Title

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Journal

American Journal of Physiology, 270(2)

ISSN

0002-9513

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Publication Date

1996-02-01

DOI

10.1152/ajpcell.1996.270.2.c608

Peer reviewed

Effects of Na-K-2Cl cotransport inhibition on myocardial Na and Ca during ischemia and reperfusion

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Anderson, S. E., C. Z. Dickinson, H. Liu, and P. M. Cala. Effects of Na-K-2Cl cotransport inhibition on myocardial Na and Ca during ischemia and reperfusion. *Am. J. Physiol.* 270 (*Cell Physiol.* 39): C608–C618, 1996.—In the context of the "pump-leak" hypothesis (37), changes in myocardial intracellular Na (Na_i) during ischemia and reperfusion have historically been interpreted to be the result of changes in Na efflux via the Na-K pump. We investigated the alternative hypothesis that changes in Na_i during ischemia are the result of changes in the Na "leak" rather than changes in the pump. More specifically, we hypothesize that the increase in Na_i during ischemia is in part the result of increased Na uptake mediated by Na/H exchange. Furthermore, we present data consistent with the interpretation that the Na-K-2Cl cotransporter is active (or, alternatively, displaced from equilibrium) during ischemia and may contribute an additional Na efflux pathway during reperfusion. Thus inhibition of Na efflux via Na-K-2Cl cotransport during ischemia and reperfusion could result in increased Na_i and therefore decreased force driving Ca efflux via Na/Ca exchange and ultimately increased intracellular Ca concentration ($[Ca]_i$). Na_i (in meq/kg dry wt) and $[Ca]_i$ (in nM) were measured in isolated Langendorff-perfused rabbit hearts using nuclear magnetic resonance spectroscopy. Except during the 65 min of ischemia, hearts were perfused with *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid-buffered Krebs-Henseleit solution equilibrated with 100% O_2 at 23°C and pH 7.4 ± 0.05. During ischemia, Na_i rose from 16.6 ± 0.3 to 62.9 ± 5.1 ($\Delta Na_i \simeq 46$) meq/kg dry wt and decreased during subsequent reperfusion (mean ± SE, $n = 3$ hearts). To measure Na uptake ("leak") in the absence of efflux via the Na-K pump, in all of the protocols described below, the perfusate was nominally K-free solution containing 1 mM ouabain for 10 min before ischemia and during the 30-min reperfusion. After K-free perfusion, Na_i rose from 20.2 ± 0.5 to 79.1 ± 5.3 ($\Delta Na_i \simeq 59$) meq/kg dry wt ($n = 3$) during ischemia and decreased during K-free reperfusion. When amiloride (1 mM) was added to the K-free perfusate to inhibit Na/H exchange, Na_i rose from 16.3 ± 0.9 to 44.7 ± 5.1 ($\Delta Na_i \simeq 28$) meq/kg dry wt ($n = 3$) during ischemia; i.e., amiloride decreased Na uptake. When bumetanide (20 μM) was added to the nominally K-free perfusate to inhibit Na-K-2Cl cotransport, Na_i rose from 22.5 ± 3.9 to 83.8 ± 13.9 ($\Delta Na_i \simeq 61$) meq/kg dry wt ($n = 3$) during ischemia and did not decrease during reperfusion; i.e., bumetanide inhibited Na recovery during reperfusion ($P < 0.05$ compared with bumetanide free). For the same protocol, the presence of bumetanide resulted in increased $[Ca]_i$ during ischemia and reperfusion ($P < 0.05$); these increases in $[Ca]_i$ are interpreted to be the result of increased Na_i. Thus the results are consistent with the hypotheses.

myocardial ischemia; sodium/hydrogen exchange; sodium-potassium-chloride cotransport; bumetanide

INCREASES IN INTRACELLULAR Na (Na_i) during ischemia have been ascribed to a decrease in Na efflux via the Na-K-ATPase (pump) secondary to a decrease in ATP. Conversely, decreases in Na_i observed during reperfusion have been thought to be the result of increased Na efflux via the Na-K pump as ATP availability increases. In other words, in the context of the "pump-leak" hypothesis (37), changes in Na_i have been interpreted as the result of changes in Na fluxes through the "pump." Here, we test the alternate hypothesis that, in the heart, increases in Na_i during ischemia are the result of an increase in Na influx or leak (as opposed to a decrease in pump efflux) and that, during reperfusion, both Na-K-ATPase and the Na-K-2Cl cotransporter act to extrude the Na gained during ischemia. In addition, the hypothesis implies that ischemia-induced changes in Na "leak" precede and precipitate increased Na-K pump activity.

We have previously presented evidence that the chain of events leading to myocardial hypoxic injury is *1*) increased anaerobic metabolism, *2*) decreased intracellular pH, *3*) increased intracellular H concentration-stimulates pH-regulatory Na/H exchange, *4*) increased Na uptake increases Na_i concentration ($[Na]_i$), *5*) decreased or reversed Na/Ca exchange, *6*) increased intracellular Ca concentration ($[Ca]_i$), and *7*) stimulation of a host of Ca-dependent mechanisms that contribute to cell injury (2, 4, 5). Others have presented evidence consistent with this hypothesis for myocardial ischemia (21, 23, 32, 35, 38).

In contrast to our hypothesis that ascribes increases in Na_i during hypoxia and/or ischemia to increased Na uptake, the historically accepted point of view has been that increases in Na_i are the result of decreased Na extrusion due to substrate (ATP) limitations of the pump (26, 34). The Na-K pump has long been considered to be the major, if not the only, pathway for Na efflux from myocardial cells. However, evidence for the presence of an Na-K-2Cl cotransporter in heart cells has been presented (8, 15), and, although most studies suggest that net flux via this pathway is directed into the cell under normal conditions (7, 12, 28), recent studies demonstrating that the Na-K-2Cl cotransport inhibitor furosemide diminishes increases in extracellular K early during ischemia suggest that net flux via the cotransporter may be directed out of the cell under these conditions (20). Thus the cotransporter may act as an auxiliary net Na efflux pathway during ischemia and reperfusion (6).

Here, we present evidence that, contrary to the view that Na_i accumulation during ischemia is a result of decreased pump-mediated efflux, increases in Na_i are the result of increased influx. Additionally, our data suggest that, early during myocardial ischemia, Na efflux via Na-K-ATPase, rather than being decreased, is actually increased and increases in Na_i are the result of an imbalance in influx (by dissipative Na flux

pathways) and efflux (by conservative Na flux pathways that include but are not limited to the pump), which are both increased. (The terms "dissipative" and "conservative" are used in reference to energy stored in the Na gradient: dissipative pathways cause a decrease in this energy; conservative pathways cause an increase.) Furthermore, as previously shown (38), the increase in Na_i during ischemia is amiloride sensitive, suggesting that a major portion of the increase is mediated by Na/H exchange. Also, as previously shown (28), the increase in Na_i during ischemia is bumetanide sensitive, suggesting that another portion of the increase is mediated by Na-K-2Cl cotransport. Finally, our studies demonstrate that, when Na-K-ATPase is inhibited (by K-free perfusate containing 1 mM ouabain) during reperfusion, a bumetanide-sensitive pathway mediates net, conservative, Na efflux consistent with the interpretation that Na-K-2Cl contributes to net Na efflux during reperfusion after ischemia. It should be noted, however, that net Na flux via this pathway must be in the same direction as the force that drives the flux. Therefore, a change in the direction of the driving force for Na-K-2Cl cotransport (arising from protocol-dependent changes in the cotransporter substrate gradients) will change the direction of net Na flux via the pathway.

METHODS

General. The methods used have been modified slightly from those previously reported (2). New Zealand White rabbits were anesthetized with pentobarbital sodium (65 mg/kg) and heparinized (1,000 United States Pharmacopeia U/kg). Hearts were removed and perfused at a constant rate of 27–29 ml/min at 22–24°C. Control perfusate contained (in mmol/l) 133 NaCl, 4.75 KCl, 1.25 $MgCl_2$, 1.82 $CaCl_2$, 20 *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 8 NaOH, and 11.1 dextrose. Perfusates were titrated to pH 7.35–7.45 and equilibrated with 100% O_2. To measure Na_i, 15 mM dysprosium triethylenetetraminehexaacetic acid 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 (1). 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 5 μM (2, 19, 32). FBAPTA was then washed out of the extracellular space with control solution for 15 min before measurement of $[Ca]_i$. Global ischemia was achieved by clamping the perfusion line for 65 min, and reperfusion was achieved by reinstating normal flow with the preischemic solution for 20–25 min. Please note that, to discriminate between changes in Na uptake and Na efflux, unless otherwise stated, Na efflux via Na-K-ATPase was inhibited by removal of KCl from the perfusate (osmotic substitution with sucrose) and addition of 1 mM ouabain (2, 31) for 10 min before ischemia and throughout reperfusion. According to protocol, amiloride or bumetanide was added to the perfusate 10 min before ischemia and during reperfusion. Bumetanide was first solubilized in ethanol at a concentration of 100 mM and then added to the perfusate to a final concentration of 20 μM. After perfusions were complete, hearts were weighed wet and dried for at least 48 h at 65°C to determine dry weight.

Unless otherwise noted, all hearts were perfused with the same solution before and after ischemia. One set of experiments, however, was conducted to test the subhypothesis that the Na-K-2Cl cotransporter acts as a pathway for net Na efflux during reperfusion with normal K (4.75 mM) solution. This set of experiments required different solutions before and after ischemia: K-free plus ouabain perfusion before ischemia to achieve maximal Na loading and normal K plus ouabain solution during reperfusion to assess the role of the cotransporter under normal K conditions. All protocols used in this study are ischemic protocols. For ease of reference, throughout the remainder of this report, the protocols are referred to by the solution used before ischemia; e.g., hearts perfused with K-free plus ouabain solution before and after ischemia comprise the K-free plus ouabain protocol. It must be remembered, however, that, since the intra- and extracellular contents cannot be controlled during ischemia, the names of the protocols do not necessarily reflect the status of any compartment in the heart. For example, the extracellular space is not expected to be K free during ischemia in the K-free plus ouabain protocol. (It is only nominally K free during perfusion, for that matter.) Thus the protocols are named for the perfusates used and (especially during ischemia) are not meant to describe the ionic distribution in the heart.

Unless otherwise stated, results are reported as means ± SE. Analysis of variance for repeated measures was used to test for differences among treatments. If differences among treatments were found, the Tukey's test for multiple comparisons was used to determine the times at which differences between treatments occurred. It should be noted that comparisons using the Tukey's test were not made across time, only across treatments for a particular time. Differences were considered significant when $P < 0.05$.

^{23}Na and ^{19}F nuclear magnetic resonance spectroscopy. ^{23}Na experiments were conducted using a Nicolet NT 200 spectrometer, and ^{19}F experiments were conducted using a GE Omega 300 horizontal bore system. ^{23}Na and ^{19}F spectra were generated from the summed free induction decays of 1,000 and 1,500 excitation pulses (90° and 45°) with ±4,000- and ±5,000-Hz sweep widths, respectively, using 2K word data files. For both nuclei, data files were collected over 5-min intervals. To improve the signal-to-noise ratio for ^{19}F measurement of $[Ca]_i$, the free induction decays from two 5-min ^{19}F files were added together. Because the nuclear magnetic resonance (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 content in milliequivalents per kilogram dry weight was calculated from the calibrated area under the unshifted peak of the ^{23}Na spectrum after subtracting out the extracellular peak (1). $[Ca]_i$ in nanomoles per liter of cell water was calculated as the product of the ratio of the areas of the Ca-bound and Ca-free peaks in the FBAPTA spectrum and the 500 nM Ca-FBAPTA dissociation constant (2, 19, 32).

RESULTS

Na uptake and Na efflux increase during ischemia. The data in Fig. 1 depict Na content of the heart during 65 min of ischemia followed by 25 min of reperfusion. When the pump was allowed to function during the ischemic interval (by virtue of perfusion with normal K concentration medium before ischemia), Na_i rose from 16.6 ± 0.3 to 62.9 ± 5.1 meq/kg dry wt. In contrast, when Na-K-ATPase was inhibited by 10 min of perfu-

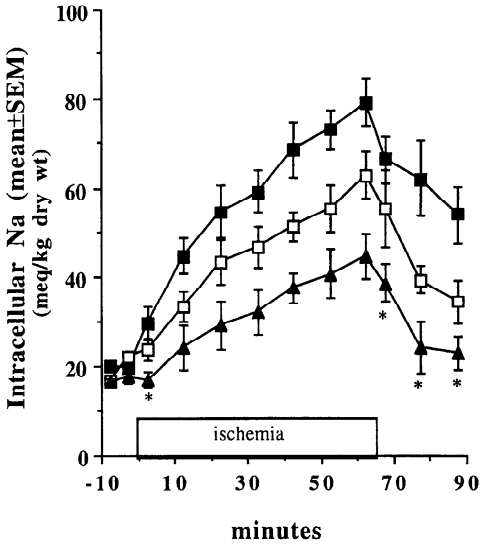

Fig. 1. Ischemia stimulates Na uptake more than Na extrusion. Intracellular Na (Na_i, in meq/kg dry wt) is plotted vs. minutes. Data are represented in time as corresponding to midpoint of interval in which they were acquired. All hearts are perfused with K-free plus 1 mM ouabain solution for 10 min before ischemia and during reperfusion except those in normal K protocol (□, $n = 3$). When Na-K-ATPase was inhibited by K-free plus ouabain preperfusion (■, $n = 3$), addition of 1 mM amiloride (▲, $n = 3$) decreased changes in Na_i otherwise observed during ischemia and improved recovery during reperfusion. Data are consistent with hypothesis that ischemia increases both Na uptake and Na-K-ATPase-mediated Na extrusion and that a portion of Na uptake is via Na/H exchange. $^*P < 0.05$ compared with K-free plus ouabain.

sion with K-free solution containing 1 mM ouabain (2, 31) before ischemia, Na_i rose from 20.2 ± 0.5 to 79.1 ± 5.3 meq/kg dry wt during ischemia. To reemphasize the point initially raised under METHODS, to the extent that the cells lose K during ischemia, extracellular K concentration ($[K]_o$) is increased above zero and the Na-K pump may be permitted to function (albeit at a reduced rate in the presence of ouabain). Consequently, Na_i accumulation during the K-free plus ouabain protocol will underestimate the Na uptake to the extent that the pump is functional and Na entering the cells via dissipative routes is extruded by the pump. Furthermore, after K-free plus ouabain perfusion, the force driving Na-K-2Cl cotransport is directed out of the cell, and the cotransporter may also contribute to net Na efflux during ischemia (see below and DISCUSSION), leading to a further underestimate of Na uptake. Within this context, during ischemia, the difference between the normal K curve and K-free plus ouabain curve represents a lower limit for the amount of Na extruded by the Na-K-ATPase. To assess the rate of Na efflux via Na-K-ATPase, we compared the rate of increase in Na_i early during ischemia (while the rate of increase in Na_i is greatest and most linear) in the presence and absence of functional Na-K-ATPase. When the pump is inhibited after perfusion with K-free, ouabain-containing medium, ischemia results in an increase in Na_i of 2.0 meq·kg dry $wt^{-1}\cdot min^{-1}$ over the first 12.5 min of ischemia. In contrast, the rate of increase in Na_i during ischemia after perfusion with normal K concentration medium and no ouabain (pump functional) is 0.91 meq·kg dry $wt^{-1}\cdot min^{-1}$. Thus, during ischemia after normal K perfusion, Na efflux via the Na-K pump must be at least 1.09 meq·kg dry $wt^{-1}\cdot min^{-1}$. Using a similar experimental model, we have previously shown that, during 65 min of K-free, normoxic perfusion (Na-K-ATPase inhibited), Na_i rose almost linearly from 11 to 39 meq/kg dry wt (2). Because Na_i does not change over this interval during normoxic perfusion with normal K media (normal Na-K pump function), net Na efflux via Na-K-ATPase is at least 0.43 meq·kg dry $wt^{-1}\cdot min^{-1}$; a value that is <50% of that calculated for Na efflux via the Na-K pump during ischemia. Clearly then, these data demonstrate that, early during ischemia, the Na-K pump rate is increased, presumably in response to increased $[Na]_i$ (25). Furthermore, when the value of 0.43 meq·kg dry $wt^{-1}\cdot min^{-1}$ measured for Na_i uptake (and therefore efflux under steady-state normoxic conditions) is compared with the uptake rate of 2.0 meq·kg dry $wt^{-1}\cdot min^{-1}$ during ischemia, it is apparent that, early during ischemia, both dissipative Na uptake and efflux are increased.

Figure 1 also demonstrates the effect of adding the Na/H exchange inhibitor amiloride (11, 24) to the K-free perfusate for 10 min before ischemia and during reperfusion. When both the Na/H exchanger and the pump are inhibited by preperfusion with K-free perfusate containing ouabain and amiloride (1 mM), Na_i increases from 16.3 ± 0.9 to 44.7 ± 5.1 meq/kg dry wt during 65 min of ischemia, a reduction of more than 30 meq/kg dry wt from that in the absence of amiloride (>50% less uptake in the presence of amiloride). Thus, as shown previously in rat hearts, ischemia-induced Na uptake is inhibited by amiloride (23, 35), suggesting that a portion of the increase in Na uptake is via Na/H exchange. The fact that Na_i increases during ischemia in the presence of amiloride suggests that a portion of Na uptake occurs via a pathway or pathways other than Na/H exchange under these conditions (30, 38). This possibility is considered further in the DISCUSSION.

Na efflux via Na-K-2Cl cotransport during K-free reperfusion. Although all treatments presented in Fig. 1 were associated with increases in Na_i during ischemia, they were also associated with decreases in Na_i during reperfusion. This latter observation was surprising, since net Na extrusion is generally attributed to the activity of Na-K-ATPase that should have been inhibited in the hearts reperfused with K-free media containing 1 mM ouabain. Thus it appears that, under these conditions, a Na-K-ATPase-independent pathway is able to mediate net, conservative, Na efflux during postischemic reperfusion. On the basis of the above observation, reports by others on the presence of Na-K-2Cl cotransport in myocytes (15, 20, 28), and a consideration of thermodynamic constraints (presented in the DISCUSSION), we postulated that after ischemia Na-K-2Cl cotransport provides an additional route for net Na efflux. This hypothesis was tested by measuring changes in heart cell Na_i during ischemia and reperfusion while the Na-K pump was inhibited by ouabain with or without K in the presence and absence of the

cotransport inhibitor bumetanide (20 μM) (9, 22). Figure 2 shows the results of these experiments; for each protocol, the heart was perfused with the identified solution for 10 min before ischemia and during reperfusion. These data clearly illustrate that, when the Na-K pump is inhibited by K-free plus ouabain perfusion, bumetanide (20 μM) significantly inhibits Na efflux during reperfusion ($P < 0.05$). (Results shown in Fig. 1 for the K-free plus ouabain protocol without bumetanide are included in Fig. 2 for comparison.) Thus the data demonstrate that, when the Na-K pump has been inhibited by K-free plus ouabain perfusion, significant Na efflux occurs via a bumetanide-inhibitable pathway (presumably the Na-K-2Cl cotransporter) during postischemic reperfusion. Furthermore, the fact that no measurable Na recovery occurs when Na-K-2Cl cotransport is inhibited suggests that all of the decrease in Na_i observed during reperfusion with K-free plus ouabain solution is mediated by Na-K-2Cl cotransport.

Na_i during ischemia and reperfusion with normal K solutions. Figure 2 also summarizes the effects of bumetanide on Na_i during ischemia and reperfusion

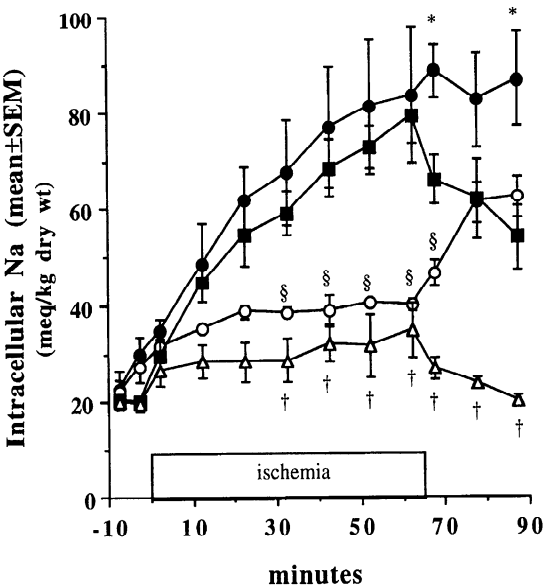

Fig. 2. When Na-K-ATPase is inhibited (K-free plus 1 mM ouabain), bumetanide (20 μM) inhibition of Na-K-2Cl cotransport prevents Na_i efflux during reperfusion. Na_i (in meq/kg dry wt) is plotted vs. minutes. Within each of 4 treatments, hearts are perfused with identical solutions for 10 min before ischemia and during reperfusion. When K-free plus ouabain treatments are compared, Na_i is greater during reperfusion after addition of bumetanide (●, $n = 3$) than without bumetanide (■, $n = 3$). When treatments with bumetanide plus ouabain are compared, during ischemia and reperfusion, Na_i is greater for K-free treatment (●, $n = 3$) than for normal K treatment (○, $n = 3$). When normal K without ouabain treatments are compared, Na_i is greater during ischemia and reperfusion after addition of bumetanide (△, $n = 3$) than without bumetanide (Fig. 1, □). Data are consistent with the hypothesis that Na-K-2Cl cotransport is active in myocardium during ischemia and reperfusion and that, when the driving force for the cotransporter is directed out of cell, it acts as a pathway for net Na efflux. $P < 0.05$: *vs. K-free plus ouabain; †vs. normal K (Fig. 1, □); §vs. K-free plus ouabain plus bumetanide. [Data shown for K-free plus ouabain protocol (without bumetanide) were previously shown in Fig. 1; see text for further explanation.]

under normal K (4.75 mM) conditions. Although the K-free plus ouabain experiments were aimed at quantifying changes in Na uptake during ischemia (most easily assessed in the absence of efflux via the Na-K pump), nominally K-free perfusion would cause the force driving the Na-K-2Cl cotransporter to be directed outward and may additionally cause changes in the kinetics of transport via the cotransporter. For this reason, experiments were conducted using normal K perfusion to assess Na accumulation under more physiological conditions. Under the more physiological conditions, Na accumulation does not reflect uptake to the extent that efflux via the pump is able to match uptake. Therefore, the contributions of the various pathways to Na transport are more difficult to determine from changes in Na_i. Nevertheless, the following results may provide useful insight into the relative contributions of the Na-K pump and the Na-K-2Cl cotransporter to changes in Na_i during ischemia and reperfusion as well as demonstrate the limits of the experimental model.

The effect of bumetanide on Na_i accumulation during ischemia under normal K conditions can be assessed by comparing the open squares in Fig. 1 with the open triangles in Fig. 2. The data show that, when the Na-K pump is allowed to run (normal K without ouabain), the presence of 20 μM bumetanide results in lower values for Na_i after 30 min of ischemia and throughout reperfusion ($P < 0.05$). These data support the interpretation that, when $[K]_o$ is normal (or greater during ischemia) and $[Na]_i$ is comparatively low (thus the force driving Na-K-2Cl cotransport remains directed into the cell), bumetanide inhibits net Na influx via the cotransporter. It is important to remember, however, that under normal K conditions Na efflux via the Na-K-ATPase may depend on Na uptake. Therefore, differences in Na_i between the normal K plus bumetanide protocol and the normal K protocol may represent differences in Na transport both via the cotransporter and via Na-K-ATPase. To assess the contribution of the Na-K pump to Na transport under these conditions, ouabain (1 mM) was added to the normal K plus bumetanide perfusate (Fig. 2, open circles). The results demonstrate that Na_i is less ($P < 0.05$) during the last 35 min of ischemia and the first reperfusion interval for the normal K plus ouabain plus bumetanide protocol than for the K-free plus ouabain plus bumetanide protocol. This suggests that addition of ouabain alone to the perfusate is not sufficient to completely inhibit the Na-K pump during ischemia and the first reperfusion interval. Here again, the effects of bumetanide on Na_i during ischemia cannot be used to quantitatively assess cotransport-mediated flux for the normal K plus ouabain protocol due to an indeterminate contribution from the Na-K pump (see the DISCUSSION for further explanation of these results).

Finally, one set of experiments was conducted to test the subhypothesis that, after maximal Na loading during ischemia, the Na-K-2Cl cotransporter mediates net Na efflux during reperfusion with normal K solution. The data summarized in Fig. 3 demonstrate that, when ischemia follows perfusion with K-free plus oua-

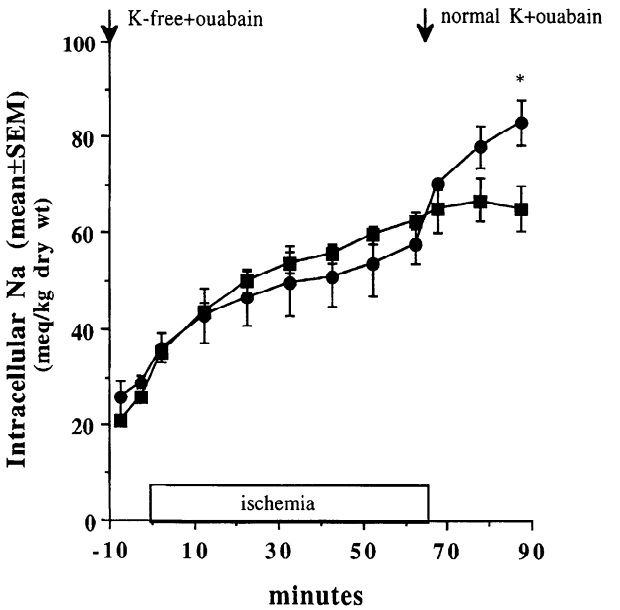

Fig. 3. When Na-K-ATPase is inhibited by K-free solution containing 1 mM ouabain before ischemia, bumetanide (20 μM) inhibition of Na-K-2Cl cotransport causes an increase in Na_i during reperfusion with normal K plus ouabain solution. Na_i (in meq/kg dry wt) is plotted vs. minutes. All hearts were perfused with K-free solution containing ouabain for 10 min before ischemia and reperfused with normal K solution containing ouabain with (●, n = 3) or without (■, n = 3) addition of bumetanide. Data support the hypothesis that Na-K-2Cl cotransport mediates net Na efflux during reperfusion. *$P < 0.05$ compared with normal K without bumetanide.

bain solution, Na_i is greater after 20 min of reperfusion with normal K perfusate containing bumetanide than normal K perfusate without bumetanide. The simplest interpretation of these data is that during reperfusion with normal K solution a bumetanide-sensitive pathway mediates net Na efflux. That is, when Na_i is maximized during ischemia (by 10 min of K-free plus ouabain perfusion before ischemia), bumetanide inhibits net Na efflux during reperfusion with normal K solution. This is consistent with the hypothesis that, under these conditions, Na-K-2Cl cotransport is mediating net Na efflux during reperfusion.

$[Ca]_i$ during ischemia with or without Na-K-2Cl cotransport. We and others have demonstrated that the increase in Na_i during hypoxia and ischemia is accompanied by an increase in $[Ca]_i$, apparently the result of a decrease in the transmembrane Na gradient and thus decreased or reversed net Ca flux through the Na/Ca exchanger (2, 32). If our hypothesis is correct and Na-K-2Cl cotransport provides a significant Na efflux pathway, then bumetanide inhibition of the cotransporter should result in a further increase in $[Na]_i$ during ischemia and thus exacerbate the increase in $[Ca]_i$ mediated by Na/Ca exchange. Figure 4 shows the results of studies designed to test this hypothesis. During ischemia after 10 min of perfusion with K-free plus 1 mM ouabain solution, mean $[Ca]_i$ increased 663 nM (from 438 ± 48 to 1,101 ± 148 nM), but, after bumetanide inhibition of Na-K-2Cl cotransport, mean $[Ca]_i$ increased 1,590 nM (from 1,410 ± 510 to 3,000 ± 1,129 nM). If our hypothesis is correct, then during ischemia the difference between $[Ca]_i$ in hearts preperfused with or without bumetanide is the result of, and should be reflected by, qualitatively similar differences in Na_i. That is, as Na_i increases so too does $[Ca]_i$. Although the Na data from hearts preperfused with K-free plus ouabain solution with and without bumetanide (Fig. 2) are not significantly different during ischemia, the trend in the data suggests that Na_i could be greater after cotransport inhibition. Thus the trend in the data is consistent with the hypothesis that bumetanide inhibition of Na efflux via Na-K-2Cl cotransport increases Na_i during ischemia and thereby decreases the driving force for Ca efflux via Na/Ca exchange, which results in Ca_i accumulation. More importantly, compared with reperfusion without bumetanide, bumetanide inhibition of Na efflux during reperfusion is associated with a relative increase in $[Ca]_i$ from 878 ± 172 to 3,286 ± 630 nM after 20 min of reperfusion.

DISCUSSION

Ischemia increases both Na influx and Na efflux. The RESULTS demonstrate that both Na uptake and Na efflux are increased in rabbit myocardium early during ischemia (Fig. 1), whereas ATP remains >50% of control (2, 23). Because a significant fraction of Na uptake is inhibited by amiloride under these circumstances, the data provide additional support for the hypothesis (4, 23, 35) that the Na/H exchanger is a major route for net Na uptake during ischemia. Previ-

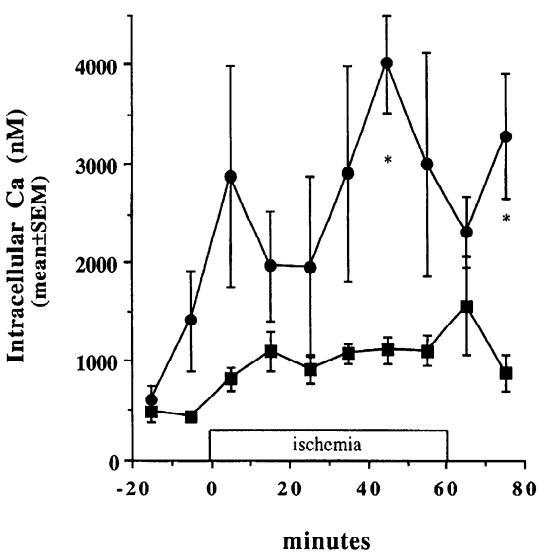

Fig. 4. When Na-K pump is inhibited by presence of ouabain and absence of K in perfusate, bumetanide inhibition of Na-K-2Cl cotransport increases intracellular Ca concentration ($[Ca]_i$) during ischemia and reperfusion. Intracellular Ca (in nM) is plotted vs. minutes. All hearts are perfused with K-free plus 1 mM ouabain solution for 10 min before ischemia and during reperfusion. $[Ca]_i$ is greater during ischemia and reperfusion after addition of 20 μM bumetanide (●, n = 3) than without bumetanide (■, n = 3). *$P < 0.05$. Data support the hypothesis that, when force driving Na-K-2Cl cotransport is directed out of cell, cotransporter mediates net Na efflux so that cotransport inhibition causes a relative increase in Na_i (Fig. 2) and thereby decreases Ca efflux and/or increases Ca uptake via Na/Ca exchange during both ischemia and reperfusion.

ous studies by ourselves and others showed that Na_i increases during ischemia. Because, in the present case, experiments have been performed with both functional and inhibited Na-K-ATPase (K-free plus ouabain perfusion) (2, 31), we have been able to demonstrate that the rate of Na efflux via Na-K-ATPase actually increases early during ischemia. This demonstrated increase in Na-K-ATPase activity illustrates that ATP is not initially limiting Na efflux. These results are consistent with recent reports from experiments using chick myocytes in which the Na-K pump was inhibited by ouabain and K-free superfusion to assess the role of the pump in volume regulation. These studies led Smith et al. (31) to conclude that the historically accepted pump-leak hypothesis may not explain cell swelling and Na uptake during ischemia and that "swelling during ischemic injury may not result from Na^+/K^+ pump failure alone." Consistent with this view, our analysis demonstrates that increased Na_i is due to increased Na entry, which, as a result of increasing $[Na]_i$, actually stimulates Na-K-ATPase activity (25, 36). Increased Na-K-ATPase activity will in turn increase ATP consumption, which, under ischemic conditions, will lead to a vicious cycle of proton production stimulating Na/H exchange-mediated increases in $[Na]_i$ (2) and, therefore, Na-K-ATPase activity, ATP consumption, and proton production.

Amiloride inhibits Na uptake during ischemia. The fact that amiloride inhibits a major portion of ischemia-induced Na uptake is consistent with the hypothesis that the Na uptake is in part via Na/H exchange. Support for this scenario is provided by previous studies that showed that amiloride and its analogues (which inhibit Na uptake via Na/H exchange and thereby interupt the cycle) decrease high-energy phosphate depletion during hypoxia and preserve myocardial function after ischemia (2, 10, 18, 35). Our data further demonstrate that, even after perfusing the hearts with K-free media containing 1 mM ouabain plus 1 mM amiloride for 10 min before ischemia, Na uptake (although inhibited) is nevertheless measurable. This result suggests that Na/H exchange inhibition by 1 mM amiloride is incomplete and/or that Na uptake during ischemia also occurs via pathways other than Na/H exchange. For instance, a Na-dependent HCO_3 transport pathway (13, 16, 38) could mediate Na uptake under the conditions present during our experiments if extracellular CO_2 were sufficient during ischemia to allow extracellular HCO_3 concentration to rise to the levels required to support the observed Na uptake. This possibility is further supported by the results of Vandenberg et al. (38) who concluded that, under HCO_3-buffered conditions, 35% of total H efflux during reperfusion is via Na-dependent pathways and showed that, even when the perfusate is nominally HCO_3 free (HEPES buffer), ~10% of total proton efflux remains after Na/H exchange and lactate efflux is inhibited. It has also been reported that Na uptake via conductive Na pathways contributes to increases in Na_i during hypoxia, and it has been suggested that this could occur during ischemia (30).

Finally, although amiloride is neither the most active nor the most specific of the Na/H exchange inhibitors, its effects in this study may be reasonably ascribed to its inhibition of Na/H exchange. That is, although amiloride has been reported to inhibit Na/Ca exchange and L-type Ca channels, its effect on Na_i in the experiments reported here will be mostly due to its effect on Na/H exchange. More specifically, given the conditions of these experiments and assuming that the Na/Ca exchanger obligatorily exchanges three Na for one Ca and that the K-free plus ouabain protocol used in the amiloride experiment effectively "clamps" membrane potential (E_m) at −40 mV (14), we calculate that, if $[Na]_i$ rises above 15 mM (after 2.5 min of ischemia), the force driving the Na/Ca exchanger is directed so that it would mediate net Na efflux. Thus the Na uptake we observe is not via Na/Ca exchange and therefore not directly limited by possible inhibition of Na/Ca exchange by amiloride.

Effect of temperature on Na transport during ischemia. Although our studies were conducted at room temperature, they are comparable to those conducted at 37°C. For instance, Steenbergen et al. (33) used NMR to measure that in rat hearts $[Na]_i$ rises to 26.7 ± 3.8 mM after 30 min of ischemia at 37°C. Assuming 2.5 g H_2O/g dry wt, this Na concentration corresponds to 66.8 ± 9.5 meq/kg dry wt, similar to the value of 59 ± 1 meq/kg dry wt measured by Tani and Neely (35) with the use of standard chemical analysis in a similar preparation. We find Na_i rises to 46.8 ± 4.6 meq/kg dry wt at 23°C in 30 min and to 62.9 ± 5.2 after 65 min. Thus the levels of Na_i attained are similar, but the rate of accumulation is decreased at lower temperature. This difference in rate of Na accumulation could be the result of the various Na transport pathways having different increases in rate produced by raising temperature 10°C (Q_{10}); the simplest conclusion being that the Q_{10} for the influx pathways are greater than for the efflux pathway(s). On the other hand, this response to decreased temperature is as one would predict from the hypothesis that Na uptake is, to a large extent, via Na/H exchange (or other Na-dependent pH regulatory pathways), which responds to increased intracellular proton concentration. That is, during ischemia, the rate of Na_i accumulation and its temperature dependence may be functions of the rate of anaerobic ATP consumption and its temperature dependence rather than functions of the Na transport proteins themselves per se.

Na-K-2Cl cotransport inhibition prevents Na efflux during reperfusion with K-free solution. These studies also demonstrate that the Na-K-2Cl cotransport inhibitor bumetanide inhibits Na efflux during reperfusion with K-free perfusate. Previous studies have demonstrated the presence of the Na-K-2Cl cotransporter in myocardium (8, 15). However, inhibition of the cotransporter results in cell shrinkage (7), which suggests that, under control conditions (with normal Na, K, and Cl gradients), the cotransporter is directed into the cell; i.e., under control conditions, the cotransporter mediates ion and osmotically obliged water uptake. Consequently, when ion and thus water uptake is inhibited

(while the normal efflux routes remain functional), a new steady state characterized by reduced cell volume and ion content is achieved. On the other hand, recent studies have demonstrated that the Na-K-2Cl cotransport inhibitor furosemide decreases net K efflux in rat hearts during ischemia (20). This finding is consistent with the interpretation that Na-K-2Cl cotransport is directed out of the cell at some time during ischemia. That the cotransporter is able to mediate net solute uptake under control conditions yet efflux after ischemia is explicable in terms of alterations in the force driving cotransport, which are predictable consequences of changes in cell ion concentrations that occur during ischemia. That is, under control conditions in which $[Na]_i$ and intracellular Cl concentration ($[Cl]_i$) are relatively low, the force driving the cotransporter may be directed into the cell, yet large increases in $[Na]_i$, and presumably $[Cl]_i$, during ischemia (23, 27, 35) would be expected to cause the direction of the force driving Na-K-2Cl cotransport to reverse, consistent with our observations (see below for a more complete discussion). The direction of net flux through the cotransporter can be calculated from the chemical potential difference $\Delta\mu_{Na\text{-}K\text{-}2Cl} = RT(\ln[Na]_o/[Na]_i + \ln[K]_o/[K]_i + 2\ln[Cl]_o/[Cl]_i)$, where R is the ideal gas constant, T is the absolute temperature, and the subscripts o and i refer to the extra- and intracellular spaces, respectively. Because, during K-free perfusion, the K gradient is infinite and directed out of the cell, the cotransporter will necessarily be directed out of the cell and will serve as an Na efflux pathway. Under these conditions, bumetanide inhibition of Na-K-2Cl cotransport will decrease Na efflux. Thus bumetanide inhibition of net Na efflux during reperfusion with K-free solution containing ouabain (Fig. 2) is the expected result. One would also predict that, if the cotransporter is active, during ischemia after K-free perfusion it would mediate Na-K-2Cl flux out of the cell at the beginning of ischemia before significant increases in $[K]_o$ (20). If that were true, bumetanide inhibition of Na-K-2Cl cotransport would similarly decrease Na efflux during ischemia and thereby contribute to an increase in Na_i. Although the differences between the K-free plus ouabain protocols with or without bumetanide are not significant during ischemia ($P = 0.087$), for each of the data points acquired during ischemia, Na_i in hearts perfused with bumetanide is nominally greater than in hearts perfused with bumetanide-free medium. It should also be noted that the potency of bumetanide in the K-free protocols may be diminished, since bumetanide will not bind to, and thus inhibit, the cotransporter when $[K]_o$ is very low [half-maximal binding of [^{3}H]bumetanide to dog kidney membranes requires $[K]_o$ of 1 mM (9)]. If, however, as suggested by our previous studies, ischemia-induced K loss from the cells is on the order of the Na uptake (2), then K concentrations in the vicinity of the transporter are not zero and apparently sufficient to permit bumetanide binding and therefore Na-K-2Cl cotransport inhibition during reperfusion. Indeed, the effects of bumetanide during K-free reperfusion are likely the result of bumetanide binding during ischemia when $[K]_o$ is greater than during reperfusion.

One of the limitations of this study is that we have only tested one concentration of bumetanide and the effects of bumetanide on the heart, and Ca metabolism in particular, have not been well characterized. Although we have not determined whether bumetanide inhibition of the cotransporter is complete, it is sufficient (at least during reperfusion) to produce a measurable effect on Na transport in support of the hypothesis. The concentration of bumetanide we have used (20 μM) is within the range (0.6–100 μM) reported by others to inhibit Na-K-2Cl cotransport in heart myocytes (8, 12, 15). However, one study using isolated rat hearts showed that addition of 0.1 μM bumetanide resulted in decreased Na_i accumulation after 4 h of ischemia at 4°C, whereas other concentrations of bumetanide (both greater and lesser) had no significant effect (28). Questions of the specificity of bumetanide may also arise. Although bumetanide has been considered the "inhibitor of choice" for in vitro studies of the cotransporter (9), it has been shown to partially inhibit Cl/HCO_3 exchange and K-Cl cotransport in red blood cells. The required dose for this partial inhibition, however, is five times higher than those used in this study. Furthermore, the simplest hypothesis that explains how inhibition of Cl/HCO_3 exchange and/or K-Cl cotransport could result in a relative increase in Na_i requires that net Na flux is mediated by the Na-K-2Cl cotransporter consistent with the conclusions of this study (e.g., inhibition of Cl loss via Cl/HCO_3 exchange and/or K-Cl cotransport could diminish a decrease in $[Cl]_i$ and thereby relatively decrease the rate of Na-K-2Cl cotransport as described below). Bumetanide has also been reported to inhibit L-type Ca channels in rabbit ventricular myocytes (29). The effects of Ca channel inhibition could be numerous, but one would not expect it to cause the increase in $[Ca]_i$ we observe. Nonspecific toxic effects of 500 μM furosemide on isolated mouse hepatocytes have also been reported (28). The bumetanide-dependent increases in $[Ca]_i$ we observe (Fig. 4) could be due to such effects, but the fact that Na remains low and recovers during reperfusion in the normal K plus bumetanide protocol (Fig. 2) argues that 20 μM bumetanide itself is not sufficiently toxic to prevent the cells from regulating Na_i during reperfusion. Finally, there are only two possible explanations for the effect of bumetanide on Na_i during reperfusion after ischemia following K-free plus ouabain perfusion. Bumetanide either decreases net Na efflux or increases net Na influx. To our knowledge, no study has presented evidence that bumetanide increases Na flux via any transport pathway nor that it decreases Na flux via any pathway other than the Na-K-2Cl cotransporter.

Na-K-2Cl cotransport and $[Na]_i$ under normal K conditions. The results of protocols conducted using normal K perfusion are more difficult to interpret with respect to the effects of bumetanide on Na uptake because, when the Na-K pump is allowed to act as an Na efflux pathway, changes in Na_i cannot be interpreted to be the result of changes in Na uptake alone.

Specifically with reference to the protocols used in this study, 1 mM ouabain perfusion with normal K solution before ischemia is not sufficient to completely inhibit the Na pump (see RESULTS and compare open and closed circles in Fig. 2). Nevertheless, the significant differences among the various protocols can provide insight into the relationships among the various Na transport pathways. Most interesting, perhaps, is the fact that, under K-free plus ouabain conditions, bumetanide inhibits Na efflux and thereby causes an increase in Na_i during reperfusion (Fig. 2), whereas, under normal K conditions, bumetanide appears to inhibit Na uptake and thereby causes a relative decrease in Na_i. That is, when hearts are perfused with normal K solution before and after ischemia, during the last 35 min of ischemia and throughout reperfusion Na_i is less in the hearts perfused with solution containing bumetanide than those without (open triangles in Fig. 2 vs. open squares in Fig. 1). This suggests that, under these conditions (relatively low Na_i), the driving force for the cotransporter (see below) is directed into the cell; therefore, cotransport inhibition by bumetanide decreases net Na uptake and thereby decreases Na_i accumulation during ischemia. Decreasing Na_i accumulation during ischemia would allow the driving force for the cotransporter to remain directed into the cell so that, during reperfusion, bumetanide would continue to inhibit the cotransporter from acting as a net influx pathway. Inhibiting net influx via the cotransporter would thus allow Na_i to remain lower during reperfusion with media containing bumetanide (as demonstrated by the data).

Finally, one set of experiments was conducted to test the subhypothesis that, after maximal Na loading during ischemia, the Na-K-2Cl cotransporter acts as a pathway for net Na efflux during reperfusion with normal K solution. The data demonstrate that, after maximal Na loading (K-free plus ouabain solution before ischemia), addition of bumetanide to the normal K plus ouabain solution increases net Na uptake during reperfusion (Fig. 3). Again, bumetanide is either inhibiting an Na efflux pathway or stimulating an Na influx pathway. Given that bumetanide is a fairly specific inhibitor of Na-K-2Cl cotransport at this concentration (and not known to stimulate any Na transport pathway) and the force driving Na-K-2Cl cotransport is calculated to be directed out of the cells under these conditions (see below), the data are consistent with the hypothesis that the cotransporter is acting as a net Na efflux pathway during reperfusion with normal K solution. Comparison of normal K and K-free reperfusion with the same solutions containing bumetanide raises another interesting question. In both cases, Na_i is greater after addition of bumetanide. This is consistent with the hypothesis that bumetanide is inhibiting Na-K-2Cl cotransport while it is functioning as a net Na efflux pathway (the latter being as predicted using the thermodynamic arguments described below). However, whereas bumetanide prevents a decrease in Na_i during K-free reperfusion, during normal K reperfusion, addition of bumetanide results in an increase in Na_i. There are at least two ways to interpret these results. Either K-free reperfusion is increasing the rate of Na efflux via the cotransporter or decreasing the rate of Na uptake via some other pathway. At this point, we cannot say whether one or both of these effects is occurring; however, at least two scenarios are consistent with the postulate that the rate of efflux via the cotransporter is increased. First, the force driving the cotransporter is greater in magnitude during K-free perfusion. If the rate of flux through the cotransporter increases with the magnitude of the force, then Na efflux should be faster during K-free perfusion. Second, if K-free perfusion increases the rate of net efflux via the cotransporter, positive feedback could arise as a result of increased Cl loss and thereby decreased $[Cl]_i$, which has been shown, in the squid giant axon, to stimulate the cotransporter (3).

Force driving the Na-K-2Cl cotransporter. One can calculate the direction of the net force acting on, and therefore the direction of net transport by, the Na-K-2Cl cotransporter under more physiological conditions, such as during ischemia and reperfusion with normal K solution without ouabain. (A detailed explanation of the following calculations is given in the APPENDIX.) As a first approximation, we use the Na_i measurements made during the normal K protocol (Fig. 1), and, based on previously published values (2), we assume that within 2 min after control perfusion intracellular volume (V_i) is 2.37 l/kg dry wt and extracellular volume (V_o) is 2.25 l/kg dry wt. We further assume that *1*) at the beginning of ischemia, extracellular ion concentrations equal perfusate values and $[Cl]_i$ is equal to 40 mM and $[K]_i$ is equal to 130 mM (17); *2*) at the end of ischemia, $[K]_o$ is equal to 14 mM (20); *3*) cells act as perfect osmometers; and *4*) all intra- and extracellular contents remain within the spectrometer's active volume. The latter assumption is made to allow changes in extracellular content to be calculated from changes in intracellular content; i.e., changes in cell solute or water content are equal and opposite to changes in the extracellular content for the same substance. Preliminary experiments have been conducted using ^{19}F NMR to measure V_o/V_i and the chloride equilibrium potential (E_{Cl}) (27). V_o/V_i is measured as the ratio of extra- to intracellular trifluoroacetamide content (TFM_o/TFM_i) assuming the extra- and intracellular concentrations are equal. E_{Cl} is calculated from the ratio of intra- to extracellular trifluoroacetate content (TFA_o/TFA_i) using V_o/V_i to calculate the ratio of the respective concentrations and assuming the TFA equilibrium potential (E_{TFA}) is equal to E_{Cl}. (Note: this does not assume that $E_{Cl} = E_m$.) Thus $E_{Cl} = RT/z\mathrm{Fln}[(TFA_o \times TFM_i)/(TFA_i \times TFM_o)]$ in which the ratios of TFA content and TFM content are equal to the ratios of the corresponding ^{19}F NMR spectral peak areas. We find E_{Cl} approaches zero ($[Cl]_i = [Cl]_o = 90$ mM) and V_o/V_i of 0.83 at the end of ischemia. Using these values and the NMR measured value of $[Na]_i$ of 24.8 mM at the end of ischemia, we calculate that $[Na]_o$ falls from 141 to 130 mM and $[K]_i$ falls from 130 to 114 mM during 65 min of ischemia.

The energy for Na-K-2Cl cotransport ($\Delta\mu_{Na\text{-}K\text{-}2Cl}$ = −1,093 J/mol) is thus directed out of the cell and remains directed out during reperfusion. In fact, even though $[Na]_o$ and $[Cl]_o$ increase during reperfusion, the magnitude of the force directed out of the cell increases ($\Delta\mu_{Na\text{-}K\text{-}2Cl}$ = −1,267 J/mol) when $[K]_o$ falls from 14 to 4.75 mM (as K lost from the cells during ischemia is washed out of the extracellular space). The calculated values are consistent with our results and suggest that, even after normal K perfusion without ouabain, the cotransporter acts as an Na efflux pathway late during ischemia and during reperfusion.

Na-K-2Cl cotransport inhibition increases $[Ca]_i$ during ischemia and reperfusion. During ischemia as well as during reperfusion, bumetanide inhibition of Na-K-2Cl cotransport resulted in a significant increase in $[Ca]_i$ when the Na-K pump is inhibited by K-free plus ouabain perfusion (Fig. 4). These observations are consistent with our hypothesis that, during postischemic reperfusion, the cotransporter can be a significant pathway for Na efflux. That is, any event that leads to increased $[Na]_i$ and therefore decreases the transmembrane Na gradient will cause an increase in $[Ca]_i$ due to decreased extrusion or, in the extreme, increased Ca entry via the Na/Ca exchanger. Thus, if the bumetanide-dependent increase in $[Ca]_i$ is the result of an increase in Na_i, bumetanide must either stimulate Na uptake or inhibit Na efflux. Because the established action of bumetanide is to inhibit Na-K-2Cl cotransport, the data are consistent with the interpretation that bumetanide is inhibiting Na-K-2Cl cotransport-mediated Na efflux during ischemia and reperfusion in the K-free plus ouabain protocol. Although the effect of bumetanide on Na_i during ischemia after K-free plus ouabain perfusion is not statistically significant, the significant increase in $[Ca]_i$ (which is measured by NMR with much higher resolution than Na_i), together with predictions based on thermodynamic considerations, provides inferential support for the postulate that the nominal increase in Na_i is the result of inhibition of net Na efflux via the Na-K-2Cl cotransporter. This notion gains further support from the results of the K-free plus ouabain protocol in which both Na_i and $[Ca]_i$ increase as a result of the presence of bumetanide during reperfusion when, as a result of the exceedingly large intracellular-to-extracellular K gradient, the Na-K-2Cl cotransporter must function as an Na efflux pathway.

These results also support the comprehensive hypothesis that asserts that increases in Na_i cause increases in $[Ca]_i$ as a result of decreased Ca efflux via Na/Ca exchange. Using the assumptions described above to calculate V_o/V_i for the K-free plus ouabain plus bumetanide protocol in which $[Ca]_i$ was measured, we calculate that, at the end of ischemia, $[Na]_o$ is equal to 133.5 mM and $[Na]_i$ is equal to 31.2 mM. With the assumptions that $[Ca]_o$ is equal to 1.8 mM, E_m is equal to −40 mV (14), and the Na/Ca exchanger exchanges three Na for one Ca, the exchanger would be "at equilibrium" if $[Ca]_i$ were 4.7 μM. The $[Ca]_i$ we measure reaches a maximum of 4.01 ± 0.49 μM during ischemia. Thus, for most of the ischemic interval, the forces on the Na/Ca exchanger would be driving net Ca flux into the cell as predicted by the hypothesis.

In light of the thermodynamic calculations for the normal K conditions discussed above, these results are particularly interesting with respect to the practice of prescribing furosemide for patients who have high blood pressure or congestive heart failure and who may also be at risk for episodes of myocardial ischemia. Under these conditions, furosemide may be beneficial in that, when the force driving Na-K-2Cl cotransport is directed into the cell, furosemide may act to inhibit Na (and Cl) uptake (Fig. 2) (28). On the other hand, if, for instance, the ischemic interval is lengthy, the Na, K, and Cl gradients may be altered enough to change the direction of the force driving the cotransporter so that it mediates net Na efflux (Figs. 2 and 3). In the latter case, furosemide could inhibit net Na efflux during reperfusion after extended ischemia (Fig. 2), thus exacerbating increases in $[Na]_i$ and thereby $[Ca]_i$ (Fig. 4) and increasing the risk of myocardial injury and infarction.

Conclusions. Together with previous work (2), these data demonstrate that both Na uptake and Na efflux are increased during ischemia and that, as previously demonstrated (23, 35), Na uptake increases more than Na efflux. In the rabbit heart, as in rat and ferret hearts, the ischemia-induced increase in Na_i can be greatly inhibited by amiloride, which supports the hypothesis that a significant portion of the Na uptake is via Na/H exchange (23, 38). The increase in Na_i observed during ischemia is accompanied by an increase in $[Ca]_i$, which is predicted by our comprehensive hypothesis, since the energy available for Na extrusion via Na/Ca exchange is reduced or, in the worst case (>2-fold increase in $[Na]_i$), reversed. Furthermore, when the Na-K pump is inhibited by K-free plus ouabain perfusion before ischemia, the Na-K-2Cl cotransport inhibitor bumetanide inhibits Na efflux during reperfusion after ischemia (under both K-free and normal K conditions). In addition, after K-free plus ouabain perfusion, $[Ca]_i$ is increased by Na-K-2Cl cotransport inhibition during ischemia and reperfusion. This too is consistent with the hypothesis that, under these conditions, the cotransporter acts as an Na efflux pathway such that its inhibition results in a relative increase in $[Na]_i$ and an Na-dependent increase in $[Ca]_i$ (presumably by Na/Ca exchange).

APPENDIX

The following provides a more detailed explanation of the way in which the net driving force for Na-K-2Cl cotransport was calculated from data acquired using ^{23}Na and ^{19}F NMR spectroscopy. Values corresponding to the beginning of ischemia are indicated by subscript 1 and after 65 min of ischemia by subscript 2. Intra- and extracellular compartments are indicated by subscripts i and o, respectively. We assume that the cells act as perfect osmometers (i.e., water follows solute in an isotonic fashion) and that there is no change in total solute or fluid volume within the active volume of the NMR probe. Thus the total amount of any substance (indicated by subscript t) is constant and equal to the sum of the intra- and

extracellular amounts. Intra- and extracellular Na contents (Na_i and Na_o) in milliequivalents per kilogram dry weight are measured as previously described (2). At the beginning of ischemia, we further assume the following: V_{o1} = 2.25 ml/g × dry wt, V_{i1} = 2.37 ml/g × dry wt (2), $[Na]_{o1}$ = 141 mM, $[Na]_{i1}$ = Na_{i1}/2.37 ml/g = 7 mM, $[K]_{i1}$ = 130 mM (17), $[K]_{o1}$ = 4.75 mM, $[Cl]_{i1}$ = 40 mM (17), and $[Cl]_{o2}$ = 143 mM. At the end of ischemia, we assume $[K]_{o2}$ = 14 mM (20).

We used ^{19}F NMR to measure E_{Cl} and V_i/V_o after 65 min of ischemia using the method described by Ramasamy et al. (27) who presented evidence consistent with the assumptions we make: TFA is distributed identically to Cl, TFM is distributed so that its intra- and extracellular concentrations are identical, and each of these fluorinated compounds is 100% NMR visible in both the intra- and extracellular spaces. Thus the ratio of the ^{19}F NMR spectral areas TFM_i/TFM_o is equal to V_i/V_o, since the ratio of the spectral areas is equal to the ratio of the respective contents $|([TFM]_i \times V_i)/([TFM]_o \times V_o)|$ is equal to V_i/V_o, assuming $[TFM]_i$ is equal to $[TFM]_o$. Similarly, the ratio of spectral areas TFA_o/TFA_i is equal to the ratio of the respective contents $|([TFA]_o \times V_o)/([TFA]_i \times V_i)|$, which allows us to calculate that $E_{TFA} = (RT/zF)\ln([TFA]_o/[TFA]_i) = (RT/zF)\ln[(TFA_o/V_o)/(TFA_i/V_i)] = (RT/zF)\ln[(TFA_o \times V_i)/(TFA_i \times V_o)] = (RT/zF)\ln[(TFA_o \times TFM_i)/(TFA_i \times TFM_o)] = E_{Cl}$, assuming E_{Cl} is equal to E_{TFA}.

From E_{Cl} and V_i/V_o we can calculate $[Cl]_i$, $[Cl]_o$, V_i, and V_o relative to the initial values, assuming conservation of all components within the active volume. Thus, at the end of ischemia, $Cl_{t2} - ([Cl]_{o2} \times V_{o2}) + ([Cl]_{i2} \times V_{i2}) = [Cl]_{i2} \times (|([Cl]_{o2}/[Cl]_{i2}) \times V_{o2}| + V_{i2})$ and, by rearrangement, $[Cl]_{i2} = Cl_{t2}/(|([Cl]_{o2}/[Cl]_{i2}) \times V_{o2}| + V_{i2})$. However, Cl_{t2} is equal to Cl_{t1}, which is equal to a constant and $[Cl]_{o2}/[Cl]_{i2} = 10\exp(E_{Cl2}/-58.5)$ at room temperature, so $[Cl]_{i2} = |(V_{o1} \times [Cl]_{o1}) + (V_{i1} \times [Cl]_{i1})|/(|[10\exp(E_{Cl2}/-58.5)] \times V_{o2}| + V_{i2})$ and $[Cl]_{o2} = [Cl]_{i2}/[10\exp(E_{Cl2}/58.5)]$. To calculate V_{o2} and V_{i2}, we assume $V_{o1} + V_{i1} = V_{o2} + V_{i2}$. Multiplying both sides by the known value $V_{o2}/V_{i2} = TFM_{o2}/TFM_{i2}$ and rearranging $V_{o2} = (V_{o1}+V_{i1}) \times (TFM_{o2}/TFM_{i2})/[1 + (TFM_{o2}/TFM_{i2})]$ and $V_{i2} = V_{o1} + V_{i1} - V_{o2}$. Having calculated V_{o2} and V_{i2}, $[Na]_{i2} = Na_{i2} \times$ dry wt/V_{i2} (Na_{i2} is measured), and $[Na]_{o2}$ can be calculated as the difference between the initial amount of extracellular Na and the change in the amount of Na_i divided by the extracellular volume at the end of ischemia: $[Na]_{o2} - |([Na]_{o1} \times 2.25\ ml/g) - (Na_{i2} - Na_{i1})| \times$ dry wt/V_{o2}. $[K]_{i2}$ is likewise calculated as the difference between the total amount of K and the amount of extracellular K at the end of ischemia divided by the intracellular volume at that time: $[K]_{i2} = |([K]_{i1} \times V_{i1}) + ([K]_{o1} \times V_{o1}) - ([K]_{o2} \times V_{o2})|/V_{i2}$. The chemical potential energy available to drive Na-K-2Cl cotransport at the end of ischemia after normal K perfusion was then calculated $\Delta\mu_{Na\text{-}K\text{-}2Cl} = RT(\ln[Na]_o/[Na]_i + \ln[K]_o/[K]_i + 2\ln[Cl]_o/[Cl]_i) = -1{,}093$ J/mol.

We wish to thank Nanah Suk for her technical assistance.

This work was supported by the University of California Hibbard Williams Fund, the Cigarette and Tobacco Surtax Fund of the State of California through the Tobacco-Related Disease Research Program of the University of California, Grant 3RT-0112, and National Heart, Lung, and Blood Institute Grant HL-21179. NMR spectrometer expense was funded in part by Division of Research Resources Grant RR02511 and National Science Foundation Grant PCM-8417289.

This work was done during the tenure of a research Fellowship from the American Heart Association, California Affiliate.

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Received 6 September 1994; accepted in final form 25 August 1995.

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