Innovative Methodology

Novel method for measuring junctional proton permeation in isolated ventricular myocyte cell pairs

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Swietach, Pawel, and Richard D. Vaughan-Jones. Novel method for measuring junctional proton permeation in isolated ventricular myocyte cell pairs. Am J Physiol Heart Circ Physiol 287: H2352–H2363, 2004.—Partial exposure of single ventricular myocytes to membrane-permeant weak acids or bases, using a dual-microperfusion technique, generates large and stable intracellular pH (pHi) gradients. In this study, we have investigated the feasibility of using the technique to estimate junctional proton permeability. This was done by recording the pHi gradient developed across the junctional region of a pair of conjoined ventricular myocytes, isolated enzymically from a guinea pig heart when one of the cells was partially exposed to acetate or -glycyrrhetinic acid, exposure of the proximal cell to weak acid or base produced no pHi change in the distal cell, confirming that distal changes were normally caused by acid-base flux through connexons assembled into junctional channels. The validity of the dual-microperfusion method was tested further by using a diffusion-permeation–reaction model for intracellular protons, designed to highlight possible errors in the estimates of $P_{\text{app}}$. Our technique for measuring $P_{\text{app}}$ provides a useful alternative to the previous, more invasive technique of locally loading acid through a cell-attached patch pipette. The technique may provide a simple method for investigating the factors regulating cell-to-cell proton transmission.

$P_{\text{app}}$.

Intracellular pH (pHi) is a major modulator of cell function, particularly in the heart, where pHi affects contractility and electrical excitability (e.g., 3, 23, 24). Changes of cardiac pHi will occur during whole body acid-base disturbances (25) and during changes in the metabolic workload placed on the heart (2, 8). A large drop in pHi also occurs during myocardial ischemia, where it accounts for much of the observed inotropic decline (1, 9).

Previous studies (19) have identified sarcolemmal H+–ion transporters that regulate pHi in the heart. In addition, spatial movements of intracellular protons are regulated by mobile buffer molecules, such as inorganic phosphate, certain dipeptide molecules, and CO2–HCO3- buffer (31, 34, 37, 46). In effect, these molecules reversibly bind intracellular H+ ions and shuttle them within the cytoplasmic compartment, a form of facilitated H+ diffusion predicted originally by Junge and McLaughlin (17). The process has been described mathematically by diffusion equations incorporating the concept of an apparent proton diffusion coefficient ($D_{\text{app}}$) (37, 46) or by diffusion-reaction algorithms (34) that incorporate the mobility, concentration, kinetics, and acid-dissociation constants of the intracellular mobile buffers.

To understand the factors governing the spatial regulation of pHi in larger multicellular structures such as the myocardium, it is necessary to quantify not only sarcolemmal transport and H+ mobility, but also any cell-to-cell H+ transmission. Gap junctions provide a major pathway for the transmission of intracellular solutes between cells. These are hydrophilic channels that comprise a hexameric arrangement of connexin proteins. Ventricular myocytes, for example, express connexin 43 (44). Although intracellular proton mobility is relatively slow, gap junctions may impose a further rate-limiting barrier to the spread of a local pHi disturbance. It is noteworthy that junctional conductance to various solutes, unlike diffusion, can be regulated by channel-gating reactions (20, 21, 32, 41).

Recently, cell-to-cell proton transmission has been measured in terms of an apparent junctional proton permeability constant ($P_{\text{app}}$) (45). Acid was loaded locally through a patch-pipette into enzymically isolated ventricular myocyte cell pairs, and its passage across the cell junction was imaged confocally using AM-loaded carboxy-seminaphthorhodafluor-1 (SNARF-1). The mean $P_{\text{app}}$ obtained experimentally by Zaniboni and co-workers (45, 46) was 2.03 ± 10^{-4} cm/s. Intracellular proton transmission was blocked by the connexin inhibitor α-glycyrrhetinic acid (α-GA), suggesting that acid was indeed moving between myocytes via gap junctions. It is proposed that proton flux through the gap junction is mediated by “proton porter” molecules, most likely the cytoplasmic mobile buffers highlighted previously. These possess a range of values up to a molecular mass of ~500 Da (see Table 1 of Ref. 37). Because gap junctions permit the flux of solutes up to 1 kDa (14, 20), almost all cytoplasmic mobile buffers in cardiac myocytes are likely to be permeant.

Unfortunately, the pipette-based method for estimating $P_{\text{app}}$ (45) is highly invasive and suffers from the drawback that local time courses for acid loading need to be analyzed using complex diffusion-permeation equations. The method may be susceptible to errors because the calculation procedure relies on pHi data gathered from only a few small regions of interest in the cell. In this study, we present a novel experimental method for measuring $P_{\text{app}}$. This is a far less invasive technique based on a dual-microperfusion apparatus (29, 30) used to generate pHi gradients across the junctional region of a myocyte cell pair. We show that the sharp discontinuity in the longitudinal pHi profile coincident with the junctional region...
represents an H⁺ permeation phenomenon. We develop a method for using the discontinuity to estimate \( P_{\text{H}^+} \). We also develop a diffusion-permeation reaction model for intracellular protons and use this to verify our experimental approach. The new method is technically easier to use and more readily applied to cardiac cell pairs than the patch-pipette method.

METHODS

Myocyte cell pair isolation. Ventricular myocytes were isolated from guinea pigs (~400 g wt) that were euthanized by cervical dislocation. The procedure was done according to United Kingdom Home Office Regulations. Hearts were subjected to a combination of mechanical and enzymatic dispersal (0.7 mg/ml collagenase, Roche; and 0.04 mg/ml protease, Sigma); see Refs. 19 and 45 for details. On average, 1 in 15 cells was coupled in the side-by-side orientation and ~1 in 40 was coupled in the end-to-end orientation. Cells were stored in DMEM culture medium at room temperature. All experiments were carried out in a glass-bottomed Plexiglas chamber mounted on an inverted confocal microscope (Leica DM IRBE). Before experiments, the bottom of the bath was coated with 400 μl of 1% poly-l-lysine to promote cell adhesion.

Confocal measurement of \( \text{pH}_i \). Cells suspended in culture medium were mixed with the AM ester of carboxy-SNARF-1 and applied to the bath (final dye concentration: 10 μM). Cells were allowed to load with dye for 10 min. Despite being a mobile buffer, intracellular carboxy-SNARF-1 does not, at the concentrations used, significantly affect \( D_{\text{H}^+} \) or \( P_{\text{H}^+} \) measured in myocytes. During experiments, cells were superfused with HEPES-buffered normal Tyrode solution at 37°C. Intracellular dye was excited by an argon laser at 514 nm, and fluorescence was imaged confocally at 580 and 640 nm (the ratio of which determines intracellular pH according to a calibration curve, obtained using the nigericin method) with the use of a Leica ×40, 1.25 numerical aperture, oil immersion, plano-apochromat objective lens. For details of confocal measurement and calibration of pH, with the ratiometric fluorophore SNARF, see Ref. 46.

Microperfusion system. To create regional differences in \( \text{pH}_i \), we used a microperfusion system (29, 30). It consisted of a short (~7 mm) length of custom-designed, double-barreled, square-bored glass tubing (Wilmad Glass; Buena, NJ) pulled by hand in a Bunsen flame into tapering micropipettes. The glass barrel was attached to a micro-tubing (Wilmad Glass; Buena, NJ) pulled by hand in a Bunsen flame. The glass barrel was attached to a micro-perfusion system (29, 30). It consisted of a short (~7 mm) length of custom-designed, double-barreled, square-bored glass tubing (Wilmad Glass; Buena, NJ) pulled by hand in a Bunsen flame into tapering micropipettes. The glass barrel was attached to a micro-tubing (Wilmad Glass; Buena, NJ) pulled by hand in a Bunsen flame.

The quality of dual microperfusion membranes, so that the only source of \( \text{pH}_i \) change generated in the proximal cell. In this way, a concentration gradient of protons was generated across the junction that would drive an intercellular proton flux. Figure 1A illustrates the protocol for an end-to-end and side-by-side cell pair. In a few cases, the boundary was moved to the distal cell, to confirm that the fluorescence signal at the junction did not introduce pH artifacts per se.

Solutions and drugs. Normal Tyrode contained (in mM) 135 NaCl, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 20 HEPES, and 11 glucose. Solutions containing weak acid or base were osmotically compensated by appropriately reducing the concentration of NaCl. Ammonium Tyrode (30 mM) contained (in mM) 105 NaCl, 30 NH₄Cl, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 20 HEPES, and 11 glucose. Ammonium Tyrode (20 mM) contained (in mM) 115 NaCl, 20 NH₄Cl, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 20 HEPES, and 11 glucose. Acetate Tyrode (80 mM) contained (in mM) 55 NaCl, 80 NaCH₃CO₂, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 20 HEPES, and 11 glucose. Acetate Tyrode (120 mM) contained (in mM) 15 NaCl, 120 NaCH₃CO₂, 4.5 KCl, 1 MgCl₂, 2 CaCl₂, 20 HEPES, and 11 glucose. The pH of all solutions was adjusted to 7.4 with 4 M NaOH or 5 M HCl.

Drugs. Cariporide, a sodium-hydrogen exchange inhibitor (27), was a kind gift of Dr. H.-W. Kleeman (Aventis). 18a-GA, a gap-junctional blocker (26), was obtained from Sigma.

Derivation of \( P_{\text{H}^+} \). Intracellular proton diffusion and junctional permeation can be quantified in terms of \( D_{\text{H}^+} \) and \( P_{\text{H}^+} \), as shown mathematically by Irving et al. (16) and Zamboni et al. (45), respectively. \( D_{\text{H}^+} \) and \( P_{\text{H}^+} \) are related to the mobile buffer diffusion coefficient \( D_{\text{mob}} \) and mobile permeability constant \( P_{\text{mob}} \), respectively, and both depend on the mobile-to-total buffering capacity ratio \( \beta_{\text{mob}}/\beta_{\text{tot}} \):

\[
D_{\text{H}^+} = \frac{D_{\text{mob}} \beta_{\text{mob}}}{\beta_{\text{tot}}} \tag{1}
\]
Innovative Methodology

MEASURING JUNCTIONAL H⁺ PERMEATION

\[ P_{\text{tot}}^{\text{app}} = \frac{P_{\text{mob}} \cdot \beta_{\text{mob}}}{\beta_{\text{fix}}} \]  

(2)

\[ D_{\text{mob}}^{\text{app}} \text{ and } P_{\text{tot}}^{\text{app}} \text{ have been measured by pipette acid-loading experiments (37, 45, 46). These two parameters are, in fact, related by the boundary condition equation, which describes the concentration of protons at the junctional region (45):} \]

\[ -D_{\text{mob}}^{\text{app}} \cdot \nabla[H^+] = P_{\text{mob}}^{\text{app}} \Delta[H^+] \]  

(3)

where \( \Delta[H^+] \) is the difference in proton concentration on either side of the junction, and \( \nabla[H^+] \) denotes the mean slope of the proton concentration profile on either side of the junction. With the use of Eq. 3, any parameter can be estimated if the remaining three are known. Because \( D_{\text{mob}}^{\text{app}} \) has been measured independently of Eq. 3 (46), an experimental protocol that develops a pHi gradient across a cell pair can yield a measure of \( \Delta[H^+] \) and \( \nabla[H^+] \), and hence \( P_{\text{tot}}^{\text{app}} \). Dual microperfusion with weak acids or bases produces large and stable pHi gradients, which, under appropriate conditions, can be used to derive \( P_{\text{tot}}^{\text{app}} \) using Eq. 3 (see Fig. 1B). If we assume that all the mobile buffers involved in facilitating intracellular diffusion also participate in intercellular proton transmission, it is possible to combine Eq. 1 and Eq. 2 with Eq. 3, giving

\[ -D_{\text{mob}}^{\text{app}} \cdot \frac{\beta_{\text{mob}}}{\beta_{\text{fix}} + \beta_{\text{mob}}} \cdot \nabla[H^+] = P_{\text{mob}}^{\text{app}} \Delta[H^+] \]  

(4)

The significance of Eq. 4 is that the pHi, dependence of mobile and fixed buffering capacity (46) is eliminated, and the parameters relate only to mobile buffer permeation and diffusion.

Estimating \( P_{\text{tot}}^{\text{app}} \). Each experiment was recorded as a stack of confocal images of carboxy-SNARF-1 fluorescence ratio measured at 580 to 640 nm. A subset of the stack corresponding to the resting state (R, i.e., no dual microperfusion) during uniform superfusion with normal Tyrode was averaged into a single spatial map of fluorescence ratio. A similar procedure was performed for the subset of the stack representing the dually perfused state (DP) during the pHi steady state. A plotting vector was positioned along the cell pair so that it contained the best R and DP files on either side of the junction. With the use of concentration profiles taken over several micrometers, is used to approximate the junctional \( P_{\text{mob}}^{\text{app}} \) calculated in step 4 will only be valid for junctional pH values near 7.0 because our assumed value of \( D_{\text{mob}}^{\text{app}} \) was derived near resting pH. If the method fails at step 1, the flux between the cells is beyond the resolving power of the technique.

Diffusion-permeation-reaction algorithms for two coupled cells. A diffusion-permeation reaction algorithm for intracellular H⁺ ions was used to simulate cell-to-cell transmission of protons in a model end-to-end cell pair. The simulation was performed using the one-dimensional solver, pdepe, supplied by MATLAB (The MathWorks). Because of the nature of this mechanistic solver, only end-to-end cell pairs of comparable cell lengths could be considered. The details and formulation of the model are presented in the Appendix.

Statistics. Results are presented as means ± SE. Two-tailed unpaired Student’s t-tests at 5% significance level were used to test the significance of two populations. Single-factor ANOVA at 5% significance level was used to test when more than two populations were compared simultaneously.

RESULTS

Observing pHi discontinuities in the junctional region. Figure 2 shows a dual microperfusion experiment performed on a triplet of coupled cells isolated from the guinea pig ventricle. The cell assembly was partially exposed to 20 mM ammonium included in the right-hand microstream (Fig. 2A,i). Initially the cells were exposed uniformly to normal HEPES-buffered Tyrode solution. Dual microperfusion was then applied at two different boundary positions denoted by microstream boundary position 1 (BP1) and then boundary position 2 (BP2) in Fig. 2A,i. Local pHi was averaged in four ROIs positioned longitudinally down one cell pair (Fig. 2A,ii), and the time courses of pHi in each region were plotted in Fig. 2B. The period of dual microperfusion has been indicated by the solid bars above the time-course traces in Fig. 2B. The time courses were used to identify the moment when pHi had reached a steady state during dual microperfusion. This was necessary to plot time-averaged longitudinal profiles (Fig. 2C) along the plotting vector (shown in Fig. 2A,iii) and to display steady-state spatial pHi maps (Fig. 2D). With BP1, the proximal cell was half perfused with ammonium. The pHi change in the distal cells is due to junctional proton transmission down a concentration gradient from the distal to the proximal cell. The large discontinuity in the longitudinal profile (Fig. 2C, BP1) represents the rate-limiting step for junctional proton transmission. This was used to derive an estimate of \( P_{\text{mob}}^{\text{app}} \) (see METHODS). In contrast, with BP2, the entire proximal cell and part of the distal cells were exposed to ammonium. Because both the pre- and postjunctional regions were perfused with extracellular weak base, no junctional pHi discontinuity was recorded (Fig. 2C, BP2). This latter experiment shows that our method does not record junctional discontinuity where it is not expected to occur. The spatial pHi maps shown in Fig. 2D illustrate the distribution of pH before and during dual microperfusion. As expected from a passive diffusion-permeation process, the pHi gradient within individual cells is smooth and continuous (all three maps), whereas in the case of BP1, there is a sharp discontinuity due to the rate-limiting junctional permeation event (second map).

It was more difficult to obtain a satisfactory cell-to-cell pHi profile in side-by-side cell pairs because the microstream...
boundary width is usually \( \approx 8 \mu m \), which is a significant fraction of cell width (typically 25 \( \mu m \)). Figure 3A,i shows a side-by-side cell pair where the proximal (right) cell was unusually wide. Part of the proximal cell was exposed to 20 mM ammonium under HEPES-buffered conditions. A large junctional discontinuity was evident (Fig. 3C) when pH\(_i\) had reached a steady state during dual microperfusion (Fig. 3B).

The spatial pH\(_i\) maps in Fig. 3D again confirm the passive properties governing the pH\(_i\) distribution and show that junctional permeation is a rate-limiting event, despite slow intracellular proton diffusion (46).

Dual microperfusion experiments were also performed with the membrane-permeant weak acid acetate. Figure 4 illustrates a dual microperfusion experiment performed on an end-to-end guinea pig cell pair. Acetate (120 mM) was confined to the right microstream. Both microstreams contained 30 \( \mu M \) cariporide to inhibit any Na\(^+\)/H\(^+\) exchange-mediated pH\(_i\) recovery associated with acidosis (27). The boundary between microstreams was placed at two positions along the length of the proximal cell (Fig. 4A,i). The four ROIs drawn in Fig. 4A,ii were used to generate pH\(_i\) time courses shown in Fig. 4B. During the period of steady-state pH\(_i\), the longitudinal pH\(_i\) profiles were measured along the plotting vector (shown in Fig. 4A,iii). Both microstream boundary positions generated a junctional discontinuity of pH\(_i\). This is shown in the longitudinal profiles (Fig. 4C) and the spatial pH\(_i\) maps (Fig. 4D). Because the dual microperfusion technique is not as invasive as other methods for measuring junctional transmission [e.g., local acid injection from a cell-attached pipette (45); or rupturing the distal cell (21, 22)], several experiments can be performed on the same cell pair. By varying boundary position, the junctional pH\(_i\) could be varied: as the microstream boundary was positioned closer to the junction, the junctional pH\(_i\) displacement increased (e.g., Fig. 4C).

Because it was easier to perform experiments on end-to-end cell pairs, this geometry was chosen when estimating the mean \( P_{app}^{+H} \). Partial microperfusion with 20 mM ammonium (\( n = 5 \),...
30 mM ammonium \((n = 9\) experiments\), 80 mM acetate \((n = 15\) experiments\), and 120 mM acetate (also contained 30 \(\mu\)M cariporide; \(n = 14\) experiments) gave a mean \(P_{ij}^{\mathrm{app}}\) of 4.45 \((0.21) \cdot 10^{-4}\) cm/s \((n = 43\) experiments\). ANOVA tests did not reveal any significant difference in the means of these four categories \((P > 0.05)\). To calculate \(P_{ij}^{\mathrm{app}}\), we used the value of \(D_{ij}^{\mathrm{app}}\) estimated by Zaniboni et al. (Ref. 46; \(D_{ij}^{\mathrm{app}} = 12.1 \cdot 10^{-7}\) \(\mathrm{cm}^2/s\)). This value holds true when junctional \(\mathrm{pH}_i\) is \(\sim 7.0\), although it may possibly increase with \(\mathrm{pH}_i\) (19, 46). In the experiments analyzed in this section, the average junctional \(\mathrm{pH}_i\) was 7.04 \pm 0.02 and ranged from 6.83 to 7.28. This validates the use of \(12.1 \cdot 10^{-7}\) \(\mathrm{cm}^2/s\) as our estimate of \(D_{ij}^{\mathrm{app}}\) in Eq. 3.

**Parajunctional contribution to intercellular proton flux.** The dual microperfusion technique uses a local application of membrane-permeant weak acid or base that may itself contribute to cell-to-cell proton flux by diffusing through a parajunctional pathway, i.e., by means of a flux across the lipid bilayer rather than through the gap junctions. Significant parajunctional flux would complicate the interpretation of our results in terms of gap junction-mediated proton transmission. Experimental evidence for the importance of this parajunctional flux component was explored by blocking gap junctions pharmacologically. A suitable blocker is 18\(\alpha\)-GA shown to inhibit 90% of junctional transmission in guinea pig myocyte pairs at a dose of 60 \(\mu\)M (26, 45). Cell pairs were perfused with solutions containing 60 \(\mu\)M \(\alpha\)-GA for a minimum of 5 min before dual microstream flow was switched on. Figure 5A shows an end-to-end cell pair superfused with normal Tyrode containing 60 \(\mu\)M \(\alpha\)-GA (HEPES-buffered solutions). The cell
The pair was partially exposed to 80 mM acetate (right microstream, Fig. 5A, i). Both microstream solutions also contained 60 uM H9262/H9251-GA for persistent junctional inhibition. The time courses of pH_i shown in Fig. 5B suggest the pH_i change in the distal cell was minimal (see ROIs 3 and 4 in Fig. 5A, ii). This was confirmed in the longitudinal profiles shown in Fig. 5C where, despite a large acid-load in the proximal cell, junctional proton transmission was negligible. This and other experiments with H9251-GA (n = 17 with 80 mM acetate, and n = 10 with 20 mM ammonium; mean junctional pH_i; 6.97 ± 0.03) have suggested that the parajunctional route is not a significant pathway for transcellular proton flux in our experimental protocol. The residual $P_{ij}^{pp}$ of 0.42 ± 0.07·10^{-4} cm/s (n = 17) represents 9% of control $P_{ij}^{pp}$ ($P < 0.05$). This residual transmission is more likely a reflection of the inability of the drug to produce total block (26, 45) rather than parajunctional transmission mediated by the weak acid or base short circuiting the gap-junctional pathway.

Verification of dual microperfusion technique by computer simulation. Computer simulations were run to model diffusion-permeation phenomena for H^+ ions in cell pairs. A “model” cell pair (shown Fig. 6A) was partially perfused with 30 mM ammonium or 80 mM acetate, such that 75% of the proximal (right) cell was exposed to the weak acid-base. Two types of intracellular H^+ buffer were included in the computational algorithm. Fixed buffer was characterized by the parameters derived by Zaniboni and others (46). The mobile buffering component was fitted with a one-component buffering capacity curve to simplify computation. Concentrations and acid-dissociation constants assumed for the fixed and mobile buffers are listed in Table 1. The average $P_{ij}^{pp}$ determined experimentally was 4.45( ± 0.21)·10^{-4} cm/s. Therefore, from Eq. 2, our modeling used a value of 10^{-3} cm/s for $P_{pob}$, which is close to that predicted from a mobile-to-total buffering ratio of 0.44 (46). The values of junctional acetate and ammonium permeation constants were selected as described in the APPENDIX. The
remaining parameters were derived from Table 1. Typical guinea pig myocyte dimensions were 120 μm in length and 25 μm in width. Figure 6B shows the simulated pH i time courses in four ROIs (Fig. 6A(ii)) during a 4.5-min exposure to the dual microstream when ammonium was used (Fig. 6B, i) or acetate (Fig. 6B, ii). The vertical dashed lines indicate the moment when the longitudinal pH i profiles were generated (shown in Fig. 6C). It is clear from the pH i profiles that a large pH i discontinuity is predicted to coincide with the junctional region. The junctional pH i profiles predicted in Fig. 6C were then used to derive $P_{mob}^{app}$ in a manner identical to that described for experimental data in Fig. 1B.

The purpose of the model was to assess the sensitivity of $P_{mob}^{app}$ (measured from the pH i discontinuity simulated by the computational model) to changes in particular parameters, namely junctional and parajunctional permeation, extracellular acetate/ammonium concentration, boundary position, cell geometry, and starting pH i. In each of eight tests for acetate and ammonium partial perfusion, one parameter in the model was varied at a time within a certain range while the remaining parameters were kept at their default values (see Tables 2 and 3 for acetate and ammonium dual microperfusion tests, respectively). The simulated junctional pH i discontinuity can be used to derive an estimate of $P_{mob}$ by a procedure identical to that for calculating $P_{mob}$ from experimental data (see Fig. 1B, Eq. 4, and METHODS). If the value of $P_{mob}^{app}$ is similar to the value of $P_{mob}$ set in the algorithm, the experimental method of calculating $P_{mob}$ employed in the present work is likely to be valid. Otherwise, the simulation would indicate that artifacts produce detrimental effects on the estimate of junctional proton permeability. In each of the tests described in Tables 2 and 3, the slope of the relationship between estimated $P_{mob}^{app}$ and the varied parameter was calculated. The closer the slope was to zero, the less sensitive was $P_{mob}^{app}$ to variations in the parameter under test. Ideally, simulations should produce a slope of zero for all parameters, except $P_{mob}$ (which should be related to $P_{mob}^{app}$ with a slope of around unity, i.e., $P_{mob} = P_{mob}^{app}$). It may be inferred from the computational results summarized in
Tables 2 and 3 that $P_{mob}$ was most sensitive by far to variations of $P_{mob}$, with a slope relating the two parameters equal to $\approx 1.0$. The simulations show that our method of deriving $P_{H^{\text{app}}}$ is not detrimentally affected by changes in starting pH, cell geometry, junctional permeability to the weak acid-base, or the parajunctional pathway (slope 0). This supports the dual perfusion method as a valid approach for elucidating junctional $H^+$ permeation.

DISCUSSION

A full understanding of the mechanisms underlying the spatio-temporal distribution of pH in a syncytial network of cells requires a study of both intracellular $H^+$ diffusion and junctional $H^+$ permeation. Research into the spatial aspects of pH in heart has gained impetus from the observations of pH gradients at the border zones of localized ischemic areas (4, 42). Similar border zones have been simulated experimentally in cultured confluent myocyte monolayers (13). To understand and simulate spatial pH regulation in the heart, including that occurring at ischemic border zones, it is necessary to have an accurate estimate of $P_{H^{\text{app}}}$ for protons. With the advent of confocal imaging techniques for pH, it has been possible to obtain accurate estimates for $D_{H^{\text{app}}}$ within a given cell type (28, 46).

Table 1. Constants and default values used for modeling guinea pig myocyte cell pairs

<table>
<thead>
<tr>
<th>Constant</th>
<th>Definition</th>
<th>Default Value</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>$C_{mob}$</td>
<td>Mobile buffer concentration</td>
<td>23.8 mM</td>
<td>46</td>
</tr>
<tr>
<td>$C_{fix}$</td>
<td>Fixed buffer concentration</td>
<td>68.3 mM</td>
<td>46</td>
</tr>
<tr>
<td>$K_{mob}$</td>
<td>Mobile buffer dissociation constant</td>
<td>$10^{-7.102}$</td>
<td>46</td>
</tr>
<tr>
<td>$K_{fix}$</td>
<td>Fixed buffer dissociation constant</td>
<td>$10^{-6.098}$</td>
<td>46</td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>Fast protonation rate constant</td>
<td>$10^{10}$ M$^{-1}$ s$^{-1}$</td>
<td>7</td>
</tr>
<tr>
<td>$K_{am}$</td>
<td>Ammonium dissociation constant</td>
<td>$10^{-8.03}$</td>
<td>19</td>
</tr>
<tr>
<td>$K_{ac}$</td>
<td>Acetate dissociation constant</td>
<td>$10^{-4.528}$</td>
<td>19</td>
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<tr>
<td>$P_{NH4}$</td>
<td>$NH_4^+$ membrane permeability constant</td>
<td>$1.408 \times 10^{-7}$ cm$^2$/s</td>
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<tr>
<td>$P_{Ac}$</td>
<td>$Ac^-$ membrane permeability constant</td>
<td>$6.77 \times 10^{-9}$ cm$^2$/s</td>
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</tr>
<tr>
<td>$P_{NH3}$</td>
<td>$NH_3$ membrane permeability constant</td>
<td>$5.45 \times 10^{-4}$ cm$^2$/s</td>
<td>*</td>
</tr>
<tr>
<td>$P_{HAc}$</td>
<td>$HAc$ membrane permeability constant</td>
<td>$3.81 \times 10^{-4}$ cm$^2$/s</td>
<td>*</td>
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<tr>
<td>$D_{mob}$</td>
<td>Mobile buffer diffusion coefficient</td>
<td>$2.8 \times 10^{-6}$ cm$^2$/s</td>
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<td>$D_{fix}$</td>
<td>Fixed buffer diffusion coefficient</td>
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<td>$D_{NH3/NH4}$</td>
<td>$NH_3/NH_4^+$ diffusion coefficient</td>
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<td>$D_{Ac}$</td>
<td>$HAc/Ac^-$ diffusion coefficient</td>
<td>$7.08 \times 10^{-6}$ cm$^2$/s</td>
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<tr>
<td>$D_{H}$</td>
<td>Free proton diffusion coefficient</td>
<td>$6.1 \times 10^{-5}$ cm$^2$/s</td>
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Innovative Methodology

MEASURING JUNCTIONAL H⁺ PERMEATION

Table 2. Results of ammonium simulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0–100</th>
<th>10</th>
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<th>10</th>
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<th>10</th>
<th>10</th>
<th>10</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_{\text{mob}} ) (10⁻⁴ cm/s)</td>
<td>51</td>
<td>0–100</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
<td>51</td>
</tr>
<tr>
<td>( P_{\text{para}} ) (10⁻⁴ cm/s)</td>
<td>53.5</td>
<td>53.5</td>
<td>0–100</td>
<td>53.5</td>
<td>53.5</td>
<td>53.5</td>
<td>53.5</td>
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<td>53.5</td>
</tr>
<tr>
<td>([\text{NH}_4^+]_o) mM</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
<td>30</td>
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<tr>
<td>Boundary, %</td>
<td>75</td>
<td>75</td>
<td>75</td>
<td>75</td>
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</tr>
<tr>
<td>Cell length, μm</td>
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<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Starting ( \text{pH}_i )</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Mean ( P_{\text{mob}} )</td>
<td>48.04</td>
<td>10.21</td>
<td>10.13</td>
<td>10.06</td>
<td>9.99</td>
<td>10.09</td>
<td>10.07</td>
<td>10.38</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.95</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
<td>0.00</td>
<td>0.00</td>
<td>0.01</td>
</tr>
</tbody>
</table>

\( P_{\text{mob}} \), mobile buffer permeability constant; \( P_{\text{para}} \), parameter buffer permeability constant; \([\text{NH}_4^+]_o\), extracellular \text{NH}_4^+ concentration; \( \text{pH}_i \), intracellular \text{pH}; *, estimate. Test parameters are shown in bold.

Table 3. Results of acetate simulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>0–100</th>
<th>10</th>
<th>10</th>
<th>10</th>
<th>10</th>
<th>10</th>
<th>10</th>
<th>10</th>
<th>10</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P_{\text{mob}} ) (10⁻⁴ cm/s)</td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
<td>19.3</td>
</tr>
<tr>
<td>( P_{\text{para}} ) (10⁻⁴ cm/s)</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
<td>37</td>
</tr>
<tr>
<td>([\text{Ac}^-]_o) mM</td>
<td>80</td>
<td>80</td>
<td>80</td>
<td>10–100</td>
<td>80</td>
<td>80</td>
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<tr>
<td>Boundary, %</td>
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<td>75</td>
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<td>75</td>
<td>75</td>
<td>75</td>
</tr>
<tr>
<td>Cell length, μm</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
<td>120</td>
</tr>
<tr>
<td>Starting ( \text{pH}_i )</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
<td>7.1</td>
</tr>
<tr>
<td>Mean ( P_{\text{mob}} )</td>
<td>48.64</td>
<td>10.19</td>
<td>10.05</td>
<td>9.99</td>
<td>9.98</td>
<td>10.04</td>
<td>10.03</td>
<td>10.01</td>
<td></td>
</tr>
<tr>
<td>Slope</td>
<td>0.96</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
</tr>
</tbody>
</table>

\( P_{\text{para}} \), permeability of uncharged solute; \([\text{Ac}^-]_o\), extracellular negative charged solute concentration. Parameters under test are shown in bold; default parameter values are shown in normal type. The mean \( P_{\text{mob}} \) was calculated over the entire test range. The slope was calculated by normalizing the test range to 0–100%.

33, 37). Only recently has attention been drawn to measuring \( P_{\text{pp}}^{\text{app}} \) using the same imaging technology (45).

One common method for studying junctional transmission of various ions has traditionally involved measurements of electrical conductance across the junction (e.g., 5, 21, 22, 43). This approach, however, largely reflects \( \text{K}^+ \) and \( \text{Cl}^- \) flux through gap junctions because these ions are the major permeant intracellular charge carriers. Such a current will not reflect the permeation of mobile buffer molecules that translocate \( \text{H}^+ \) ions because such molecules have a higher molecular weight and exist in various ionization states, including a possible electrically silent form. Electrical measurements of junctional conductance therefore cannot give a measure of junctional proton transmission.

Two methods have now been developed for measuring \( P_{\text{pp}}^{\text{app}} \) in myocyte pairs. In both, confocal \text{pH}_i imaging is used to characterize the discontinuity in a \text{pH}_i profile across the junctional region. This discontinuity is the result of a rate-limiting step in the shuttling of protons between opposite ends of a cell pair when a region within one of the cells is subjected to a localized \text{pH}_i change. In the first method, the \text{pH}_i disturbance is brought about by pipette loading of acid. The \( P_{\text{pp}}^{\text{app}} \) measured using this approach is 2.03(± 0.66)·10⁻⁴ cm/s for isolated guinea pig myocyte cell pairs (45). Taking the cell-averaged \text{pH}_i at the midpoint of acid loading (\text{pH}_i = 6.95 after 30 s of acid loading), the corresponding mobile-to-total buffering capacity ratio has been estimated as ~0.44. With the use of Eq. 2, the value of \( P_{\text{pp}}^{\text{app}} \) suggests a \( P_{\text{mob}} \) of 4.6(± 1.5)·10⁻⁴ cm/s. Such a permeability constant is 10- to 25-fold lower than that reported for \( \text{K}^+ \) (36, 39) but of a similar order of magnitude to \( \text{K}^+ \) but of a similar order of magnitude to \( \text{K}^+ \) (36, 39) but of a similar order of magnitude to

The average \( P_{\text{pp}}^{\text{app}} \) estimated in end-to-end guinea pig myocyte pairs using dual microperfusion was 4.45(±0.21)·10⁻⁴ cm/s. There was no significant difference in the estimate of \( P_{\text{pp}}^{\text{app}} \) from experiments using (in mM) 80 acetate, 120 acetate, 20 ammonium, or 30 ammonium. With the use of an appropriate value for the mobile-to-total buffering capacity, the junctional permeability to those mobile buffers that mediate proton transmission (\( P_{\text{mob}} \)) was estimated as ~10.11(±0.48)·10⁻⁴ cm/s. This value of \( P_{\text{mob}} \) is consistent with the general trend observed previously between molecular weight and junctional permeability (14). Figure 7 shows junctional permeability data obtained from cardiac preparations (compiled from 6, 12, 14, 15, 35, 38–40, 45). The trend can be approximated by a best-fit exponential equation

\[
P_{\text{junct}} = 0.0059 \cdot \exp(-0.008 \cdot \text{MW})
\]

where MW is molecular weight. On the basis of a list of intracellular mobile buffers assembled previously by Vaughan-Jones et al. (37), the concentration-weighted average molecular...
mass of mobile buffers is 197 Da. With the use of Eq. 5, mobile buffers are predicted to have a junctional permeability constant of 12.2 \times 10^{-4} \text{ cm/s}, in close agreement with the present experimental findings.

The present estimate of \( P^\text{mob}_{\text{HI}} \) is of the same order of magnitude as that measured recently by Zaniboni et al. (45), although there is a statistically significant difference (\( P = 0.007 \)). One reason for the difference may be due to a degree of anisotropy between end-to-end and side-by-side \( H^+ \) permeability. Indeed, the spread of electrical activity in myocyte monolayers has been shown to display anisotropic conduction properties, with faster conduction occurring in the end-to-end orientation (10, 11). The previous estimate of \( P^\text{mob}_{\text{HI}} \) (45) was obtained from a pooled population of side-by-side and end-to-end cell pairs, whereas the present estimate was derived from end-to-end cell pairs only. Anisotropy for \( H^+ \) transmission, if it occurs, may possibly be explained in terms of differences in membrane folding and/or gap junction expression density in the side-by-side versus end-to-end configuration (e.g., 18).

It might be argued that dual microperfusion may permit a parajunctional flux of protons between cells, shuttled via membrane-permeant ammonia or acetic acid, thus bypassing connexin channels and leading to an overestimate of junctional \( P^\text{mob}_{\text{HI}} \). The present study, however, presents experimental and computational evidence for the validity of our dual microperfusion approach. First, pharmacological inhibition of gap junctions with 60 \( \mu \text{M} \) 18\( \alpha \)-GA reduced \( P^\text{mob}_{\text{HI}} \) by 91\%. A similar drug efficacy has been measured previously (26, 45). This suggests the parajunctional proton transmission route is not of major importance. Second, the computational simulations summarized in Tables 2 and 3 provide evidence that the measurement of \( P^\text{mob}_{\text{HI}} \) is far more sensitive to \( P^\text{mob}_{\text{HI}} \) than any other parameter. Displacing the starting \( \text{pH} \) or changing the concentration or the degree of perfusion with weak acid-base had a minimal effect on the estimate of \( P^\text{mob}_{\text{HI}} \) (and hence of \( P^\text{mob}_{\text{HI}} \)). Varying the junctional and parajunctional permeability to the weak acid-base also had a negligible effect on the estimate of \( P^\text{mob}_{\text{HI}} \). These inferences validate the dual microperfusion approach as a method for calculating \( P^\text{mob}_{\text{HI}} \).

The dual perfusion technique should be useful for studying the factors that regulate junctional proton transmission. First, it measures intracellular proton concentration directly, without resorting to electrical measurement, but at the same time, it is far less invasive than previously used local acid-injection protocols. Second, the analysis is based on time-averaged \( \text{pH} \) profiles, which therefore have a significantly improved signal-to-noise ratio. Finally, dual microperfusion experiments are reversible and can thus be performed at different starting values of \( \text{pH} \) or with different microstream boundary positions. It may, for example, be feasible to use this approach to study the \( \text{pH} \) dependence of junctional acid transmission.

**APPENDIX**

The computational problem consists of a system of diffusion and reaction equations describing the behavior of solute concentration

\[
\frac{\partial u}{\partial t} = D \cdot \nabla u + R(u) \tag{A1}
\]

where \( u \) is the vector with the concentrations of participating solutes, \( D \) is a vector of diffusion coefficients, and \( R \) is a vector of kinetic equations. Because the problem of cell pairs involves two compartments coupled at the junctional boundary, the system has 2\( \times \)\( n \) equations. For the purpose of modeling, all buffers are pooled into a mobile (M) and fixed (F) component. The fixed component was estimated in Zaniboni et al. (46) to have a concentration of 68.3 mM and a \( pK \) of 6.098. By best fitting the residual buffering, the corresponding fitting parameters for the mobile buffering are 23.8 mM and 7.102. If “A” and “B” describe the two cells of the pair

\[
\begin{align*}
\text{u} & = (\text{H}^+)_a, [\text{HM}]_a, [\text{M}]_a, [\text{H}^+\text{M}]_a, [\text{M}^+]_a, [\text{M}^-]_a, [\text{NH}_3]_a, [\text{NH}_2^-]_a, [\text{Ac}^-]_a, [\text{HAc}]_a, [\text{Ac}^-]_a, [\text{Ac}^-]_a
\end{align*}
\]

where \( \text{HAc}/\text{Ac}^- \) is acetic acid/acetate.

The diffusion vector of Eq. A1 is based on the diffusion coefficients calculated from Table 1. The reaction component of Eq. A1 is defined as follows:

\[
\begin{align*}
R_1 &= R_5 + R_8 + R_7 + R_8 \\
R_2 &= -(k_{\text{H}^+]\text{NH}_3^+u_5 - k_{\text{NH}_3^-}u_7) \\
R_3 &= -(k_{\text{H}^+]\text{M}^-u_5 - k_{\text{M}^+}u_7) \\
R_4 &= -(k_{\text{H}^+]\text{Ac}^-u_5 - k_{\text{Ac}^-}u_7) \\
R_5 &= (k_{\text{H}^+]\text{M}^-u_5 - k_{\text{M}^+}u_7) \\
R_6 &= (k_{\text{H}^+]\text{Ac}^-u_5 - k_{\text{Ac}^-}u_7) \\
R_7 &= -k_{\text{M}^+}u_5 - k_{\text{M}^+}u_7 \\
R_8 &= -k_{\text{Ac}^-}u_5 - k_{\text{Ac}^-}u_7 \\
R_9 &= -k_{\text{H}^+]\text{NH}_3^+u_5 - k_{\text{NH}_3^-}u_7 \\
R_{10} &= k_{\text{H}^+]\text{NH}_3^+u_5 - k_{\text{NH}_3^-}u_7 \\
R_{11} &= -(k_{\text{H}^+]\text{M}^-u_5 - k_{\text{M}^+}u_7) \\
R_{12} &= -k_{\text{M}^+}u_5 - k_{\text{M}^+}u_7 \\
R_{13} &= -(k_{\text{H}^+]\text{Ac}^-u_5 - k_{\text{Ac}^-}u_7) \\
R_{14} &= -(k_{\text{H}^+]\text{NH}_3^+u_5 - k_{\text{NH}_3^-}u_7) \\
R_{15} &= -(k_{\text{H}^+]\text{M}^-u_5 - k_{\text{M}^+}u_7) \\
R_{16} &= -(k_{\text{H}^+]\text{Ac}^-u_5 - k_{\text{Ac}^-}u_7) \\
R_{17} &= -(k_{\text{H}^+]\text{NH}_3^+u_5 - k_{\text{NH}_3^-}u_7) \\
R_{18} &= -(k_{\text{H}^+]\text{M}^-u_5 - k_{\text{M}^+}u_7) \\
R_{19} &= -(k_{\text{H}^+]\text{Ac}^-u_5 - k_{\text{Ac}^-}u_7)
\end{align*}
\]

Although intrinsic buffering is often referred to as instantaneous, intrinsic buffers are in fact fast buffers with a finite rate constant. The rate of protonation (\( k_{\text{NH}_3^-} \)) varies with chemical entity, but it is typically as fast as 10^{10} M^{-1}s^{-1} (7). It appears that taking any value > 10^{8} M^{-1}s^{-1} produces a converging solution; therefore, it is not critical to use an accurate value for \( k_{\text{NH}_3^-} \) for rapid buffers. Boundary conditions were set to zero for protons and mobile and fixed buffers. NH_{3}^{+}, NH_{2}^{+}, Ac^{-}, and HAc were assigned flux conditions as follows (19):
Innovative Methodology

MEASURING JUNCTIONAL H⁺ PERMEATION

\[
-D_{NH₄} \cdot \nabla [NH₄⁺] = P_{NH₄} \cdot V_m \cdot F \left( \frac{[NH₄⁺] - [NH₄⁺]}{1 - \epsilon} \right)
\]

\[
-D_{H⁺} \cdot \nabla [H⁺] = P_{H⁺} \cdot (\epsilon [H⁺] - [H⁺])
\]

\[
-D_{Ac⁻} \cdot \nabla [Ac⁻] = P_{Ac⁻} \cdot V_m \cdot F \left( \frac{[Ac⁻] - [Ac⁻]}{1 - \epsilon} \right)
\]

where \( V_m \) is membrane potential, \( R \) is the universal gas constant, \( T \) is the absolute temperature, \( F \) is the Faraday constant, and \( \epsilon \) is \( \exp(-V_m/F(R/T)) \). The values of the parameters are available in Table 1. To incorporate compartmentalization of extracellular weak acid/base, boundary conditions varied depending on locus. Extracellular concentrations of the solutes were multiplied by a step function taking values of 1 where the boundary was exposed to the solute and 0 otherwise.

The final step in the formulation of the problem is to consider related concentration changes to account for intercellular permeability using the general boundary condition

\[
-D_{\text{X}} \cdot \nabla [\text{X}] = P_{\text{X}} \cdot \Delta [\text{X}] \]

where \( \text{X} \) is a solute, \( \nabla [\text{X}] \) is the pre- and postjunctional slope of the concentration profile, and \( \Delta [\text{X}] \) is the concentration difference at the junction. Two types of solute flux occur at the junctional boundary. Charged solutes (e.g., \( NH₄⁺ \) and \( Ac⁻ \)) and mobile buffers cross between cells via the junctional pathway, mediated by gap junctional channels. Uncharged solutes (\( NH₃ \), \( HAc \)) can permeate through a faster, parajunctional pathway by virtue of their lipid solubility. The latter is not mediated by gap junctions but involves permeation across a pair of apposed membranes. Because free proton concentration is very low, its flux as free ions is negligible. Fixed buffer, by definition, cannot cross junctions. Junctional transmission of the mobile buffer (HM/M) is assigned a \( P_{\text{mobile}} \). Junctional permeability to the charged form of weak acid-base can be estimated from the molecular weight using Eq. 5. Parajunctional flux, on the other hand, is determined by the membrane permeability of the uncharged species, as listed in Table 1. Because two membranes must be traversed in the parajunctional route, the permeability constants are halved.

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