Ionic diffusion in transverse tubules of cardiac ventricular myocytes

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Shepherd, Neal, and Holly B. McDonough. Ionic diffusion in transverse tubules of cardiac ventricular myocytes. Am. J. Physiol. 275 (Heart Circ. Physiol. 44): H852–H860, 1998.—We have estimated the rate of diffusion of calcium ions in the transverse tubules of isolated cardiocytes by recording changes in peak calcium current (I_{Ca}) caused by rapid changes of the extracellular calcium concentration ([Ca]_o) at various intervals just preceding activation of I_{Ca}. Isolated ventricular cells of guinea pig heart and atrial cells from rabbit heart were voltage-clamped (whole cell patch), superfused at a high flow rate, and stimulated continuously with depolarizing pulses (0.5 Hz, 200- or 20-ms pulses from a holding potential of −45 or −75 mV to 0 mV). In ventricular cells, the change in peak I_{Ca} following a sudden change of [Ca]_o increased rapidly as the delay between the solution change and depolarization was increased, up to a delay of 75 ms (time constant (τ) = 20 ms, 30–40% of total current change), and then increased more slowly (τ = 200 ms, 60–70% of total current change); 400–500 ms were needed to achieve 90% of the total current increase. In atrial cells, a clear separation into two phases was not possible and 90% of the current change occurred within 85 ms. The slow phase of current change, which was unique to the ventricular cells, presumably reflects the slow equilibration of ions between the bulk perfusate and the lumina of the transverse tubules. If the length of the transverse tubules were equal to the cell thickness, the slow rate of change of current would be consistent with an apparent diffusion coefficient for calcium ions of 0.95 × 10^{-6} cm^2/s, considerably smaller than the value in bulk solution (7.9 × 10^{-6} cm^2/s). Most likely, this discrepancy is due to a high degree of tortuosity in the transverse tubular system in guinea pig ventricular cells or possibly to ion binding sites within the tubular membranes and glycocalyx.

guinea pig ventricular cells; calcium; excitation-contraction coupling

THE TRANSVERSE TUBULES of cardiac ventricular muscle cells provide passageways for the exchange of solutes between the interstitial fluid and the interior of the cell. This exchange is important because nearly two-thirds of the cell membrane is in the form of transverse tubules (7), and excitation-contraction (E-C) coupling is mediated by structures residing mainly in the tubular membranes (i.e., the close apposition of plasmalemma and junctional sarcoplasmic reticulum; Refs. 12 and 20). Although the exchange of solutes between the interstitium and the interior of the cell is generally assumed to be rapid, the tubular network is known to be tortuous, consisting of both transverse and axial components (8, 7), and could impose a significant barrier to diffusion. A recent theoretical analysis suggests that substantial depletion or accumulation of ions could occur within the tubules during the passage of ionic current through the cell membrane, especially if the diffusion is slower than in bulk solution (2). Experimentally, slow movement of calcium ions within the transverse tubules of guinea pig ventricular cells has been observed in the presence of calcium-sensitive dyes (4), and slow exchange of ions between bulk perfusate and the cell surface has been observed in both rabbit and rat ventricular myocytes (30).

To attempt to determine the rate at which ions in the interstitium can exchange with those in the transverse tubules, we have studied the rate at which the peak magnitude of a rapidly activating and rapidly inactivating membrane current is changed by a very rapid change of the extracellular ion concentration. We compare the responses of guinea pig ventricular cells, which have a well-developed transverse tubular system (7), with those of rabbit atrial cells, which are similar in shape to the ventricular cells but lack transverse tubules (12, 16, 25, 27). Our results are consistent with a degree of tortuosity in the transverse tubular system that is similar to that found in skeletal muscle (1). A preliminary account of these results has been published (23).

METHODS

Preparation of cells. Male guinea pigs weighing between 250 and 500 g were deeply anesthetized with 75 mg/kg pentobarbital sodium. The heart was rapidly excised and the aorta cannulated for coronary perfusion with oxygenated solutions (35–36°C). The heart was first perfused for 5 min with nominally calcium-free, low-sodium solution containing (in mM) 100 NaCl, 10 KCl, 1.2 KH2PO4, 50 taurine, 4 MgSO4, 20 glucose, and 10 HEPES at pH 6.9. The perfusate was then switched to a solution of the same composition with the addition of 1 mg/ml collagenase (type L, Sigma Chemical), 1 mg/ml fatty acid-free serum albumin (A-6003, Sigma Chemical), and 100 µM of CaCl2. CaCl2 (50 mM) was added in three aliquots during the enzyme perfusion to bring the total added calcium concentration to 200 µM. The heart was then minced and further incubated, with continuous stirring, in 5–10 ml of the same enzyme solution with the addition of 0.07 mg/ml protease (type XIV, Sigma Chemical). At 5- to 10-min intervals, the supernatant, containing dissociated cells, was drawn off and replaced by more enzyme solution. The cell suspensions were diluted into low-sodium solution to which 0.5 mM CaCl2 and 1 ml/500 ml kanamycin (Sigma Chemical) had been added and were stored at 23°C.

Atrial cells were obtained from the hearts of adult male rabbits by essentially the same method, with two changes: the perfusion solutions were prepared with calcium-free Tyrode solution rather than the modified Tyrode solution (see Voltage clamp) and the stirring times were approximately

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doubled. This investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health [DHHS Publication No. (NIH) 85–23, Revised 1985].

Voltage clamp. For experimentation, cells were placed in a Lucite chamber (3 ml) and continuously superfused with a prewarmed (35°C) modified Tyrode solution composed of (in mM) 150 NaCl, 5.4 KCl, 1.2 MgCl₂, 5 HEPES, 1.8 CaCl₂, and 5.5 glucose, with pH adjusted to 7.4 with NaOH. The details of our technique have been described in previous papers (22, 29). In short, the ends of an isolated cell were glued to the tips of two glass rods (diameter 76 µm) with alcian blue (25). Cells were attached in either a longitudinal or a transverse orientation (Fig. 1A). The length, width, and thickness of the cell were measured (to the nearest one-half division of the eyepiece graticule (1.25 µm)) by rotating the rod pair so that the cross-sectional area and the volume of the cell could be estimated. The mean dimensions of the principal groups of ventricular cells were (length × width × thickness) 121 ± 19 × 32 ± 5 × 12 ± 2 µm (longitudinal) and 139 ± 37 × 26 ± 4 × 13 ± 1 µm (transverse) (n = 5 in both cases). Atrial cells used in the study of calcium current (I_Ca) were 121 ± 20 × 16 ± 2 × 11 ± 3 µm. Most, although probably not all, of the portion of the cell lying over the glass surface was firmly attached (see Discussion).

After a cell was successfully fixed on the rods, a patch pipette was attached near the center of the cell, compensation was made for the pipette capacitance, and the whole cell-clamp condition was established. Patch pipette tips were ~2 µm in diameter, giving a resistance between 1.6 and 3 MΩ when filled with a solution containing (in mM) 130 KCl, 10 HEPES, 5 K-oxaloacetate, 5 K-succinate, 1 MgCl₂, 0.02 EGTA, and 5 mM Na₃ATP, pH 7.4. The cell capacitance was estimated by applying a symmetrical ±5 mV pulse while holding at −75 mV and again at −45 mV. One to two minutes after membrane rupture, the cell was stimulated continuously at 0.5 Hz with depolarizing pulses to 0 mV. The holding potential was −45 mV for the study of I Na and −75 mV for the study of sodium currents (I Na). After a steady peak current was established, solution-changing protocols were initiated. For the study of I Na, we added 250–350 µM lidocaine (6) to the control and experimental solutions to reduce the current to a level that could be controlled.

Solution changes. Very fast extracellular solution changes were applied to a cell by means of a double-jet system (22). The position of the jets was monitored by an LED/phototransistor pair, the output of which was recorded simultaneously with the electrical and mechanical signals from a cell. In all experiments the flow velocity was ~50 mm/s, permitting a fast and highly reproducible solution change. The temperature of the perfusate was kept at 35°C throughout the experiment by switching between continuously flowing prewarmed solutions and keeping the flow rates identical for all solutions. The solution temperature was monitored by means of a thermistor placed 0.2 mm below the cell-bearing rods, 0.2 mm above the floor of the chamber, and 1 mm distal to the rod ends.

The values for the delay between the solution change and the depolarization given in Figs. 1–4 represent the difference between the first motion of the perfusion jets and the upstroke of the depolarizing pulse. The actual instant at which the solution change begins at the cell is ~19 ms following the jet motion, as estimated from exponential fits to the points describing the change in difference current as a function of the delay (see Figs. 2 and 3, described in RESULTS). This means that, for the nominally 15-ms delay (Fig. 2A), the actual solution change began ~4 ms after the peak inward current, so the difference current in that case was always zero and is omitted from the data plots.

In a preliminary study we found that the change in the membrane potential following a step of extracellular potassium concentration ([K] o) from 5.4 to 10.8 mM was 90% complete in 63 ± 5 ms (n = 6), whereas the change in the holding current under similar conditions (resting potential) needed 202 ± 28 ms to reach 90% completion (n = 4, P = 0.0014). It seems likely that this discrepancy is due to electrotonic spread of depolarization, resulting in the depolarization of a cell due to a change in [K] o, proceeding much faster than the concentration change itself. Furthermore, the rates of change of both potential and current were proportional to the flow rate of the solution, up to at least the flow rate used here. Thus we feel that the rate of change of solution at the cell surface is limited by diffusion through a slow-flowing
boundary layer, the thickness of which depends on the bulk flow rate. Diffusion through such a layer could be quite slow if the layer includes caveolae, a significant amount of glycosyl- 
other extracellular elements that might slow diffusion.

Recording and analysis. Current signals were digitized (Instrutech VR-100; 9-kHz channel, 4 channels) and stored on videotape with concurrent strip-chart recording (Gould 2400).

Off-line analysis was made by recreating the analog signal, videotape with concurrent strip-chart recording (Gould 2400). Parameter values obtained from the fits to averaged data are given as values ± SE of the fit. When fits to data sets from individual cells are summarized, the results are given as means ± SE. Two-tailed t-tests were used to determine statistical significance (Excel).

Fig. 2. Time course of $\Delta I_{Ca}$ following a rapid change in [Ca], A: $I_{Ca}$ (left) and a cell from guinea pig ventricle (right) (records digitized at 2.5 kHz). B: difference currents ($\Delta I_{Ca}$) plotted versus delay following a rapid change of [Ca], from 0.45 to 1.8 mM. Values are means ± SE. Lines represent the function $\Delta I = \theta_4 I_{Ca} \exp [(t - t_2)/t_5] + \theta_1 I_{Ca} \exp [(t - t_3)/t_5]$, where zero time ($t_0$), fast and slow time constants ($t_1$ and $t_2$), and fast and slow fractional current contributions ($\theta_1$ and $\theta_2$) were determined by the fitting program. For longitudinal atrial cells (A, long), $\Delta I_{Ca}/I_{Ca} = 1.01 ± 0.06 (n = 5)$. For longitudinal ventricular cells (V, long), $\Delta I_{Ca}/I_{Ca} = 1.24 ± 0.11 (n = 5)$. For transverse ventricular cells (V, tran), $\Delta I_{Ca}/I_{Ca} = 1.05 ± 0.05 (n = 5)$. For transverse V with holding potential of -75 mV (V, -75), $\Delta I_{Ca}/I_{Ca} = 1.12 ± 0.13 (n = 4)$. inset: relationship between [Ca], and $\Delta I_{Ca}$ for a delay of 1,900 ms for atrial cells ($\Delta I_{Ca}/I_{Ca} = 1.09 ± 0.02, n = 5$) and ventricular cells ($\Delta I_{Ca}/I_{Ca} = 1.06 ± 0.05, n = 5$) (separate groups of cells from those used to define time course of $\Delta I_{Ca}$). $\Delta I_{Ca}$ was calculated as described above and in text. Line is derived from a least-squares fit with Hill equation, where Hill number is 1, $K_{1/2} = 0.97$ mM, and peak $\Delta I_{Ca} = 1.55$ (where $\Delta I_{Ca} = 1$ for a change of [Ca], from 0.45 to 1.8 mM, and $K_{1/2}$ is concentration at half-maximal $I_{Ca}$).

Fig. 3. Time course of $\Delta I_{Na}$ following a rapid change of extracellular Na concentration ([Na],). A: difference current ($\Delta I_{Na}$) following a rapid change of [Na], for an atrial myocyte (left) and a ventricular myocyte (right) (digitized at 10 kHz). Note that current waveforms, although much faster, show same kinds of changes with increasing delay as those observed for $I_{Ca}$ waveforms (Fig. 2A). B: $\Delta I_{Na}$ in 5 ventricular cells following a reduction of [Na], from 150 to 105 mM (replacing NaCl with CsCl, in presence of 0.35 mM lidocaine to reduce current to a controllable level), and $\Delta I_{Na}$ in 5 atrial cells following a reduction of [Na], from 150 to 75 mM in absence of lidocaine. For delays between 25 and 285 ms, differences between ventricular and atrial responses were statistically significant (P < 0.05, Wilcoxon). Lines are drawn as in Fig. 2B.
ing the time course of changes in the holding current cannot readily provide a quantitative measure of diffusion times because there are multiple potassium channels with conductances that depend in complex ways on the membrane potential, the potassium equilibrium potential, the external concentration, and time. Thus the current at a given point in time after a solution change will depend on the current changes up to that time. This makes it virtually impossible to translate the time course of the current into a time course of ionic concentration, even for cells lacking transverse tubules. In accordance, our approach to exploring the extracellular space with rapid solution changes is to look at the effect of a “step” change of ionic concentration on a subsequent “delta function” conductance change in, for the present case, \( I_{Ca} \) and \( I_{Na} \). Although neither the concentration changes nor the conductance changes used in the present study are ideally rapid, both are sufficiently so to allow us to observe compartmentalization of the extracellular space in ventricular myocytes.

A second important feature of our method is that we compare results obtained from experiments with ventricular cells with results obtained with atrial cells. This takes into account the uncertainty in the actual time course of the ionic concentration changes at the most superficial parts of the sarcolemma, as mentioned earlier. Without this comparison we could not, with any certainty, ascribe the apparently slow exchange of calcium and sodium observed in the ventricular myocytes to cellular components.

**RESULTS**

Effect of a rapid change of \([Ca]_o\). At various intervals preceding every fifth depolarization (delay; Fig. 1B), the calcium or sodium concentration surrounding a cell ([Ca] or [Na]) was rapidly changed (Ca replacing Mg or, vice versa, Cs replacing Na; Fig. 1B). As the delay between the solution change and the depolarization was increased, the difference between the current in the test pulse \( I_{test} \) and that in the preceding conditioning pulse \( I_{control} \) (Fig. 1C) also increased (Fig. 2A). The change in peak \( I_{Ca} \) following an increase of \([Ca]_o\) from 0.45 to 1.8 mM was much more rapid in atrial cells than in ventricular cells (Fig. 2). The difference current in the atrial cell reached 90% of maximum by 85 ms but only 50% of maximum in that time in ventricular cells. The mean ratio (+SE) of the extrapolated maximum \( I_{Ca} \) to the control current \( (\Delta I_{Ca}/I_{control}) \) was 1.01 ± 0.06 (n = 5) for atrial cells and 1.24 ± 0.11 (n = 5) for longitudinally oriented ventricular cells; i.e., the current was approximately doubled in each cell type by increasing \([Ca]_o\) from 0.45 to 1.8 mM. For descriptive purposes, the data points describing \( I_{Ca} \) as a function of the delay were fitted by the sum of two exponential functions (Fig. 2B; see details in METHODS). For longitudinally oriented ventricular cells, the rate coefficients were 45 ± 12 and 5.1 ± 0.6 s⁻¹, with 36 ± 5% of the current change in the fast phase and 64 ± 4% in the slow phase (estimates from fit ± SE of estimate). For atrial cells, neither the rate coefficients (58 ± 39 and 19 ± 20 s⁻¹) nor the fractional divisions of the overall current change (66 ± 62 and 34 ± 62%) were sufficiently certain for us to be confident of the separation into a fast and slow phase. On the other hand, the atrial data were well fitted by a single exponential with a rate coefficient of 36 ± 2 s⁻¹. Irrespective of the type of fit, the ventricular and atrial currents at each delay between 35 and 285 ms were significantly different (P < 0.01, Wilcoxon test).

The waveform of the difference currents did not vary with their magnitudes or with the delays in either cell type; i.e., the waveforms in Fig. 2A, left, were essentially scaled versions of a single waveform, and the same holds for Fig. 2A, right. Thus it is likely that 1) all current increases were due mainly to factors other than altered gating, and 2) for the ventricular cells, the slow and fast components reflect changes in the same kind of current.

The rabbit atrial cells used in this study were similar in shape to the guinea pig ventricular cells but were smaller [apparent surface areas of longitudinally attached cells were 6,700 ± 1,800 and 11,000 ± 2,100 µm² for atrial and ventricular cells, respectively (n = 5)] and had a smaller apparent specific capacitance (1.04 ± 0.08 and 1.69 ± 0.40 µF/cm²). The difference between the measured specific capacitances presumably reflects the relative paucity of transverse tubules in the atrial cells (12, 16, 25, 27). Thus the slow change in \( I_{Ca} \) in ventricular cells may reflect a slow exchange of calcium between the transverse tubules and extracellular space. Alternative explanations include 1) a biphasic change in the calcium concentration of the solution surrounding the cell, 2) ventricular cells and atrial cells having a different relationship between \( I_{Ca} \) and \([Ca]_o\), 3) slow changes in the calcium channel itself, and 4) \([Ca]_o\)-dependent changes in the calcium content of the sarcoplasmic reticulum (SR).

First, the change in the extracellular concentration of calcium itself might be biphasic due to, e.g., slow exchange of solutes in the region between the glass rods and the cell. The more rapid response of the atrial cells to changing \([Ca]_o\) would, on the basis of this hypothesis, be due to their smaller area of attachment to the glass rods. However, the slow component in the ventricular cells constituted 64% of the overall current change, whereas only 34 ± 3% (n = 5) of the ventricular cell surface was directly apposed to the rod surface in the longitudinal orientation. Furthermore, the relationship between \( I_{Ca} \) and the delay was very similar for cells attached in either a transverse or longitudinal orientation (Fig. 2B), and just 20 ± 4% (n = 5) of the cell surface was apposed to the rod surface in the transverse orientation (Fig. 2B). Finally, for the two smallest ventricular cells used in the experiments summarized in Fig. 2B (transverse orientation), the mean area of attachment of the cell to the glass was smaller than the mean area of attachment of two of the atrial cells (longitudinal orientation), but the time to reach 90% of total current change in these cells was 350 ms for the ventricular cells and 80 ms for the atrial cells. Because the experiments on atrial and ventricular cells were identical except for the cell type, a biphasic change of \([Ca]_o\) is not a likely explanation for the markedly biphasic change in \( I_{Ca} \) found only in ventricular cells.

Steady-state relationship between \([Ca]_o\) and \( I_{Ca} \). Hypothetically, a difference between the time course of current change in atrial and ventricular cells could...
reflect a very different relationship between \([Ca]_o\) and \(I_{Ca}\) in the two cell types. For example, the apparently rapid change of \(I_{Ca}\) in atrial cells could be due to saturation of \(I_{Ca}\) at a very low \([Ca]_o\). However, the measured relationship between \([Ca]_o\) and \(I_{Ca}\) in atrial cells is very similar to that in ventricular cells (Fig. 2B, inset). This relationship was found in a separate group of cells by measuring the difference currents for changes of \([Ca]_o\) from 0.45 mM to either 0.9, 1.35, or 1.8 mM at a delay sufficiently long that \(I_{Ca}\) reached a steady state in both cell types [1,900 ms; for the change to 1.8 mM, \(\Delta I_{Ca}/I_{control} = 1.09 \pm 0.02 (n = 5)\) for atrial cells and 1.06 \(\pm 0.05 (n = 5)\) for ventricular cells]. We conclude that the different time courses of change in \(I_{Ca}\) after a rapid concentration change are not due to different relationships between \(I_{Ca}\) and \([Ca]_o\).

Slow changes in \(I_{Ca}\)? The slow phase of current change could be due to a slow change in the calcium channel itself, e.g., a slow shift in the level of steady-state inactivation (17). A difference between the atrial and ventricular cell in this regard is plausible due to the markedly faster waveform of \(I_{Ca}\) in atrial cells, presumably due to different gating parameters of \(I_{Ca}\) in the two cell types (Fig. 2A). However, the time course of change in \(I_{Ca}\) was the same at a holding potential of either −45 or −75 mV, at which potential any shifts in inactivation should be too small to affect \(I_{Ca}\) (Fig. 2B, filled circles and diamonds, respectively) (17). A less direct kind of slow change in \(I_{Ca}\) could occur due to changes in SR calcium content as \([Ca]_o\) is changed. However, there is no reason to expect a difference between atrial and ventricular cells in this respect, and, in any case, an increase in the SR calcium content and release would tend to reduce rather than increase \(I_{Ca}\) (21). This would make the change in \(I_{Ca}\) after a change of \([Ca]_o\) appear faster rather than slower.

Changes in \(I_{Na}\) due to rapid changes of \([Na]_o\). All explanations of the slow change in \(I_{Ca}\) that invoke slow changes in the calcium channel itself, either directly or indirectly, were rendered much less plausible by the finding that \(I_{Na}\) changes in the same way, qualitatively, following a change of \([Na]_o\) as does \(I_{Ca}\), following a change of \([Ca]_o\), in both atrial and ventricular cells (Fig. 3). Unfortunately, \(I_{Na}\) is too large to damp in the absence of a channel blocker, and the presence of the blocker complicates analysis of the results due to possible interactions between sodium ions and the blocking agent. Nevertheless, the qualitative similarity of the results of the experiments with \(I_{Na}\) and \(I_{Ca}\) suggests that the different rates of change of ionic currents following a concentration change are not ascribable to the properties of a particular ion channel but rather to the properties of the cells, presumably to the difference in relative density of transverse tubules.

Estimating diffusivity of calcium ions in transverse tubules. Because the physical basis of the slow phase of current change in ventricular cells seems likely to be diffusion in the transverse tubules, appropriate solutions of the one-dimensional diffusion equation (1, 2, 5) should account for our data (Fig. 4). With the assumption that \([Ca]_o\) in the solution surrounding the cell changes exponentially with a rate coefficient of \(\beta\), the solution to the diffusion equation has the form (5)

\[
\frac{[Ca]_i(t, x) - [Ca]_i(0, x)}{[Ca]_i(\infty, x) - [Ca]_i(0, x)} = 1 - \frac{2}{\sqrt{\pi}} e^{-\beta t} \int_0^\infty e^{-x^2} dx
\]

\[
= \frac{16\beta L^2}{\pi} \sum_{n=0}^\infty (-1)^n \cos (2n + 1)\pi x/2L
\]

\[
\frac{2n + 1)^2(4\beta L^2 - \pi^2D(2n + 1)^2)}{2n + 1)^2(4\beta L^2 - \pi^2D(2n + 1)^2)} e^{-(2n + 1)^2\pi^2Dt/4L^2}
\]

where \([Ca]_i(t, x)\) is the concentration of calcium in the transverse tubules at time \(t\) and distance \(x\) (expressed as a fraction of \(L\) from the center of the cell). \(L\) is one-half the tubule length (i.e., the distance from the cell center to the cell surface via the tubules), and \(D\) is
the diffusivity of the calcium ion, \([\text{Ca}]_t(0, x)\) is assumed to be constant for all \(x\). Current was calculated from Eq. 1 by dividing \(L\) into 100 equal segments, computing the current from the calcium concentration in each segment at 16 ms intervals (using the relationship between \(I_{\text{CA}}\) and \([\text{Ca}]_t\), in Fig. 2B, inset), and summing the currents in the segments (1). The tubular currents were summed with those of the superficial membrane with the assumption of a uniform distribution of \(I_{\text{CA}}\) throughout all of the cell membrane area. Note that the fits yield only the ratio \(D/L^2\) rather than values for both \(D\) and \(L\). The tubules are assumed to have a simple, idealized geometry in which each tubule runs from the side of the cell facing the glass rods to the opposite side, roughly normally but not necessarily straight (20). In reality, the cell cross section is elliptical (9) rather than rectangular, and the actual tubular geometry is likely to be complex (8).

We fitted the atrial cell data first with the assumption that we could obtain from this fit estimates of both \(\beta\) and the instant at which the extracellular solution change actually began near the cell surface (Fig. 2) and then fitted the data with these values for \(\beta\) and \(t_0\) (Fig. 4, filled squares). The fitted line corresponded to a current change that was almost entirely attributable to the superficial cell membrane (98%) and to a change in \([\text{Ca}]_o\) that began at a delay of 19 ms and proceeded at a rate of 26 s\(^{-1}\). These values for \(t_0\) and \(\beta\) are used in all subsequent analyses, although it does seem unlikely to us that the atrial cells are actually entirely lacking in transverse tubules. Nevertheless, fits to the ventricular data with these values for \(t_0\) and \(\beta\) were reasonable, and fits with other values did not significantly change the fitted values of \(D/L^2\), although the fraction of current in the transverse tubules (\(\phi_{\text{tt}}\)) varied inversely with \(\beta\).

The data from ventricular cells were fitted in two ways. First, we averaged the data from each group of ventricular cells (holding potential −45 mV) in the manner described earlier (Fig. 2) and then fitted the cumulative data points with Eq. 1 (Fig. 4). \(D/L^2\) for the transverse cells was significantly larger than \(D/L^2\) for the longitudinal cells (1.57 ± 0.19 vs. 1.13 ± 0.17 s\(^{-1}\), respectively; \(P < 0.05\)), whereas values for \(\phi_{\text{tt}}\) were similar (0.60 and 0.58, respectively). In the second method, nearly identical results were obtained by fitting data sets from individual cells and then averaging the fitted values of the parameters \(D/L^2 = 1.60 ± 0.28\) and 1.13 ± 0.30 s\(^{-1}\) (means ± SD), respectively, \(P = 0.03\); \(\phi_{\text{tt}} = 0.59\) and 0.60, despite a nearly twofold range of individual values for \(D/L^2\). The identity of the results from the two methods implies a linear addition of the values of \(D/L^2\) from different cells and, therefore, from different regions of the same cell. This result can be used to understand the different values for \(D/L^2\) obtained for different cell orientations and to estimate the value of \(D/L^2\) for cells that are not attached to any surface.

It is likely that the difference between \(D/L^2\) for transverse and longitudinal cells reflects the fact that the longitudinally oriented cells have one entire side of the cell facing the glass surface, restricting access of the extracellular solution to the tubule openings on that surface, whereas the transversely oriented cells have only 66% of one side occluded in this way. Extrapolating the fitted values of \(D/L^2\) to the hypothetical case in which 0% of the cell surface is occluded gives a value for \(D/L^2\) of 2.42 s\(^{-1}\) (Fig. 4B).

However, if one side of the idealized cell were really totally attached to the glass, and if that attachment completely occluded the extracellular solution from that cell surface, at least on the timescale of our solution changes, then the length of the diffusion path through the transverse tubules would be twice that for the completely unattached cell. Thus \(D/L^2\) for a cell attached over 100% of its length should be one-quarter its value when both sides of the cell are free, i.e., 2.42 s\(^{-1}\)/4 = 0.61 s\(^{-1}\), rather than the value we find, 1.13 s\(^{-1}\) (Fig. 4B). This presumably means that the portion of the cell that overlaps the rods is not actually completely occluded. To estimate the degree of occlusion, we can imagine the cell as having two regions, one in which both ends of the tubules are exposed to the extracellular solution (\(D/L^2 = 2.42 \text{ s}^{-1}\)) and the other having one end totally occluded (\(D/L^2 = 0.61 \text{ s}^{-1}\)). To account for our data in this way, it is necessary to assume that only 71% of the cell length overlapping the rods is actually attached, with the rod-facing tubules of that portion of the cell totally occluded, whereas the remainder has all cell surfaces and tubule mouths readily accessible to the extracellular solution (Fig. 4B, dashed line).

This analysis does not take into account that the transversely oriented cells were thicker on average (13 µm) than the longitudinally oriented cells (12 µm, see METHODS). Although this difference was not statistically significant, if it is taken into account by multiplying \(D/L^2\) for the transverse cells by the square of the ratio of the thicknesses, the extrapolated value of \(D/L^2\) for an unattached cell becomes 3.23 s\(^{-1}\), and the fitted values can be accounted for if 13% of the cell length that overlaps the rods is actually unattached.

**DISCUSSION**

**Origin of fast phase of current change.** The apparent rate of change of ion concentration at the atrial cell surface (26 s\(^{-1}\), Fig. 4) is surprisingly slow given that the linear velocity of the cell perfusate is two to three cell widths (50 µm) per millisecond. One reason for this is that the atrial cells almost certainly are not entirely devoid of transverse tubules, and some of what the curve-fitting program finds as rapid phase (Fig. 4A) is probably actually a small slow phase, as suggested by the exponential fits to the data (Fig. 2B) and by cell capacitance measurements (see below). A second reason is that the concentration change at the cell surface depends to some extent on diffusion through a boundary layer, because the initial rate of change of current or potential depends linearly on the flow velocity (see METHODS). We assume in our analyses that the rate of change of ionic concentration at the cell surface is the same for both atrial and ventricular cells.

**Origin of slow phase of current change.** Our results are consistent with the idea that the slow component of current change in ventricular cells reflects a physical
property of the cells, which we hypothesize is the restricted diffusion space constituted by the lumens of the transverse tubular network. It is unlikely that the small caveolae found in cardiac cell membranes are responsible for the slow exchange of extracellular calcium near the sarcolemma, because they are quite small (90 nm in diameter; Ref. 13) and are present in both atrial and ventricular cells (19). On the other hand, caveolae are more prevalent in the transverse tubules than elsewhere on the cell surface (13) and may contribute to the apparently slow diffusion in the transverse tubules.

The value for D/L^2 obtained, 2.42 s^-1, was obtained from cells with a mean thickness of 12.5 µm. If we were to assume that 2L = thickness, D would have a value of 0.95 x 10^-6 cm^2 s^-1, less than one-eighth its value in bulk solution (7.9 x 10^-6 cm^2 s^-1; Ref. 14). The transverse tubules would need to be ~2.8 times the cell thickness in length to account for the fitted value of D/L^2. This degree of tortuosity is similar to that of skeletal muscle, as derived from a study of potassium depletion (1) and estimated from electron micrographs (11). The tortuous nature of the transverse tubules in cardiac muscle has been observed in morphological studies that document both longitudinal and transverse segments of the “transverse” tubular system (8). Unfortunately, the length of the transverse tubules in guinea pig ventricular cells cannot be determined even approximately from morphometric data because the diameter of the tubules is known to vary widely within a cell (7) and because the number of tubules per unit of surface membrane area has not been reported for these cells. Although the tubules are certainly at least somewhat tortuous, diffusion within the tubules might also be slowed by calcium binding sites (3), which Bers and Peskoff (2) have shown could account for as much as a fivefold slowing of diffusion. However, the degree of tortuosity necessary to account for our results is within a plausible range, and it would be necessary to postulate sodium binding sites as well to account fully for our results.

On the other hand, it is possible that we have overestimated the value of D in this study because of either SR calcium loading or calcium-dependent inactivation of I_{Ca}. The elevation of [Ca]o before depolarization could load the SR with calcium in proportion to the delay between the solution change and depolarization, and the extra calcium release would inhibit I_{Ca} (21). Thus ΔI_{Ca} for the longer delays would be underestimated and the apparent rate of change of I_{Ca} overestimated. However, in the experiments with transversely oriented cells, we could measure the force of contraction and compare the change in twitch force with the change in I_{Ca} following a change of [Ca]o. In those experiments, for a delay of 885 ms, the rate of rise of twitch force was increased by a mean of 105 ± 34%, and the difference current was 105 ± 10% of control for that delay. This result is consistent with there being little or no SR loading, because the rate of rise of tension is approximately proportional to I_{Ca} for constant SR loading (28). Calcium-dependent inactivation is unlikely to be very important for the reasons discussed earlier. However, there were small changes in the apparent rate of inactivation of I_{Ca} induced by the rapid change in [Ca]o, which can be seen as a slight positive overshoot of the difference current in Fig. 1. Such inactivation would affect the shape of the curves for I_{Ca} versus delay in the same way as does SR loading, resulting again in the overestimation of D.

Comparison with previous studies. A recent study using methods similar to ours (30) reported rates of change of membrane currents and potential similar to those reported here but differing in important ways. I_{Ca} in rabbit ventricular myocytes was found to decline to 10% of its initial value (t50) within 241 ms of the application of a calcium-free solution (with 2 mM EGTA). Although this seems much faster than what we find in guinea pig myocytes, the results cannot be directly compared, because the relationship between current magnitude and calcium concentrations was not given. Nevertheless, our calcium exchange rate (t50 < 600 ms) does compare well with the decline of I_{Ca} in rat myocytes in the same report (t50 = 910 ms) (30).

Yao et al. (30) also measured the rate of change of membrane potential following an increase in [K]o, finding a t50 of only 80 ms in rabbit myocytes, and concluded that monovalent ions diffuse more rapidly in the extracellular space than do calcium ions. However, if the threefold difference between the rates of change of potential and current is taken into account, the rates of change of potassium and calcium currents would be nearly identical (see METHODS). Likewise, we find no difference between the rates of change of I_{Ca} (Fig. 2) and I_{Na} (Fig. 3) in both atrial and ventricular cells. Thus there is as yet no strong evidence for a difference between the extracellular rates of diffusion of monovalent and divalent cations.

Comparison with morphometric studies and capacitance measurements. The surface area-to-volume ratio of transverse tubular membrane in guinea pig ventricle is 0.42 µm^-1 (7), and the ratio of external membrane area to volume in our cells, excluding the attached fraction described above, was 0.20 µm^-1. Thus the fraction of membrane area immediately accessible to the extracellular solution would be 0.20/0.62 = 0.32, less than the value of 0.40–0.42 we find from the fitted data (1 – ΦV; Fig. 4A). The discrepancy is not large given that we estimate the cell surface area by assuming it to be a smooth surface without caveolae or wrinkles that would increase the true area.

The measured capacitance in our preparations probably excludes the portion of the cell attached to the rods due to the high extracellular resistance in the attached regions. Correcting for this on the basis of the cell dimensions and the estimated 71% attachment of cell membrane overlapping the rods, we find a specific capacitance of 1.32 µF/cm² for atrial cells and 2.25 µF/cm² for ventricular cells. The usual estimate of the specific capacitance of cell membranes per se is 1 µF/cm². Thus the intracellular membranes should be 0.32/1.32 = 0.24 of the total membrane area in atrial cells and 1.25/2.25 = 0.56 in ventricular cells. The
discrepancy for the atrial cells probably arises from the curve fitting, as mentioned in Origin of fast phase of current change. The number for the ventricular cells is very close to the values of 0.58–0.60 found from the data fits (Fig. 4A).

The similarity among the results from morphometry, capacitance measurements, and current changes due to rapid extracellular solution changes supports the notion that we have actually measured the rate of interstitial-tubular exchange and is consistent with the assumption that the membrane channels conducting the currents are uniformly distributed in the cell membrane.

Significance of ionic exchange in transverse tubules. Whatever the mechanism, slow tubular diffusion is likely to have functional significance, because the structures mediating E-C coupling are located in the tubules (12, 20). The accumulation and depletion of potassium and calcium ions could profoundly affect E-C coupling, particularly when the heart hypertrophies and the tubules apparently elongate (18). In pressure-overload hypertrophy, ventricular cell thickness has been shown to increase by as much as 50% (9, 15), which would slow diffusion by a factor >2 if the transverse tubules elongate to the same degree. From the experimentalist’s point of view, a slow diffusion space surrounding 60% of the cell membrane would complicate the interpretation of experiments involving fast solution changes and could make it more difficult to voltage clamp whole cells.

Potential uses of this method. The method described here may provide a relatively simple means, perhaps the only means, for estimating the length of transverse tubules in living cardiac muscle cells. This information is difficult or impossible to obtain through morphometry and could provide insight into morphological changes occurring during development or in pathological conditions.

Also, the ability to isolate changes of ionic conductance in the tubular membranes from those in the superficial membrane in live, intact cells should be useful in identifying the cellular location of other ion transporters in the cell membrane, such as sodium-calcium exchange, which information could deepen our understanding of E-C coupling in heart muscle. This should be particularly helpful in studying the changes in transporter distribution that may occur under pathological or experimental conditions (16).

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REFERENCES


