Calcium Current Measurements in Acutely Isolated Neonatal Cardiac Myocytes

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ABSTRACT. Action potentials and voltage clamp-induced ionic currents were recorded in acutely isolated neonatal rabbit cardiac myocytes using the whole-cell voltage clamp technique. Time- and voltage-dependent Ca^{2+} currents in neonatal myocytes were elicited by depolarizations from a holding potential of -80 mV to various clamp potentials. The maximal measured inward Ca^{2+} current was 206 ± 10 pA (mean $\pm$ SEM, $n = 51$). The peak current occurred at a mean membrane potential of 7.8 ± 1.3 mV ($n = 51$). The Ca^{2+} current voltage relation was shifted 26 mV in the positive direction when the external Ca^{2+} concentration was increased 10-fold. Ca^{2+} current rundown was observed with a half-time of approximately 20 min. Cells dialyzed with solution containing the Ca^{2+} chelating agent, EGTA (0.04 mM), had action potential durations similar to those previously reported in papillary muscle. In contrast, a higher concentration of EGTA (14 mM) prolonged the action potential duration. Control of the cell internal ionic composition was achieved by dialysis of the cell with a time constant for Na^+ ions of 1.2 to 2.6 min. Tetrodotoxin (10 μM), included in some experiments to block Ca^{2+} entry via Na^+ channels, was shown to be more than 98% effective. These results characterize the whole-cell voltage clamp technique as applied to immature heart cells. (*Pediatr Res* 30: 83–88, 1991)

Abbreviations

TTX, tetrodotoxin
 I_{peak} , peak current
I-V, current-voltage
 R_s , series resistance
 τ_{Na} , time constant for Na^+ current
 V_{clamp} , clamp potential
 V_{hold} , holding potential
HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid

Calcium ions play a central role in the generation of action potentials and the development of tension in myocardial cells. Changes in myoplasmic calcium concentration are thought to be mediated by sarcolemmal calcium transport as well as release of Ca^{2+} from intracellular stores (1, 2). The relative contribution of these two processes to tension generation demonstrates both species and developmental variability (3–6). In this regard, age-

related changes in cellular ultrastructure (7–9) and the function of the sarcoplasmic reticulum (10–12) have been described. Developmental aspects of sarcolemmal Ca^{2+} channels, however, remain largely unexplored.

The refinement of the whole-cell voltage clamp technique (13) has stimulated significant advances in the study of cellular ionic currents and mechanisms of tension generation (for review see reference 14). Investigators using this technique have directly measured Ca^{2+} channel characteristics in isolated adult cardiac myocytes (15–17). In contrast, there is little experience with freshly isolated neonatal mammalian myocytes. Ca^{2+} currents have been recorded in embryonic chick (18), cultured fetal rat (19–21), and neonatal rabbit (22) myocytes. Other investigators have described the action potential configuration in cell aggregates and papillary muscles (23, 24).

We report the first measurement of action potential characteristics and time- and voltage-dependent Ca^{2+} currents in acutely isolated neonatal mammalian myocytes. The methods used in these experiments are similar to those described for studies of Ca^{2+} channels in acutely isolated adult cardiac myocytes. However, because differences between neonatal and adult myocytes may influence the interpretation of experimental results using this method, a detailed validation of this technique as applied to immature cells is presented. This experimental approach allows for direct comparison of Ca^{2+} currents between neonatal and adult myocytes using similar methodologies.

MATERIALS AND METHODS

Cell isolation. Cardiac myocytes were prepared from the hearts of neonatal (1- to 5-d-old, 50- to 100-g) New Zealand White rabbits by enzymatic dissociation as described previously (25). Animals were anticoagulated with 1000 U of heparin and anesthetized with pentobarbital (50 mg) by intraperitoneal injection. The intact heart was excised rapidly and allowed to beat spontaneously in warmed (37°C) oxygenated 3.5 mM Ca^{2+} Tyrode's solution to facilitate removal of blood from the ventricles [solution composition (concentrations expressed in mM): NaCl 136, KCl 5.4, NaH_2PO_4 0.33, MgCl_2 1, HEPES 10, mannitol 4, thiamine HCl 0.6, glucose 10, pyruvic acid 2, CaCl_2 3.5; pH 7.35]. Tyrode's solution was filtered through a 0.45- μm plain nylon filter (Micron Separations Inc., Westborough, MA).

The aorta was cannulated and the heart perfused (Minipuls peristaltic pump, Gilson, Middleton, WI) with warmed, oxygenated 0 mM Ca^{2+} Tyrode's solution at a flow rate of 2.2 mL/minute for 3 min. The pericardium and atrial appendages were removed. The heart was then perfused with enzyme solution for 7 to 9 min until the heart began to soften. The enzyme solution consisted of 0 mM Ca^{2+} Tyrode's solution containing collagenase (1 mg/mL = 300 U/mL, Type I; Sigma Chemical Co., St. Louis, MO) and protease (0.07 mg/mL = 0.35 U/mL, Type XIV; Sigma) that had been filtered through a 0.8- μm filter. The perfusate was then changed to 0.1 mM Ca^{2+} Tyrode's solution for 3 min. The ventricles were removed, opened, and shaken in 0.1

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mM Ca^{2+} Tyrode's solution to disperse individual myocytes. The myocytes were then maintained in 0.1 mM Ca^{2+} Tyrode's solution without further manipulation. The yield of viable myocytes ranged from 30 to 60%.

Viable neonatal cells were spindle-shaped and had recognizable striations and nuclei (Fig. 1). They excluded trypan blue dye and did not contract spontaneously. However, they contracted in response to depolarizing currents (25). Nonviable cells frequently had spontaneous contractions and rapidly lost identifiable intracellular organization. All experiments were performed within 6 h of cell isolation.

Experimental preparation. Isolated cells were placed in a 1-mL experimental chamber on the stage of an inverted microscope (Nikon, Garden City, NY). Microelectrodes were prepared from no. 7052 glass capillary tubing (Corning, Horsehead, NY) using a double-pull technique (26). The pipette tip resistance measured 5 to 8 M Ω in Tyrode's solution. Cells were voltage clamped using an EPC-7 (Adams/List, Great Neck, NY) patch clamp amplifier (13). The current signal was filtered at 1 kHz using an eight-pole Bessel filter (Frequency Devices, Haverhill, MA). Membrane potential and current were recorded on a dual beam storage oscilloscope (Tektronix, Beaverton, OR) and photographed for later analysis. All experiments were performed at room temperature (maintained between 23 and 24°C).

Cell seal resistance was calculated by dividing V_{hold} by the holding current. Current capacitive transients were minimized by adjusting the transient cancellation circuitry of the patch clamp amplifier. Cell access R_s was calculated as the reciprocal of the passive conductance of the preparation (" G_{series} " on the patch clamp amplifier) (27).

Because the capacitance of biologic membranes is reasonably constant at 1 $\mu\text{F}/\text{cm}^2$, measurement of the cell capacitance yields an estimate of the cell membrane surface area (26). Cell capacitance was taken as the value of the slow capacitive transient circuit during optimum transient cancellation.

Action potentials were stimulated by brief depolarizations (10 ms) and recorded with the clamp amplifier in the current clamp mode. The composition of the internal (pipette) solution was in mM: KCl 120, EGTA 14, HEPES 20, NaH_2PO_4 10, MgCl_2 1, CaCl_2 1, and ATP-Mg 10, titrated to pH 7.1 with KOH. The external bath was Tyrode's solution with 1.8 mM Ca^{2+} .

Measurement of Ca^{2+} currents. Ca^{2+} I-V relations were determined by employing sequential 100-ms depolarizations ($V_{\text{clamp}} = -40$ to 60 mV) from a V_{hold} of -80 mV at 5-s intervals. I_{peak}

was measured for each V_{clamp} and net current (I_{net}) calculated as $I_{\text{net}} = I_{\text{peak}} - I_{\text{leak}}$ where $I_{\text{leak}} = V_{\text{clamp}}/R_c$ (where R_c is cell resistance). Voltage- and time-dependent Na^+ and K^+ channels were blocked with TTX (10 μM ; Sigma) and Cs^+ , respectively. For measurement of Ca^{2+} currents, the composition of the internal solution was (in mM): CsCl 96, HEPES 20, EGTA 14, CaCl_2 1, MgCl_2 1, and ATP-Mg 5, titrated to pH 7.1 with CsOH; the external solution contained (in mM): CsCl 135, HEPES 10, CaCl_2 10, MgCl_2 1, glucose 10, and TTX 10 μM , titrated to pH 7.3 with CsOH. After penetration of a cell, a 5-min equilibration period allowed for dialysis of the cell interior with pipette solution.

In a separate set of experiments, Ca^{2+} current rundown was determined by recording currents during sequential depolarizations at 5-min intervals. The first clamp was performed shortly after cell penetration ($t = 0$ min). The mean current at each time point was calculated as a fraction of the initial peak current for each cell.

Time constant of dialysis. The interior of the cell was dialyzed with internal pipette solution via the open pipette tip, allowing control of intracellular ionic composition. Because experiments were performed with smaller pipette tip diameters than are commonly used (13, 26), the effectiveness of dialysis of the cell interior by internal pipette solution was determined. The rate of dialysis was studied by measuring changes in Na^+ current as intracellular Na^+ concentration came into equilibrium with the pipette solution. The decay of peak inward Na^+ current after cell penetration was measured with equal Na^+ concentration in the internal pipette and external solutions. The internal solution contained (in mM): NaCl 60, CsCl 45, HEPES 20, EGTA 14, CaCl_2 1, MgCl_2 1, and ATP-Mg 5, titrated to pH 7.1 with CsOH. The external solution contained (in mM): NaCl 60, CsCl 75, HEPES 10, MgCl_2 1, CaCl_2 1, and glucose 10, titrated to pH 7.3 with CsOH. Repeated depolarizations (10 ms in duration) were performed at 1-min intervals from a resting potential of -80 mV to a clamp potential of 0 mV. For each clamp step, the inward Na^+ current (normalized to the Na^+ current at 1 min after cell penetration) was measured. The decay time constants (τ_{Na}) were calculated as the slope of the regression line of the logarithm of the mean Na^+ current versus time.

Na^+ current inhibition by TTX. To measure Ca^{2+} channel currents, TTX was included in the external solution to block Ca^{2+} influx through Na^+ channels. Cardiac Na^+ channels are known to be less sensitive to TTX than neuronal Na^+ channels (23, 24). Accordingly, the concentration of TTX required to block Na^+ current in isolated neonatal heart cells was determined. The peak Na^+ current was measured for depolarizations from a holding potential of -80 mV to a clamp potential of -20 mV in increasing concentrations of TTX. The internal solution contained (in mM): NaCl 10, CsCl 96, HEPES 20, EGTA 14, CaCl_2 1, MgCl_2 1, and ATP-Mg 5, titrated to pH 7.1 with CsOH. The external solution contained (in mM): NaCl 40, CsCl 95, HEPES 10, MgCl_2 1, CaCl_2 1, and glucose 10, titrated to pH 7.3 with CsOH, to which various concentrations of TTX were added. For each of nine cells, the fractional response to TTX, $(I_0 - I_{\text{peak}})/I_0$, was calculated where I_0 was the peak Na^+ current in the absence of TTX. The apparent K_m for TTX was then derived from Scatchard analysis of the mean fractional response for each concentration of TTX.

Statistical analysis. Measured values are reported as mean $\pm$ SEM. The K_m for TTX is reported with 95% confidence intervals because its distribution is non-Gaussian.

RESULTS

Whole cell data. In a total of 51 neonatal whole cell recordings, mean cell resistance measured 1.8 ± 0.1 G Ω (ranging from 0.7 to 5.0 G Ω) and pipette series resistance averaged 14 ± 1 M Ω . Cell surface area was $24.7 \pm 0.8 \times 10^{-6}$ cm^2 . This value agrees well with the 16×10^{-6} cm^2 calculated from planimetric data for

Fig. 1. Photograph of a typical neonatal ventricular myocyte isolated from a 4-d-old rabbit with micropipette in continuity with the cell interior. Note the cylindrical shape, well-defined striations, and intracellular ultrastructure. No vacuoles or other abnormal morphologic features are present. The cell is approximately 70 μm long and 7 μm wide with a calculated surface area of 16×10^{-6} cm^2 .

the single cell in Figure 1, assuming a right cylinder 70 μm long and 7 μm wide.

Depolarization-induced action potentials in neonatal cells (Fig. 2) had a resting potential of -71 ± 2 mV ($n = 11$), an overshoot potential of 44 ± 6 mV ($n = 11$), and an action potential amplitude of 115 ± 2 mV ($n = 11$). The action potential duration was 620 ± 100 ms ($n = 11$). This value is significantly longer than that of 402 ± 32 ms ($n = 9$, $p < 0.001$) found in neonatal papillary muscle (unpublished data from this laboratory) using the single sucrose gap preparation (4) or in a previous report using conventional microelectrodes (24). However, when the concentration of EGTA in the pipette was decreased to 0.04 mM with no Ca²⁺ added to the internal solution, the action potential duration decreased to 397 ± 14 ms ($n = 6$, $p < 0.001$ versus isolated cells in 14 mM EGTA). Thus, the action potential duration may be prolonged in isolated myocytes as a result of buffering of intracellular Ca²⁺ by EGTA.

Ca²⁺ currents. Depolarization of isolated neonatal cardiac myocytes in 10 mM Ca²⁺ solution induced time- and voltage-dependent inward currents. Original records demonstrate typical inward Ca²⁺ currents that peak at 10 to 15 ms and are largely inactivated by the end of the 100-ms clamp step (Fig. 3).

The mean I-V relation of the peak current for 51 neonatal cells is shown in Figure 4. Significant activation of inward Ca²⁺ current occurs above -30 mV. The membrane potential at which

Fig. 2. Action potential measured from a cardiac myocyte isolated from a neonatal rabbit. Note the normal resting potential (-75 mV), overshoot potential (30 mV), and long action potential duration (700 ms). All are indicators of the physiologic integrity of the isolated cell. The cell was bathed in Tyrode's solution containing 3.5 mM Ca²⁺. The internal electrode solution contained 120 mM KCl.

the largest current occurred ranged from -10 to 30 mV (mean 7.8 ± 1.3 mV). The average value of these peak currents is 206 ± 10 pA. Of note, no second inward peak or hump in the I-V relation between membrane potentials of -30 to 0 mV is seen. This region corresponds to the peak current of the T-type Ca²⁺ channels reported in preparations of mature myocytes (16).

Further, as shown in Figure 4, the depolarization-induced Ca²⁺ current is dependent on the external Ca²⁺ concentration. Decreasing external [Ca²⁺] decreased the amplitude of the net current and shifts the I-V relation toward less positive values. Regression lines calculated to fit the linear portions of the ascending limb of the I-V relation curves have similar slopes and are separated by 26 mV. This value is comparable to the 29-mV separation expected for a 10-fold increase in concentration gradient predicted by the Goldman-Hodgkin-Katz equation.

A progressive "rundown" of Ca²⁺ current has been well described in chromaffin cells (28) and preparations of adult myocytes (29). Accordingly, serial recordings of voltage-gated Ca²⁺ currents were made (every 5 min) in 49 cells. The mean peak inward current was found to decrease progressively, reaching an amplitude of approximately 50% of its initial value after 20 min (Fig. 5). This progressive, and apparently irreversible, fall in Ca²⁺ current may influence measures of maximal Ca²⁺ conductance. However, 90% of the current remained 5 min after the initial recordings. It therefore appears that allowing a 5-min period for cell dialysis with pipette solution has only a small effect on measurements of peak inward Ca²⁺ current.

Rate of intracellular dialysis. The rate of dialysis of the cell interior was determined using the driving force for Na⁺ current as a measure of intracellular Na⁺ concentration. Voltage clamp steps to 0 mV with equal internal and external Na⁺ concentrations should produce no Na⁺ current because the driving force for Na⁺ movement across the cell membrane is zero. Therefore, after penetration of the cell with a pipette containing an Na⁺ concentration equal to the bath solution, the time course of the decay of inward Na⁺ current during sequential voltage clamp steps provides an estimate of the time required for equilibration of pipette Na⁺ with intracellular Na⁺ (28). In six neonatal cells, the time course of cell dialysis appeared to be a combination of an early rapid fall in Na⁺ current ($\tau_{\text{Na}} = 1.2$ min) and a slower, later decay ($\tau_{\text{Na}} = 2.6$ min) (Fig. 6). The magnitude of the Na⁺ current at 5 min was 10% of the Na⁺ current at 1 min. Thus, equilibration of low molecular weight solutes between the sarcoplasm and the pipette solution was largely accomplished within 5 min after penetration of the cell.

TTX sensitivity. To prevent Ca²⁺ influx through Na⁺ channels (15, 16), TTX was added to the experimental solution. To determine the concentration of TTX needed to inhibit current

Fig. 3. Original records of the current response of a neonatal myocyte to a series of voltage steps. The panel on the left shows transsarcolemmal Ca²⁺ current (top) and cell membrane potential (bottom) as a function of time. Voltage clamp steps ranging from 0 mV to 50 mV are superimposed at the left ($V_{\text{hold}} = -80$ mV). The panel on the right displays current and voltage traces from the same cell during voltage clamp steps ranging from -40 mV to 0 mV. Inward current is displayed as downward (negative) deflections. Zero current and potential lines are also displayed. After a brief capacitive spike, an inward current rapidly activates and then gradually inactivates over the course of a 100-ms voltage clamp. Cell no. 160.

Fig. 4. Peak Ca^{2+} I-V relation and the effect of external $[\text{Ca}^{2+}]$. In 10 mM Ca^{2+} external solution, Ca^{2+} channel activation occurs at clamp potentials greater than -30 mV ($V_{\text{hold}} = -80$ mV, values represent mean $\pm$ SEM, $n = 8$, curves drawn by hand). Maximal inward current occurs at a clamp potential of 10 mV. The Ca^{2+} I-V relation in 1 mM Ca^{2+} ($n = 7$) has a smaller peak amplitude, and the linear portion of the curve is shifted toward more negative potentials. This is consistent with the predicted behavior of a current carried mainly by Ca^{2+} ions (see text for further discussion).

Fig. 5. Ca^{2+} current rundown. Depolarization-induced Ca^{2+} current (expressed as a fraction of the current at time $t = 0$ min) is plotted as a function of time for repeated stimulations. Values are means $\pm$ SEM of measurements from 16 to 49 cells. The curve is drawn by hand ($V_{\text{hold}} = -80$ mV, $V_{\text{clamp}} = 10$ mV). The current decreased to 55% of its initial value in 20 min.

flow through Na^{+} channels in neonatal myocytes, the K_m for TTX inhibition of Na^{+} current was determined. TTX added to the external solution inhibited depolarization-induced Na^{+} current ($V_{\text{hold}} = -80$ mV, $V_{\text{clamp}} = -20$ mV) in a dose-dependent fashion (Fig. 7). TTX ($10 \mu\text{M}$) inhibited Na^{+} channel conductance by greater than 98%. Using Scatchard analysis, the K_m for inhibition was $0.37 \mu\text{M}$ (95% confidence limits = 0.26 to $0.62 \mu\text{M}$, $n = 9$). This value is similar to that reported for Na^{+} channel inhibition (concentration required for half-maximal response = $0.94 \mu\text{M}$) in intact fetal rat ventricle (23).

DISCUSSION

We have described the measurement of voltage-gated Ca^{2+} currents in acutely isolated myocytes from neonatal rabbit hearts. The whole-cell voltage clamp preparation for isolated myocytes has many advantages over multicellular preparations used in studying excitable cell membranes. It provides voltage clamp

Fig. 6. Time course of cell dialysis. Na^{+} current is used as an indication of dialysis of the cell interior with pipette solution. Depolarization-induced inward Na^{+} current (without TTX in the external solution) is plotted as a function of time (mean of six cells). This current decreases logarithmically as the transmembrane Na^{+} concentration gradient decreases as a result of dialysis of the cell. The measurements are expressed as a percentage of the current at time $t = 1$ min after cell penetration. Internal and external solutions contained 60 mM Na^{+} . $V_{\text{hold}} = -80$ mV and $V_{\text{clamp}} = 0$ mV. The τ_{Na} for the initial component of dialysis (defined by the first two points, dotted line) is 1.2 min, and that of the slower component (five points, dashed line) is 2.6 min. See text for more detailed analysis.

Fig. 7. Scatchard plot of the inhibition of peak Na^{+} current by TTX. Depolarization-induced inward Na^{+} current was inhibited by the addition of 0.2 to $10 \mu\text{M}$ TTX to the external solution ($V_{\text{hold}} = -80$ mV, $V_{\text{clamp}} = -20$ mV). Consistent with simple Michaelis-Menten kinetics, Scatchard analysis (fractional inhibition/ $[\text{TTX}]$ plotted vs fractional inhibition) of the dose-dependent inhibition of Na^{+} current produces a linear plot. However, because of the scatter of the experimental points, more complex kinetic behavior cannot be excluded. Values represent means $\pm$ SEM for two to six cells. The apparent K_m ($-1/\text{slope}$) for inhibition of Na^{+} current by TTX in neonatal cells is $0.37 \mu\text{M}$ ($n = 9$). In $10 \mu\text{M}$ TTX, greater than 98% of depolarization-induced Na^{+} current is blocked. Voltage clamps performed in the absence of internal or external Na^{+} or TTX occasionally demonstrated transient inward currents. These currents were blocked by $10 \mu\text{M}$ TTX (data not shown) and may reflect movement of ions other than Na^{+} through the Na^{+} channel. Therefore, $10 \mu\text{M}$ TTX was included in the external solution during all studies of Ca^{2+} currents.

control of the cell membrane and allows for direct measurement of transsarcolemmal currents. It also permits experimental control of the intracellular ionic environment of the cell. Furthermore, rapid changes in solutions bathing the exterior of the cell can be accomplished with only a minimal diffusion barrier. In

contrast, in multicellular preparations, the interstitial space has been shown to allow large localized concentration gradients. Our results suggest that the whole-cell voltage clamp technique may be used to explore developmental differences in cardiac Ca²⁺ current.

Isolated cell characteristics. Isolated neonatal rabbit myocytes retain many characteristics of intact viable cardiac cells including cell morphology, gross cellular ultrastructure, the exclusion of trypan blue, and the ability to remain stable without spontaneous contractions in high Ca²⁺ medium. Moreover, they develop action potentials and contract in response to depolarizing currents (25).

The morphology of action potentials recorded from isolated cells (Fig. 2) is similar to that recorded in isolated papillary muscles (4, 24), and the action potential amplitude is virtually identical (24). The effects of changes in external K⁺ on resting membrane potential in newborn rabbit papillary muscle has been previously measured at two concentrations (24). Recently, we have described the K⁺-dependence of resting membrane potential of isolated neonatal myocytes and its relation to inwardly rectifying K⁺ channels with comparable results (30).

The action potential duration of isolated myocytes was 620 ± 100 ms when the cell was dialyzed with solution containing 14 mM EGTA plus 1 mM CaCl₂. However, in low EGTA internal solution (40 μ M EGTA with no added Ca²⁺), the action potential duration was not significantly different from that recorded in isolated papillary muscles in the single sucrose gap apparatus. The increased action potential duration with high EGTA pipette solution may indicate the presence of a Ca²⁺-dependent repolarizing current (perhaps carried by a Ca²⁺-dependent K⁺ channel) in neonatal cardiac myocytes. The presence of a long action potential duration has been considered an "important indication of the robustness of the cell, and this is required for recording the very slow inward current" (30). These results suggest that isolated myocytes retain the electrophysiological properties of native ventricular tissue.

Ca²⁺ currents. A depolarization-induced time- and voltage-dependent inward Ca²⁺ current was measured in neonatal myocytes. The mean current voltage relation exhibited channel activation at potentials positive to -30 mV and maximal inward current near 10 mV. Experimental conditions were adjusted to insure that measured currents were carried exclusively by Ca²⁺ ions. To provide additional evidence that the measured current resulted from the movement of Ca²⁺, the effect of changes in external Ca²⁺ concentration was determined. The results shown in Figure 4 demonstrate that the magnitude of the inward current is dependent on external Ca²⁺ concentration. As predicted by the Goldman-Hodgkin-Katz equation, the I-V relation is shifted toward more negative values when external Ca²⁺ is decreased. As a result of buffering by EGTA, Ca²⁺ concentration in the cell is very low (less than 0.1μ M) and Ca²⁺ current does not become outward even at very positive potentials.

To exclude Ca²⁺ movement via Na⁺ and K⁺ channels, these channels were blocked with TTX and Cs⁺, respectively. TTX (10μ M) was shown to decrease inward current through Na⁺ channels by 98%. Na⁺ channel blockade is crucial in studies of voltage-gated Ca²⁺ channels because T-type Ca²⁺ channels exhibit time and voltage dependence very similar to that of Na⁺ channels. However, the Scatchard analysis shown in Figure 7 suggests that in the presence of 10μ M TTX, the measured current is almost certainly carried by Ca²⁺ ions passing through Ca²⁺ selective channels.

The Ca²⁺ current measured in these experiments exhibited a time and voltage dependence similar to that reported for L-type Ca²⁺ channels in adult myocardium. However, the magnitude of the peak current (210 pA) was much smaller than previously reported for preparations of adult myocytes from other species (1 – 2 nA) (15, 29). In addition, the I-V relation did not show the characteristic second hump corresponding to the activation of

T-type Ca²⁺ channels found in adult myocytes at relatively negative membrane potentials (16).

Other technical considerations. Because of the smaller size of neonatal myocytes, higher resistance microelectrodes were used in our experiments as compared with those used in single-cell studies of isolated adult myocytes. Despite the fact that the reduced tip diameter of these electrodes resulted in an increased series resistance, series resistance compensation was not necessary. Because of the small magnitude of the measured currents, the maximum potential drop across the cell access resistance ($14 \text{ M}\Omega \times 200 \text{ pA} = 2.8 \text{ mV}$) was within acceptable limits (30).

The rate of dialysis of the cell interior by internal pipette solution was also affected by the smaller pipette size. The rate of dialysis is an important factor in studies using the whole-cell voltage clamp technique, which takes advantage of dialysis of the cell to control the intracellular environment. When the decay of the inward Na⁺ current was used as an indicator of the rate of equilibration of the sarcoplasm with internal pipette solution (Fig. 6), the decay displayed an exponential time course with two time constants (initial $\tau_{\text{Na}} = 1.2$ min, final $\tau_{\text{Na}} = 2.6$ min). These values are substantially longer than that reported for chromaffin cells (0.1 min) using larger, 1 – $3 \text{ M}\Omega$ pipettes (26). The relationship between diffusional rates, pipette and cell size, and the molecular weight of the diffusing substance has been recently described by Pusch and Neher (27). Using the initial dialysis time constant (τ_{Na}) and the measured pipette series resistance, the calculated normalized diffusional rate for Na⁺ from our experiments, $k_{\text{Na}} \times C_m^{1.5}$ (where $k_{\text{Na}} = R_s/\tau_{\text{Na}}$ and C_m is cell membrane capacitance), is $1340 \times 10^{-14} \Omega \times \text{F}^{1.5} \times \text{s}^{-1}$, which is similar to $1966 \pm 831 \times 10^{-14} \Omega \times \text{F}^{1.5} \times \text{s}^{-1}$ measured by Pusch and Neher (27). Similarly, using equation 18 from their article,

$$\tau (\text{s}) = (V/V_0) \times (0.6 \pm 0.17) \times R_s \times (\text{molecular wt})^{1/3}$$

where V is the cell volume ($2700 \mu\text{m}^3$ for a cylinder $7 \times 7 \times 70 \mu\text{m}$) and $V_0 = 1897 \mu\text{m}^3$, time constants of 0.6 , 0.7 , and 1.5 min are calculated for Na⁺, Ca²⁺, and EGTA, respectively. The calculated time constant for Na⁺ is similar to the τ_{Na} that we measured for Na⁺ dialysis. These values compare favorably with those estimated for larger adult myocytes ($20 \times 26 \times 150 \mu\text{m}$, see reference 7). Cells of this size have a volume approximately 20 times that of neonates and, thus, similarly rapid dialysis requires very low resistance pipettes (R_s less than $1 \text{ M}\Omega$).

In our experiments, cells were allowed to equilibrate for a minimum of 5 min after cell penetration before the start of data collection. Of note, however, no transient component of Ca²⁺ current rundown with a time constant similar to that expected for cell dialysis is seen (Fig. 5). Thus, sufficient dialysis occurs within the first few minutes after cell penetration to avoid major artifactual effects on measured Ca²⁺ currents.

CONCLUSIONS

The results of our investigation suggest that neonatal rabbit myocytes may be isolated by enzymatic dissociation and that they demonstrate time- and voltage-dependent inward Ca²⁺ currents similar to those described for L-type Ca²⁺ channels in preparations of mature myocytes. However, these currents are much smaller than those reported for adult cells, perhaps in part because of the smaller size of the neonatal cells. Additionally, T-type Ca²⁺ currents are not readily apparent in these immature cells and may emerge in an age-related fashion. The application of the whole-cell voltage clamp technique to acutely isolated rabbit cells provides a more complete description of voltage-gated Ca²⁺ channels than has previously been possible in immature mammalian cardiac tissue and allows for direct comparison of membrane Ca²⁺ currents between neonatal and adult myocytes using similar isolation techniques.

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