Intracellular proton mobility and buffering power in cardiac ventricular myocytes from rat, rabbit, and guinea pig

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Zaniboni, Massimiliano, Pawel Swietach, Alessandra Rossini, Taku Yamamoto, Kenneth W. Spitzer, and Richard D. Vaughan-Jones. Intracellular proton mobility and buffering power in cardiac ventricular myocytes from rat, rabbit, and guinea pig. Am J Physiol Heart Circ Physiol 285: H1236–H1246, 2003. First published May 15, 2003; 10.1152/ajpheart.00277.2003.—Intracellular pH (pHi) is an important modulator of cardiac function. The spatial regulation of pH within the cytoplasm depends, in part, on intracellular H+ (H+) mobility. The apparent diffusion coefficient for H+, Dapp, was estimated in single ventricular myocytes isolated from the rat, guinea pig, and rabbit. Dapp was derived by best-fitting predictions of a two-dimensional model of H+ diffusion to the local rise of intracellular pH, recorded confocally (ratiometric seminaphorhodafluor fluorescence) downstream from an acid-filled, whole cell patch pipette. Under CO2/HCO3−-free conditions, Dapp was similar in all three species (mean values: 8–12.5 × 10−7 cm2/s) and was over 200-fold lower than that for H+ in water. In guinea pig myocytes, Dapp was increased 2.5-fold in the presence of CO2/HCO3− buffer, in agreement with previous observations in rabbit myocytes. H+ mobility is therefore low in cardiac cells, a feature that may predispose them to the generation of pH gradients in response to sarcolemmal acid/base transport or local cytoplasmic acid production. Low H+ mobility most likely results from H+ shuttling among cytoplasmic mobile and fixed buffers. This hypothesis was explored by comparing the pH dependence of intrinsic, intracellular buffering capacity, measured for all three species, and subdividing buffering into mobile and fixed fractions. The proportion of buffer that is mobile will be the main determinant of Dapp. At a given pH, this proportion appeared to be similar in all three species, consistent with a common value for Dapp. Over the pH range of 6.0–8.0, the proportion is expected to change, predicting that Dapp may display some pH sensitivity.

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not been estimated for any other cardiac cell. Confocal seminaphorhoradfluor (SNARF) imaging of the spread of intracellular acid from a cell-attached micropipette indicated that, for rabbit myocytes, H\(^+\) mobility was \(\sim250\)-fold lower than in simple unbuffered solution. Intracellular mobility was severalfold faster in the presence of carbonic buffer \((\sim2 \times 10^{-6} \text{ cm}s^{-1})\), but estimates of \(D_{\text{mob}}^{\text{pp}}\) were still nearly two orders of magnitude lower than for unbuffered H\(^+\) (28).

In the present work, we compared H\(^+\) mobility in ventricular cells isolated from the guinea pig, rat, and rabbit. One aim was to establish whether low intrinsic H\(^+\) mobility, and its enhancement by carbonic buffer, is a general phenomenon. We also compared \(\beta_{\text{ref}}\) and its dependence on pH\(_i\) in the three species. Although there have been many reports of intracellular buffering power in cardiac cells, there have been few quantitative comparisons. Using our estimate of the H\(^+\)-binding capacity of mobile buffers in cardiac tissue, we mapped the likely intracellular concentration and average \(pK_a\) of fixed intrinsic buffer in cardiac cells. We then considered how the relative concentrations of fixed and mobile buffer contribute to the regulation of H\(^+\) mobility.

A preliminary report of this work has appeared in abstract form (41).

**METHODS**

**Cell Isolation**

Rat and guinea pig ventricular myocytes were enzymatically isolated according to a previously described procedure (see Ref. 17). Briefly, single ventricular myocytes were isolated from albino guinea pigs and from Wistar rats (killed by cervical dislocation) weighing 400 and 300 g, respectively, using a combination of enzymatic and mechanical dispersion (0.7 mg/ml collagenase, Boehringer Mannheim, and 0.04 mg/ml protease, Sigma; St. Louis, MO). Rats and guinea pigs were killed humanely, according to UK Home Office recommendations, by concussion and cervical dislocation. The cells were finally suspended in HEPES-buffered Dulbecco’s modified Eagle’s medium (culture medium) and kept at room temperature until use.

As previously described (26), adult rabbit ventricular myocytes were obtained from New Zealand White rabbits (2–3 kg). Animals were anesthetized with an intravenous injection of pentobarbitone sodium (50 mg/kg) and 0.5 ml heparin to prevent clotting, in accordance with national guidelines. The heart was rapidly removed and attached to a Langendorff perfusion system. The heart was digested with a solution containing 1 mg/ml collagenase (class II, Worthington Biochemical; Freehold, NJ), 0.1 mg/ml protease (type XIV, Sigma), and 0.1 mM CaCl\(_2\). The cells were stored until use at room temperature in the normal HEPES-buffered solution.

All myocytes used in this study had a rod shape, well-defined striations, and did not spontaneously contract. Experiments were performed within 24 h after isolation.

**Solutions**

Superfusion solutions were held at 37°C and delivered by means of a peristaltic pump to the cell chamber. The 1-ml Plexiglas chamber had a clear glass bottom and was mounted on the stage of an inverted microscope (Leica DM IRBE). The temperature of the solutions in the bath was kept at 37°C by an electrical temperature control circuit. Bathing solutions continuously flowed through the bath at \(\sim2\) ml/min, and the solution depth in the chamber was held at \(\sim200\) \(\mu\)m. The bottom of the bath was coated with poly-l-lysine (Sigma) to improve cell adhesion.

HEPES-buffered Tyrode solution contained (in mM) 135 NaCl, 4.5 KCl, 1 MgCl\(_2\), 2 CaCl\(_2\), 11 glucose, and 20 HEPES adjusted to pH 7.4 with 1 M NaOH at 37°C. Bicarbonate-buffered Tyrode solution was identical except for the NaCl concentration, which was reduced to 120 mM, and for 22 mM NaHCO\(_3\), which was added instead of HEPES. Bicarbonate solution was equilibrated with 5% CO\(_2\)-95% air.

For ammonium- or acetate-containing solutions, NaCl was replaced osmotically by ammonium chloride and sodium acetate. For chloride-free solutions, all chloride-containing salts (except ammonium) were replaced with salts of gluconic acid and pH was adjusted to 7.4 using 1 M NaOH (NH\(_4\)Cl was replaced with NH\(_4\)SCN). For sodium-free solutions, sodium salts were osmotically replaced by N-methyl-D-glucamine and pH was adjusted to 7.4 with 5 M HCl.

In some experiments, 10 mM 1 hydrogen ion exchanger (NHE)-1 inhibitor (24), structurally related to a previous inhibitor compound, HOE-694 (Aventis; 20), was added to the Tyrode solution.

The fluorophore stock solution was prepared by dissolving 1 mg SNARF-1-AM (Molecular Probes) in 1 ml DMSO to reach a concentration of 1.7 mM.

To acid load myocytes, suction pipettes were filled with an unbuffered isotonic solution containing (in mM) 140 KCl, 0.5 MgCl\(_2\), 5.5 dextrose, and 1 HCl (pH 3.0). To pipette load myocytes with unesterified carboxy-SNARF-1 (Molecular Probes), it was added to a final concentration of 400 \(\mu\)M in a filling solution containing (in mM) 140 KCl, 1.0 MgCl\(_2\), and 10 HEPES adjusted to pH 7.1 with 1 M NaOH. No attempt was made to compensate for the small reduction in pH of the filling solution caused by the acidic nature of the fluorophore.

**Whole Cell Epifluorescence Measurement of pH\(_i\)**

Whole cell pH\(_i\) was measured at 37°C in single myocytes previously equilibrated at room temperature for 10 min in culture medium containing 10 \(\mu\)M of the AM form of carboxy-SNARF-1 as the fluorescent pH indicator (see, e.g., Ref. 18). Cells were superfused in a chamber mounted on an inverted epifluorescence microscope. The dye was excited by light from a 100-W xenon lamp at 540 nm. Emission at 590 and 640 nm was measured by two photomultiplier tubes, filtered at 10 Hz, and digitized by an analog-digital converter (CED 1401, Cambridge Electronic Design). The ratio was averaged at 2 Hz (in-house software) and converted off-line to pH using the calibration curve obtained by the nigericin calibration technique (see Ref. 31).

**Confocal Measurement of pH\(_i\)**

The pH\(_i\) was measured at 37°C in single myocytes using the carboxy-SNARF-1 dye (5). A laser scanning confocal microscope, Leica DM IRBE, with Leica TCS NT software and a Leica \(	imes40\), 1.25 numerical aperture, oil immersion, planapo-achromat objective lens were used to confocally image the cells. SNARF excitation was achieved with the 514-nm laser line of an air-cooled argon laser. Emitted fluorescence was simultaneously collected by two photomultiplier tubes equipped with band-pass filters at 640 and 580 nm. A transmitted light detector also provided a nonfluorescent image of the cell. The two fluorescence x-y images plus the transmission image were acquired on-line at a rate of one frame every
2 s. No z scan was performed, and pinhole size was kept constant at a fixed value of 1.29.

Confocal images were processed, and the fluorescence ratio (580/640 nm) was calculated off-line using NIH Image and Transform (Fortner Software; Sterling, VA) software packages. The ratio metric signal was converted to pH, as previously described, using the nigericin calibration technique (31). This requires bathing solutions of varying pH that also contain 10 μM of the ionophore nigericin. The best-fit equation for the calibration curves obtained from several myocytes was used to calculate pH of the cells. Local pH was measured in predefined regions of interest (ROIs) that were drawn as circles of 10 μm diameter, avoiding nuclei and the pipette tip. To derive space-profiles for pH, only one ROI was drawn, with a long rectangular shape that fitted most of the cell area, avoiding the pipette tip.

**Acid and SNARF pipette-loading procedure.** Myocytes were exposed to acid via whole cell patch pipette attachment for pHi, only one ROI was much smaller than that along the other two axes.

Myocytes were used to acidize via whole cell patch pipette attachments, as previously described (34). The pipettes were made from borosilicate capillary tubing (Harvard Apparatus; Edenbridge, UK) and had a resistance, when filled, of 1–2 MΩ. Transmembrane potential was monitored by means of the bridge circuit of an Axoclamp 2B amplifier (Axon Instruments; Union City, CA). Before a cell was contacted with the pipette tip, the pipette potential was set to zero and the voltage drop across the pipette was compensated with the bridge balance. Cells, previously loaded with SNARF-AM as described above, were introduced into the perfusion chamber and continuously bathed with Tyrode solution. Fluorescence (580 and 640 nm) and transmission confocal images were recorded before (~1 min) and during pipette attachment and break in. Pipette attachments were made as close to the end of a cell as possible and lasted from 1 to 7 min. A similar technique was used to pipette load carboxy-SNARF-1 into myocytes.

**Finite Element Method Model**

The spatiotemporal characteristics of the intracellular proton concentration during pipette loading were analyzed by solving diffusion equations. Two-dimensional rather than three-dimensional, time-dependent equations are solved because the depth of the cell on a coverslip is small; hence variation of H⁺ concentration along the z-axis is likely to be much smaller than that along the other two axes.

The geometry of a myocyte was used to define a two-dimensional array of points (x,y) called domain Ω, with boundary points being on ∂Ω. This array of points was generated by Delaunay triangulation (MATLAB PDE Toolbox), whereby the density of the mesh depended on the regularity of the geometry (32), such that density decreased where the structure had regular, rectangular geometry and increased in regions where the boundary (i.e., cell membrane) became irregular. The diffusion problem was solved using the finite element method (FEM) approach. In FEM, piecewise approximations with simple functions at each point are combined to provide a solution for the whole domain. Unlike previous approaches (finite difference method; see Ref. 34), FEM permits considerable flexibility in mesh density and distribution and optimizes computational time in regions where it is not necessary to maintain a high mesh density. The method is particularly well suited to our type of boundary condition.

The variable u is the concentration of protons above baseline [change in H⁺ concentration (Δ[H⁺])]. The diffusion partial differential equation (PDE) is

\[ \frac{\partial u}{\partial t} = D \cdot \nabla^2 u + P(x, y) \quad (x, y) \in \Omega \]

where \( P \) is the apparent acid injection rate and \( D \) is the apparent proton diffusion coefficient. The PDE is conditioned to have a reflection boundary condition (i.e., zero flux of proton equivalents across the sarcolemma)

\[ \nabla u = 0 \quad (x, y) \in \partial \Omega \]

and an initial condition of zero

\[ u(t = 0) = 0 \quad (x, y) \in \Omega \]

The geometry and pipette location are defined according to the transmission image. The FEM algorithm implemented in the MATLAB PDE Toolbox (for an overview, see Ref. 32) is used to solve the diffusion PDE for a particular value of diffusion coefficient, \( D \). Solutions can be obtained as a function of time or space, depending on the nature of the data. Because this diffusion PDE is linear, the solution is scaled by a constant to fit the magnitude of the results (equivalent to scaling the injection rate \( P \)). This is repeated for different values of \( D \) until the best fit is obtained according to the least-squares method. This best-fitting value of \( D \) is selected as the apparent proton diffusion coefficient. Fitting procedures are limited to the first 60 s of acid injection due to possible sarcolemmal acid extrusion and changes in the mobile-to-total buffer ratio (Eq. 2), which will alter the value of \( D \) (32).

**Measuring Intrinsic Buffering Capacity**

\( \beta_{int} \) was measured while cells were superfused in HEPES-buffered solutions (nominally free of CO₂/bicarbonate, containing various concentrations of a salt of a weak acid (acetate) or a weak base (ammonium)). For experiments with guinea pig and rat myocytes, ammonium-containing solutions were chloride free (plus 30 μM cariporide; Aventis); they contained 40, 30, 20, 15, 10, 5, and 2.5 mM NH₄SCN. Acetate solutions contained 80, 60, 40, 20, and 10 (plus 30 μM cariporide). For experiments on rabbit myocytes, solutions were sodium free (no cariporide added) and contained 15, 12, 9, 5, 3, 2.5, or 1 mM NH₄Cl. No acetate experiments were performed with rabbit cells.

Cells were superfused in normal Tyrode followed by a switch to one of the ammonium or acetate solutions. A series of solution changes was then made, in which the extracellular weak acid or base concentration was stepped to progressively lower levels, eventually returning to normal Tyrode solution. This produced step changes of pHi in the alkaline and acid directions, respectively (see Fig. 5A). The changes approached a steady state after 2–4 min. The pHi before and after the weak acid/base removal step (pHₘₐₜₐₜ and pHₘₜₚₜₚₜₚₜ, respectively) was noted. During ammonium removal, NH₄⁺ leaves the cell in the form of uncharged NH₃, leaving behind H⁺. The change in [H⁺], is assumed to be equal to the change in intracellular NH₄⁺ concentration ([NH₄⁺]), which can be estimated using the following rearranged Henderson-Hasselbalch equation

\[
[\text{NH}_4^+] \text{H} = [\text{NH}_3] \times 10^{pH_0 - p\text{H}}
\]

where [NH₄⁺] in the extracellular solution ([NH₄⁺]) is calculated from the total ammonium concentration (C_{tot} = NH₃ + NH₄⁺) divided by \( 1 + 10^{pH_0 - p\text{H}} \), where pH₀ is extracellular pH. \( \beta_{int} \) may then be computed (23)

\[
\beta_{int} = \frac{[\text{NH}_4^+]_{\text{H}_\text{start}} - [\text{NH}_4^+]_{\text{H}_\text{stop}}}{p\text{H}_{\text{H}_\text{stop}} - p\text{H}_{\text{H}_\text{start}}} - \frac{C_{\text{tot}} \times 10^{p\text{H}_0}}{1 + 10^{p\text{H}_0 - p\text{H}_{\text{H}_\text{start}}}}
\]

**References**
where start and stop indicate before and after the weak acid/base removal step. An analogous approach can be applied to acetate removal experiments. During the removal of acetate, intracellular acetate takes up H⁺ and leaves the cell as acetic acid.

\[
\beta_{\text{nat}} = \frac{[\text{Ac}^-]_{\text{stop}} - [\text{Ac}^-]_{\text{start}}}{\text{pH}_{\text{stop}} - \text{pH}_{\text{start}}} \\
= \frac{C_{\text{tot}} \cdot 10^{-\text{pK}_a \cdot 10 \text{mM}}}{1 + 10^{\text{pK}_a - \text{pH}_{\text{stop}}}} \left( \frac{1}{[\text{H}^+]_{\text{stop}}} - \frac{1}{[\text{H}^+]_{\text{start}}} \right)
\]

where \([\text{Ac}^-]_{\text{i}}\) is the intracellular acetate concentration. The pKₐ values for ammonium and acetate are assumed to be 9.03 and 4.53, respectively. The pH dependence of buffering is approximated by referring the value of \(\beta_{\text{nat}}\) so determined to the midpoint in the change of pH, induced by the step decrease in extracellular weak acid/base.

**Mobile buffering capacity.** Cardiac myocytes have recently been proposed (34) to contain the following mobile buffers (see DISCUSSION).

**RESULTS**

**Imaging Intracellular Acid Diffusion**

Figure 1 illustrates the experimental protocol applied to a rat ventricular myocyte. The patch pipette was filled with buffer-free, isotonic KCl adjusted to pH 3.0 and positioned close to one end of the cell (Fig. 1A). The superfusate contained no added CO₂/HCO₃ and was buffered with 20 mM HEPES (pH 7.4). It also contained 30 μM cariporide, a high-affinity, selective inhibitor of sarcolemmal NHE-1 to prevent acid extrusion during the course of the acid-loading protocol. A ratiometric image of the cell loaded with SNARF is shown in Fig. 1B. Superimposed on this image are three ROIs positioned longitudinally downstream of the pipette.

After break in by the pipette, the myocyte acidified, as shown in Fig. 2. Figure 2 compares the time course of pH averaged in the three ROIs. The greater the distance of the ROI from the pipette, the slower was the rate of acidification. After break in, acidification was detected almost immediately above the noise level.
in the proximal ROI, but it was not detected in the most distal ROI, 52 μm downstream, until ~30 s later. This indicates a slow spreading of acid within the cytoplasmic compartment.

In Fig. 2B, changes of pH in the three regions over the first 60 s have been converted into increments in H⁺ concentration and best fitted by the FEM model of two-dimensional diffusion described in METHODS. The triangulated image of the cell used for the fitting procedure is illustrated in Fig. 1C. The value for $D_{pp}$ obtained by the model was $2.5 \times 10^{-7}$ cm²/s.

Figure 2C illustrates results from a similar experiment, but this time on a guinea pig ventricular myocyte. The longitudinal profile for $[H^+]_i$ was extracted from the data at different times after break in. A gradient of up to 0.3 pH units was apparent during the course of acid loading, with the greatest $[H^+]_i$ occurring at the site of the pipette. These data were best fitted by the diffusion model assuming a value for $D_{pp}$ of $5.0 \times 10^{-7}$ cm²/s. The good spatiotemporal agreement between the model and the data shown in Fig. 2, B and C, indicates that acid movement in guinea pig and rat cells conforms to a passive diffusive process, as reported previously for the rabbit ventricular myocyte (34).
carboxylic acid form of the fluorophore. Figure 4 illustrates longitudinal profiles of fluorescence obtained at different time intervals after break in by the pipette. The profiles have been best fitted by the FEM model. In three experiments, a mean value for the diffusion coefficient of SNARF of $3.22 \pm 0.86 \times 10^{-7}$ cm$^2$/s was obtained, comparable to the value of $\sim 1 \times 10^{-7}$ cm$^2$/s measured recently for SNARF mobility in the rabbit ventricular myocyte. Assuming the AM-loading procedure for SNARF results in $\sim 400$ M/M of the acid form of the dye inside the cell, as it does in the rabbit myocyte (34), the fluorophore's low buffering capacity ($\sim 170$ M/M pH unit; estimated assuming one H$^+$ binding site per SNARF molecule) and low intracellular mobility means that it will spatially shuttle only small quantities of H$^+$ during an acid injection procedure. With the use of the formalism derived by Junge and McLaughlin (15) and by Irving et al. (14), the presence of the intracellular fluorophore would increase $D_{H}^{app}$ in accordance with the function

$$\Delta D_{H}^{app} \approx \frac{D_{SNARF}^{app} \cdot \beta_{SNARF}}{\beta_{int} + \beta_{SNARF}}$$

where $D_{SNARF}^{app}$ is the apparent diffusion coefficient of SNARF, $\beta_{SNARF}$ is the buffering capacity of the dye, and $\beta_{int}$ is 24 mM at pH 7.1 (see Fig. 5). The increase would be $0.02 \times 10^{-7}$ cm$^2$/s, i.e., an increase in $D_{H}^{app}$ of $\sim 1\%$. This increase will therefore have a negligible effect on the experimental measurements of H$^+$ mobility. Furthermore, the similar SNARF mobility measured in guinea pig and rabbit myocytes strongly suggests that mobility in rat myocytes will also be comparable. If so, the fluorophore will have a negligible impact on H$^+$ mobility in ventricular myocytes from all three species.

Many previous studies have used AM-loaded 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) instead of SNARF to evaluate pH$_i$ regulation in cardiac cells. Without knowledge of the intracellular concentration and mobility of BCECF, it is not possible to estimate whether it significantly affects H$^+$ mobility. Given the similarity in the molecular weight of SNARF and BCECF, however, and given the similar loading protocols, it would not seem unreasonable to assume that both fluorophores will influence H$^+$ mobility by similar amounts.

**Estimating Total, Mobile, and Fixed Intrinsic Buffering Capacity**

Figure 5, A and B, illustrates the experimental protocol used for investigating the pH$_i$ dependence of total $\beta_{int}$. A wide range of pH$_i$ values was achieved by superfusing various concentrations of salts of a membrane-permeant weak base or weak acid (ammonium and acetate salts, respectively). Successive reduction in the concentration of these compounds in the superfusate results in a step-wise titration of the cytoplasm by acid and base, respectively. The procedure for calculating $\beta_{int}$ is detailed in METHODS.

The graph shown in Fig. 5B plots data on $\beta_{int}$ gathered from guinea pig myocytes. Also plotted (blue curve) is the pH$_i$ dependence predicted for intrinsic mobile buffer capacity ($\beta_{mob}$). This has been assembled by summing the capacity of the mobile buffers identified in cardiac tissue, listed recently by Vaughan-Jones et al. (34; see also present study, METHODS). The capac-

![Fig. 3. H$^+$ mobility in three species. Average values for $D_{H}^{app}$ were derived using the FEM algorithm best fitted to time courses of the rise of [H$^+$], in guinea pig, rat, and rabbit myocytes (number of cells is indicated in parentheses). For the guinea pig and rat, data determined with and without 30 M cariporide were combined, as the drug had no significant effect on $D_{H}^{app}$ (P > 0.05; see text). Results for the rabbit were determined in the presence of 1 mM amiloride. Values of $D_{H}^{app}$ among species not significantly different (P > 0.05; unpaired t-test).](image)

![Fig. 4. Intracellular SNARF mobility. Longitudinal SNARF profiles were recorded at different times (2, 11, 19, and 35 s) after cell break in by a pipette filled with unesterified SNARF into a guinea pig ventricular myocyte (length 108 M, width 32 E). The spatial profiles are shown along a rectangular ROI (length 97 M, width 7 M) positioned centrally along the cell and averaged along the width of the cell. The pipette was attached 7 M from the end of the cell.](image)
ity of any individual mobile buffer was estimated from its concentration (C) and pKₐ:

$$\beta = \frac{-\ln(10) \cdot C \cdot 10^{pH-pK_a}}{(1 + 10^{pH-pK_a})^2}$$  \hspace{1cm} (1)

For polyprotic species, overall buffering capacity is the sum of Eq. 1 for each pK. Subtraction of the resulting curve for $\beta_{mob}$ from the data points plotted for $\beta_{int}$ produces points predicted for fixed intrinsic buffer capacity ($\beta_{fix}$). Interestingly, the characteristics of these latter data conform to a buffer with a concentration of 68.3 mM, with a single pKₐ of 6.1, as illustrated by the red curve in Fig. 5B, which is a least squares, best fit to the data. Summing this curve with that for the mobile buffer gives the pH dependence of $\beta_{int}$, plotted as the continuous black curve in Fig. 5B.

Figure 5C illustrates the experimental results obtained for $\beta_{int}$ in all three species. Superimposed on the data are the individual curves for $\beta_{mob}$, and the pH dependence predicted for $\beta_{int}$ has been calculated for all the mobile buffers listed in Methods and plotted (blue curve) versus pH. The difference between $\beta_{int}$ and $\beta_{mob}$ has also been plotted as the red squares. These latter points were best-fitted using Eq. 1. The best fit was obtained for a one-component buffer population (pKₐ 6.1, concentration 68.3 mM). Note that the two-component and higher-component buffer fits gave unrealistic estimates of buffer concentration (>100 mM) or physiologically irrelevant pKₐ values below 5. The best-fit curve (red) is therefore proposed to describe the pH dependence of the fixed intracellular buffer ($\beta_{fix}$). When summed with $\beta_{mob}$, this generates the black line ($\beta_{int}$). This analysis shown in B was repeated for rat (red; n = 12–27 data points/0.1 pH unit bin except for the bins’ midpoints of 6.15–6.35, 7.55, and 7.65, where n = 5) and rabbit (green; n = 11–25 data points/0.1 pH unit bin) myocytes and has been plotted together with that for the guinea pig (blue). The best-fit concentrations and pKₐ for the fixed buffer components in the three species are shown in Table 1.
similar in all three cases (6.1–6.2), as is the effective concentration (varying from 53 to 68 mM).

**Effect of Carbonic Buffer on \( H^+ \) Mobility**

Figure 6 summarizes the results for the estimation of \( D_{\text{H}^+}^{\text{pp}} \) in guinea pig ventricular myocytes superfused with Tyrode solution equilibrated with carbonic buffer (5% CO\(_2\)/22 mM HCO\(_3^-\); pH 7.4). The experimental protocol and analysis in these experiments were identical to that employed previously for estimating intrinsic \( H^+ \) mobility, with the exception that the pipette filling solution was saturated at 37°C with 5% CO\(_2\), to match the PCO\(_2\) inside and outside the cell. The time course of the rise of \([H^+]_i\) in proximal and distal downstream regions, when best fitted by the FEM model, produced values for \( D_{\text{H}^+}^{\text{pp}} \) that were up to threefold larger than in the absence of carbonic buffer (i.e., with HEPES-buffered superfusate). This indicates that, in the guinea pig ventricular myocyte, the presence of a CO\(_2\)/bicarbonate buffer system facilitates intracellular proton mobility, as reported previously for rabbit myocytes (34).

**DISCUSSION**

**Low Intrinsic \( H^+ \) Mobility in the Ventricular Myocyte**

Intrinsic, \( H^+ \) mobility, defined as that measured in the absence of carbonic buffer, appears similar in rat, guinea pig, and rabbit ventricular myocytes, average values for \( D_{\text{H}^+}^{\text{pp}} \) being 8–12 × 10\(^{-7}\) cm\(^2\)/s. A previous estimate in rabbit ventricular myocytes was somewhat lower ([4 × 10\(^{-7}\) cm\(^2\)/s (34)] but still falls within the range of individual values recorded in the present study (2.9–22 × 10\(^{-7}\) cm\(^2\)/s). The value for \( D_{\text{H}^+}^{\text{pp}} \) is more than two orders of magnitude lower than for \( H^+ \) in unbuffered aqueous solution (1.2 × 10\(^{-4}\) cm\(^2\)/s). A low \( H^+ \) mobility is therefore likely to be a universal feature of mammalian ventricular cells.

Low values for \( D_{\text{H}^+}^{\text{pp}} \) are proposed to be caused by the presence of intracellular buffers that bind almost all acid or base introduced into or generated within the cell. Depending on net electrical charge and molecular size, intrinsic buffer molecules are likely to exhibit a range of mobilities lower than for \( H^+ \). Large macromolecules, like proteins, display extremely low intracellular mobility with apparent diffusion coefficients of ~10\(^{-8}\) cm\(^2\)/s (37). They are, therefore, effectively immobile (i.e., fixed) on the time scale of the present experiments. Lower molecular weight intrinsic buffers like homocarnosine (a cytoplasmic dipeptide containing histidine) and inorganic phosphate are likely to have higher mobility coefficients. Because the overall capacity of intrinsic buffer is high (20–60 mM depending on pH\(_i\)), the free diffusive flux of \( H^+ \) will be negligible compared with that of \( H^+ \) conjugated to mobile buffers. \( H^+ \) will therefore be moved from regions of high to low concentration by means of a buffer shuttle, protonated buffer diffusing to more alkaline regions where deprotonation occurs.

Geometric factors are also likely to play a role in determining a low intrinsic \( H^+ \) mobility. In muscle cells, macromolecular crowding within the cytoplasmic compartment is thought to reduce the effective volume for diffusion by up to 50%, thus reducing solute mobility (16). This may be accentuated further by extensive sarcolemmal invagination, as occurs with the t-system. It will be of interest, therefore, to explore whether \( H^+ \) mobility is faster in cardiac cells that lack a t-system, such as atrial (13) and Purkinje myocytes (8).

**Estimates of \( D_{\text{H}^+}^{\text{pp}} \) are unaffected by sarcolemmal NHE.** Sarcolemmal acid extrusion, by removing \( H^+ \) from the cytoplasm, will limit their spatial spread. This could potentially lead to an underestimate of \( D_{\text{H}^+}^{\text{pp}} \). While \( H^+ \) transport undoubtedly occurs, transport proteins such as NHE did not appear to distort the measurement of \( D_{\text{H}^+}^{\text{pp}} \) as the estimate was unaffected by adding NHE inhibitors to the superfusate (e.g., amiloride or cariporide), a result similar to that obtained previously in rabbit ventricular myocytes (34). These observations are consistent with a recent mathematical analysis indicating that NHE activity should not significantly distort estimates of \( D_{\text{H}^+}^{\text{pp}} \), provided fitting algorithms are restricted to the first 60 s of acid loading, the time period used in the present work (32). Effects on acid loading caused by subcellular sequestration of \( H^+ \) into organelles such as mitochondria also cannot be excluded but, over the time scale of our experiments, such a mechanism, if sufficiently fast and large, would appear as a cytoplasmic \( H^+ \) sink that contributes to our overall estimate of buffering capacity and hence would be accommodated by the present analysis.
Facilitation of $H_+^+$ Mobility by Carbonic Buffer

Carbonic buffer contributes to spatial pH$_i$ regulation. We found that the $H_+^+$ mobility coefficient in guinea pig myocytes is enhanced two- to threefold in the presence of carbonic buffer, an effect that will help to reduce the magnitude and duration of pH$_i$ gradients associated with local acid/base disturbances within the cell. This result is similar to that reported recently in rabbit ventricular myocytes where carbonic facilitation of $H_+^+$ mobility was up to sixfold, suggesting this is a general phenomenon in cardiac cells (28). Similar observations have also been made in intestinal enterocytes (30). Carbonic facilitation of $H_+^+$ mobility has been questioned recently in neuronal cells (25), although interpretation of the evidence for this has been disputed (32, 35).

The mechanism whereby CO$_2$/bicarbonate buffer facilitates $H_+^+$ mobility is proposed to depend on a buffer shuttle, much as occurs with intracellular pH$_i$ regulation. In this case, however, there is diffusion and hydration of intracellular CO$_2$ (or diffusion and dissociation of H$_2$CO$_3$), leading to the appearance of H$^+$ and HCO$_3$ in regions distal to the local acid disturbance, with back diffusion of HCO$_3$ to neutralize some of the original acid load (28, 35). The shuttle thus effectively mediates a passive spread of $H_+^+$ from proximal to distal regions. In rabbit ventricular myocytes and murine duodenal enterocytes, the enzyme carbonic anhydrase has been proposed to play a key role in maximizing the turnover rate of this shuttle (28, 30). The participation of carbonic anhydrase in rat and guinea pig myocytes was not investigated in the present work, although the expectation is that the enzyme will play a similar role in these cells.

Under physiological conditions, the intrinsic and carbonic buffer shuttles will independently mediate spatial movements of intracellular acid. Their effects should be combined when computing contributions to $D_{H+}^{pp}$ (28).

Intrinsic Buffers and the Regulation of Intrinsic $H_+^+$ Mobility

The values for intrinsic buffering power reported here are within the range of most previous estimates in cardiac tissue (4, 36), including ventricular myocytes isolated from the guinea pig (17, 18, 40), rat (11, 39), and rabbit (21). More importantly, our quantitative estimates of the pH$_i$ dependence of $\beta_{int}$ in the three species are also similar. This would be consistent with the intracellular concentration and pH$_a$ of individual constituent buffers being comparable among the species, thus also accounting for the comparable values of $D_{H+}^{pp}$.

**Fractional mobile buffer capacity.** The influence of mobile and fixed intrinsic buffer shuttles on $H_+^+$ mobility is described by the function (14, 15, 32)

$$D_{H+}^{pp} = \frac{\ln (10) \cdot [H^+] \cdot D_H + \sum_i (D_{mob,i} \cdot \beta_{mob,i})}{\sum_i \beta_{mob,i} + \sum_j \beta_{fix,j}}$$

where $D_H$ is the $H^+$ diffusion coefficient, $D_{mob,i}$ is the diffusion coefficient of the $i$th mobile buffer of capacity $\beta_{mob,i}$, and $\beta_{fix,j}$ is the capacity of the $j$th fixed intrinsic buffer. At physiological pH$_i$ and pooling all mobile buffers under one diffusion coefficient and all fixed buffers under zero mobility, this equation can be approximated as

$$D_{H+}^{pp} = \frac{D_{mob} \cdot \beta_{mob}}{\beta_{int}}$$

where $D_{mob}$ is the mobile buffer diffusion coefficient and $\beta_{int}$ equals the summed capacity of all mobile and fixed intrinsic buffers (equal to $\beta_{mob} + \beta_{fix}$). This has been dubbed the “proton mobility” equation (32).

*Equation 2* indicates that a major determinant of intrinsic $H_+^+$ mobility will be the fraction of $\beta_{int}$ that is mobile ($\beta_{mob}/\beta_{int}$). As illustrated in the present work, $\beta_{int}$ may be estimated experimentally from measurements of pH$_i$, whereas $\beta_{mob}$ may be computed theoretically from the list of mobile buffers identified in cardiac tissue (Ref. 34 and the present study, methods). While this list may not be entirely comprehensive, it will nevertheless provide a reasonable estimate of the pH$_i$ dependence of mobile buffering power (see blue curve in Fig. 5B). With the use of data shown in Figure 5, B and C, one may compute the mobile buffer fraction ($\beta_{mob}/\beta_{int}$) for a range of pH$_i$ values. This has been compared in Fig. 7 for rat, rabbit, and guinea pig myocytes. Over the range of pH$_i$ from 8.0 to 6.0, the mobile fraction of intrinsic buffering in all three species is predicted to decline by ~80%. If buffer mobility ($D_{mob}$) were independent of pH$_i$ (see below), the proton mobility equation would predict that the same acidosis should produce an 80% decline of $H_+^+$ mobility.

At present, there is no experimental evidence for the effects of pH$_i$ on $H_+^+$ mobility in cardiac cells, but...
measurements of $D_{\text{app}}$ in extruded samples of molluscan axoplasm suggest that it is decreased by acidosis (1). Mobile buffers in axoplasm differ significantly from those in cardiac cytoplasm (the former contains high concentrations of amino acids such as glycine (180 mM) and aspartic acid (75 mM), whereas the latter contains more modest concentrations of dipeptides (2–9 mM) such as homocarnosine and anserine (34)). Nevertheless, as discussed above, there is a strong theoretical argument for a qualitatively similar pH$_i$ dependence of $D_{\text{app}}$ in the heart. While it may not be feasible to extrude cytoplasm from cardiac myocytes without affecting constituent buffers (particularly the fixed buffers), it may be possible to estimate $D_{\text{app}}$ in an intact cell after pipette loading of an intracellular base rather than acid, thereby exploring more alkaline regions of pH$_i$.

**Fixed intrinsic buffer.** A striking observation in the present study is that the fraction of $\beta_{\text{nat}}$ attributed to fixed buffer can be represented by a single component of pK$_a$ 6.1–6.2 and a concentration of 50–70 mM (e.g., red curve in Fig. 5B). This is in contrast to the predicted mobile buffer curve that comprises contributions from several individual buffers of much lower concentration, with overlapping pK$_a$ values such that summed mobile capacity is relatively constant over the physiological pH range. We conclude that fixed buffers in the cardiac myocyte constitute a relatively homogeneous population. The pK$_a$ of 6.1–6.2 would, for example, be consistent with imidazole groups on histidine residues. Given their effective concentration, these would most likely be components of proteins that are of low cytoplasmic mobility or anchored within the cell.

The fixed buffer curve (i.e., fixed buffer capacity vs. pH$_i$) predicted in the present study is comparable to the intrinsic buffer component of low pK$_a$ first identified by Leem et al. (18). These authors approximated the pH$_i$ dependence of intrinsic buffering in the guinea pig ventricular myocyte by assuming two principal buffer populations, one of low average pK (6.03) and high concentration (84 mM) and one of higher average pK$_a$ (7.57) and more modest concentration (29 mM). These subdivisions are therefore reminiscent of the present proposals for fixed and mobile buffers (e.g., Fig. 5B). Indeed, Swietach et al. (32) have recently adopted the Leem description of intrinsic buffering in a computer model of two-dimensional diffusion of acid within a cardiomyocyte. This was done by defining the low pK$_a$ component of buffering as fixed and the higher pK$_a$ component as mobile. The success of the simulation suggests that the broad division of intrinsic buffering into mobile and fixed components with differing pH-dependent characteristics may be correct.

**Intracellular buffer mobility.** Apart from the mobile buffer fraction, $D_{\text{mob}}$ is the other principal factor influencing $D_{\text{app}}$. The proton mobility equation predicts that, at a pH$_i$ of 7.1, $D_{\text{mob}}$ in guinea pig myocytes will be $2.6 \times 10^{-6}$ cm$^2$/s, a value pooled for all mobile buffers. Possible effects of pH$_i$ on $D_{\text{mob}}$ have not been investigated, but mobility will be independent of pH$_i$ only if the protonated and nonprotonated forms of any buffer display identical diffusion coefficient values.

Given that protonation alters the net electrical charge on a molecule, this may influence the mobility of a buffer within the intracellular compartment, so that a pH sensitivity of $D_{\text{mob}}$ cannot be excluded.

**Relevance to Physiological Acidosis and Ischemia**

A $D_{\text{app}}$ of sufficient magnitude is necessary to promote uniformity of pH, in the face of a local acid/base disturbance. A reduced $D_{\text{app}}$ during intracellular acidosis will therefore increase the possibility of pH$_i$ nonuniformity, at a time when acid efflux transporters operating locally at the sarcolemma are strongly stimulated. At present, there are no experimental data regarding possible pH$_i$ heterogeneity induced by acid transport in cardiac myocytes, although nonuniformity is readily produced by other maneuvers (27, 34). The physiological consequences of pH$_i$ nonuniformity are also not known, but the spatial coordination of local, intracellular processes such as sarcocomere contraction is likely to be compromised.

It is tempting to speculate that a decline of $D_{\text{app}}$ may occur during the severe metabolic acidosis associated with myocardial ischemia. Such speculation, however, would be premature. Complex metabolic disturbances, such as occur during ischemia, are associated with changes in the concentration of certain intracellular mobile buffers, an effect independent of changes in pH$_i$.

For example, during myocardial ischemia, there is a considerable rise (from 2 to 30 mM) in the levels of inorganic phosphate (see, e.g., Ref. 2), a molecule with a principal pK$_a$ of 6.9. This might raise rather than reduce H$_i$ mobility. In contrast, as noted recently (28), because acidosis during ischemia is also associated with a fall of [HCO$_3^-$]$_i$, this would tend to reduce overall H$_i$ mobility. Direct measurements of $D_{\text{app}}$ under conditions that simulate ischemic acidosis will therefore be required to explore these possibilities further.

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