Changes in intracellular Na\(^+\) and pH in rat heart during ischemia: role of Na\(^+\)/H\(^+\) exchanger

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Park, Choon-Ok, Xiao-Hui Xiao, and David G. Allen. Changes in intracellular Na\(^+\) and pH in rat heart during ischemia: role of Na\(^+\)/H\(^+\) exchanger. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H1581–H1590, 1999.—The role of the Na\(^+\)/H\(^+\) exchanger in rat hearts during ischemia and reperfusion was investigated by measurements of intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) and intracellular and extracellular pH. Under our standard conditions (2-Hz stimulation), 10 min of ischemia caused no significant rise in [Na\(^+\)]\(_i\), but an acidosis of 1.0 pH unit, suggesting that the Na\(^+\)/H\(^+\) exchanger was inactive during ischemia. This was confirmed by showing that the Na\(^+\)/H\(^+\) exchange inhibitor methylisobutyl amiloride (MIA) had no effect on [Na\(^+\)]\(_i\) or on intracellular pH during ischemia. However, there was a short-lived increase in [Na\(^+\)]\(_i\) of 8.2 ± 0.6 mM on reperfusion, which was reduced by MIA, showing that the Na\(^+\)/H\(^+\) exchanger became active on reperfusion. To investigate the role of metabolic changes, we measured [Na\(^+\)]\(_i\) during anoxia. The [Na\(^+\)]\(_i\) did not change during 10 min of anoxia, but there was a small, transient rise of [Na\(^+\)]\(_i\) on reoxygenation, which was inhibited by MIA. In addition, we show that the Na\(^+\)/H\(^+\) exchanger, tested by sodium lactate exposure, was inhibited during anoxia. These results show that the Na\(^+\)/H\(^+\) exchanger is inhibited during ischemia and anoxia, probably by an intracellular metabolic mechanism. The exchanger activates rapidly on reperfusion and can cause a rapid rise in [Na\(^+\)]\(_i\).

A large increase in the total myocardial Ca\(^{2+}\) on reperfusion of the ischemic heart was first shown by Shen and Jennings in 1972 (28). Subsequent studies of the free intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) have demonstrated that [Ca\(^{2+}\)]\(_i\) rises during ischemia, but the rise on reperfusion is greater and its magnitude correlates with cellular damage and the appearance of arrhythmias (15, 20, 29). Thus it is widely believed that the increase in [Ca\(^{2+}\)]\(_i\) during ischemia and reperfusion has an important role in the pathological changes that occur (19, 30).

However, the mechanism of the increases in [Ca\(^{2+}\)]\(_i\) remains uncertain. Early experiments showed a large influx of Ca\(^{2+}\) on reperfusion that was blocked by Ni\(^{2+}\) or reduced extracellular Ca\(^{2+}\) but not by Ca\(^{2+}\) channel blockers (26). This has led to the view that Ca\(^{2+}\) influx during reperfusion is by the Na\(^+\)/Ca\(^{2+}\) exchanger and is associated with elevated intracellular Na\(^+\) concentration ([Na\(^+\)]\(_i\)) (19, 30). For this reason, there is great interest in how [Na\(^+\)]\(_i\) changes in ischemia and reperfusion. In principle, [Na\(^+\)]\(_i\) is maintained below electrochemical equilibrium by the Na\(^+\) pump, which requires ATP for its operation. During ischemia, ATP inevitably declines, so eventually the Na\(^+\) pump will be impaired and [Na\(^+\)]\(_i\) will rise. In keeping with this analysis, many studies have shown that [Na\(^+\)]\(_i\) rises during ischemia (25, 31), and the rise in [Na\(^+\)]\(_i\) has been correlated with a decline in the metabolic status and reduced turnover of the Na\(^+\) pump (3). After 10 min of ischemia the reported rise in [Na\(^+\)]\(_i\) is quite variable, with many studies showing large rises while some show relatively small rises or even a fall (for review see Refs. 12 and 35).

It is also possible that [Na\(^+\)]\(_i\) might rise in ischemia through increased influx as opposed to the decreased efflux discussed above. Lazzunski et al. (18) pointed out that lactic acid accumulation during ischemia causes a large intracellular acidosis, and if the Na\(^+\)/H\(^+\) exchanger were active, this would be expected to minimize the acidosis and cause an increase in [Na\(^+\)]\(_i\). Instead they suggested that the protons leaving the cell accumulate and produce an extracellular acidosis that is sufficiently great to inhibit the Na\(^+\)/H\(^+\) exchanger (18). During reperfusion the extracellular protons are flushed away, and the activity of the Na\(^+\)/H\(^+\) exchanger would then lead to rapid recovery of intracellular pH (pHi) and a rise in [Na\(^+\)]\(_i\)]. The latter could then lead to Ca\(^{2+}\) entry by means of the Na\(^+\)-/Ca\(^{2+}\) exchanger. There is considerable evidence in support of this hypothesis, and a number of studies have shown that inhibitors of the Na\(^+\)/Ca\(^{2+}\) exchanger protect against some of the damaging effects of ischemia (for review see Ref. 24).

In earlier work from this laboratory, we used application of extracellular sodium lactate to cause an intracellular acidosis in isolated ventricular cells (2) and perfused hearts (32). As expected, we found that intracellular acidosis activated the Na\(^+\)/H\(^+\) exchanger, which caused a subsequent recovery of pHi, a rise in [Na\(^+\)]\(_i\)], and a rise in [Ca\(^{2+}\)]\(_i\)]. However, an extracellular acidosis comparable to that observed in the ischemic heart did not inhibit the exchanger, and in fact the increases in [Na\(^+\)]\(_i\) and [Ca\(^{2+}\)]\(_i\)] were larger when an extracellular acidosis was also present. These findings have led us to reinvestigate in the perfused heart whether or not the Na\(^+\)/H\(^+\) exchanger is inhibited during ischemia and, if so, by what mechanism. We measured [Na\(^+\)]\(_i\) and pHi in the perfused heart using the fluorescent indicators Na\(^+\)-binding benzoafuran isophthalate (SBFI) and carbosynaminaphthorhodafluor (SNARF), which were loaded into the heart in their membrane-permeant forms. In addition, we used SNARF in the extracellular solution to measure the extracellular pH (pHe).
METHODS

Heart preparation. The preparation and monitoring of Langendorff-perfused rat hearts have been described previously (32). Briefly, female Sprague-Dawley rats were anesthetized with pentobarbital, and the hearts were excised and perfused retrogradely at a flow rate of 9–13 ml·min⁻¹·g wet wt⁻¹ at 35°C. The hearts were stimulated at 2 Hz after the sinoatrial node and right atrium were excised and the atrioventricular node was crushed. Left ventricular function was assessed by recording the pressure developed in a balloon in the left ventricle (LVDP). The hearts were placed in a chamber that was mounted on the stage of an inverted microscope modified for fluorescence measurements. A × 10 objective was used to illuminate the heart and collect the fluorescence emitted from the surface of the heart.

Solutions, drugs, and experimental interventions. The perfusate had the following composition (in mM): 118.5 NaCl, 5 KCl, 1.2 NaH₂PO₄, 1.2 MgSO₄, 40 HEPES, 1 CaCl₂, and 11 glucose. The high concentration of HEPES was chosen to simulate the extracellular pH buffering capacity of HCO₃⁻/CO₂ Tyrode solution; the solutions were nominally HCO₃⁻ free to limit the pH regulatory mechanisms to the Na⁺/H⁺ exchanger and the lactate transporter (33). The pH was adjusted to 7.4 with NaOH, and total [Na⁺] after pH adjustment was ~140 mM. The solutions were continuously bubbled with 100% O₂. Probenecid (1 mM) was added to all solutions to minimize the loss of tetracarboxylate fluorescent indicators from cells and to reduce the degree of compartmentalization of the dyes (4). The Na⁺/H⁺ exchange inhibitor 5-(N-methyl-N-isobutyraminomethyl)amiloride (MIA) was supplied by Research Biochemicals (Natick, MA), and a stock solution of 10 mM in DMSO was prepared. The progesteron U-44069 was supplied by Cayman Chemical (Ann Arbor, MI), and a stock solution was prepared at 100 mg/ml in DMSO.

Ischemia was achieved by switching off the peristaltic pump to stop the perfusion inflow to the heart. The temperature of the chamber was maintained at 35°C during ischemia. Anoxia was produced by equilibrating the perfusate with 100% N₂ instead of O₂ (glucose still present in perfusate). To induce intracellular acidosis, 20 mM sodium lactate was added to the perfusate, and pHₕ was readjusted to 7.4.

Loading with fluorescent indicators. The hearts were loaded with perfusion with SBFI-AM or SNARF-AM to measure [Na⁺]ᵰ, or pHᵰ, (for details see Refs. 22 and 32). Before the hearts were loaded with dye, the autofluorescence was measured. The hearts were then perfused with the loading solution until the signal from the heart had reached a plateau. With SBFI-AM, loading took 60–90 min, and the fluorescence signal after loading was usually five to eight times the original autofluorescence signal. With SNARF-AM, loading took 15–20 min, and the fluorescence signal was increased up to 150–200 times the autofluorescence signal. To measure pHᵰ, SNARF (10 µM) was added to the perfusate.

The Na⁺-dependent signal of SBFI was obtained by illuminating at 340 and 380 nm and dividing the emitted light signals from the heart at 530 nm. Filters of 340 and 380 nm in front of the illuminating light were changed at 40 Hz by using a spinning rotor to minimize the movement artifacts associated with each contraction and with ischemia. The signals were filtered at 0.3 Hz, and the ratio was calculated after subtraction of the autofluorescence. The pH-dependent signal of SNARF was obtained by illuminating at 540 nm, and the fluorescence emitted at 590 and 640 nm was measured using two photomultiplier tubes; from the output of these tubes, the ratio was calculated.

Calibration of the fluorescence signals. We performed in vivo calibration according to the methods described earlier (22, 32). The SBFI- or SNARF-loaded hearts were perfused with each calibration solution at the end of experiments. The SBFI calibration solutions consisted of various [Na⁺] and gramicidin D (0.2 µg/ml) and monensin (40 µM) as Na⁺ ionophores and strophanthidin (100 µM) as an inhibitor of the Na⁺-K⁺ pump. From this, in vivo calibration values of the ratio at 0 mM Na⁺ (R_min), the ratio at a saturating [Na⁺] (R_max), the signal from 0 mM Na⁺ at 380 nm (S₂₈), the signal from 140 mM Na⁺ at 380 nm (S₂₈), and the apparent dissociation constant (Kₐ) were determined. In 11 hearts, R_min = 0.43 ± 0.01, R_max = 1.03 ± 0.05, S₂₈/S₂₈ = 2.00 ± 0.14, and Kₐ = 17.8 ± 1.9. These values can then be used to convert the ratio (R) found in experiments to [Na⁺], (9).

Calibrations of intracellular SNARF signals were performed in a strongly buffered K⁺-based solution set to pH 5.0–9.0. The pH was set to the pH₉ with 10 µM nigericin, a proton and K⁺ ionophore. The mean values obtained (n = 9) were as follows: R_min = 0.21 ± 0.03, R_max = 1.17 ± 0.07, and S₂₈/S₂₈ = 0.58 ± 0.06. All SNARF ratios were converted to pH units with use of these constants.

A different in vitro calibration was used to convert extracellular SNARF signals to the pH₉. In three experiments, R_min = 0.14 ± 0.01, R_max = 1.97 ± 0.02, S₂₈/S₂₈ = 0.18 ± 0.01, and Kₐ = 7.40 ± 0.01.

Sensitivity of SBFI to acidosis. During ischemia the heart develops a substantial intracellular acidosis, and most ionic indicators are at least partly affected by acidosis. In a previous study, no significant effect of pH (6.5–7.0) on SBFI sensitivity was observed (32); in contrast, a large inhibitory effect of acidosis on the Kₐ of SBFI for Na⁺ was reported (14). We therefore reassessed this issue over a larger range of pH (6.0–7.5). This in vitro calibration was performed on the microscope stage under conditions similar to those used for the heart. Two solutions were prepared: 150 mM KCl, 1 mM MgCl₂, 5 mM HEPES, and 5 mM 2-(N-morpholinio)ethanesulfonic acid and the equivalent solution with 150 mM NaCl. The pH was adjusted to 6.0, 6.5, 7.0, or 7.5. These solutions were mixed in proportions to give 0, 10, 20, 50, and 150 mM Na⁺ with (Na⁺ + K⁺) = 150 mM at the four different pH levels. SBFI was added to each solution, and the 380-nm, 360-nm, and ratio signals were determined under each condition. The ratio was measured at 340 nm (S₂₈), which was significantly different from zero (P < 0.01, by repeated-measures ANOVA). However, Kₐ and S₂₈/S₂₈ showed significant changes as a function of pH (P < 0.01 and P = 0.4, respectively, by repeated-measures ANOVA). The slope of the linear regression of Kₐ against pH was −8.8 ± 2.7 mM/pH unit, which was significantly different from zero (P < 0.0005), whereas the slope of S₂₈/S₂₈ against pH was 0.24 ± 0.08 (P < 0.01). Because Kₐ decreases with increasing pH while S₂₈/S₂₈ increases, the apparent affinity (Kₐ · S₂₈/S₂₈) changes only modestly. The effect of pH on the relation between SBFI ratio and [Na⁺] is shown in Fig. 1C. For this purpose, the global mean of R_min and R_max was used, and the values of Kₐ and S₂₈/S₂₈ provided by the linear regression fit were used. These curves can be used to correct for the effect of pH on the SBFI ratio (see DISCUSSION).
Minimization of movement artifacts and autofluorescence correction. The heart was carefully located above the objective and moved in the horizontal plane to maximize the fluorescence signals and minimize any movement artifacts associated with contraction or ischemia.

An important component of autofluorescence arises from mitochondrial NADH, which fluoresces between 400 and 550 nm when illuminated at 340–380 nm. Thus for SBFI (illuminated at 340 and 380 nm) autofluorescence was substantial and changed in ischemia and anoxia, whereas for SNARF (illuminated at 540 nm) autofluorescence was negligible. In the experiments with SBFI the original fluorescence measurements were corrected for autofluorescence before calculation of the ratio. During ischemia and anoxia, additional correction was required, inasmuch as experiments on unloaded hearts showed that, during ischemia, autofluorescence increased by 75% at 340 nm and by 30% at 380 nm \( (n = 12) \). These changes developed on ischemia and declined on reperfusion, with a time constant of \( \tau = 2 \) min, and they were corrected with a function with the same properties. In anoxia, autofluorescence increased by 120% at 340 nm and by 70% at 380 nm \( (n = 6) \). Control experiments showed that the changes in autofluorescence during lactate and MIA exposures were not significant.

Distribution of SBFI. In a previous study we showed that saponin, which permeabilizes the surface membrane but not the mitochondrial membrane, released 69% of the SBFI fluorescence, suggesting that 31% of the SBFI was in the mitochondria \( (22) \). It is also possible that SBFI may load into endothelial cells, as has been shown to occur with indo1 when loaded into whole hearts in the AM form \( (21) \). To test this possibility, we made use of the fact that a brief exposure to detergent selectively damages the endothelial cells \( (17) \). In these experiments a pressure transducer was attached to the perfusate inflow to measure the perfusion pressure. We first established that endothelial cells could release a relaxing factor by measuring the fall in perfusion pressure on exposure to ACh in hearts pretreated with 9,11-dideoxy-9\( \alpha\),11\( \alpha\)-methanoepoxy prostaglandin \( (U-44069) \). Second, we perfused

**Table 1. Sensitivity of SBFI to Na\(^+\) as a function of pH**

<table>
<thead>
<tr>
<th>pH</th>
<th>6.0</th>
<th>6.5</th>
<th>7.0</th>
<th>7.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>( R_{\text{min}} )</td>
<td>1.28 ± 0.05</td>
<td>1.25 ± 0.04</td>
<td>1.31 ± 0.03</td>
<td>1.30 ± 0.01</td>
</tr>
<tr>
<td>( R_{\text{max}} )</td>
<td>3.67 ± 0.18</td>
<td>4.04 ± 0.21</td>
<td>3.83 ± 0.35</td>
<td>3.90 ± 0.23</td>
</tr>
<tr>
<td>( S_{\text{f2}}/S_{\text{b2}} )</td>
<td>1.17 ± 0.12</td>
<td>1.42 ± 0.06</td>
<td>1.31 ± 0.05</td>
<td>1.61 ± 0.09</td>
</tr>
<tr>
<td>( K_d )</td>
<td>33.1 ± 4.4</td>
<td>26.1 ± 1.8</td>
<td>25.5 ± 3.3</td>
<td>18.7 ± 2.6</td>
</tr>
</tbody>
</table>

Values are means ± SE for 4 determinations. \( R_{\text{min}}, \) ratio at 0 mM Na\(^+\); \( R_{\text{max}}, \) ratio at saturating Na\(^+\) concentration; \( S_{\text{f2}}, \) signal from 0 mM Na\(^+\) at 380 nm; \( S_{\text{b2}}, \) signal from 140 mM Na\(^+\) at 380 nm; \( K_d, \) apparent dissociation constant; SBFI, Na\(^+\)-binding benzofuran isophthalate.
the heart for 60 s with various concentrations of Triton X-100 added to the perfusate (0.01–0.1% by volume). Third, we established the lowest concentration of Triton X-100 that abolished the relaxing effect of ACh. This concentration was assumed to permeabilize the endothelial cells. Finally, we measured the fractional fall in SBFI fluorescence when the minimum permeabilizing concentration of Triton X-100 was perfused for 1 min into an SBFI-loaded heart.

Statistics. Statistical significance was tested with Student’s paired or unpaired t-test (unless otherwise stated), and P < 0.05 was accepted as statistically significant. Values are means ± SE of n hearts.

RESULTS

Distribution of SBFI in loaded hearts. These preliminary experiments were designed to measure the fraction of SBFI in endothelial cells as opposed to myocytes (see METHODS). Under our control conditions the perfusion pressure was 115 ± 12 mmHg, and this was increased by 22 ± 4 mmHg (n = 8) by perfusion with the prostaglandin (100 nM U-44069). In the prostaglandin-pretreated hearts, ACh (1 µM) caused a fall in perfusion pressure of 14 ± 3 mmHg, which could be repeated several times. The smallest concentration of Triton X-100 that when perfused for 1 min abolished this effect was 0.02%, and after this treatment, ACh caused an increase in perfused pressure of 3.5 ± 1 mmHg (n = 7). Four hearts were loaded with SBFI-AM in the usual way and then perfused with this concentration of Triton X-100 for 1 min. This caused a small fall in fluorescence at 340 and 380 nm. At 340 nm, which is relatively [Na⁺]i insensitive, this reduction was 17 ± 2%, suggesting that this fraction of the SBFI was located in the endothelial cells. This exposure to Triton X-100 also substantially reduced LVDP to 54 ± 7% of control, suggesting that ventricular myocytes were damaged or affected by the changed endothelial or capillary function, so these hearts were not used for any other purpose.

Changes in [Na⁺]i and pH during ischemia. Figure 2 shows the results of a representative experiment in which 10 min of ischemia were followed by reperfusion. LVDP decreased to very low levels within 2 min of the cessation of flow. After 5 min of global ischemia, a contracture became apparent and increased for the duration of ischemia. On reperfusion the contracture rapidly resolved, but then the pressure record showed low-amplitude oscillations, and on inspection the heart was fibrillating. Fibrillation ceased spontaneously after ~7 min, and normal spontaneous contractions returned, although only at ~30% of the control amplitude. The [Na⁺]i record shows a small decline during the 10 min of ischemia, but the most obvious feature is a large and rapid rise of [Na⁺]i, starting within 10 s of reperfusion. The [Na⁺]i reached a peak after 2 min and then declined toward control over 20 min. In nine hearts the control [Na⁺]i was 10.2 ± 0.7 mM and the change during 10 min of ischemia was insignificant (~0.3 ± 0.8 mM). On reperfusion, [Na⁺]i showed a large, transient increase of 8.2 ± 0.6 mM (P < 0.001), which returned to control levels within 20 min of reperfusion.

As noted in the introduction, the rise in [Na⁺]i, during short periods of ischemia is variable in different studies. One feature of our study is that we stimulated at a low rate (2 Hz), whereas in many studies the hearts were spontaneously active (heart rate ~5 Hz). We therefore performed a series of experiments in which the stimulation rate was raised from 2 to 5 Hz ~10 min before the period of ischemia. In this group of four hearts the control (2 Hz) [Na⁺]i was 7.2 ± 1.2 mM. At 5 Hz the [Na⁺]i increased significantly to 11.5 ± 1.3 mM (P < 0.05), as previously demonstrated in this preparation (22). Under these conditions, [Na⁺]i rose by 6.0 ± 0.8 mM (P < 0.005) after 10 min of ischemia. The [Na⁺]i measurements with SBFI are from cells within 100 µm of the surface (32); it is therefore possible that these cells receive some O₂ by diffusion from the oxygenated perfusate that surrounds the ischemic heart. To determine whether oxidative phosphorylation in these cells might alter the [Na⁺]i response, we performed three experiments in which 2 mM cyanide was added to the extracellular perfusate when ischemia was induced and one experiment in which 2 mM cyanide was added to the perfusate 1 min before ischemia. These experiments gave similar results. The control [Na⁺]i was 9.5 ± 0.8 mM, and the rise in [Na⁺]i after 10 min of ischemia was not significant (0.4 ± 0.3 mM).

It is well established that ischemic hearts develop an intracellular acidosis (Fig. 3). If the Na⁺/H⁺ exchanger were operational, extrusion of H⁺ during the ischemia would be expected to increase the [Na⁺]i. Thus the absence of a rise in [Na⁺]i during ischemia suggests that the Na⁺/H⁺ exchanger was inoperative at this time; conversely, the transient rise of [Na⁺]i, on reperfu-
tion would be compatible with operation of the exchanger during reperfusion (18). To test this hypothesis, the Na\(^+\)/H\(^+\) exchange inhibitor MIA was used. The dashed trace in Fig. 2 shows the [Na\(^+\)]\(_i\) from another heart after treatment with 10 µM MIA. The transient rise in [Na\(^+\)]\(_i\) on reperfusion was greatly reduced under these conditions. In nine hearts treated with MIA the control [Na\(^+\)]\(_i\) after 10 min of exposure was 7.9 ± 1.1 mM, which was not significantly different from the concentration in untreated hearts. In the MIA-treated hearts there was no significant change in [Na\(^+\)]\(_i\) during ischemia, but on reperfusion [Na\(^+\)]\(_i\) increased by 2.5 ± 0.8 mM (P < 0.05), which was significantly smaller than the increase observed in the absence of MIA (8.2 ± 0.6 mM).

Figure 3 shows the LVDP and pH\(_i\) (continuous trace) in a rat heart during 5 min of ischemia and the subsequent recovery. After 5 min of ischemia, recovery of LVDP was complete, and it was therefore possible to directly compare the pH\(_i\) changes from two periods of ischemia in the same heart. In nine hearts the resting pH\(_i\) was 7.38 ± 0.03 pH units and pH\(_i\) declined during ischemia, giving an acidosis of 0.54 ± 0.07 pH unit after 5 min. On reperfusion, pH\(_i\) recovered to the control level (7.36 ± 0.04) within a few minutes. In a separate series of experiments (n = 8), the pH\(_i\) change during 10 min of ischemia was 1.0 ± 0.1 pH unit. To determine whether activity of the Na\(^+\)/H\(^+\) exchanger was modifying these pH\(_i\) changes, 10 µM MIA was applied. MIA produced a slowly developing acidosis of ~0.02 pH U/min, suggesting that the Na\(^+\)/H\(^+\) exchanger operates at a slow rate under control (2-Hz stimulation) conditions. During the 5 min of ischemia in the presence of MIA, an intracellular acidosis of 0.45 ± 0.06 pH unit was produced, which is not significantly different from the control value. However, pH\(_i\) recovery on reperfusion was slowed in the presence of 10 µM MIA (dashed trace). On average, the initial rate of pH\(_i\) recovery after ischemia was 0.77 ± 0.16 pH unit/min (n = 9), whereas in the presence of MIA the initial rate of pH\(_i\) recovery was significantly reduced to 0.28 ± 0.07 pH unit/min (n = 5).

The fact that MIA had no significant effect on the magnitude of acidosis during ischemia again suggests that the Na\(^+\)/H\(^+\) exchanger was inactive during ischemia. Conversely, the slowing of the recovery of pH\(_i\) during reperfusion in the presence of MIA confirms earlier studies (33) showing that the Na\(^+\)/H\(^+\) exchanger is one of the routes for proton extrusion after ischemia.

Changes in pH\(_i\) and [Na\(^+\)]\(_i\) during lactate exposure. Ischemic hearts develop an intracellular acidosis mainly because of accumulation of lactic acid (8). To investigate the regulation of pH\(_i\) associated with acidosis, 20 mM sodium lactate was added to the perfusate. Lactic acid in the uncharged form rapidly enters the cells and has been shown to produce an intracellular acidosis of ~0.2 pH unit in isolated myocytes (2).

Figure 4 shows pH\(_i\) and LVDP measured simultaneously in one heart during 5 min of lactate exposure. Initially, there was a rapid fall of pressure with a decline of pH\(_i\). In the subsequent 5 min, there was a
slower recovery of LVDP and pHi with a similar time course. In 15 hearts, pHi fell by $0.16 \pm 0.01$ pH unit at the time when LVDP was maximally reduced to $22 \pm 4\%$. In the subsequent 5 min, during the maintained exposure to lactate, pHi recovered by $0.14 \pm 0.01$ pH unit and LVDP returned toward the control level. When lactate was washed off, pHi increased to a more alkaline value than the original control, reaching $7.54 \pm 0.04$; thereafter, pHi recovered toward the initial control values. The LVDP increased substantially, overshooting the original control, and then returned to control levels. Note the increase in arrhythmias associated with the sodium lactate exposure, which was particularly striking in this preparation.

This recovery of pHi during the lactate challenge suggests that a proton-regulating process has been active, such as the Na\textsuperscript{+}/H\textsuperscript{+} exchanger. The dashed trace demonstrates the effects of applying lactate in the same heart in the presence of 10 µM MIA. Under these conditions the rapid acidosis associated with sodium lactate application was unchanged ($0.16 \pm 0.01$ pH unit), whereas the recovery of pHi was reduced to $0.02 \pm 0.01$ pH unit. Thus pHi recovery was reduced to $\sim 15\%$ of control ($n = 12$) and the LVDP recovery was almost abolished. On washout of lactate, the alkalinization and the overshoot in the LVDP were largely abolished. These features indicate that the recovery of pHi and the alkalinization on sodium lactate removal involve the Na\textsuperscript{+}/H\textsuperscript{+} exchange, as previously demonstrated (34).

If the removal the intracellular H\textsuperscript{+} during sodium lactate exposure involves the Na\textsuperscript{+}/H\textsuperscript{+} exchanger, then there should be a coupled entry of Na\textsuperscript{+}, which should cause a rise in [Na\textsuperscript{+}]. (32). The effects of lactate exposure on [Na\textsuperscript{+}] are shown in Fig. 5. In six hearts the control [Na\textsuperscript{+}] was $8.3 \pm 1.0$ mM and [Na\textsuperscript{+}] increased by $3.1 \pm 0.9$ mM during lactate exposure. The time course of this rise paralleled the recovery of pressure. On washout of lactate, [Na\textsuperscript{+}] returned to its control value. In the presence of MIA, the [Na\textsuperscript{+}] rise was abolished (dashed trace), as was the recovery of pHi (Fig. 4). This experiment demonstrates that a sodium lactate exposure that produces a much smaller acidosis than that which occurs in ischemia (Fig. 3) is capable of producing a substantial and measurable rise in [Na\textsuperscript{+}], by operation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger.

Changes in pHo during ischemia. The reason Na\textsuperscript{+}/H\textsuperscript{+} exchange is not operative under ischemic condition is not clear. One mechanism, which was proposed by Lazdunski et al. (18), is inhibition of operation of the Na\textsuperscript{+}/H\textsuperscript{+} exchanger by the extracellular acidosis associated with H\textsuperscript{+} extrusion. During myocardial ischemia the pHo falls drastically, because protons can no longer be removed by the circulation. In contrast to this proposal, our earlier studies showed that lactate exposure in the presence of an extracellular acidosis of 1 pH unit (pH 6.4) stimulated, rather than inhibited, the exchanger (2, 32). However, it is possible that the extracellular acidosis during ischemia might be $>1$ pH unit, in which case the Na\textsuperscript{+}/H\textsuperscript{+} exchange might be inhibited. To examine this possibility, we measured the magnitude of the pHo change during ischemia.

Figure 6 shows pHo and LVDP in one heart during 10 min of ischemia. In five hearts the resting pHo was $7.36 \pm 0.03$ and during the ischemia it fell by $0.82 \pm 0.04$ pH unit. Thus the extracellular acidosis of 1.0 pH unit (32) used in our earlier studies (2) adequately simulated the ischemic situation. It follows that the...
pH changes during myocardial ischemia are not sufficient to inhibit Na\(^+\)/H\(^+\) exchange. This suggests that some other changes that occur during ischemia, such as changing metabolite levels, might be the cause of the inhibition of the exchanger.

Changes in [Na\(^+\)] and pH during anoxia. To simulate some of the metabolic changes that occur during ischemia, we used anoxia. Figure 7 shows the effects of anoxia on [Na\(^+\)] and LVDP. Reoxygenation in this heart caused a 12-min period of fibrillation, then a spontaneous return to normal rhythm. There was no obvious change in [Na\(^+\)] during the period of anoxia, but on reoxygenation there was a rapid rise in [Na\(^+\)], which recovered to control levels over ~10 min. In 13 hearts, resting [Na\(^+\)] was 8.8 ± 0.6 mM. During 10 min of anoxia, the [Na\(^+\)] did not change significantly, and at the end of 10 min, [Na\(^+\)] was 9.8 ± 0.9. On reperfusion, [Na\(^+\)] showed a transient rise of 1.7 ± 0.4 mM (P < 0.001), which was similar in time course, although smaller in amplitude, than that observed in the ischemia experiments. When we applied the Na\(^+\)/H\(^+\) exchange inhibitor MIA (10 µM), the transient rise was abolished, and there were no significant changes in [Na\(^+\)] at any stage in anoxia or reoxygenation. Because it is known that there is an intracellular acidosis during anoxia (6), this result strongly suggests that Na\(^+\)/H\(^+\) exchange was inhibited during anoxia but reactivates to extrude intracellular H\(^+\) during reoxygenation. The smaller acidosis during anoxia than during ischemia (6) is consistent with the smaller rise in [Na\(^+\)] on reoxygenation than on reperfusion.

To confirm this issue, we used sodium lactate exposures to investigate the activity of the Na\(^+\)/H\(^+\) exchanger during anoxia. Figure 8 shows the pressure and the control pH\(_i\) records (continuous trace) during sodium lactate exposure under control conditions and shows the features previously described in Fig. 4. Then the heart was perfused with N\(_2\), which caused a slowly developing acidosis of 0.08 ± 0.1 pH unit (n = 7) and a gradual fall in developed pressure. The pH\(_i\) record from the lactate exposure during N\(_2\) exposure shows a greatly reduced slow pH\(_i\) recovery, which is indicative of reduced Na\(^+\)/H\(^+\) exchange activity (Fig. 4). In seven hearts the immediate acidosis caused by lactate exposure was unaffected (0.13 ± 0.03 pH unit in O\(_2\) vs. 0.15 ± 0.04 pH unit in N\(_2\)). However, the subsequent slow recovery of pH\(_i\) fell from 0.11 ± 0.2 pH unit in O\(_2\) to 0.05 ± 0.02 pH unit in N\(_2\). Thus the pH\(_i\) recovery attributable to the Na\(^+\)/H\(^+\) exchanger fell from 87 ± 7% of the initial pH\(_i\) change over 5 min to 29 ± 6% (P < 0.02). This result shows that the pH buffering is not significantly affected by the small change in pH\(_i\), or by the metabolic changes associated with anoxia (33, 38) but that the activity of the exchanger is reduced by a factor of about three.

**DISCUSSION**

SBFI as an [Na\(^+\)] indicator in the perfused heart. SBFI has been widely used as an Na\(^+\) indicator in single cells and has proved a reliable and valuable indicator. Loading of the SBFI-AM into intact hearts involves the additional problem that the indicator may be hydrolyzed in other cell types, particularly the endothelial cells that lie between the capillaries and the myocytes. In the present study we have developed a
new method of assessing this problem, and this approach suggests that ~17% of the SBFI resides in the endothelial cells. This may be an overestimate, since the reduction in developed pressure suggested that some ventricular myocytes were also damaged during this procedure. Thus most of the fluorescent signal appears to derive from cells other than the endothelial cells, of which cardiac myocytes are by far the greatest in number and volume. This confirms the previous arguments that most of the SBFI fluorescence arises from cardiac myocytes on the basis of the observations that the [Na\(^+\)], the rise in [Na\(^+\)] during recovery from acidosis, and the increase in [Na\(^+\)] during stimulation are comparable, with equivalent measurements in isolated single ventricular myocytes (22, 32). An additional problem is that the signal we measure derives principally from the outermost 100 µm of the myocardium (32) and is therefore especially sensitive to [Na\(^+\)] in the epicardial myocytes.

In the present study we have explored the Na\(^+\) sensitivity of SBFI over a large range of pH and showed that SBFI shows a small reduction in sensitivity as pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases. How does correction for the probable effects of pH on SBFI affect our estimates of [Na\(^+\)] during ischemia? During 10 min of ischemia the pH decreases.

[Na\(^+\)] during ischemia. The present experiments show that under our standard conditions [Na\(^+\)] showed little change during 10 min of global ischemia. The published reports of changes in [Na\(^+\)], during ischemiapen, show considerable variation. For instance, measurements of total intracellular Na\(^+\) from myocardial biopsies showed 40% increase after 10 min (31). Measurements using Na\(^+\)-NMR are also variable, with some studies showing very large increases in [Na\(^+\)] (+230%) after 10 min of ischemia (25) or quite small increases (+20%) (23). Some measurements using Na\(^+\)-sensitive microelectrodes have shown a small (~20%) fall in [Na\(^+\)] during ischemia (16). Reasons for these differences have been discussed, but there is no consensus (12, 35). Our experiments performed at 5 Hz showed a higher initial [Na\(^+\)] and [Na\(^+\)] increased during ischemia by 6 mM, representing a 50% increase (or 80% after correction for the effect of acidosis). These values are in the middle of the range of reported [Na\(^+\)] increases. Interestingly, the decline of [Na\(^+\)] after 10 min of ischemia (16) was in a preparation stimulated at 1–2 Hz. Thus our results suggest that differences in the stimulation frequency contribute to the variation of reported rises of [Na\(^+\)] during ischemia.

Why does stimulation at a higher frequency cause [Na\(^+\)] to rise faster during ischemia? A likely explanation is that the metabolic status of the preparation is reduced by high-frequency stimulation (5). Consequently, the metabolic status during ischemia will reach lower levels more rapidly, and cellular functions dependent on metabolic status will fail earlier. The Na\(^+\) pump is inhibited by metabolic changes, particularly an increase in phosphate and ADP as well as decreases in ATP (1). Furthermore, the Na\(^+\) pump has been shown to turn over at a lower rate during ischemia, and this occurs simultaneously with the beginning of the rise in [Na\(^+\)]. (3). Thus we propose that the early rise in [Na\(^+\)], in preparations stimulated at physiological frequencies (5 Hz) is principally caused by metabolic inhibition of the Na\(^+\) pump.

Na\(^+\)/H\(^+\) exchange activity during ischemia and reperfusion. Our results show clearly that the Na\(^+\)/H\(^+\) exchanger was functionally inhibited during ischemia. The degree of intracellular acidosis induced by ischemia (1 pH unit in 10 min) would be expected to produce a clearly detectable increase in [Na\(^+\)] on the basis of the results with lactate application in perfused conditions, but none was observed. Furthermore, the [Na\(^+\)] and pH during ischemia were unaffected by MIA, again showing that the exchanger was not functional. The latter result confirms earlier reports (11, 23) showing that Na\(^+\)/H\(^+\) inhibitors did not affect the acidosis observed in ischemia. Conversely, the exchanger appeared to reactivate rapidly during reperfusion. The rise in [Na\(^+\)], was clear within 1 min of reperfusion and rose rapidly to peak after ~2–4 min, suggesting that the exchanger was substantially reactivated within 1 min of reperfusion. Much of the rise of [Na\(^+\)] results from Na\(^+\)/H\(^+\) exchange activity, since it was largely inhibited by MIA.

In contrast to our results, which suggest that the Na\(^+\)/H\(^+\) exchanger is inhibited during ischemia, are a number of results showing that the increase in [Na\(^+\)], or [Ca\(^+\)], observed during ischemia can be reduced by amiloride or its derivatives (23, 31), implying that the Na\(^+\)/H\(^+\) exchanger is active during ischemia. A possible alternative interpretation of these experiments is that the amiloride-like drugs are acting by inhibiting Na\(^+\) influx through Na\(^+\) channels (10).

Mechanism of inhibition of the Na\(^+\)/H\(^+\) exchanger during ischemia. In 1985, Lazdunski et al. (18) suggested that the Na\(^+\)/H\(^+\) exchanger would be inhibited during ischemia by the extracellular acidosis that develops. Although it is generally agreed that extracellular acidosis inhibits the Na\(^+\)/H\(^+\) exchanger (18, 34, 37), the situation during ischemia is considerably more complex. Specifically, there is a large intracellular acidosis, which activates the exchanger that is inhibited by the extracellular acidosis, which inhibits the exchanger. Our earlier data on single cells with [Ca\(^+\)], and pH, measurements (2) and with [Na\(^+\)] measurements in perfused hearts (32) suggested that, under the conditions of extracellular acidosis and intracellular acidosis likely to occur during ischemia, the exchanger would not be inhibited. This leads to the paradoxical situation that the exchanger is clearly inhibited during ischemia, but the evidence suggests that this is not
caused by extracellular acidosis. This conclusion is further supported by our observation that the Na\(^{+}/H\(^{+}\) exchange is also inhibited during anoxia when flow continues, and any extracellular acidosis would be expected to be very small. Thus the most likely conclusion is that some consequence of ischemia and anoxia other than extracellular acidosis causes the exchanger to be inhibited.

The present experiments give only limited clues as to the mechanism of inhibition of the exchanger. The cardiac Na\(^{+}/H\(^{+}\) exchanger is known to be activated by a range of neurohumoral agents (for review see Ref. 27). It seems instead that some metabolic or other change common to ischemia and anoxia inhibits the exchanger. Our finding that anoxia inhibits the Na\(^{+}/H\(^{+}\) exchange confirms a brief report on isolated guinea pig ventricular myocytes (7). However, a subsequent study from the same laboratory reported that cyanide had only a small effect on the pH changes caused by the exchanger, and they attributed these to a concomitant change in the intracellular buffering. Thus they concluded that the activity of the Na\(^{+}/H\(^{+}\) exchanger was unaffected by inhibition of oxidative phosphorylation. A number of studies (37, 38) have shown that deoxyglucose leads to inhibition of the Na\(^{+}/H\(^{+}\) exchange probably by reducing the intracellular ATP concentration. This suggests a phosphorylation site on the Na\(^{+}/H\(^{+}\) exchanger, which is required for activity, but published data on the kinase(s) that might control this site are contradictory. In 1989, Weissberg et al. (37) found that calmodulin inhibitors reduced the activity of the exchanger, whereas Wu and Vaughan-J ones (38) found that calmodulin inhibitors were ineffective unless the activity of the exchanger had been inhibited with a phosphodiesterase inhibitor. Another possibility is that protein kinase C controls the phosphorylation site. Although there have been studies suggesting that protein kinase C is involved in \(\alpha\)-adrenergic stimulation of the Na\(^{+}/H\(^{+}\) exchanger (36), others have failed to find effects of phorbol esters on activity and believe that the cardiac isoform of the Na\(^{+}/H\(^{+}\) exchanger lacks sites suitable for phosphorylation by protein kinase C (27).

Even if we assume that there is a phosphorylation site on the cardiac Na\(^{+}/H\(^{+}\) exchanger that regulates activity, it is not clear that ATP could fall fast enough during ischemia to allow dephosphorylation of the site and inhibition of activity. Our data suggest that the exchanger is inhibited early during 10 min of ischemia, because there was no difference in the rate of fall of pH during ischemia in the presence or absence of an Na\(^{+}/H\(^{+}\) exchange inhibitor. Measurements of ATP in the rat heart during ischemia show that ATP has fallen to \(\sim\)40–50% over 10 min (8). In the first 5 min of ischemia, ATP concentrations are protected by consumption of phosphocreatine, so it seems doubtful whether changes in ATP could underlie the rapid reduction in activity of the exchanger that we observe. We conclude that the activity of the exchanger is clearly inhibited rapidly by ischemia or anoxia and is reactivated rapidly on reperfusion or reoxygenation, but the mechanism involved is not clear.

Significance of our findings for ischemia. One of the attractive features of the original hypothesis by Lazdunski et al. (18) was that it explained why the total Ca\(^{2+}\) was constant during ischemia but increased during reperfusion and why the rise in free [Ca\(^{2+}\)] was greater during reperfusion than during ischemia (20). Our new findings confirm that the Na\(^{+}/H\(^{+}\) exchanger is inhibited during ischemia and rapidly reacts on reperfusion but suggest that the mechanism of inhibition is not extracellular acidosis. Thus this attractive feature of the hypothesis of Lazdunski et al. is retained, but the underlying mechanism is different.

The importance of the rise in [Na\(^{+}\)], that we observe during reperfusion, but not during short periods of ischemia, is that it will cause increased Ca\(^{2+}\) influx on the Na\(^{+}/Ca\(^{2+}\) exchanger and lead to an elevated [Ca\(^{2+}\)]. If it is accepted that the rise in [Ca\(^{2+}\)] during reperfusion is one of the cytotoxic aspects of ischemia-reperfusion, then this provides a therapeutic approach to minimizing reperfusion damage. Of particular interest are a number of studies in which inhibitors of the Na\(^{+}/H\(^{+}\) exchanger provide some protection from reperfusion damage when administered before ischemia (13, 23; for review see Ref. 24). An important feature of our study is that it suggests that the time at which Na\(^{+}/H\(^{+}\) exchange inhibitors would offer the most protection is the moment of reperfusion, confirming some experimental studies (31).

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