Mechanisms of pHᵢ Recovery After Global Ischemia in the Perfused Heart

Jamie I. Vandenbeng, James C. Metcalfe, and Andrew A. Grace

A Na⁺-HCO₃⁻ coinfusion carrier and the Na⁺-H⁺ antiport have both been shown to contribute to recovery from intracellular acidosis in cardiac tissue. We have investigated the participation of these mechanisms as well as metabolite (lactate and CO₂) washout in the recovery of pHᵢ after myocardial ischemia. Isovolumic ferret hearts were Langendorff-perfused with either HCO₃⁻-buffered or nominally HCO₃⁻-free (HEPES-buffered) medium at 30°C. pHᵢ was estimated from the chemical shift of the 3¹H-nuclear magnetic resonance signal of intracellular PO₄⁻₃ and net H⁺ efflux rates were calculated at pH 6.80. The H⁺ efflux rate during reperfusion, after 10 minutes of global ischemia, was 15.5±1.9 mmol·1⁻¹·min⁻¹ (n=10) in hearts perfused with HCO₃⁻-buffered medium and 8.2±1.5 mmol·1⁻¹·min⁻¹ (n=9, p<0.01) in hearts perfused with HEPES-buffered medium. HCO₃⁻ influx, assessed either by inhibition by 4,4'-diisothioanostilbene-2,2'-disulfonic acid (20 μM) or by initially perfusing hearts with HEPES-buffered medium but reperfusing with HCO₃⁻-buffered medium, accounted for 3.5-4.9 mmol·1⁻¹·min⁻¹, and CO₂ efflux accounted for 3.8-6.2 mmol·1⁻¹·min⁻¹ of the additional H⁺ efflux in HCO₃⁻-buffered medium. H⁺-coupled lactate efflux, measured by NAD⁺-linked spectrophotometric assay and inhibited by the sarcolemmal monocarboxylate transport inhibitor 4,4'-dibenzamidostilbene-2,2'-disulfonate (0.25 mM), contributed 3.7-6.2 mmol·1⁻¹·min⁻¹. H⁺ efflux via the 5-(N-ethyl-N-isopropyl)amiloride-sensitive Na⁺-H⁺ antiport was 1.0-2.9 mmol·1⁻¹·min⁻¹. pHᵢ recovery after ischemia is therefore principally mediated by metabolite (lactate and CO₂) washout. Na⁺-coupled acid extrusion contributed approximately 35% of total H⁺ efflux in this system. However, the associated Na⁺ entry (=5 mmol·1⁻¹·min⁻¹) may contribute to Ca²⁺ overload after reperfusion. (Circulation Research 1993;72:993–1003)

KEY WORDS  •  pHᵢ  •  ischemia  •  reperfusion  •  nuclear magnetic resonance spectroscopy

The deleterious effects of ischemia on the mechanical and electrical activity of the heart are well described.¹⁻³ These effects are reversible if reperfusion is initiated within a few minutes; however, it may take several days before normal function is regained.⁴⁻⁶ Although the role of intracellular acidosis in ischemic contractile failure has been extensively studied,³⁻⁶,⁷ the mechanisms of pHᵢ recovery after reperfusion and the relation between pHᵢ recovery and restoration of cardiac contraction have not been established.

Regulation of pHᵢ in cardiac tissue is mediated by intracellular buffers and transmembrane ion flux processes, including the Cl⁻-HCO₃⁻ exchanger,⁸ the Na⁺-H⁺ antiport,⁹ and a recently described acid extrusion mechanism that is both Na⁺ and HCO₃⁻ dependent.¹⁰⁻¹² However, the regulation of pHᵢ during ischemia and reperfusion may not be mediated by the same mechanisms as in well-perfused cardiac preparations.¹⁴ For example, the hydrolysis of phosphocreatine (PCr) and accumulation of inorganic phosphate (Pi) will alter the buffering capacity of the heart,¹⁴,¹⁵ and the accumulation of CO₂ and lactate may, when washed out during reperfusion, contribute to pHᵢ recovery.¹⁶⁻²⁰

In this study, we report on the mechanisms of pHᵢ recovery after global ischemia in the Langendorff-perfused ferret heart and correlate the activity of these mechanisms with recovery of cardiac contraction. The role of H⁺-coupled lactate efflux has been estimated by assaying lactate in the myocardial effluent during reperfusion and by using 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS) to inhibit lactate efflux.²⁰⁻²² The contribution of the Na⁺-H⁺ antiport has been assessed using the high-affinity Na⁺-H⁺ antiport inhibitor 5-(N-ethyl-N-isopropyl)amiloride (EIPA).²³ The role of HCO₃⁻-dependent mechanisms has been investigated by comparing pHᵢ recovery after ischemia in hearts perfused with nominally HCO₃⁻-free medium with recovery in hearts perfused with HCO₃⁻-buffered medium and by using 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) as an inhibitor of HCO₃⁻ transport mechanisms.²⁴ From these protocols, the relative contributions of metabolite washout (lactate and CO₂) and Na⁺-coupled acid extrusion (Na⁺-H⁺ exchange and Na⁺-HCO₃⁻ coinfusion) to the recovery of pHᵢ after global ischemia have been estimated, and the relation between recovery of pHᵢ and contractile function is considered.

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### Materials and Methods

#### Heart Preparation

The preparation and monitoring of Langendorff-perfused ferret hearts have been described previously. Briefly, male ferrets (n=43), 3–12 months of age, were anesthetized with pentobarbital (250 mg·kg⁻¹ i.p.) and heparinized (2,000 units i.p.). The heart was excised and immediately arrested in ice-cold HCO₃⁻-buffered medium (see below). The aorta was cannulated, and perfusion was commenced with HCO₃⁻-buffered medium at 30°C. Perfusion was at constant flow (5–6 ml·g⁻¹·min⁻¹) using a peristaltic pump (Watson-Marlow 502S) without recirculation of the perfusate at any stage. This resulted in an initial perfusion pressure of 50–90 mm Hg; perfusion pressure was monitored with a pressure transducer (Statham P23XL) connected via polyethylene tubing to a side arm on the aortic cannula. Isovolumic left ventricular developed pressure (LVPD, calculated by subtracting end-diastolic from systolic pressure) was monitored using a saline-filled latex balloon inserted in the left ventricular cavity. The initial left ventricular end-diastolic pressure was adjusted to 5–10 mm Hg. Pressures were recorded on a four-channel chart recorder (Gould 2400S), and LVPD was calculated by subtracting end-diastolic pressure from systolic pressure. The atrioventricular node was crushed to abolish atrioventricular conduction. Agar-filled polyethylene electrodes were sutured to the right ventricular epicardium, and hearts were paced at 1.0 Hz by use of square-wave stimuli of 10-msec duration at twice threshold voltage.

#### Solutions

The two perfusion media used were as follows: 1) HCO₃⁻-buffered medium containing (mM) NaCl 119, NaHCO₃ 25, KCl 4, KH₂PO₄ 1.2, MgCl₂ 1.0, CaCl₂ 1.8, and glucose 10, equilibrated with 95% O₂-5% CO₂ at 30°C (pH 7.42–7.44), and 2) nominally HCO₃⁻-free HEPES-buffered medium containing (mM) NaHCO₃ 0, HEPES 5, and sodium gluconate 22, equilibrated with 100% O₂ and pH-adjusted to 7.42–7.44 at 30°C with 2N NaOH. Sodium gluconate was added to HEPES-buffered media to maintain chloride and sodium at the same concentrations in both perfusion media. All perfusion media reagents were analytical reagent grade.

EIPA (a gift from Hoechst AG, Germany) was dissolved in dimethyl sulfoxide, 2-mM stock concentration, and added to the perfusate to achieve a final concentration of 1 μM. In the Langendorff-perfused ferret heart, this dose of EIPA reduces flux through the Na⁺-H⁺ antiport by >90%.[13] DIDS (Sigma Chemical Co., Poole, UK) was added directly to media to a final concentration of 20 μM, a dose that has previously been estimated to reduce flux via HCO₃⁻-dependent acid extrusion mechanisms by 70–80%.[13] DBDS (a generous gift from Dr. Robert Poole, Department of Biochemistry, University of Bristol, UK) was dissolved in dimethyl sulfoxide: methanol (4:1), 50-mM stock concentration, and added to the perfusate to a final concentration of 0.25 mM. This concentration of DBDS causes >10-fold reduction in the initial rate of transport of l-lactate (0.5 mM) across the erythrocyte plasma membrane[21] and 50–80% inhibition of lactate transport across the cardiac sarcolemma of rat and guinea pig ventricular myocytes.[22] The perfusion media and pharmacological agents used to assess the relative contributions of CO₂ efflux, lactate efflux, Na⁺-H⁺ exchange, and HCO₃⁻ influx to net H⁺ efflux after reperfusion are summarized in Table 1.

Hearts were perfused initially with HCO₃⁻-buffered medium for 30–60 minutes until LVPD was stable. Perfusion was then switched to either HEPES-buffered or HCO₃⁻-buffered P₃-free medium (KH₂PO₄ replaced with

### Table 1. Experimental Protocols

<table>
<thead>
<tr>
<th>Hearts perfused with HCO₃⁻-buffered medium</th>
<th>Lactate efflux</th>
<th>CO₂ efflux</th>
<th>HCO₃⁻ influx</th>
<th>Na⁺-H⁺ exchange</th>
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<tr>
<td>Control</td>
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<td>+</td>
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<tr>
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<tr>
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<td>+DBDS+EIPA (after DIDS)</td>
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<td>+</td>
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<th>CO₂ efflux</th>
<th>HCO₃⁻ influx</th>
<th>Na⁺-H⁺ exchange</th>
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<tbody>
<tr>
<td>Control</td>
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<tr>
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</tr>
<tr>
<td>+EIPA</td>
<td>+</td>
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<tr>
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<thead>
<tr>
<th>Hearts perfused with HEPES- or HCO₃⁻-buffered medium before ischemia and reperfused with HCO₃⁻ or HEPES-buffered medium, respectively</th>
<th>Lactate efflux</th>
<th>CO₂ efflux</th>
<th>HCO₃⁻ influx</th>
<th>Na⁺-H⁺ exchange</th>
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<tr>
<td>HCO₃⁻ to HEPES</td>
<td>+</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>HEPES to HCO₃⁻</td>
<td>+</td>
<td>-</td>
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</table>

DBDS, 4,4'-dibenzamidostilbene-2,2'-disulfonate; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; DIDS, 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid; +, active; –, inactive; ±, partially active.

*DBDS (0.25 mM) inhibits sarcolemmal monocarboxylate transport.[21,22]
†EIPA (1 μM) inhibits Na⁺-H⁺ exchange.[23]
§DIDS (20 μM) inhibits anion exchangers including HCO₃⁻ influx mechanisms.[24]
an equimolar concentration of KCl) and allowed to equilibrate for a further 30 minutes before experimental interventions. In control hearts, perfusion with P₄-free medium for up to 4 hours had no significant effect on LVDP, pH₁, PCR, or ATP levels. During pharmacological interventions, hearts were equilibrated with perfusion medium containing the relevant drug for 2–10 minutes before ischemia and for the first 5 minutes of reperfusion. The time required for changes of perfusion media was determined with methylene orange and took ~1.5 minutes to complete.

**Ischemia Procedure**

Hearts were made globally ischemic by switching off the peristaltic pump and clamping the perfusion inflow line for 10 minutes. Circulation of water through the water jacket and probe head was continued during ischemia to ensure that the temperature of the heart was maintained at 30°C. A second ischemic episode was induced, 60–90 minutes after the first episode, only in those hearts in which metabolic and functional parameters had returned to preischemic values within 60 minutes.26 In a separate series of experiments, hearts were perfused in the probe head outside the magnet and made ischemic using the above procedure, except that immediately before reperfusion the heart was removed from the probe head so that the myocardial effluent could be collected during the first 2 minutes of reperfusion. Effluents were collected in Eppendorf tubes and frozen immediately, or perchloric acid was added at a final concentration of ~1 M to precipitate DBDS, which is insoluble at low pH. After centrifugation, excess perchlorate was precipitated by neutralizing the supernatant with K₂CO₃. Supernatants from the neutralized samples were stored at ~20°C, and all samples were later analyzed for lactate by an NAD⁺-linked spectrophotometric assay.28 Phosphate content of effluents was analyzed by a nuclear magnetic resonance (NMR) spectroscopic assay.29

**NMR Spectroscopy**

In well-perfused hearts, the signal from intracellular Pᵢ is very small, making it an insensitive indicator for measurement of pH₁.3 However, during ischemia and the first 1.5–2 minutes of reperfusion, the accumulated intracellular Pᵢ permits rapid sequential estimation of pH₁ from the chemical shift between the PCR and Pᵢ resonances. pH₁ values were obtained from the chemical shift of the Pᵢ resonance using a standard calibration curve.30

Hearts were housed in a purpose-built dual-tuned (¹H/²H) probe head31 and placed in a Bruker AM400 wide-bore spectrometer. ³¹P-NMR spectra were recorded at 162 MHz with 40° pulses delivered at 0.9-second intervals. Control spectra, acquired 5 minutes before ischemia, were averaged from 300 transients. During ischemia and the first 2.5 minutes of reperfusion, 16–32 transients were averaged for each spectrum. For pH₁ measurements, NMR spectra were processed with a gaussian broadening factor of 0.02 and a line broadening of ~40 Hz to enhance resolution. For assessment of bioenergetic status, NMR spectra were processed with a line broadening of 20 Hz. Relative phosphate metabolite concentrations were determined from the areas of the ³¹P-NMR resonances after correcting for the differential saturation of resonances due to incomplete relaxation of magnetization at high pulsing frequencies.25 Saturation factors (mean±SEM) were determined from eight experiments by comparing areas of resonances in fully relaxed spectra obtained using an interpulse delay of 15 seconds with the corresponding areas when the interpulse delay was 0.9 seconds. The saturation factors used were as follows: Pᵢ, 1.3±0.3; PCR, 1.4±0.1; and β-ATP, 1.1±0.2.

**Data Analysis**

The net proton efflux rate (Jₜₜ) during reperfusion was calculated (in millimoles per liter per minute) by the following formula:

\[ Jₜₜ = \beta_{\text{tot}} \cdot \frac{\text{d}pH/\text{dt}}{ \text{d}t} \]

where \( \beta_{\text{tot}} \) is total H⁺ buffering capacity. We have previously estimated the intrinsic H⁺ buffering capacity of the Langendorff-perfused ferret heart to be 37–42 mmol·1⁻¹ at pH 6.85–6.95 and the buffering capacity due to HCO₃⁻/CO₂ (\( \beta_{\text{tot}} \)) to be ~14 mmol·1⁻¹.13 During ischemia and reperfusion, the hydration and resynthesis of PCR will also contribute to H⁺ buffering, which we have estimated to be ~8 mmol·1⁻¹, assuming 80–90% hydration of 15–20 mmol PCR and a pKₐ for Pᵢ of 6.8.3,24 Therefore, the values for \( \beta_{\text{tot}} \) used in this study were 62 mmol·1⁻¹ for hearts perfused with HCO₃⁻-buffered medium and 48 mmol·1⁻¹ for hearts perfused with HEPES-buffered medium. Values for dPₐH/dt were calculated from the slope of a linear least-squares regression line fitted to pH₁ recovery data. All results are quoted as mean±SEM. Statistical comparisons use the two-tailed unpaired Student’s t test,32 with significance being taken at \( p<0.05 \).

**Results**

**Effects of 10 Minutes of Ischemia**

Ischemia leads to rapid cessation of oxidative phosphorylation. The concentration of ATP is initially maintained by a compensatory acceleration of anaerobic glycolysis and hydrolysis of PCR, accompanied by accumulation of Pᵢ (see Figure 1a). After reperfusion, PCR was resynthesized, reaching 90% of the preischemic value within 1.5 minutes, and there was a corresponding decrease in Pᵢ to preischemic levels in 1.5–2 minutes. The changes in phosphate metabolite concentrations during ischemia and reperfusion were similar in all hearts described in this study and are illustrated for hearts perfused with HCO₃⁻-buffered medium in Figure 2a.

The chemical shift of the Pᵢ resonance (control position indicated by dashed line, Figure 1a) indicates the fall in pH₁ during 10 minutes of ischemia and its subsequent recovery during reperfusion. The corresponding time course of change in pH₁ obtained from the spectra illustrated in Figure 1a is shown in Figure 1b. In hearts perfused with HCO₃⁻-buffered medium, there was no significant change in pH₁, from the control value of 7.15±0.02 (n=10) during the first 2 minutes of ischemia; thereafter, there was a monotonic fall in pH₁ to 6.73±0.03 (see Figure 2b). During the first 15 seconds of reperfusion, there was no significant recovery of pH₁. Subsequently, pH₁ increased approximately linearly (dashed line, Figure 2b) at a rate of 0.25±0.03 pH units·min⁻¹. The rate of pH₁ recovery appeared to slow after the first 1–1.5 minutes, but accurate pH₁ determination during this period was not
FIGURE 1. Effects of 10 minutes of ischemia and 5 minutes of reperfusion with HCO$_3$-buffered medium at 30°C on pH$_i$ and left ventricular developed pressure (LVDP) in the Langendorff-perfused ferret heart. Panel a: Serial $^{31}$P nuclear magnetic resonance spectra obtained at the times indicated. The peaks in the spectra correspond to (from left to right) intracellular inorganic phosphate (P$_i$; dashed line at 5.03 ppm indicates a pH$_i$ of 7.17), phosphocreatine, and the $\gamma$, $\alpha$, and $\beta$-phosphates of ATP. Spectra are averages of 300 transients (preischemia and +5 minutes of reperfusion) or 16–32 transients (during ischemia and the first 2.5 minutes of reperfusion). Panel b: Plot of pH$_i$ during 10 minutes of ischemia and the first 5 minutes of reperfusion; measurements were obtained from the chemical shift of the P$_i$ peak. Panel c: LVDP recording during the first 5 minutes of ischemia and first 5 minutes of reperfusion.

always possible because of the small P$_i$ signal. Measurements of pH$_i$ from spectra acquired 2.5–7.5 minutes after reperfusion indicated that pH$_i$ had returned to preischemic levels in all hearts.

During the first minute of ischemia, LVDP declined to $-50\%$ of the preischemic value and then declined more slowly, with contraction ceasing after $\approx5$ minutes (see Figure 1c). After reperfusion, there was an initially rapid partial recovery of LVDP to $80\%$ after 2 minutes. LVDP subsequently recovered more slowly and did not return to the preischemic value for 30–60 minutes (see Figure 2c).

pH$_i$, Recovery After Reperfusion

Contribution of lactate efflux. Lactate efflux during the first 2 minutes in hearts perfused outside the magnet

![Figure 1](image1.png)

![Figure 2](image2.png)
(see “Materials and Methods”) was 5.4±1.0 μmol·g wet wt⁻¹ (n=5) in hearts reperfused with HCO₃⁻-buffered medium and 4.1±0.5 μmol·g wet wt⁻¹ (n=5) in hearts reperfused with HEPES-buffered medium. These values represent total lactate efflux, i.e., lactate that has accumulated in the extracellular space during ischemia in addition to lactate leaving cells during reperfusion. In hearts perfused with HEPES-buffered medium, the presence of 0.25 mM DBDS reduced lactate efflux after reperfusion by 45% during the first 30 seconds and by 65–70% during the subsequent 60 seconds (see Figure 3). Perfusion of hearts placed in the NMR spectrometer with either HCO₃⁻-buffered or HEPES-buffered medium containing DBDS also caused a slowing of pHᵢ recovery (see Figure 4 and Table 2), consistent with H⁺-coupled lactate efflux contributing to acid extrusion after reperfusion.

**Contribution of Na⁺-H⁺ antiport.** To test whether the residual pHᵢ recovery seen in hearts perfused with HEPES-buffered medium with lactate efflux inhibited by DBDS was due to Na⁺-H⁺ exchange, hearts were perfused with medium containing 0.25 mM DBDS and 1 μM EIPA for 2 minutes before ischemia and the first 5 minutes of reperfusion. A typical series of NMR spectra obtained from a heart during blockade of the Na⁺-H⁺ antiport and inhibition of lactate efflux is illustrated in Figure 5. During ischemia, PCr decreased and subsequently recovered during reperfusion with corresponding changes in Pᵢ (compare with Figure 1a). The Pᵢ resonance shift during ischemia indicated a fall in pHᵢ from 6.98 (dashed line in Figure 5) to 6.59. During reperfusion, the chemical shift of the Pᵢ resonance did not alter, indicating that pHᵢ had not recovered in this experiment. Simultaneous inhibition of lactate efflux and Na⁺-H⁺ exchange slowed pHᵢ recovery from 0.17±0.02 pH units·min⁻¹ (hearts perfused with HEPES-buffered medium) to 0.03±0.02 pH units·min⁻¹ (see Figure 6 and Table 2). The contribution of the Na⁺-H⁺ antiport to recovery of pHᵢ with lactate efflux mechanisms intact was assessed by adding EIPA to HCO₃⁻- or HEPES-buffered medium for 2 minutes before ischemia and during the first 5 minutes of reperfusion. EIPA (1 μM) caused a small reduction in the rates of pHᵢ recovery under both conditions (hearts perfused with HCO₃⁻-buffered medium, 0.1<p<0.2; hearts perfused with HEPES-buffered medium, 0.05<p<0.1; see Table 2).

**Contribution of external HCO₃⁻-dependent mechanisms.** In three experiments, hearts were perfused with HCO₃⁻-buffered medium containing 20 μM DIDS for 2–10 minutes before ischemia and during 5 minutes of reperfusion. In the presence of DIDS, pHᵢ fell from 7.07±0.03 to 6.64±0.04 during ischemia, and after reperfusion, pHᵢ recovered at an initial rate of 0.17±0.03 pH units·min⁻¹ (p<0.05 compared with initial rate of pHᵢ recovery in the absence of DIDS). In a second series of experiments, five hearts were perfused with HEPES-buffered medium before ischemia but reperfused with HCO₃⁻-buffered medium. After 10 minutes of ischemia in HEPES-buffered medium, there should be minimal accumulation of CO₂ and therefore minimal CO₂ efflux after reperfusion. However, reperfusion with HCO₃⁻-buffered medium will permit HCO₃⁻ influx to contribute to pHᵢ recovery. By use of this protocol, illustrated in Figure 7, pHᵢ fell from 6.98±0.03 to 6.64±0.02 during ischemia. Reperfusion with HCO₃⁻-buffered medium caused an immediate further decrease in pHᵢ to 6.62±0.02, consistent with an initial influx of CO₂, before pHᵢ recovered at 0.19±0.02 pH units·min⁻¹ (see Table 2).

**Contribution of CO₂ efflux.** The contribution of CO₂ efflux was assessed by the inhibition of lactate efflux by use of DBDS, Na⁺-H⁺ exchange by use of EIPA, and HCO₃⁻ influx by preperfusion with 20 μM DIDS for 30 minutes in hearts perfused with HCO₃⁻-buffered medium. The iso-thiocyanate group on DIDS reacts with free amino groups, such that DIDS will precipitate with EIPA if they are mixed in solution. DIDS binds covalently to HCO₃⁻ transporters, causing irreversible inhibition of HCO₃⁻ transport. Simultaneous inhibition of HCO₃⁻ transport and Na⁺-H⁺ exchange can therefore be achieved by preperfusion of hearts with DIDS, followed by addition of EIPA or amiloride. During simultaneous inhibition of lactate efflux, Na⁺-H⁺ exchange, and HCO₃⁻ influx, pHᵢ recovery after reperfusion was reduced to 0.11±0.03 pH units·min⁻¹ (p<0.05 compared with hearts perfused with HCO₃⁻-buffered medium).

An indirect estimate of the potential contribution of CO₂ efflux to pHᵢ recovery was also obtained using the reverse perfusion sequence to that described above for estimating HCO₃⁻ influx. That is, hearts were perfused with HCO₃⁻-buffered medium before ischemia but reper-

**FIGURE 3.** Bar graph showing the effect of 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS, 0.25 mM) on lactate efflux during the first 90 seconds of reperfusion in hearts perfused with HEPES-buffered medium (n=4, both groups).

**FIGURE 4.** Graph showing the effect of 4,4'-dibenzamidostilbene-2,2'-disulfonate (DBDS, 0.25 mM) on pHᵢ recovery during the first minute of reperfusion in hearts perfused with HCO₃⁻ (BICARB)-buffered (alone [ ] and with DBDS []) or HEPES-buffered (alone [ ] and with DBDS [ ]) medium.
TABLE 2. Changes in pH During Ischemia and Reperfusion

<table>
<thead>
<tr>
<th>Perfusion medium</th>
<th>$n$</th>
<th>Preischemic pH$_i$</th>
<th>End-ischemic pH$_i$</th>
<th>dpH/dt (at pH 6.80)</th>
<th>$J_{H_+}$ (at pH 6.80)</th>
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<tr>
<td>HCO$_3^-$</td>
<td>10</td>
<td>7.15±0.02</td>
<td>6.73±0.04</td>
<td>0.25±0.02</td>
<td>15.5±1.4</td>
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<td>HCO$_3^-$+DBDS</td>
<td>4</td>
<td>7.13±0.01</td>
<td>6.75±0.05</td>
<td>0.19±0.02</td>
<td>11.8±1.2</td>
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<td>HCO$_3^-$+EIPA</td>
<td>5</td>
<td>7.12±0.01</td>
<td>6.77±0.01</td>
<td>0.20±0.03</td>
<td>12.7±1.8</td>
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<td>HCO$_3^-$+DIDS</td>
<td>3</td>
<td>7.07±0.03*</td>
<td>6.64±0.04</td>
<td>0.17±0.03</td>
<td>10.6±1.9</td>
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<td>HCO$_3^-$+DBDS+EIPA</td>
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<td>7.09±0.02</td>
<td>6.72±0.02</td>
<td>0.11±0.03*</td>
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<td>Hearts reperfused with HEPES-buffered medium</td>
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<tr>
<td>HEPES</td>
<td>9</td>
<td>7.07±0.02</td>
<td>6.64±0.03</td>
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<td>7.09±0.01</td>
<td>6.67±0.03</td>
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<td>7</td>
<td>6.98±0.02*</td>
<td>6.62±0.02</td>
<td>0.13±0.02</td>
<td>6.2±0.9</td>
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<tr>
<td>HEPES+EIPA+DBDS</td>
<td>5</td>
<td>6.97±0.02*</td>
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<td>0.03±0.02†</td>
<td>1.4±1.0*</td>
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<td>Hearts perfused with HEPES- or HCO$_3^-$-buffered medium before ischemia and reperfused with HCO$_3^-$ or HEPES-buffered medium, respectively</td>
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<tr>
<td>HCO$_3^-$ to HEPES‡</td>
<td>4</td>
<td>7.15±0.02</td>
<td>6.70±0.03</td>
<td>0.25±0.02</td>
<td>12±1.0</td>
</tr>
<tr>
<td>HEPES to HCO$_3^-$§</td>
<td>5</td>
<td>6.98±0.04</td>
<td>6.64±0.02</td>
<td>0.19±0.02</td>
<td>12±1.1</td>
</tr>
</tbody>
</table>

$J_{H_+}$, net H$^+$ efflux rate; DBDS, 4,4'-dibenzenamidostilbene-2,2'-disulfonate; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; DIDS, 4,4'-disothiocyanostilbene-2,2'-disulfonic acid. Values are mean±SEM. $J_{H_+}$ was calculated by multiplying the measured dpH/dt by the calculated total H$^+$ buffering capacity (see text for discussion).

*p<0.05 compared with control in each group (Student’s unpaired t test).

†pH$_i$ did not recover to 6.80 during the first 2 minutes of reperfusion; therefore, the dpH/dt reported is that calculated during 1-2 minutes of reperfusion.

‡For these groups, total H$^+$ buffering capacity was assumed to be the same as that in hearts perfused with HEPES-buffered medium (see text for discussion).

§For these groups, total H$^+$ buffering capacity was assumed to be the same as that in hearts perfused with HCO$_3^-$-buffered medium (see text for discussion).

fused with HEPES-buffered medium, which prevented HCO$_3^-$ influx during reperfusion but still permitted CO$_2$ efflux. In this series of experiments, pH$i$ fell from 7.15±0.02 to 6.70±0.03 (n=4) during ischemia and subsequently recovered at 0.25±0.03 pH units · min$^{-1}$ after reperfusion with HEPES-buffered medium.

H$_2$PO$_4^-$ efflux. To test the hypothesis that Na$^+$-H$_2$PO$_4^-$ efflux contributes to net H$^+$ efflux during reperfusion, P$_i$ was assayed in effluents. In control hearts (n=5) equilibrated with P$_i$-free buffer for 60 minutes, P$_i$ efflux was <0.1 μmol · g wet wt$^{-1} ·$ min$^{-1}$. P$_i$ in effluent collected during the first 2 minutes of reperfusion in hearts ranged from <0.1 μmol · g wet wt$^{-1}$ (n=5) to 0.3 μmol · g wet wt$^{-1}$.


**FIGURE 7.** Time course of averaged changes in pH; in five hearts perfused with HEPES-buffered medium before ischemia followed by reperfusion with HCO$_3^-$-buffered medium. This protocol was used to estimate the contribution of HCO$_3^-$ influx to the recovery of pH, (see text for details).

$\mu$mol·g wet wt$^{-1}$. The corresponding H$^+$ efflux rate of $<0.26$ mmol·l$^{-1}$·min$^{-1}$ is small compared with the contributions from the other mechanisms (see below).

Relative Contributions of Lactate Efflux, Na$^+$-H$^+$ Exchange, HCO$_3^-$ Influx, and CO$_2$ Efflux to pH Recovery

The changes in pH$\alpha$ during ischemia and reperfusion for all hearts included in this study are shown in Table 2. Where possible, net H$^+$ efflux rates have been calculated (as described in "Materials and Methods") at a pH of 6.80 rather than at the pH immediately after reperfusion. Although the values for buffering capacity were obtained at slightly higher pH$\alpha$, there should not be a significant discrepancy over this small range of pH$\alpha$ values. The H$^+$ efflux rates are shown in the final column of Table 2. The relative contributions of the different acid extrusion mechanisms to net H$^+$ efflux were estimated by comparing the differences in H$^+$ efflux rates in the presence or absence of inhibitors of each mechanism. CO$_2$ and lactate efflux each accounted for 30–50% of net H$^+$ efflux at pH 6.80, whereas Na$^+$-H$^+$ exchange and HCO$_3^-$ influx each contributed 10–30% (see Table 2).

Recovery of LVDP After Reperfusion

During the first 5 minutes of reperfusion, LVDP recovered to 30–80% of the preischemic LVDP (see Figure 8 and Table 3). The most striking differences occurred between hearts perfused with HCO$_3^-$-buffered medium (±EIPA or DBDS) and those perfused with HEPES-buffered medium (±EIPA or DBDS). For example, in HCO$_3^-$-buffered medium, LVDP recovered to 79±3% (n=10) after 2 minutes of reperfusion compared with 32±2% (n=9) at the same time point in HEPES-buffered medium.

In hearts perfused with HEPES-buffered medium in the presence of DBDS and EIPA, there was a significant recovery of LVDP to 29±2% (n=5) of the preischemic value after 2 minutes of reperfusion, in the absence of any significant recovery of pH$\alpha$ (see Figure 6). During the same period, however, there was recovery of PCr and P$_i$ to preischemic levels (see Figure 5). LVDP did not reach preischemic levels during the first 5 minutes of reperfusion in any experimental group, despite the recovery of pH$\alpha$ and restoration of PCr and P$_i$ to preischemic levels during this time. Full recovery of LVDP took 30–90 minutes.

**Discussion**

Metabolic Effects of Ischemia

In the experiments reported here, [ATP], remained unaltered from the preischemic value after 10 minutes of global ischemia. Therefore, it is very likely that the hearts have suffered only mild and reversible damage (see Reference 2 for review). In addition, there was no splitting of the P$_i$ resonance observed in these experiments, which suggests that there was no substantial inhomogeneity with respect to pH$\alpha$, within the globally ischemic myocardium, a phenomenon that has been observed after more prolonged periods of ischemia.

**pH$\alpha$ During Ischemia and Reperfusion**

During the first 1–2 minutes of ischemia, pH$\alpha$ remained unaltered from the preischemic pH$\alpha$ in all experiments before falling =0.4 pH units after 10 minutes of ischemia. This pH$\alpha$, fall is less than has been reported from in vivo studies and in isolated hearts perfused at 37°C (0.6–1.0 pH units after 10 minutes). This may be explained by the...
slower rates of ATP utilization and anaerobic glycolysis during ischemia at 30°C. Most groups have seen a monophasic fall in pH during early ischemia, but others have noted that the fall in pH is slower in the first minute than subsequently, and during hypoxia a transient alkalosis has been described. The discrepancy in these observations has been attributed to differences in the relative rates of proton production by ATP hydrolysis and breakdown of Pcr, which absorbs protons and produces P1, which increases the buffering capacity of the heart.

**Buffering Capacity After Reperfusion**

To compare H+ efflux rates under different perfusion conditions, it is necessary to consider both the rate of pH recovery as well as the buffering capacity. The value for βmetr (62 mM in hearts perfused with HCO3−-buffered medium) was obtained from the sum of intrinsic buffering capacity (=40 mmol·l−1), βCO2 (=14 mmol·l−1), and biochemical buffering due primarily to accumulation of P1 (=8 mmol·l−1). The βmetr value used in our study is similar to that obtained by Wolfe et al, (=58 mmol·l−1 between pH 6.4 and 6.9) in the ischemic rat myocardium. These values were derived from hearts exposed to an acid load (i.e., either using an NH4Cl prepulse or lactic acid accumulation during ischemia). During reperfusion, however, the ability of the heart to resist a rise in pH will not necessarily be the same as its ability to resist a fall in pH.

Intrinsic buffering capacity primarily reflects the presence of histidyl residues of intracellular proteins. We have assumed that after a brief episode of ischemia this has not changed, and so we used the value derived from the well-perfused ferret heart, i.e., 40 mM. During reperfusion, the ability of intracellular CO2/HCO3− to resist a rise in pH, (i.e., via the dissociation of H2CO3 to H+ and HCO3−) is dependent on the presence of a constant extracellular CO2. This is similar to the situation in the well-perfused heart, and so we have used the value derived during normal perfusion, i.e., 14 mM. This possibly represents an underestimate of the βCO2 during reperfusion, because the [HCO3−] in the heart at end ischemia is likely to be higher at a given pH than the value in the well-perfused heart (see discussion in Reference 15). Nevertheless, our estimate of the contribution of CO2/HCO3− (23% of βmetr) is similar to that estimated by Wolfe et al during ischemia in the rat heart (21% at pH 6.9). The [HCO3−], estimated in cardiac tissue superfused with HEPES-buffered medium is 6.9. At this concentration, βCO2 would be small (<1.2 mM); therefore, we have ignored this value in our estimate of βmetr in hearts reperfused with HEPES-buffered medium. The contribution of Pcr hydrolysis and P1 accumulation was based on estimates of concentrations of these metabolites at the time of reperfusion and shortly thereafter. Because we did not find any significant differences in phosphate levels in hearts perfused with different media, we used the same value for all hearts. During ischemia, lactate will contribute to βmetr and its contribution will be greater than would be suggested by the low PK of lactate (~3.8), because lactate is distributed in the intracellular as well as extracellular space. However, during reperfusion the ability of lactate to resist increases in pH would be dependent on the presence of a constant extracellular lactate, which is not the case; therefore, its contribution to βmetr will be negligible during reperfusion.

**Recovery of pH, After Reperfusion**

**Contribution of lactate efflux**. Conversion of lactate efflux (in micromoles per gram wet weight) to a corresponding H+ efflux (in millimoles per liter intracellular fluid) was calculated by assuming that the stoichiometry of H+–lactate efflux is 1:1 and that the intracellular fluid volume is 0.43 ml·g wet wt−1. We have also taken into account that total lactate efflux includes intracellular...
lactate that is washed out during reperfusion as well as lactate that accumulated in the extracellular space during ischemia, which Wolfe et al.\(^{36}\) measured to be 15–50% of total lactate efflux. In hearts perfused with HCO\(_3^-\) -buffered medium, lactate efflux during the first 2 minutes corresponded to a net H\(^+\) efflux of 6.4–10.8 mmol·l\(^{-1}\) intracellular fluid\(^{-1}\), and in hearts perfused with HEPES-buffered medium, the value was 4.8–8.2 mmol·l\(^{-1}\) intracellular fluid\(^{-1}\). If the rate of lactate efflux was constant during this time, then the corresponding H\(^+\) efflux rates would be =4.3 and =3.3 mmol·l\(^{-1}\)·min\(^{-1}\) in hearts perfused with HCO\(_3^-\) - and HEPES-buffered media, respectively. The data presented in Figure 3 suggest that the rate of lactate efflux was probably constant for the first minute of reperfusion but subsequently decreased. The above estimates of the contribution of H\(^+\)-coupled lactate efflux to pH\(_r\) recovery (3.3–4.3 mmol·l\(^{-1}\)·min\(^{-1}\)) are therefore probably underestimates.

Lactate efflux is inhibited by a number of compounds, including cinnamic acid derivatives such as α-cyano-4-hydroxycinnamate\(^{39,36}\) and stilbene derivatives such as DBDS.\(^{1,22}\) DBDS does not cross the sarcolemmal membrane and inhibiting has the advantage that it will not affect mitochondrial monocarboxylate uptake while inhibiting lactate efflux.\(^{22}\) In our experiments, DBDS (0.25 mM) reduced lactate efflux by 45% during the first 30 seconds and 65–70% during the subsequent 60 seconds after reperfusion. The lactate assayed in the first 30 seconds will include lactate washed out from both the extracellular and intracellular spaces. Although DBDS will slow the rate of lactate efflux during ischemia over a 10-minute period, one would still anticipate a substantial accumulation in the extracellular space. Therefore, it is likely that the latter values (65–70%) are a better estimate of the extent of inhibition of lactate efflux by 0.25 mM DBDS after reperfusion. This is intermediate between the values estimated by Wang et al.\(^{22}\) for inhibition of sarcolemmal lactate transport by DBDS in rat and guinea pig ventricular myocytes (50% and 80%, respectively). Incomplete inhibition of lactate efflux by DBDS could be due to simple diffusion of undissociated lactic acid or to the presence of other lactate transporters that are not inhibited by DBDS, as appears to be the case in the cardiac sarcolemma of rats and guinea pig.\(^{22}\) The contribution of lactate efflux to pH\(_{recovery}\) is therefore likely to be =80% of net H\(^+\) efflux in hearts reperfused with HEPES-buffered medium and =40% in hearts reperfused with HCO\(_3^-\)-buffered medium.

**Contribution of Na\(^+\)-H\(^+\) antiport.** EIPA (1 μM) slowed net H\(^+\) efflux by =2 mmol·l\(^{-1}\)·min\(^{-1}\). This dose of EIPA has been shown to cause significant inhibition of the Na\(^+\)-H\(^+\) antiport in the ferret heart after acid loading using the NH\(_4\)Cl prepulse technique.\(^{15}\) Furthermore, in hearts perfused with HEPES-buffered medium containing DBDS to inhibit lactate efflux, blockade of the Na\(^+\)-H\(^+\) antiport almost completely abolished pH\(_r\) recovery (see Figure 7). The residual pH\(_r\) recovery under these conditions may be explained in a number of ways. First, there is likely to have been incomplete inhibition of lactate efflux (as discussed above). Second, oxidation of lactate trapped within the cell would also remove protons. Third, although these hearts are nominally HCO\(_3^-\) free in cardiac tissue perfused with HEPES-buffered medium, [HCO\(_3^-\)] has been estimated to be =0.5 mM\(^{26}\) and if all this HCO\(_3^-\) reacted with H\(^+\) and was then washed out as CO\(_2\) during the first minute of reperfusion, it would contribute 0.5 mmol·l\(^{-1}\)·min\(^{-1}\) to total net H\(^+\) efflux.

**HCO\(_3^-\)-Dependent pH recovery.** In hearts perfused with HCO\(_3^-\)-buffered medium, pH\(_r\) recovery was significantly faster than in hearts perfused with HEPES-buffered medium (p < 0.01). The faster rate of lactate efflux in hearts perfused with HCO\(_3^-\)-buffered medium can account for only 10–15% of the additional net H\(^+\) efflux compared with hearts perfused with HEPES-buffered medium, which suggests that CO\(_2\) efflux and/or HCO\(_3^-\) influx contribute to pH\(_r\) recovery. It is important to separate the relative contributions made by CO\(_2\) efflux and HCO\(_3^-\) influx, because the HCO\(_3^-\)-influx mechanism is Na\(^+\) dependent\(^{1,13}\) and thus may contribute to Na\(^+\) overload after reperfusion.\(^{39}\)

Inhibition of HCO\(_3^-\) efflux by use of DIDS (20 μM) reduced net H\(^+\) efflux by 4.9 mmol·l\(^{-1}\)·min\(^{-1}\). However, DIDS also partially inhibits lactate transport\(^{21,22}\); therefore, some of the effect of DIDS on net H\(^+\) efflux may have been mediated by inhibition of lactate efflux. When hearts were preperfused with DIDS and then perfused with EIPA and DBDS, pH\(_r\) recovery after reperfusion, which may be attributed predominantly to CO\(_2\) efflux, was 6.2±2.6 mmol·l\(^{-1}\)·min\(^{-1}\). However, this value is likely to be an overestimate because of the incomplete inhibition of other H\(^+\) extrusion mechanisms (see above).

Estimates of HCO\(_3^-\) influx and CO\(_2\) efflux were also obtained from hearts perfused with either HEPES or HCO\(_3^-\)-buffered medium before ischemia but reperfused with HCO\(_3^-\)- and HEPES-buffered media, respectively (see Figure 7 and Tables 1 and 2). These estimates are associated with uncertainties that are due to the time taken to change perfusion media and also to the additional acid load or washout caused by changes in PC\(_O_2\) when switching between HEPES- and HCO\(_3^-\)-buffered media.\(^{12,40}\) This latter problem has to an extent been taken into account by including the presence or absence of CO\(_2\)/HCO\(_3^-\) during reperfusion in the calculation of pH\(_r\). For example, in hearts reperfused with HCO\(_3^-\)-buffered medium, CO\(_2\) influx at the point of reperfusion would resist rises in pH\(_r\), thereby increasing the effective buffering capacity of the heart. Estimates of the relative contributions of CO\(_2\) efflux and HCO\(_3^-\) influx from the different protocols discussed above (3.5–6.2 and 3.5–4.9 mmol·l\(^{-1}\)·min\(^{-1}\), respectively) would suggest that CO\(_2\) efflux and HCO\(_3^-\) influx are of similar importance, each contributing 20–25% to total H\(^+\) efflux.

Under the conditions of our experiments, pH\(_r\) recovery after a brief episode of ischemia is principally mediated by washout of metabolites, specifically lactate and CO\(_2\) (see Figure 9), as suggested by Bailey et al.\(^{17}\) However, we have also shown that Na\(^+\)-H\(^+\) exchange and HCO\(_3^-\) influx may contribute as much as 20–35% of net H\(^+\) efflux. At 30°C, the rate of H\(^+\) efflux is slower than at 37°C (data not shown), but in four experiments performed at 37°C, the relative rates of H\(^+\) efflux in hearts perfused with HEPES- or HCO\(_3^-\)-buffered medium were similar to those at 30°C. Therefore, it is likely that at 37°C the relative contributions to H\(^+\) efflux of the four mechanisms described above will be similar to that in our study. Extrapolating our results to reperfusion after prolonged ischemia is more difficult. For example, ATP depletion may have differential effects on the
active H+ extrusion mechanisms (Na+-H+ exchange and Na+-HCO3 coinfux) compared with passive H+ extrusion mechanisms (lactate and CO2 efflux).

Ischemic Contractile Failure

LVDP has been shown in many studies to be determined by multiple factors,29,42 including pH4,42 In our experiments, the fall in LVDP during the first 1–2 minutes occurred before there was a significant fall in pH, (Figures 1 and 2). The accumulation of P, during this initial period (Figure 2a) could probably account for 20–30% of the observed fall in LVDP.2,28 In the isovolumic Langendorff-perfused heart, there is evidence to suggest that vascular collapse contributes to early contractile failure during ischemia41; this could explain the fall in LVDP seen during the first 30–60 seconds of ischemia in our experiments. Our results are therefore consistent with the hypothesis of Lee and Allen3 that the sequence of events leading to ischemic contractile failure is vascular collapse followed by P, accumulation, with a significant contribution from pH, only occurring later.

Recovery of LVDP After Reperfusion

In hearts perfused with HEPES-buffered medium with lactate efflux and Na+-H+ exchange inhibited, there was partial recovery of LVDP despite minimal pH recovery (see Figure 9). This demonstrates that factors other than pH, such as vascular refilling51 and decreasing P,29 also contribute to LVDP recovery. In hearts perfused with HCO3-,buffered medium (±EIPA, DIDS, and DBDS), there was an initially rapid recovery of LVDP (reaching >60% of the preischemic level within 2 minutes in all groups), which suggests that pH, recovery due to CO2 efflux contributes to the initial recovery of LVDP. It is difficult to estimate the possible contributions of other acid extrusion mechanisms to the initial recovery of LVDP from our data. For example, EIPA caused a slowing of LVDP recovery in hearts perfused with HEPES-buffered medium but augmented the initial recovery of LVDP in hearts perfused with HCO3-,buffered medium. Conversely, inhibition of lactate efflux augmented the initial recovery of LVDP in hearts perfused with HEPES-buffered medium but slowed recovery of LVDP in hearts perfused with HCO3-,buffered medium. Simultaneous measurement of [Na+] and [Ca2+] in addition to pH and P, will be required to determine the relative contributions of these mechanisms to the recovery of LVDP during reperfusion.

In hearts under all experimental conditions, recovery of LVDP was never complete before 30 minutes of reperfusion, despite the recovery of pH, and high-energy phosphates after 5 minutes. This phenomenon, termed stunning, has been extensively studied.2 In 1985, Lazdunski et al3 hypothesized that, during reperfusion, activation of the Na+-H+ antiport would lead to Na+ overload and consequently Ca2+ overload, the putative mediator of stunning (see Reference 5 for review). Although our results show that H+ efflux during reperfusion is principally mediated via lactate and CO2 efflux, there is a contribution from Na+-coupled net H+ efflux (Na+-HCO3 coinfux and Na+-H+ exchange). A small increase in [Na+], will lead to a significant rise in [Ca2+], because of the 3:1 Na+:+Ca2+ stoichiometry of the Na+-Ca2+ exchanger44 unless there is a compensatory increase in Na+-H efflux via Na+-K+-ATPase. In our experiments, Na+ influx, coupled to acid extrusion, during reperfusion was ≈5 mmol·l-1·min-1 (sum of Na+-H+ exchange and Na+-HCO3 coinflux). During normal perfusion, Na+ efflux via Na+-K+-ATPase is proportional to [Na+], and will rise from 0.4 mmol·l-1·min-1 (at a control value for [Na+] of ≈6 mM) to 4 mmol·l-1·min-1 at [Na+] of ≈12 mM (calculated using the computer program heart, version 3.6, Oxsoft Ltd., Oxford, UK*). Pike et al38 showed that [Na+] increases by ≈100% after 10 minutes of ischemia in the Langendorff-perfused ferret heart, which would be consistent with an increase from ≈6 to ≈12 mM. Assuming that Na+-K+-ATPase has not been inhibited, then these rough calculations would suggest that after reperfusion the myocyte may be able to extrude Na+ as rapidly as it is entering. However, even if Na+ influx linked to acid extrusion does not contribute to a further rise in [Na+], it will cause [Ca2+] to remain elevated during early reperfusion.

Many previous studies have shown that inhibitors of Na+-H+ exchange have beneficial effects on the recovery of cardiac contractility and normalization of [Ca2+], after myocardial ischemia (e.g., see References 46–48), thus suggesting that Na+-coupled acid extrusion may contribute significantly to [Ca2+] overload after reperfusion. Although no studies have specifically looked at the effects of inhibition of Na+-HCO3 influx on the recovery of contractile function and normalization of [Ca2+], after reperfusion, our results suggest that this may be a more significant route for Na+ entry after reperfusion. Unfortunately, DIDS would not be an appropriate inhibitor to use in such studies, because it has nonspecific deleterious effects on contraction if used in high doses for long periods.13

Conclusions

After brief episodes of ischemia, the recovery of pH, is principally mediated by metabolite washout, i.e., lactate and CO2. Nevertheless, Na+-coupled acid extrusion does contribute to net H+ efflux, with Na+-HCO3- coinfux likely to be more important than Na+-H+ exchange. The associated Na+ entry may contribute to a
rise in [Na⁺], and therefore Ca²⁺ overload via Na⁺-Ca²⁺ exchange after repuffusion.

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