations of NMR Methods and Pharmacological Probes

The NMR methods used to measure Na_i and $[Ca]_i$ have been evaluated and used previously by numerous investigators (6, 22, 31, 37, 44). Although their limitations may be significant, they do not preclude the conclusions we have drawn from our experiments. When blood or perfusate [Ca] is maintained within the normal range (3), no significant effects of DyTTHA on myocardial physiology have been reported in vivo or in vitro (4–6, 37). The most serious limitation in using the DyTTHA method for measuring Na_i with NMR is its relatively low resolution. Because the differences between treatments reported in this study are large, we were not limited by this concern.

Marban et al. (31) critically evaluated the FBAPTA method for measuring $[Ca]_i$ in myocardium and concluded that it offers advantages over fluorescent indicators loaded by the AM method, in that compartmentalization into intracellular organelles is negligible, FBAPTA-AM is promptly and completely deesterified, and signals from endothelial cells do not dominate the spectra. They also report that FBAPTA-loaded hearts have normal high-energy phosphates, and their metabolic response to ischemia is similar to hearts without FBAPTA (31). The most serious drawback in using FBAPTA is that it was designed with a dissociation constant in the range of resting $[Ca]_i$ and, as a significant Ca buffer, tends to limit changes from that value (22). To the extent that this occurs in our experiments, FBAPTA would limit increases in $[Ca]_i$ during hypoxia. However, even if the magnitude of the changes in $[Ca]_i$ we report was limited by FBAPTA, this potential artifact would have no effect on the validity of our conclusions, since they are predicated on the observation that in hearts with identical FBAPTA loading (i.e., same baseline FBAPTA buffer capacity), during hypoxia, $[Ca]_i$ is significantly less for the hypertonic group than for the isotonic control.

Finally, we have used EIPA as an inhibitor of Na uptake. Although EIPA is a potent inhibitor of Na/H exchange (5, 14, 27, 29, 48), on the basis of the fact that it also inhibits veratridine-induced hypercontracture and Ca_i loading, Haigney et al. (16) suggested that EIPA inhibits noninactivating Na channels during hypoxia and ischemia. Thus we cannot rule out the possibility that Na uptake during hypoxia is mediated by pathways other than Na/H exchange.

Perspectives

Hypertonic perfusion and Na uptake. PREDICTED RESPONSE. To reiterate, exposure of cardiac myocytes to hypertonic solutions stimulates Na/H exchange (54), and this response has been shown to be volume regulatory in *Amphiuma* RBCs (8). If cardiac myocytes are perfect osmometers and volume regulation is accomplished by Na/H exchange exclusively, the volume-regulatory Na uptake in milliequivalents per liter cell water will be approximately equal to the imposed increase in perfusate osmolarity. However, to the extent that Na uptake and proton efflux via volume-regulatory Na/H exchange entrain net flux of additional ions through other pathways (e.g., Cl uptake via Cl/HCO_3 exchange), the Na uptake required to regulate cell volume will be diminished (9). For example, one-to-one coupling of Na/H exchange with Cl/HCO_3 exchange

would result in no change in pH_i, but for each cycle of the Na/H exchanger the net osmotically active ion uptake would be two ions. That is, Na and Cl would be taken up while H and HCO_3 could be recycled as CO_2 and water (9). Because it has been reported that intracellular HCO_3 concentration is ~0.5 mM in HEPES-buffered sheep Purkinje fibers and the Michaelis-Menten constant for HCO_3 to support Cl uptake via Cl/HCO_3 exchange is ~1 mM (50), we cannot rule out the possibility that Na/H and Cl/HCO_3 exchange are functionally coupled in our studies of the heart. If they are coupled one to one, volume regulation could thus be achieved by increasing $[Na]_i$ and intracellular Cl concentration equally, and the sum of the increases in concentrations of these two ions would be approximately equal to the imposed increase in perfusate osmolarity. (Accordingly the increase in $[Na]_i$ would be about one-half the imposed increase in perfusate osmolarity.)

Thus, for the studies described here, volume-regulatory responses mediated by functionally coupled Na/H and Cl/HCO_3 exchangers would be expected to cause changes in $[Na]_i$ between one-half and one times the imposed change in perfusate osmolarity. In response to 10, 30, and 50 mosM increases in perfusate osmolarity, we predict that volume-regulatory Na/H exchange would mediate increases in $[Na]_i$ with upper limits of 10, 30, and 50 mM, respectively. With the assumption that control cell water is 2.5 l/kg dry wt (5), Na_i would increase by up to 25, 75, and 125 meq/kg dry wt to achieve volume regulation for the three respective hypertonic perfusates. On the other hand, if Na uptake via volume-regulatory Na/H exchange is coupled one to one with Cl uptake via Cl/HCO_3 exchange, the corresponding increases in Na_i would be decreased by 50%. As described below, data shown in Fig. 2 are qualitatively consistent with the latter prediction.

OBSERVED RESPONSE. After perfusate osmolarity is increased by 10 mosM (Fig. 2), Na_i increases slowly during the first 30 min of hypoxia and then increases more rapidly. To achieve volume regulation during 10 mosM hypertonic perfusion, an increase in Na_i of 12.5–25 meq/kg dry wt is predicted. This is close to the increase of 12 (from 21 to 33) meq/kg dry wt observed in the first 25 min of hypertonic hypoxic perfusion. We postulate that after volume regulation had been completed the Na/H exchanger will begin to function in its pH-regulatory mode. Indeed, Fig. 2 shows that, after 25 min of hypoxic perfusion with 10 mosM hypertonic solution, Na uptake began to proceed at a more rapid rate, similar to that observed during isotonic hypoxic perfusion (Fig. 2).

After perfusate osmolarity is increased by 30 mosM [sucrose (open triangles) and NaCl (filled triangles) in Fig. 3*A*], Na_i increases by 23–27 meq/kg dry wt during 60 min of hypoxic perfusion. Because an increase in Na_i of 37.5–75 meq/kg dry wt was predicted to achieve volume regulation under these conditions, we postulate that, for this protocol, volume regulation is not achieved within 60 min. Therefore, the Na/H exchanger remains in its volume-regulatory mode and does not respond to the decrease in pH_i that occurs during hypoxic perfusion. This hypothesis is further supported by the fact that addition of the Na/H exchange inhibitor EIPA (50–100 μM) to the 30 mM hypertonic solution decreases Na uptake by 44% compared with the same treatment without EIPA (Na_i rises from 21 to 30 meq/kg dry wt with hypertonic EIPA; ANOVA for two treatments: +15 mM NaCl ± EIPA, $P = 0.0365$; data not shown). That is, if Na_i uptake during hypertonic perfusion is mediated by Na/H exchange, it should be, and is, inhibited by EIPA.

After perfusate osmolarity is increased by 50 mosM (open circles in Fig. 2), Na_i increases by 54 (from 24 to 78) meq/kg dry wt during 60 min of hypoxia. Because we predicted an increase in Na_i of 62.5–125 meq/kg dry wt to achieve volume regulation under these conditions, we postulate that, for this protocol, volume regulation likewise was not achieved within 60 min. However, because the increase in Na_i observed in response to hypoxia alone (78 meq/kg dry wt; filled squares in Fig. 2) is within the range predicted to achieve volume regulation during 50 mosM hypertonic perfusion, we would not expect the magnitude of Na uptake in response to this level of hypertonic perfusion to be different from hypoxia alone.

On the other hand, if our hypothesis is correct and the response of the Na/H exchanger to decreased cell volume and pH are prioritized by the order of the stimulus and mutually exclusive, Na uptake after exposure to hypertonic perfusion will be independent of subsequent exposure to acidifying conditions (e.g., hypoxia). Preliminary studies are consistent with this prediction. When hearts were perfused under hypertonic normoxic conditions, 60 min after addition of 58.5 mM sucrose + 1 mM ouabain to the perfusate, Na_i had increased by 48 (from 27 ± 2 to 75 ± 8) meq/kg dry wt ($n = 3$), indicating that hypertonic perfusion stimulated Na uptake under normoxic conditions [data not shown; $P < 0.05$ vs. isotonic normoxic perfusion with K-free perfusate (5)]. However, this Na uptake is not significantly different from that observed during 60 min of isotonic hypoxic perfusion (filled squares in Fig. 2) or 50 mM hypertonic hypoxic perfusion (open circles in Fig. 2). Thus, as expected, if responses are prioritized and mutually exclusive, responses to pH and volume stimuli were not additive.

In summary, our data are consistent with the interpretation that, for hypertonic perfusion to decrease Na uptake during hypoxia, the increase in osmolarity must be large enough (>10 mosM) to maintain the Na/H exchanger in the volume-regulatory mode throughout the hypoxic interval but small enough (<50 mosM) so that the Na uptake required for volume regulation is less than would otherwise occur during isotonic hypoxic perfusion. This interpretation also explains our observation that moderately hypertonic (+30 mosM) perfusion decreases Na uptake after normoxic acidification (Fig. 4), whereas other investigators have shown that increasing perfusate osmolarity by 100–150 mosM increases Na uptake after normoxic acidification (54). For the latter studies, the Na uptake required to achieve volume regulation would be up to 375 meq/kg

dry wt (3–15 times greater than calculated for the 10–50 mosM hypertonic solutions used here), thus causing Na uptake to be greater than that required to regulate pH_i.

Hypertonic perfusion and cell volume. Although the experiments reported here were not designed to test the hypothesis that the response of the Na/H exchanger to hypertonic perfusion is volume regulatory, our Na data allow measurement of changes in cell volume associated with hypertonic perfusion, and these are consistent with the arguments presented above [volume changes were calculated from changes in the area of the extracellular Na peak (5); data not shown]. Briefly, after 2.5 min of K-free hypoxic perfusion, cell volumes for all hypertonic protocols were nominally less than the isotonic control, and those obtained from hearts exposed to 50 mosM hypertonic perfusate were significantly less ($P < 0.05$ by paired *t*-test). Furthermore, after 52.5 min of K-free hypoxic perfusion, the cell volumes for the hypertonic protocols had increased to levels not significantly different from control or from each other. However, the nominal extent of recovery (percentage of control volume) was inversely related to the tonicity of the perfusate. (Relative to their own isotonic prehypoxia cell volumes, they were 100 ± 3, 96 ± 8, 92 ± 11, and 87 ± 11% for the isotonic and +10, +30, and +50 mosM perfusions, respectively.) This is noteworthy, since it is a characteristic of a graded response, such as volume regulation (8), and consistent with our contention that cells exposed to +30 and +50 mosM hypertonic perfusion had not achieved volume regulation by the end of the hypoxic interval.

Hypertonic resuscitation. Finally, these studies provide a new explanation for some of the observed beneficial effects of hypertonic solutions used to treat hypovolemia in trauma care. That is, although a portion of the effects are undoubtedly due to transfer of volume from the intracellular to the extracellular compartment (24), additional effects consistent with our hypothesis have been reported, e.g., increases in myocardial pH_i and contractility (2, 54). In addition, our results provide a rational explanation for the finding that the optimum dose for hypovolemic resuscitation with hypertonic solutions causes an increase in extracellular osmolarity on the order of 25–30 mosM (36).

Conclusions

Over the last few years, data have accumulated in support of the hypothesis that myocardial injury due to hypoxia and ischemia is largely the result of the following chain of events: *1*) increased anaerobic metabolism increases proton production, which decreases pH_i; *2*) decreased pH_i stimulates pH-regulatory Na/H exchange; *3*) increased Na/H exchange increases cell Na uptake; *4*) increased Na uptake increases $[Na]_i$; *5*) increased $[Na]_i$ decreases or reverses the force driving Na/Ca exchange; *6*) altered Na/Ca exchange causes an increase in $[Ca]_i$; and *7*) increased $[Ca]_i$ causes a cascade of Ca-dependent processes that lead to cell injury and death.

This study presents further evidence consistent with the hypothesis. More specifically, we have demonstrated that increases in $[Na]_i$ during hypoxia (*step 4*) are due to increased Na uptake (*step 3*) and not to decreased Na efflux via Na-K-ATPase. We provide further support for the hypothesis by demonstrating that initiating hypertonic perfusion before hypoxia attenuates the hypoxia-induced increase in Na uptake and that decreased Na uptake is associated with a relative decrease in $[Ca]_i$ (predicted converse of *steps 5* and *6*). Finally, the results demonstrate that hypertonic perfusion decreases Na and Na-dependent Ca_i accumulation during hypoxia only if cells are exposed to hypertonic perfusion before hypoxia and not if the order of exposure is reversed.

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The results of the experiments described here have been presented in abstract form (19, 20).

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