Intracellular pH Regulation in Ferret Ventricular Muscle

The Role of Na-H Exchange and the Influence of Metabolic Substrates

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Aspects of pH regulation in ferret ventricular cells have been investigated by using pH- and sodium-selective microelectrodes in bicarbonate-free Tyrode's solution. An acid load was produced by the transient application of NH₄Cl (10 or 20 mmol/l). A complete recovery from an acid load was still observed after multiple applications of NH₄Cl, but amiloride (0.75 or 1 mmol/l), a blocker of the Na-H exchanger, increased the acidification and inhibited the recovery. Measurements of intracellular sodium concentration showed a transient decrease during the application of NH₄Cl and a transient increase above control values during recovery from acidification. This increase was inhibited by amiloride. Intracellular sodium loading (strophantidin [low calcium–low potassium Tyrode's solution]) did not initially cause an intracellular pH (pHᵢ) change, but the acidification induced by amiloride under those circumstances was larger. Reducing extracellular sodium concentration from 155 to 5 or to 1.5 mmol/l caused an acidification. Changing extracellular pH (pHₑ) from 6.4 to 8.4 caused an average linear change in pHᵢ in the same direction of 0.085 pH units/pHₑ units. The mean intracellular buffering capacity measured with the NH₄Cl method and with the proton extrusion mechanism blocked by amiloride was 36±15 mmol pH⁻¹·l⁻¹ (mean±SD), approximately half that of previous estimations. Changing the metabolic substrate from glucose to pyruvate in the superfusing solution caused an acidification of 0.21 pH units. This could be partially blocked by α-cyano-4-hydroxycinnamate, a finding consistent with a pyruvate-H⁺ cotransport and/or a pyruvate-OH⁻ countertransport system being present in ventricular cells. The results of the present study show that ventricular cells can effectively buffer hydrogen ions and that an Na-H exchange system plays a major role in the regulation of pHᵢ. (Circulation Research 1991;68:150–161)

Hydrogen ions play an important role in the regulation of cardiac function; however, they are not passively distributed. To maintain an intracellular pH (pHᵢ) level within narrow limits, both a proton extrusion mechanism and an effective intracellular buffering system are required. In animal cells, at least three exchange mechanisms have been described, namely the NaHCO₃:Cl⁻, the Cl⁻:HCO₃⁻, and the Na-H exchange systems (see References 2–4 for reviews), and this latter system plays a major role in the regulation of pHᵢ and in the recovery from an acid load (see References 5–7 for reviews). Although regulation of pHᵢ has been studied embryonic chick cells,8–10 Purkinje fibers,11–13 and isolated canine sarcolemmal vesicles,14 there have been few detailed studies in ventricular myocardium.1,15 Ventricular muscle cells also possess a monocarboxylate transport system that causes a net transport of hydrogen ions.15 Because of this net transport, it is to be expected that the metabolic substrate will also influence the pHᵢ in the myocardium.

The aim of this study was to characterize not only the role of the Na-H exchanger in pH regulation and to measure the intracellular buffering capacity but also to study the influence of the metabolic substrate on pHᵢ in ventricular myocardium. Sodium- and pH-sensitive microelectrodes were used, and to eliminate possible HCO₃⁻ exchange systems, the experiments were carried out in HEPES-buffered HCO₃⁻-free Tyrode's solution.

The results of the present study provide evidence that the Na-H exchange system plays an important role in the regulation of pHᵢ in ventricular cells. In

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Supported by Swiss National Science Foundation grant 3.210-0.85.

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Received December 16, 1988; accepted August 27, 1990.
the estimation of the buffer capacity, when allowance is made for proton extrusion, the value obtained is about half that of previous estimations in ventricular muscle. In cardiac cells, as well as a Na-H exchanger there is also a transmembrane Na-Ca exchanger; in all probability, calcium and hydrogen ions share common intracellular binding sites, making the regulation of pH_{i} and intracellular concentrations of calcium ([Ca]_{i}) and sodium ([Na]_{i}) interdependent. The contributions of these systems are discussed.

Pyruvate-containing Tyrode’s solution causes an intracellular acidification in comparison with a glucose-containing or a substrate-free solution. Since α-cyano-4-hydroxycinnamate (CIN) markedly reduced the acidification caused by pyruvate, it is concluded that the pyruvate uptake is carrier mediated and coupled to a cotransport of H^{+} and/or countertransport of OH^{-} ions.

Materials and Methods

General Methods

The preparation, mounting, tension measurement, and electrical recording from the ferret ventricular muscle as well as the characteristics of the perfusing system have been previously described in detail. Only a brief description including recent improvements to the method will be given here. Right ventricular trabeculae and small papillary muscles of adult ferrets were mounted in a rapid perfusion chamber, where muscle tension, membrane potential, and the potential of the ion-selective microelectrodes could be measured. The chamber was grounded over a 3 mol/l KCl agar bridge placed downstream from the preparation. A built-in pH electrode (lot 402-611, obtained from Dr. W. Ingold AG, Urdorf, Switzerland) allowed continuous monitoring of the pH of the superfusion fluid in the bath.

Temperature

Because small fluctuations in temperature can cause spurious changes in the pHi signal (L.A. Blatter and J.A.S. McGuigan, unpublished observations; see also Reference 18), accurate temperature control of the calibration chamber and the experimental chamber was essential, both being maintained at 25±0.5°C. In the calibration chamber, this was obtained by a circulating water jacket equipped with a thermostat. The temperature in the experimental chamber was controlled by passing the superfusion fluid over a Peltier device (model CP1.4-127-06L, Melcor, Trenton, N.J.). The temperature of the fluid was measured by a thermistor at the entrance to the experimental chamber. This temperature was used to control the Peltier device via a feedback circuit, developed by our electronic workshop, based on that described by Chabala et al.

Solutions

The compositions of the solutions were as follows (mmol/l): pyruvate-Tyrode solution: sodium 155, potassiurn 5, calcium 5.4, magnesium 0.5, chloride 164.5, pyruvate 5, and HEPES 5; 14 mmol/l potassium–Tyrode solution: pyruvate-Tyrode solution in which sodium was reduced by 9 and potassium was increased to 14; pH 6.4 pyruvate–Tyrode solution and pH 8.4 pyruvate–Tyrode solution: HEPES buffer was replaced by PIPES 5 or by HEPPS 5, respectively; glucose-Tyrode solution: glucose 5 replaced pyruvate 5; sodium-poor glucose solution: sodium 1.5, potassium 5, calcium 5.4, magnesium 0.5, chloride 171.5, HEPES 5, glucose 10, tetramethylammonium (TMA) 155; sodium-poor pyruvate solution: sodium 5, potassium 5, calcium 5.4, magnesium 0.5, chloride 164.5, HEPES 5, pyruvate 5, TMA 150; low potassium–low calcium Tyrode’s solution: sodium 155, potassium 0.65, calcium 0.2, magnesium 0.5, chloride 157.5, pyruvate 5, HEPES 5; NH_{4}Cl-containing Tyrode’s solution: when pyruvate-Tyrode solution contained NH_{4}Cl 10 or 20, NaCl was correspondingly reduced.

Amiloride (A 7410, Sigma Chemical Co., St. Louis) was added to the superfusion solutions to give a concentration of either 0.75 or 1 mmol/l. Strophanthidin (S-6626, Sigma) was added to pyruvate-Tyrode solution to give a concentration of 10 μmol/l or dissolved in alcohol to give a stock solution of 25 mmol/l and dissolved to give a final concentration of 50 μmol/l. This gave an alcohol concentration of 34 mmol/l.

All solutions were made from stock solutions. The potassium stock solution was a mixture of KCl and KOH; this allowed the solutions to be back-titrated to a pH of 7.4 with 1 mol/l HCl. They were gassed with pure oxygen.

Ion-Selective Microelectrodes

Fabrication. The procedure for manufacturing the single-barreled sodium- and pH-selective microelectrodes has been described previously. Sodium and pH microelectrodes were of the liquid membrane type and contained the neutral sodium carrier ETH 22720 (No. 71176, Fluka AG, Buchs, Switzerland) or the neutral proton carrier tri-n-dodecylamine21 (No. 95291, Fluka). This was initially equilibrated with 100% CO_{2} overnight. The tips of the electrodes were back-filled with these commercially available ion-selective cocktails. The shanks of the sodium-selective electrodes were filled with 100 mmol/l NaCl, and the electrodes were stored by immersing their tips in the same solution. The storage and filling solution for the pH electrodes contained 100 mmol/l NaCl, 7.8 mmol/l citric acid monohydrate, and 92.2 mmol/l trisodium citrate dihydrate at a pH of 6.22 This solution had a larger buffering capacity than the original back-filling solution of Ammann et al.

Calibration of the Electrodes

To minimize junction potentials produced by minor alterations in ionic strength and composition of the various solutions, calibration was carried out differentially between a 3 mol/l KCl microelectrode.
and the ion-selective microelectrode. The measured potential in pyruvate-Tyrode solution was defined as 0 mV. Setting the potential in pyruvate-Tyrode to zero negated any change in offset potentials between the calibration and experimental chambers.

**Calibration of the pH Microelectrodes**

**pH calibration: Primary standards.** The definition of pH as \(-\log a_{H}\), where \(a_{H}\) is the hydrogen ion activity, extends only to an ionic strength of 0.1 mol/l and assumes no change in the reference potential.\(^{23,24}\) However, mammalian physiological solutions have ionic strengths of approximately 0.15–0.17 mol/l, whereas the frequently used National Bureau of Standards (NBS) pH buffers have an ionic strength of 0.05–0.1 mol/l. Bates et al\(^{25}\) have extended the definition of pH as \(-\log a_{H}\) to an ionic strength of 0.16 mol/l, and in this study we used two such pH buffer solutions described by Bates et al to calibrate the pH electrodes. The first solution contained 0.005217 molal KH\(_2\)PO\(_4\), 0.018258 molal Na\(_2\)HPO\(_4\), and 0.1 molal NaCl, with a defined pH of 7.323 at 25°C. The second solution contained 0.01 molal HEPES, 0.02 molal NaHEPES, and 0.14 molal NaCl with a pH of 7.801 at 25°C. The chemicals, with the exception of HEPES and NaHEPES, were suprapure quality from Merck, Darmstadt, FRG.

These two buffer solutions were taken as the primary pH standards. They served to calibrate the pH macroelectrode (ROSS type 81-02, Orion Research Inc., Cambridge, Mass.) together with the pH meter (expandable ion analyzer EA490 with three-digit accuracy, Orion Research). Determination of pH in all superfusion solutions as well as of the secondary pH standards (see below) was based on these standards.

The difference in calibration between the NBS buffer solutions and the buffers described by Bates et al\(^{25}\) was determined by measuring the pH of the same Tyrode’s solution after calibrating the pH macroelectrode with either the NBS buffers or with Bates’s buffers. The average pH of Tyrode’s solution calibrated with Bates’s buffers (at 25°C) was 7.351, whereas in NBS buffers it was 7.306, indicating an average deviation by 0.045 pH units (six measurements).

**pH calibration: Secondary standards.** These calibration solutions with different pH values had an ionic background mimicking the intracellular milieu with concentrations as follows (mmol/l): potassium 141.5, sodium 14.6, and magnesium 0.5; these mean concentration values were previously measured in ferret ventricular muscle.\(^{26}\) To test the linearity of the pH microelectrode response in the expected pH range, three-point calibrations were carried out at pH 8 (2.9 in a few calibration experiments), 7.2, and 6.4. These calibrations (n=11 measurements) showed an excellent linear relation \((r=0.999)\) between measured potential and pH. In the actual intracellular experiments, because of this linear relation, only a two-point calibration was carried out using secondary standards of pH 6.8 and 8.0. These solutions had the following composition (mmol/l): pH 8.0: KCl 137.5, KOH 4, NaCl 14.6, MgCl\(_2\) 0.5, HEPPS 5; pH 6.8: KCl 133.5, KOH 8, MgCl\(_2\) 0.5, PIPES 5. The solutions were made by weighing the KCl, NaCl, and buffer substances. Both KCl and NaCl were suprapure quality (Merck). KOH (1 mol/l stock, Merck) and MgCl\(_2\) (1 mol/l stock, BDH, Poole, England) were pipetted. The actual pH of the two secondary pH standards was measured on the day of the intracellular pH measurements. The electrodes used in the experiments had an average slope of 56.1±3.8 mV/pH unit (mean±SD; n=77).

**Calibration of the Sodium Microelectrodes**

**Calibrating solutions.** The chemicals used for NaCl, KCl, KOH, and MgCl\(_2\) were the same as used for the secondary pH standards. The three calibrating solutions had the following composition (mmol/l): KCl 139, KOH 2.5, MgCl\(_2\) 0.5, HEPES 5, and NaCl 4, 10, or 20. pH was titrated to 7.15.

**Results**

**Intracellular pH and Sodium Activity at Rest in Ferret Ventricle Superfused With Pyruvate-Tyrode Solution**

Since the intracellular activities of hydrogen and sodium were measured using single-barreled ion-selective microelectrodes, the membrane potential had to be measured separately with a 3 mol/l KCl microelectrode. The criteria used to judge adequate impalement of both electrodes were similar to those previously described from this laboratory.\(^{26,27}\)

The results of the intracellular hydrogen ion activity and sodium concentration at rest are summarized in Table 1. The results are presented as mean±SD of pH and pNa, for as shown by Fry et al,\(^{28}\) this is the most accurate method to present the data.

**Influence of pHi on pH, and Hydrogen Ion Equilibrium Potential**

The effect of changes of extracellular pH (pH\(_e\)) on pH\(_i\) has been investigated by increasing or decreasing pH\(_e\) by 1 pH unit. The HEPES buffer was replaced by either PIPES (pH 6.4) or by HEPPS (pH 8.4), and a typical experiment of this kind is shown in Figure 1. As pH\(_e\) decreased, so did pH\(_i\), and vice versa. In all these experiments, pH\(_i\) reached a new steady-state value.

<table>
<thead>
<tr>
<th>pH</th>
<th>pNa</th>
<th>pH</th>
<th>No. of measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.56±3.1</td>
<td>1.873±0.071*</td>
<td>7 (n=5)</td>
<td></td>
</tr>
<tr>
<td>-78.3±6.4</td>
<td>7.26±0.16†</td>
<td>77 (n=50)</td>
<td></td>
</tr>
</tbody>
</table>

*Intracellular sodium concentration of 13.4 mmol/l.
†Intracellular hydrogen ion activity of 55.5 mmol/l.
The duration of exposure to Tyrode’s solution varied from 5 to 15 minutes at pH 6.4 and from 7 to 24 minutes at pH 8.4. As far as could be judged by eye, a steady state was reached in all but four of the 22 experiments, and in these four, a steady state had almost been reached. On returning to normal Tyrode’s solution, sufficient time was allowed so that the pH returned to its original value. The relation between pH in and pH returned to its original value. The relation between pH in solution, trough and pH was linear, with an average slope of 0.085 pH units change per one unit change of pH. This is similar to that found by Coray and McGuigan but less than that found by others in Purkinje fibers and rat myocytes and probably reflects an efficient pH regulation of ferret ventricular cells (buffer capacity combined with active extrusion of protons).

pHi and [Na], During and After Application of NH4Cl

Because changing pH in our experiments caused only minor changes in pH, use was made of the NH4Cl method to bring about larger changes in pH, to enable us to investigate the properties of the Na-H exchanger. The pH changes caused by NH4Cl exposure were first described by Boron and De Weer in squid axon; this method is convenient for applying an acid load to the preparation. As shown in Figure 2A, application of NH4Cl (10 or 20 mmol/l) caused an intracellular alkalization that slightly decreased during the exposure to NH4Cl and an acidification (acid load) after removal. This acidification slowly returned to the resting level found at the beginning of the experiment, which is the control value for this series of experiments.

The [Na] changes caused by the same intervention are shown in Figure 2B. During application of NH4Cl, [Na] decreased. On removal, the [Na] increased above the control level before returning to the normal resting value over a period of minutes.

The maximal changes in pH and [Na], during and after application of NH4Cl are tabulated in Table 2. In this table and in subsequent results, only mean values are given because we only want to emphasize general trends. The decrease and increase of [Na] and pH are in inverse relation to each other and are what would be expected from the Na-H exchange system. The relation between intracellular sodium and hydrogen ions was further investigated using amiloride, a known blocker of the exchange system.

Action of Amiloride During Acid Loading After NH4Cl Application

Amiloride has been shown to block the sarcolemmal Na-H exchange in a variety of tissues. The effect of amiloride on pH after NH4Cl removal is shown in Figure 2A, and its effect on [Na], is illustrated in Figure 2B. Amiloride (0.75 mmol/l) not only increased the degree of acidification (increase in intracellular aH of 90.4% compared with control level; n=8 measurements), but recovery was also markedly slowed. On removal of amiloride, pH returned to its original level. Figure 3 shows mean±SD from five experiments; the NH4Cl (20 mmol/l) pulse has been applied twice during the same impalement, once in the absence of amiloride and once in the presence of amiloride.

The average maximal increase in aH during application of amiloride was approximately double, and a new plateau value was reached later. Despite the presence of amiloride, some recovery from the acid load took place, but pH reached a new steady-state value at a much lower level than in normal Tyrode’s solution.

As seen in Figure 2B, amiloride (1 mmol/l) prevented the increase above the resting value for [Na] after NH4Cl removal; the average intracellular sodium remained below control values by −12.2% (n=4). When the preparation was again superfused with normal Tyrode’s solution there was a transient increase in [Na]. As seen from Figure 2A, during this phase there was a recovery from a low pH.

The application of amiloride alone (see Figures 5A and 5B) led to a small acidification. In 10 such experiments, the application of amiloride (0.75 mmol/l) for times ranging from 5 to 10 minutes, in which a quasi
steady state was reached, caused an average decrease of $pH_i$ of $-0.08$ at a control $pH_i$ of 7.28. This corresponds to an average increase of $a_{iH}$ of 20.8%.

The transmembrane hydrogen ion distribution depends on $pH_o$, being in thermodynamic equilibrium at a $pH_o$ of 8.7, and it might be expected that the effect of amiloride on $pH_i$ would be $pH_o$ dependent. Application of amiloride (1 mmol/l) at $pH_o$ of 8.7 was without effect, whereas in the same experiment, application at a $pH_o$ of 6.4 caused an acidification.

In some experiments, application of amiloride at $pH_o$ of 8.7 caused a slight intracellular alkalinization. Since the $pK$ of amiloride is 9.4, at a $pH$ of 8.7, an amount of approximately 17% is present in its deprotonated, uncharged, and biologically inactive form. The uncharged molecule could pass the cell membrane and, at a $pH_i$ of approximately 7.3, bind protons, which could lead to an intracellular alkalization.

**Effect of Alterations of the Transmembrane Sodium Gradient on $pH_i$**

*Low sodium solutions.* If the transmembrane sodium gradient is reversed by lowering the external sodium, an intracellular acidification is to be expect-

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**TABLE 2. Changes in Intracellular Sodium Concentration and Intracellular pH During and After Removal of $\text{NH}_4\text{Cl}$**

<table>
<thead>
<tr>
<th>[NH$_4$Cl]$_o$ (Control)</th>
<th>During exposure to NH$_4$Cl</th>
<th>After removal of NH$_4$Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$pH_i$</td>
<td>Max $\Delta pH_i$</td>
<td>Max $\Delta a_{iH}^+$ (%)</td>
</tr>
<tr>
<td></td>
<td>+0.15</td>
<td>-30.3</td>
</tr>
<tr>
<td></td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>20 mmol/l</td>
<td>+0.22</td>
<td>-38.5</td>
</tr>
<tr>
<td></td>
<td>(n=32)</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>[NH$_4$Cl]$_o$ (Control)</th>
<th>During exposure to NH$_4$Cl</th>
<th>After removal of NH$_4$Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 mmol/l</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$pNa_i$ (Control)</td>
<td>Max $\Delta pNa_i$</td>
<td>Max $\Delta [Na_i]$ (%)</td>
</tr>
<tr>
<td></td>
<td>+0.08</td>
<td>-16.8</td>
</tr>
<tr>
<td></td>
<td>(n=7)</td>
<td></td>
</tr>
<tr>
<td>20 mmol/l</td>
<td>+0.14</td>
<td>-27.7</td>
</tr>
<tr>
<td></td>
<td>(n=12)</td>
<td></td>
</tr>
</tbody>
</table>

$\Delta pH_i$, intracellular pH; Max $\Delta pH_i$, maximal changes in $pH_i$; Max $\Delta a_{iH}^+$, maximal changes in intracellular hydrogen ion activity; [NH$_4$Cl]$_o$, extracellular NH$_4$Cl concentration; pNa$_i$, logarithm of reciprocal of the intracellular sodium concentration; Max $\Delta p[Na_i]$, maximal changes in pNa$_i$; Max $\Delta [Na_i]$, maximal changes in intracellular sodium concentration.
ed.12,32 However, as we will show later, changing the metabolic substrate in the superfusing solution will also by itself cause a change in pH. To overcome this problem, either sodium pyruvate was maintained constant at 5 mmol/l, or glucose was used as the metabolic substrate, and the sodium was reduced to 1.5 mmol/l.

In the experiments in Figure 4A, glucose was the metabolic substrate, and the external sodium was reduced from 155 to 1.5 mmol/l, sodium being replaced by TMA chloride. Figure 4B shows the effect of reducing extracellular sodium concentration to 5 mmol/l with pyruvate as the substrate. The time of exposure to the low sodium solutions varied between 6 and 10 minutes, and the change in pH due to the low sodium solutions was measured at the end of the exposure. In glucose-Tyrode solution, there was an average decrease in pH of −0.12 (n=4) at a control pH of 7.47.

This relatively alkaline control value is due to using glucose as the metabolic substrate in the superfusion solution. In pyruvate-Tyrode solution, an acidification of −0.06 pH units (n=4) at a control pH of 7.35 was found.

Sodium loading. The transmembrane sodium gradient can also be decreased by experimental interventions that increase [Na], such as blocking the Na-K pump by cardiac glycosides or by rapid stimulation in a Tyrode’s solution containing 0.65 mmol/l potassium and 0.2 mmol/l calcium.33 Figure 5A compares the effect of amiloride before and after sodium loading with strophanthidin (10 μmol/l). Amiloride (0.75 mmol/l) caused only a small acidification. Application of strophanthidin (10 μmol/l) did not initially alter the resting pH, but the acidification on application of amiloride was markedly increased. (A longer exposure to 50 μmol/l strophanthidin did, however, cause a slight acidification.) In four measurements, the mean change in pH for amiloride alone was −0.11, starting from an average resting pH of 7.21. After strophanthidin application, the mean change in pH was −0.18 (at a control pH of 7.28). This would correspond to a percentage increase of a[12]H of 27.4% and 50.1%, respectively.

Similar effects were found when [Na] was increased by rapid stimulation in the low calcium–low potassium solution. The effects are shown in Figure 5B where amiloride by itself (0.75 mmol/l) caused a small acidification. In the low calcium–low potassium solution, a depolarization of the preparations occurred, and it was not possible to maintain a continuous impalement of both electrodes in all experiments during the period of rapid stimulation. However, reimpalement after stimulation did not show a marked change in resting pH. Despite this, application of amiloride caused a greater acidification than before. In four experiments, the average decrease of pH during application of amiloride was −0.04 pH units at a control pH of 7.38 in this series of experiments. During the low calcium–low potassium superfusion, the mean decrease was −0.08 pH units (control pH 7.36). This would correspond to an average percentage increase of a[12]H of 9.7% and
19.2%, respectively. The results presented so far provide evidence that the Na-H exchanger plays an essential role in regulating intracellular pH and that pHi is buffered within narrow limits. Thus, an attempt was made to quantify the intracellular buffering capacity of these cells.

Buffering Capacity

Intracellular buffering capacity (β) is a measure of the cell's ability to resist changes of pHi. The buffering capacity is usually defined as the amount of strong base (B) (moles per liter) that must be added to a solution to produce one unit change in pH (β=dB/dpH), and several methods have been described to quantify intracellular buffering of hydrogen ions. The buffering capacity can be estimated from the NH4Cl experiments being calculated from the formula:

$$\beta = \frac{d[NH_4^+]/dpHi}{d[NH_3] \cdot 10^{pK_{H_2O} - pH_i}}$$

where \([NH_4^+] = [NH_3] \cdot 10^{pK_{H_2O} - pH_i}\) and \([NH_4^+], \) and \([NH_3],\) are intracellular concentrations of \(NH_4^+\) and \(NH_3\), respectively. At 25°C, \(pK\) was taken as 9.38, corrected for ionic strength; dpHi is taken as the difference between the point of maximal acidification after removal of NH4Cl and the pH at the time of NH4Cl removal. The assumptions are made that pK for NH4Cl is the same intracellularly and extracellularly and that \([NH_3] equals extracellular \(NH_3\) concentration before removal of NH4Cl (see Reference 34 for details). In the experiments reported here, only nonbicarbonate-buffered solutions have been used; therefore, \(\beta\) is an estimate of the combined buffering capacity of all intracellular buffers other than \(NH_3/NH_4^+\). In 30 experiments in which the control pHi was near the average pHi measured in ferret ventricular muscle (7.26), \(\beta\) ranged from 22 to 117 mmol pH\(^{-1}\)·L\(^{-1}\), with a mean±SD value of 73±28 mmol pH\(^{-1}\)·L\(^{-1}\). However, it has to be borne in mind that this value for \(\beta\) is only a reasonable estimation of the intracellular buffering as long as there is no significant movement of hydrogen ions across the cell membrane. Since the Na-H exchanger is pH dependent with full activation at an acidic pH\(^9\) during an acid load as well as during intracellular buffering a considerable proportion of the hydrogen ions gained will be extruded from the cell. If amiloride completely blocks the Na-H exchanger, the value of \(\beta\) measured after the application of amiloride will correspond to the intracellular buffering capacity. The value found in seven experiments was 36±15 mmol pH\(^{-1}\)·L\(^{-1}\). There are two major uncertainties involved in the calculation of this buffer value. Amiloride by itself causes an acidification of 0.08 pH units; if this is allowed for, the buffer capacity would increase to 41 mmol pH\(^{-1}\)·L\(^{-1}\). On the other hand, the change in pH on the withdrawal of NH4Cl should be measured at time zero, not at the maximum obtained during the experiment (see Figure 3). If the change in pH on the recovery from an acid load, when amiloride is applied, is extrapolated...
to zero time, the buffer capacity decreases to 31 mmol pH⁻¹1⁻¹. However, both of these calculations can only be regarded as approximate, but the two errors do to some extent cancel each other out. A decrease in the buffering capacity was also found when intracellular sodium was artificially increased by blocking the Na-K pump with strophanthidin (10 μmol/l). This is substantiated in the experiment shown in Figure 7A, in which the buffering capacity was reduced by 30%. In the experiment shown in Figure 7B, there was not only a reduction in β but also an increase in the initial rate of acidification after removal of NH₄Cl.

Effects of Metabolic Substrates on pHi

The effect of changing from one metabolic sub- strate to another is shown in Figure 6A. In this experiment, a progressive alkalization occurred when the superfusing solution was switched from one containing pyruvate to one containing glucose or to a substrate-free solution. The effects were reversible. In some experiments, a slow recovery from the maximum alkalinization in substrate-free Tyrode’s solution was observed. The mean pHi±SD in the different Tyrode’s solutions in the steady state are summarized in Table 3. The pooled data indicate that the average pHi in pyruvate-superfused preparations was clearly more acidic than in preparations superfused by glucose or substrate-free Tyrode’s solution. Although Figure 6 shows that changing from glucose-containing Tyrode’s solution to glucose-free Tyrode’s solution caused an alkalinization, the mean pHi values in glucose-containing and in substrate-free Tyrode’s solutions were similar, suggesting that the pHi in the substrate-free solution drifted slowly back to the value found in glucose-containing Tyrode’s solution. This was not investigated further.

Mechanism of Intracellular Acidification by Pyruvate

The intracellular acidification caused by pyruvate could be due to the undissociated form of pyruvate crossing the cell membrane and dissociating intracellularly or to a carrier mechanism coupled to a cotransport of H⁺ and/or countertransport of OH⁻ ions.15,36 To distinguish between the two alternatives, we applied CIN, a known blocker of the carrier mechanism,15,36 to ferret ventricular muscle. Starting with a glucose-Tyrode solution (5 mmol/l), ferret ventricular preparations were exposed to a 20 mmol/l pyruvate-containing solution in the presence or absence of CIN (4 mmol/l) at a constant pHi of 7.4. Exposure to CIN alone at a concentration of 4 mmol/l caused an average acidification of −0.04 pH units (n=10 measurements). Exposure to 20 mmol/l pyruvate led to an average decrease of pH by −0.20 (n=14). As shown in Figure 6B, pretreatment of the preparations by CIN markedly but reversibly depressed the acidification caused by exposure to pyruvate (20 mmol/l). The average acidification under these conditions was −0.10 pH units (n=10 measurements). This means that the intracellular acidification caused by 20 mmol/l pyruvate was reduced by about 55% through the application of CIN.

Discussion

pH Standards

Although pH is regarded as an empirical measurement, it can, under certain well-defined circumstances, be taken as pH = −log aH⁺. This convention is valid to an ionic strength up to 0.1 molal. Physiological solutions have an ionic strength of around 0.16 mol/l, which can produce errors in pH measurements when using NBS buffers as primary standards, since these buffers have an ionic strength between 0.05 and 0.1 molal.37 In the present study, two pH buffers described by Bates et al35 of an ionic strength of 0.16 molal were used, which allows the extension of the convention (pH = −log aH⁺) to this ionic strength. These buffers are more alkaline than the usual NBS buffers (pyruvate-Tyrode as a test solution was, on average, 0.045 pH units more alkaline when calibrated against Bates’s buffer solutions than against NBS buffers), and this must be borne in mind when comparing our results with published values.

Resting Values for pHi and [Na]i in Pyruvate-Tyrode Solution

pHi values. The measured average pHi of 7.26 would correspond to a pHi of 7.21 to 7.22 on the NBS scale (see above). This value is within the range of previous published values for mammalian ventricular muscle measured with ion-selective microelectrodes,29,32,35,38–42 which range from 6.86 to 7.31. Despite the fact that the cited measurements were carried out at various temperatures in either pyruvate- or glucose-Tyrode solutions and with either bicarbonate or HEPES as a buffer, no general trend could be discovered in the published values.

[Na]i values. The mean [Na] of 13.4 mmol/l measured in this series of experiments obtained at 25°C is slightly lower than the value of 14.6 mmol/l previously reported from this laboratory.16 In the new series, a correct “sealing in” of both electrodes was emphasized, and calibration of the sodium selective microelectrodes before and after an intracellular measurement was carried out routinely. Because of these procedures, the mean value of 13.4 mmol/l is regarded as the more reliable measurement. This value is similar to more recent measurements in ferret ventricular muscle.40,43

Equilibrium Potential for Hydrogen Ions and the Intracellular Buffering Capacity

At a pHi of 7.4, an average pH of 7.26, and a membrane potential of approximately −78 mV, the equilibrium potential for hydrogen ions is −8.3 mV, indicating that the transmembrane hydrogen ion distribution is far from thermodynamic equilibrium. The driving force for protons is inward, and there is a passive leak of protons into the cell.44 Moreover, depending on the metabolic state of the cell, there is
FIGURE 6. Panel A: Recording showing changes in intracellular pH (pHi) when switching among the three Tyrode's solutions: P, pyruvate; Q, substrate free; G, glucose. UH, signal corresponding to pHi. Impalement control by increasing extracellular potassium concentration ([K+]o) from 5 to 14 mmol/l was carried out at the beginning and at the end of the experiment. Panel B: Recording showing pH changes produced by 20 mmol/l pyruvate and 20 mmol/l pyruvate plus 4 mmol/l α-cyano-4-hydroxycinnamate (CIN). The application of CIN reduced the acidification produced by pyruvate.

a continuous net production of protons. Thus, to maintain a constant pH of 7.26, there must not only be an intracellular buffering mechanism but also an effective proton extrusion mechanism.

In these experiments, an attempt was made to quantify the intracellular buffering capacity β, using the NH4Cl method. In the presence of amiloride to block the extrusion mechanism, β was estimated to be approximately 36 mmol pH⁻¹ l⁻¹. The value for the buffering capacity estimated with NH4Cl alone was 73 mmol pH⁻¹ l⁻¹, suggesting that under conditions of an acid load a considerable portion of the gain in hydrogen ions is rapidly transported out of the cell. This indicates that to measure β a clear distinction must be made between the intracellular buffering capacity and the buffering capacity combined with proton extrusion. β has recently been estimated in a single heart cell with the NH4Cl method without the application of amiloride and gave values of approximately 20–30 mmol pH⁻¹ l⁻¹. One possible and important reason for the higher values obtained by us is that amiloride does not achieve a 100% block, as would be suggested by the slow recovery of pHi under amiloride (see Figure 3). Despite this limitation of the method, it does mean that the buffering capacity of ventricular muscle is lower than previously thought, in agreement with Boutrita et al.

The Na-H Exchange Mechanism in Ventricular Muscle

The evidence in favor of the Na-H exchange system playing a major role in regulating pH can be summarized as follows: 1) There is complete recovery from an acid load, even after multiple applications of NH4Cl, and this recovery is almost wholly blocked by amiloride. The small recovery of pH under amiloride (see Figure 3) could be due to either an incomplete block of the Na-H exchanger by amiloride or due to the presence of another proton extrusion system. 2) Changes in [Na], during and after application of NH4Cl are opposite the measured changes in pH. During the recovery from an acid load, [Na], increases above the resting level; the increase does not occur under amiloride. 3) Application of amiloride at pH values more acidic than 8.7 showed that the Na-H exchanger maintained the pH values more alkaline than would be expected from a passive

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<th>Table 3. Dependence of Average Intracellular pH on Metabolic Substrate</th>
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Values are mean±SD. pH, intracellular pH; n, number of measurements.
Alterations in distribution also lead to changes in $pH_i$ that can be explained on the basis of the Na-H exchange system.

In heart muscle a reduction of the sodium gradient causes a concomitant increase in the cytosolic calcium concentration and a small increase in force$^{16}$; these increases lead to an increase in proton production.$^{48}$ Thus, it is to be expected that a reduction in the sodium gradient will cause an acidification due to a partial inhibition of the Na-H exchanger, due to an increased acid production and also due to a competition between calcium and hydrogen ions for common intracellular buffer sites (see References 12, 49, and 50, but see also Reference 51). Reversing the sodium gradient by lowering extracellular sodium concentration from 155 to 5 or 1.5 mmol/l caused an acidification. However, increasing [Na] by either strophanthidin or by low calcium–low potassium solution did not immediately cause a measurable decrease in $pH_i$, although longer exposure to higher doses of strophanthidin did. Application of amiloride at increased intracellular sodium caused a larger acidification than under control conditions. Kaila and Vaughan-Jones$^{13}$ also found in Purkinje fibers that the $pH_i$ change on application of amiloride was larger after the inhibition of the sodium pump, which they attributed to an increase in acid extrusion stimulated by the decrease in $pH_i$ found after application of strophanthidin. If there was either a direct or indirect effect of calcium ions on the Na-H exchanger,$^6$ a similar explanation (i.e., an increased extrusion rate) would also apply. Since ventricular cells also possess an intracellular buffering capability, this would allow them at least temporarily to maintain an almost normal $pH_i$ despite an effective gain in hydrogen ions (metabolic generation, proton leak). Thus, a decrease in the sodium gradient might not initially cause a marked change in $pH_i$. Moreover, as we have shown (see Figure 7), there is a reduction in buffering capacity by up to 30% under strophanthidin as measured with the NH$_4$Cl method.

Relation Between the Na-Ca and Na-H Exchange Systems and Ca-H Interaction

Although all the results summarized above are consistent with the Na-H exchange system, they could, qualitatively speaking, be explained on the basis of the Na-Ca exchange system and the competition between calcium and hydrogen ions for common intracellular buffer sites. Any change, for instance, in intracellular sodium would cause a concomitant change in intracellular calcium via the Na-Ca exchanger, and the interaction between calcium and hydrogen for common buffering sites would bring about an alteration in $pH_i$.

It has been shown that amiloride can also block the Na-Ca exchange, but the Na-Ca and Na-H exchange systems have different dose response curves for the drug. At the amiloride concentrations used in this study (0.75 and 1 mmol/l), the Na-H exchanger is almost completely blocked, whereas the Na-Ca exchanger would still be 70% or more active.$^9$ Moreover, if recovery from an acid load was simply due to sodium removing calcium and to hydrogen ions being buffered intracellularly, repeated application of NH$_4$Cl by causing a net gain of hydrogen ions should show a reduction in buffering capacity and a progressive acidification of the cytoplasm. NH$_4$Cl has been applied up to 14 times in the same preparation, and recovery from an acid load remained unaffected. As shown in Figure 7A, repeated application of NH$_4$Cl during the same impairment caused neither a reduction in buffering capacity nor a permanent acidification. Thus, it must be concluded that after an acid load hydrogen ions were removed from the cell.

There is additional evidence for the Na-H exchanger playing a major role in $pH_i$ regulation from studies by other groups. During the recovery from an acid load it has recently been shown in chick heart cells that cytosolic calcium ([Ca$^2+$_i]) increases.$^{53}$ If sodium ions were exchanging solely for calcium then it would be expected that [Ca$_2+$] would decrease. However, an increase in [Ca$_2+$] would be expected by the action of the Na-Ca exchange system as a consequence of the higher intracellular sodium level, brought about by exchanging sodium for hydrogen ions by the Na-H exchange system. Moreover, Piwnica-Worms and Lieberman$^8$ argue that a short-term exposure of several minutes (similar to the protocol in our experiments) does not affect Na-Ca exchange. Finally, the Na-H exchanger has been described as present in isolated mammalian sarclemmal vesicles.$^{14}$

$pH_i$ on Changing the Metabolic Substrate

On changing from glucose-containing or substrate-free Tyrode’s solution to pyruvate-containing Tyrode’s solution (all solutions were HEPES buffered), an intracellular acidification of around 0.2 pH units was found. It has been argued that this acidification is mainly due to a pyruvate carrier since the acidification is reduced by CIN. A pyruvate-lactate transport system has been described in various heart preparations, and CIN has been shown to at least partially block the carrier-mediated pyruvate transport system in the heart.$^{15}$ Since the acidification caused by pyruvate is reduced by CIN by more than 50%, it is concluded that such a pyruvate transport system is also present in ferret ventricular muscle. Similar effects of CIN and monocarboxylate acids on $pH_i$ have been shown in sheep Purkinje fibers$^{34}$ and in frog skeletal muscle.$^{55}$ That the acidification on changing from pyruvate- to glucose-Tyrode solution is not transient must mean that a new steady state is reached between the additional gain of hydrogen ions due to the carrier mechanism and the proton extrusion mechanism.

In summary, the presence of two ionic transmembrane exchange systems, namely Na-H and Na-Ca, combined with common intracellular binding sites for hydrogen and calcium ions (see also the discussion in References 13, 51, and 53) means that the interaction
among the intracellular levels of hydrogen, calcium, and sodium are complex and that intracellular sodium is an important factor in the regulation of the much lower intracellular levels of calcium and hydrogen ions. This is further complicated by the presence of the pyruvate-lactate transport system, which causes a net inward movement of hydrogen ions and in vivo by the presence of bicarbonate transport systems.

Acknowledgments

We wish to thank Marlis Jordi for excellent technical help and C. Cigada and D. de Limoges, who built parts of our equipment. We also wish to thank S. Weidmann, A.G. Kleber, J.S. Shiner, and R. Weingart, who kindly commented on an earlier draft of the manuscript, and J.R. Blinks for critical and helpful discussion.

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Figure 7. Panel A: Recording showing intracellular pH (pH_i) changes during repeated application of NH_4Cl (20 mmol/l). UH, signal corresponding to pH_i. The experiment shows complete recovery from intracellular acidification. The recovery is not markedly influenced by strophanthin (10 μmol/l), but the buffer capacity is reduced. The buffer capacity in strophanthin is compared each time with the average of the buffer capacity obtained before and after the application. Panel B: Recording similar to that in panel A but showing that the initial rate of acidification under strophanthin is increased.
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Key words: ferret ventricular muscle • Na-H exchange • metabolic substrates • intracellular pH • intracellular sodium concentration
Intracellular pH regulation in ferret ventricular muscle. The role of Na-H exchange and the influence of metabolic substrates.
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Circ Res. 1991;68:150-161
doi: 10.1161/01.RES.68.1.150

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