Developmental changes in time course of recovery from inactivation in L-type calcium currents of rabbit ventricular myocytes

Takao Namiki, Ronald W. Joyner, and Mary B. Wagner

Department of Pediatrics, Emory University School of Medicine, and Children’s Healthcare of Atlanta, Atlanta, Georgia

Submitted 6 July 2006; accepted in final form 24 August 2006

Namiki T, Joyner RW, and Wagner MB. Developmental changes in time course of recovery from inactivation in L-type calcium currents of rabbit ventricular myocytes. Am J Physiol Heart Circ Physiol 292: H295–H303, 2007. First published August 25, 2006; doi:10.1152/ajpheart.00719.2006.—The mechanisms of recovery from inactivation of the L-type calcium current (\(I_{Ca}\)) are not well established, and recovery is affected by many experimental conditions. Little is known about developmental changes of recovery from inactivation of \(I_{Ca}\). We studied developmental changes of recovery from inactivation in \(I_{Ca}\) using isolated adult and newborn (1–4 days) rabbit ventricular myocytes. We used broken-patch and perforated-patch techniques with physiological extracellular ionic concentrations of calcium and sodium and interpulse conditioning potentials of \(-80\) or \(-50\) mV. We also maximized \(I_{Ca}\) with forskolin. We found that recovery from inactivation did not differ between adult and newborn cells when either EGTA or BAPTA was used to buffer intracellular calcium. Maximizing \(I_{Ca}\) with forskolin slowed recovery from inactivation in newborn but not in adult cells. In contrast, when the intracellular buffering of the cell was left nearly intact (perforated patch), recovery from inactivation (half-time of recovery) in the newborn cells was significantly slower than for the adult cells when either a conditioning potential of \(-80\) mV (140 ± 9 vs. 58 ± 4 ms, newborn vs. adult; \(P < 0.05\)) or \(-50\) mV (641 ± 106 vs. 168 ± 15 ms, newborn vs. adult; \(P < 0.05\)) was used. Forskolin significantly increased half-time of recovery for both adult and newborn cells. Dialysis with no calcium buffer showed a slower recovery from inactivation in newborn cells. Intracellular dialysis with a calcium buffer masked differences in recovery from inactivation of \(I_{Ca}\) between newborn and adult rabbit ventricular cells.

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
producing the negative force-frequency relationship for cells from failing human ventricles (2, 30). In studies on ventricular cells surviving in the epicardial border zone of the canine-healed infarcted heart, Dun et al. (10) showed slowing of the rate of recovery of inactivation of \( I_{Ca} \) compared with cells from the normal zone.

Little is known about the developmental changes of rates of recovery from inactivation in the L-type Ca\(^{2+}\) channel of mammalian cells. Wetzels et al. (33) showed that recovery from inactivation had voltage dependency and that the mean time constant of recovery was greater for neonatal than for adult myocytes at each membrane potential from \(-80\) to \(-40\) mV, although more prominently different at more depolarized potentials. However, these data were obtained with a high concentration (10 mM) of Ca\(^{2+}\) in the bath solution and zero Na\(^+\) in the bath solution. The time course of recovery from inactivation at physiological extracellular [Ca\(^{2+}\)] and Na\(^+\) concentration ([Na\(^+\)]) has not yet been evaluated. In the present work, we have evaluated developmental changes in recovery from inactivation at a more physiological extracellular [Ca\(^{2+}\)] (1.8 mM) and at a physiological [Na\(^+\)] (130 mM) by using tetrodotoxin to block Na\(^+\) currents. We have also compared the effects of using either EGTA or BAPTA as an intracellular Ca\(^{2+}\) buffer, as well as the effects of a physiological buffering, utilizing the perforated-patch condition on the kinetics of recovery from inactivation.

**METHODS**

**Isolation of ventricular cells.** New Zealand White adult (1.5–2 kg) and newborn (1–4 days old) rabbits were used. All animal protocols were approved by the Institutional Animal Care and Use Committee of Emory University. The enzymatic procedure for single cell isolation was previously described (22). Briefly, after rabbits were heparinized and anesthetized with pentobarbital sodium (50 mg/kg), hearts were removed and placed in storage solution and then with nominally Ca\(^{2+}\)-free solution for 5–6 min. The perfusate was then switched to the same solution containing 0.08–0.15 mg/ml collagenase (Yakult Japan) and 0.05 mg/ml protease (type XIV; Sigma) for 5–7 min, followed by perfusion with storage solution for 4 min. The ventricles were placed in storage solution, chopped finely, and triturated to disperse isolated cells and then filtered through a 100-µm mesh. Cells were used within 8 h of dissociation.

**Solutions and drugs.** Normal Tyrode solution contained (in mmol/l) 148.8 NaCl, 4.0 KCl, 1.8 CaCl\(_2\), 0.53 MgCl\(_2\), 0.33 NaH\(_2\)PO\(_4\), 5.0 HEPES, and 5.0 glucose, pH adjusted to 7.4 with NaOH. The composition of Ca\(^{2+}\)-free solution was the same as that of normal Tyrode solution except that CaCl\(_2\) was omitted. Storage solution contained (in mmol/l) 140 potassium glutamate, 25 KCl, 10 KH\(_2\)PO\(_4\), 0.5 EGTA, 1 MgSO\(_4\), 20 taurine, 5 HEPES, and 10 dextrose. \( I_{Ca} \) test solution contained (in mmol/l) 130 NaCl, 1.8 CaCl\(_2\), 20 CsCl, 0.53 MgCl\(_2\), and 10 HEPES, pH 7.4 with KOH. Tetrodotoxin (0.01–0.03 mmol/l) was added in all test solution to block Na\(^+\) current. EGTA pipette solution was (in mmol/l) 110 CsOH, 90 aspartic acid, 20 CsCl, 10 tetraethylammonium Cl, 5 HEPES, 5 MgATP, 5 Na\(_2\) creatine phosphate, 0.4 GTP(Tris), 0.1 leupeptin, and 10 EGTA, pH 7.2 with KOH. For the BAPTA pipette solution, we substituted 10 mM BAPTA for the EGTA. For perforated-patch recording, the pipette solution contained (in mM) 110 cesium glutamate, 20 CsCl, 1.1 MgCl\(_2\), 1.8 CaCl\(_2\), and 5 HEPES (pH 7.2). Just before use, the perforated-patch pipette tip was filled with antibiotic-free pipette solution by dipping the tip for a few seconds. The rest of the pipette was backfilled with pipette solution to which had been added 240 µg/mg amphotericin B. Forskolin stock solution (10 mM) was dissolved in DMSO and then diluted to 10 µM in test solution at time of use.

**Electrophysiology.** Voltage-clamp experiments were performed in the whole cell clamp configuration of the broken-patch or perforated-patch recording by use of an Axopatch 200 patch-clamp amplifier (Axon Instruments; Molecular Devices), Sunnyvale, CA). Pipettes had resistances of 1–2 M\(\Omega\) for adult cells and 2–4 M\(\Omega\) for newborn cells. In broken-patch recordings, after high resistance was achieved, the cell membrane was disrupted by suction. Series resistance and cell capacitance compensation were done. At least 10 min were allowed for the intercellular dialysis to reach equilibrium. In perforated-patch recordings, the pipette was used in the cell attached mode, resulting in a relatively low resistance access to the cell interior, whereas the intracellular constituents remained largely undisturbed. The newborn cells were significantly smaller than the adult cells, as measured by cell capacitance (16 ± 1 pF (newborn, \(n = 34\)) vs. 78 ± 3 pF (adult, \(n = 40\)); means ± SE). Series resistance, before compensation, was 9.7 ± 0.6 (\(n = 61\)) for cells in the broken-patch and 10.7 ± 1.5 (\(n = 13\)) for cells in the perforated-patch groups.

We define the time course of recovery from inactivation using a two-pulse protocol. Cells were depolarized to +15 mV from a holding potential of −80 mV for a 400- or 800-ms conditioning pulse. The test pulse was an identical depolarization to +15 mV for the same length of time after a variable recovery interval at a conditioning voltage (\(V_{cond}\)) level of −50 mV or −80 mV. The experiments were performed at room temperature (21–23°C). The elicited \(I_{Ca}\) was filtered at a corner frequency of 2 kHz, digitized at 200-µs intervals, and stored and analyzed on a computer with pCLAMP software (Axon Instruments). The peak amplitude in \(I_{Ca}\) was measured as the difference between the inward peak and the end of the current trace. The degree of recovery from inactivation was calculated individually as the half-maximum activation time (\(t_{1/2}\)) by fitting the data to a single rising exponential equation. Data are presented as means ± SE. Statistical significance was determined by ANOVA using Systat, with pair-wise comparisons using Tukey’s post hoc test. \(P < 0.05\) was considered significant.

**RESULTS**

The time course of recovery from inactivation with EGTA internal solution. Figure 1 shows data from an adult ventricular cell (Fig. 1A) and from a newborn ventricular cell (Fig. 1B). For both cells, we superimposed data from seven current traces. Each trace includes an initial current response to a voltage step from −80 to +15 mV and then the response to a second depolarization from \(V_{cond}\) (where \(V_{cond}\) is either −80 or −50 mV) to +15 mV, which is delayed from the first voltage step by 50, 100, 200, 500, 1,000, or 2,000 ms. As this time delay is increased, the membrane current (\(I_{Ca}\)) progressively increases back to the level of the initial current response. For both cells, we included a horizontal dotted line, which indicates the current level for a return to 50% of the initial current response. In Fig. 1A, top, for the adult cell with a \(V_{cond}\) of −80 mV, the recovery to 50% of the initial current response occurs after −100 ms. When the cell is held at \(V_{cond} = −50\) mV (Fig. 1A, bottom), the time required to reach 50% recovery is increased to approximately 500 ms.

Figure 1B shows data in the same format as in Fig. 1A but for an isolated newborn ventricular cell. For \(V_{cond} = −80\) mV, the recovery of \(I_{Ca}\) to 50% of the control level occurs at 100 ms, which is very similar to that obtained in Fig. 1A for the adult cell. Increasing \(V_{cond}\) to −50 mV again results in an increase in the time to reach 50% recovery, similar to the results obtained for the adult cell. For six adult and seven newborn cells, respectively, the \(t_{1/2}\) of \(I_{Ca}\) was 105 ± 9 ms.
RECOVERY FROM INACTIVATION

Broken Patch, 10 mM EGTA, Control

Adult, $V_{\text{cond}} = -80\text{ mV}$

Newborn, $V_{\text{cond}} = -80\text{ mV}$

$V_{\text{cond}} = -50\text{ mV}$

Membrane Current (pA/µF)

Time (ms)

97 ± 6 ms (means ± SE) for $V_{\text{cond}} = -80\text{ mV}$ and 425 ± 49 vs. 482 ± 34 ms for a $V_{\text{cond}} = -50\text{ mV}$. There was no significant difference between adult and newborn $t_{1/2}$ of recovery from inactivation for either $V_{\text{cond}}$; however, for both adult and newborn, the $t_{1/2}$ for recovery was significantly prolonged with $V_{\text{cond}} = -50\text{ mV}$ vs. $V_{\text{cond}} = -80\text{ mV}$.

To examine the effect of maximizing the $I_{\text{Ca}}$ on recovery from inactivation, we added 10 μM forskolin to the external solution. With forskolin, $I_{\text{Ca}}$ density was significantly increased for both adult and newborn cells. Figure 2 shows results of the same adult and newborn cells with the EGTA internal solution as in Fig. 1 but with forskolin in the bath. For the adult cell (Fig. 2A), although the peak $I_{\text{Ca}}$ is much larger than in control, the time for recovery from inactivation of $I_{\text{Ca}}$ is almost unchanged, occurring at $\sim 100$ ms for $V_{\text{cond}} = -80\text{ mV (top)}$ and at $\sim 500$ ms for $V_{\text{cond}} = -50\text{ mV (bottom)}$. In contrast, for the newborn cell (Fig. 2B) with 10 μM forskolin, $t_{1/2}$ for recovery increased to $\sim 200$ ms for $V_{\text{cond}} = -80\text{ mV (top)}$ and dramatically increased to nearly $2,000$ ms for $V_{\text{cond}} = -50\text{ mV (bottom)}$. For four adult cells and four newborn cells with $10 \mu\text{M forskolin}, t_{1/2}$ for recovery of $I_{\text{Ca}}$ was $88 \pm 6$ vs. $193 \pm 9$ ms (adult vs. newborn) for $V_{\text{cond}} = -80\text{ mV}$ and $356 \pm 27$ vs. $1,699 \pm 131$ ms for $V_{\text{cond}} = -50\text{ mV}$.

As summarized in Table 1, $t_{1/2}$ for recovery from inactivation was not different for adult vs. newborn cells in the control external solution, determined with $V_{\text{cond}}$ of either $-80$ or $-50\text{ mV}$, although results with $-50\text{ mV}$ were longer than those for $-80\text{ mV}$. The addition of forskolin to the external solution, to maximize $I_{\text{Ca}}$, did not significantly change $t_{1/2}$ for recovery from inactivation at either $V_{\text{cond}}$ in the adult cells. However, the addition of forskolin to the external solution for the newborn cells significantly prolonged $t_{1/2}$ for recovery from inactivation with $V_{\text{cond}}$ of $-80\text{ mV}$ (2-fold) and with $V_{\text{cond}}$ of $-50\text{ mV}$ (4-fold). Additionally, the addition of forskolin resulted in a significant difference between newborns and adults.

These effects are more easily seen in Fig. 3, in which we plot the data in each part as adult vs. newborn cells. For each time interval at the $V_{\text{cond}}$ (Δt), we plot the percent recovery of $I_{\text{Ca}}$. Figure 3, top left, shows the recovery from inactivation time course for $V_{\text{cond}} = -80\text{ mV}$ in control solution. Figure 3, top right, shows the recovery from inactivation time course for $V_{\text{cond}} = -50\text{ mV}$ in control solution. As shown, in control solution, there is no difference between the adult and the newborn cells, although recovery is slower when $V_{\text{cond}} = -50\text{ mV}$.

Fig. 1. Membrane Ca$^{2+}$ current ($I_{\text{Ca}}$) recorded using the dialysis-patch-clamp technique with 10 mM EGTA in the pipette from an adult (A) and a newborn (B) ventricular cell. For both A and B, we superimposed data from 7 current traces. Each trace includes an initial current response to a voltage step from $-80$ to $+15\text{ mV}$ and then the response to a second depolarization from $-80$ to $+15\text{ mV}$, which is delayed from the first voltage step by 50, 100, 500, 1,000, or 2,000 ms. We included a horizontal dotted line that indicates the current level for a return to 50% of the initial current response. A and B, top: conditioning voltage ($V_{\text{cond}}$) level of $-80\text{ mV}$. A and B, bottom: $V_{\text{cond}}$ of $-50\text{ mV}$. Recovery from inactivation is slowed for both newborn and adult cells when $V_{\text{cond}} = -50\text{ mV}$.

Fig. 2. Data under the same conditions as Fig. 1 (dialysis patch, 10 mM EGTA) but with 10 μM forskolin in the external solution to maximize the density of $I_{\text{Ca}}$. A: recovery of $I_{\text{Ca}}$ for an adult cell for $V_{\text{cond}} = -80\text{ mV (top)}$ or $-50\text{ mV (bottom)}$. B: recovery of $I_{\text{Ca}}$ for a newborn cell for $V_{\text{cond}} = -80\text{ mV (top)}$ or $-50\text{ mV (bottom)}$. Recovery is significantly slower for the newborn cell (when compared with control) but unchanged for the adult cell.
Figure 3, bottom, shows the results when 10 μM forskolin was used to maximize \( I_{Ca} \), with \( V_{\text{cond}} = -80 \text{ mV} \) (bottom left) and \( V_{\text{cond}} = -50 \text{ mV} \) (bottom right). As shown, the time course of recovery from inactivation is significantly slower for newborn than for adult cells.

**Time course of recovery from inactivation with BAPTA internal solution.** Because \( \text{Ca}^{2+} \) buffering with EGTA may have been incomplete, we performed similar experiments with BAPTA in the pipette to achieve more complete \( \text{Ca}^{2+} \) buffering. Figure 4 shows results for an adult cell (Fig. 4A) and a newborn cell (Fig. 4B) for which we did used the same double-pulse protocol as used in Fig. 1 (with control external solution), except that the EGTA in the internal solution was replaced with BAPTA. Because inactivation of \( I_{Ca} \) is slowed when BAPTA is used as a buffer, we increased the pulse duration to 800 ms for both the first and second pulses. For the adult cell, \( t_{1/2} \) for recovery from inactivation with \( V_{\text{cond}} = -80 \text{ mV} \) (Fig. 4A, top) is somewhat less than 100 ms; for \( V_{\text{cond}} = -50 \text{ mV} \) (Fig. 4A, bottom), \( t_{1/2} \) of recovery is between 200 and 500 ms. For 10 adult cells, \( t_{1/2} \) for recovery from inactivation was 86 ± 3 and 328 ± 21 ms (\( V_{\text{cond}} \) of −80 vs. −50 mV, respectively). Similar results were seen for the newborn cell (Fig. 4B). For five newborn cells, \( t_{1/2} \) for recovery from inactivation was 61 ± 8 and 227 ± 39 ms (\( V_{\text{cond}} \) of −80 vs. −50 mV, respectively).

We again maximized \( I_{Ca} \) with 10 μM forskolin and BAPTA in the pipette, and the results are seen in Fig. 5. Figure 5A shows the results for an adult cell. The \( t_{1/2} \) of recovery at either \( V_{\text{cond}} \) is similar in the presence of forskolin to that in control solution (compare to Fig. 4A). For 10 adult cells, \( t_{1/2} \) for recovery from inactivation was 77 ± 3 and 291 ± 26 ms (\( V_{\text{cond}} \) of −80 vs. −50 mV, respectively), not significantly different from the control values. For a newborn cell (Fig. 5B), \( t_{1/2} \) for recovery from inactivation is between 100 and 200 ms (top; \( V_{\text{cond}} = -80 \text{ mV} \)) and ~500 ms (bottom; \( V_{\text{cond}} = -50 \text{ mV} \)). For five newborn cells, \( t_{1/2} \) for recovery from inactivation was 135 ± 3 and 599 ± 33 ms (\( V_{\text{cond}} \) of −80 vs. −50 mV, respectively), which is significantly longer than in control solution for both \( V_{\text{cond}} \) values.

The data for which BAPTA was used as a \( \text{Ca}^{2+} \) buffer in the internal solution are summarized in Table 2. Note that the amplitude of \( I_{Ca} \) in control tends to be larger with BAPTA.

### Table 1. Analysis of recovery kinetics for \( I_{Ca} \) with 10 mM EGTA in the pipette

<table>
<thead>
<tr>
<th>Age</th>
<th>Solution</th>
<th>( n )</th>
<th>( I_{Ca} ) peak, pA/pF</th>
<th>( V_{\text{cond}} ) = −80 mV</th>
<th>( V_{\text{cond}} ) = −50 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>Control</td>
<td>6</td>
<td>6.9 ± 0.7</td>
<td>105 ± 9</td>
<td>425 ± 49</td>
</tr>
<tr>
<td>Adult</td>
<td>Forskolin</td>
<td>4</td>
<td>19.6 ± 1.2*</td>
<td>88 ± 6</td>
<td>356 ± 27</td>
</tr>
<tr>
<td>Newborn</td>
<td>Control</td>
<td>7</td>
<td>3.3 ± 0.3†</td>
<td>97 ± 6</td>
<td>482 ± 34</td>
</tr>
<tr>
<td>Newborn</td>
<td>Forskolin</td>
<td>4</td>
<td>14.0 ± 0.6*†</td>
<td>193 ± 9†</td>
<td>1,699 ± 131††</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \), number of cells. \( I_{Ca} \), \( \text{Ca}^{2+} \) current; \( t_{1/2} \), Half-time of recovery; \( V_{\text{cond}} \), conditioning potential. *Significant from control solution of the cells of the same age (\( P < 0.05 \)). †Newborn significantly different from adult cell (\( P < 0.05 \)).
buffering than with EGTA for both adult and newborn cells, but \( t_{1/2} \) for recovery from inactivation is similar. The observations seen with EGTA buffering (Table 1) that recovery is slower with \( V_{\text{cond}} = -50 \text{ mV} \) compared with \( V_{\text{cond}} = -80 \text{ mV} \) and that forskolin increases \( t_{1/2} \) of recovery for newborns but not adults was also seen when BAPTA was used in the internal solution.

Time course of recovery from inactivation with perforated-patch technique. Because BAPTA provides a fast and strong buffer of \( \text{Ca}^{2+} \), we wanted to compare these results with those obtained when the cell’s normal cytoplasmic \( \text{Ca}^{2+} \) buffering was not modified by the dialysis of an internal solution. This would provide a more physiological comparison of recovery from inactivation between the newborn and adult cells. We therefore repeated the recovery from inactivation experiments on adult and newborn cells using the perforated-patch technique. For the adult cells, an example is shown in Fig. 6A in which we show the results obtained with the control external solution (black lines) and the forskolin external solution (gray lines) with a conditioning time of 500 ms (top: \( V_{\text{cond}} = -80 \text{ mV} \); bottom: \( V_{\text{cond}} = -50 \text{ mV} \)). For both values of \( V_{\text{cond}} \), the conditioning interval is set to 500 ms. Note that, as expected, forskolin increases the amplitude of the \( I_{\text{Ca}} \). With \( V_{\text{cond}} = -80 \text{ mV} \), in both control and forskolin, the recovery from inactivation is nearly complete by 500 ms of conditioning time. In contrast, for \( V_{\text{cond}} = -50 \text{ mV} \), the recovery from inactivation is nearly complete by 500 ms for the control solution but is \(-50\%\) for the forskolin solution. During the conditioning time, there is a small inward current apparent for both values of \( V_{\text{cond}} \).

When we applied the same perforated-patch technique to a newborn cell (Fig. 6B), we also observed a large increase in the \( I_{\text{Ca}} \) with the forskolin solution compared with the control solution. For \( V_{\text{cond}} = -80 \text{ mV} \), there is less recovery from inactivation at 500 ms for both the control and forskolin solutions compared with the adult cell (Fig. 6A, top). For \( V_{\text{cond}} = -50 \text{ mV} \), there is very little recovery from inactivation at 500 ms for either the control or the forskolin solution. Another difference when the newborn and adult cells are compared is that there is much more prominent inward current; this slowly decays during the conditioning period, is greater in magnitude at \( V_{\text{cond}} = -80 \text{ mV} \) than at \(-50 \text{ mV} \), and is not affected by the addition of forskolin.

Figure 7 shows a summary of the time course of recovery from inactivation, with the perforated-patch technique, for adult and newborn cells in the control external solution (top left and top right: \( V_{\text{cond}} = -80 \text{ and } -50 \text{ mV} \), respectively) and...
also in the forskolin solution (bottom left and bottom right: \( V_{\text{cond}} \) of −80 and −50 mV, respectively). Note that the time course of recovery from inactivation is faster under all conditions of external solution and that values of \( V_{\text{cond}} \) for adult cells compared with those for newborn cells with the perforated-patch technique. These data are summarized in Table 2 and are compared with the results obtained when BAPTA was dialyzed into the cells to buffer \( \text{Ca}^{2+} \). In control solution with the perforated-patch technique, recovery from inactivation is significantly slower in newborn than in adult cells, which was not true when BAPTA was used in the pipette. Additionally, recovery in the newborn cells with the perforated patch is significantly slower than with BAPTA buffering (Fig. 8). With the addition of forskolin, the recovery from inactivation was again slower in the newborn cell than when BAPTA was used as internal solution. In the adult, recovery tends to be faster in control but slower in forskolin with the perforated-patch technique than with buffering BAPTA, but these results did not reach statistical significance. Additionally, with forskolin, \( t_{1/2} \) of recovery was increased in the newborn cell compared with control, similar to the results seen with BAPTA buffering. In contrast, in the adult with perforated patch, forskolin (compared to control) increased \( t_{1/2} \) for recovery from inactivation, which did not occur when BAPTA was used to buffer intracellular \( \text{Ca}^{2+} \).

In the experiments described above, we compared the time course of recovery from inactivation for the \( I_{\text{Ca}} \) under different \( \text{Ca}^{2+} \) buffering conditions: adding either EGTA or BAPTA with the dialysis patch or using the cell’s intrinsic buffering with the perforated patch. Under control conditions, there is a difference between adult and newborn recovery only when the perforated patch was used. When the \( I_{\text{Ca}} \) was dramatically increased with forskolin, if \( \text{Ca}^{2+} \) was buffered with either EGTA or BAPTA, \( t_{1/2} \) of recovery from inactivation was unchanged in the adult cells but slowed in the newborn cells. In contrast, when the perforated patch was used, forskolin slowed recovery from inactivation in both the adult and newborn cells. We suggest that the difference between recovery from inactivation for newborn vs. adult cells is due to decreased intrinsic \( \text{Ca}^{2+} \) buffering in the newborn cells, which becomes unmasked when perforated patch is used. Additionally, increasing \( \text{Ca}^{2+} \) flux using forskolin in newborn cells, under all buffering conditions, slows recovery from inactivation; however, in adult cells, it slows recovery only when perforated patch is used. Using forskolin to increase the \( \text{Ca}^{2+} \) flux can be problematic because forskolin increases phosphorylation and thus could be having effects on recovery from inactivation distinct from the effects produced by increasing the size of the \( I_{\text{Ca}} \). Thus we performed additional experiments in which we used the dial-

### Table 2. Analysis of recovery kinetics for \( I_{\text{Ca}} \): effect of cell dialysis

<table>
<thead>
<tr>
<th>Age</th>
<th>External Solution</th>
<th>( n )</th>
<th>Peak ( I_{\text{Ca}} ) pA/pF</th>
<th>( V_{\text{cond}} ) = −80 mV</th>
<th>( V_{\text{cond}} ) = −50 mV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult</td>
<td>Control</td>
<td>10</td>
<td>9.6±0.4</td>
<td>86±3</td>
<td>328±21</td>
</tr>
<tr>
<td>Adult</td>
<td>Forskolin</td>
<td>10</td>
<td>18.5±1.6*</td>
<td>77±3</td>
<td>291±26</td>
</tr>
<tr>
<td>Newborn</td>
<td>Control</td>
<td>5</td>
<td>7.4±0.9</td>
<td>61±8</td>
<td>227±39</td>
</tr>
<tr>
<td>Newborn</td>
<td>Forskolin</td>
<td>5</td>
<td>21.5±1.3*</td>
<td>135±3†</td>
<td>599±33‡</td>
</tr>
<tr>
<td><strong>Strong intracellular ( \text{Ca}^{2+} ) buffering: 10 mM BAPTA in pipette</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adult</td>
<td>Control</td>
<td>4</td>
<td>7.1±0.6</td>
<td>58±4</td>
<td>168±15</td>
</tr>
<tr>
<td>Adult</td>
<td>Forskolin</td>
<td>3</td>
<td>17.3±2.1*</td>
<td>105±8</td>
<td>508±46*</td>
</tr>
<tr>
<td>Newborn</td>
<td>Control</td>
<td>6</td>
<td>5.5±0.7</td>
<td>140±9†‡</td>
<td>641±106‡‡</td>
</tr>
<tr>
<td>Newborn</td>
<td>Forskolin</td>
<td>3</td>
<td>22.2±2.2*</td>
<td>277±64*†‡§§</td>
<td>1,255±189‡‡§§</td>
</tr>
</tbody>
</table>

Values are means ± SE; \( n \), number of cells. *Significant from control solution of the cells of the same age \( (P < 0.05) \). †Newborn significantly different from adult for the same solution \( (P < 0.05) \). ‡Perforated patch significantly different from BAPTA solution \( (P < 0.05) \).

Fig. 6. \( I_{\text{Ca}} \) recovery from inactivation recordings using the perforated-patch technique to investigate conditions with undisturbed intracellular \( \text{Ca}^{2+} \) buffering. Black traces, control conditions; gray traces, 10 \( \mu \text{M} \) forskolin. Data are shown for a conditioning time interval of 500 ms. A: adult cell with \( V_{\text{cond}} = −80 \text{ mV (top)} \) or −50 mV (bottom). Recovery is complete in control solution; however, at \( V_{\text{cond}} = −50 \text{ mV} \), forskolin delays recovery. B: newborn cell with \( V_{\text{cond}} = −80 \text{ mV (top)} \) or −50 mV (bottom). Recovery is slowed by forskolin at both \( V_{\text{cond}} \).
ysis patch (as for the EGTA and BAPTA) data but simply omitted both EGTA and BAPTA from the internal solution, thus not imposing any extrinsic Ca\(^{2+}\)/H\(^{1+}\) buffering on the cytoplasm. The recovery from inactivation curves for these experiments are shown in Fig. 9 (left: \(V_{\text{cond}}\) of \(-80\) mV; right: \(V_{\text{cond}}\) of \(-50\) mV). As can be seen in Fig. 9, under conditions of dialysis patch with no pipette Ca\(^{2+}\) buffering, recovery from inactivation is slower in the newborn than in the adult, similar to the results seen with perforated patch (Fig. 7, top left and top right) but in contrast to dialysis with Ca\(^{2+}\) buffering by either EGTA (Table 1) or BAPTA (Table 2). The \(t_{1/2}\) for recovery from inactivation for \(V_{\text{cond}}\) of \(-80\) mV was significantly slower in the newborn cells (102 ± 6 ms, \(n = 5\)) than in the adult cells (63.8 ± 6 ms, \(n = 5\); \(P < 0.002\)). For \(V_{\text{cond}}\) of \(-50\) mV, recovery from inactivation in the newborn cells tended to be slower than in the adult cells, but this difference did not reach statistical significance [316 ± 41 ms (newborn: \(n = 5\)) vs. 212 ± 24 ms (adult: \(n = 5\)); \(P = 0.059\)]. These results show that the differences in the time course of recovery from inactivation of the \(I_{\text{Ca}}\) between newborn and adult cells becomes apparent with dialysis with no extrinsic Ca\(^{2+}\) buffering, even without altering the size of \(I_{\text{Ca}}\). This demonstrates that neither the perforated-patch technique nor enhanced Ca\(^{2+}\) channel phosphorylation is necessary to show developmental differences in recovery from inactivation; elimination of extrinsically supplied Ca\(^{2+}\) buffering is sufficient.
DISCUSSION

There have been a large number of comparisons of $I_{Ca}$ properties between newborn and adult rabbit ventricular myocytes (1, 15, 17, 18, 21–24, 32, 33), most of them done with the EGTA-containing dialysis-patch technique, with one recent study using the perforated-patch technique (14). There is general agreement that the amplitude (pA) of $I_{Ca}$ increases with age, with most of this difference being accounted for by increases in cell capacitance, although the current density (pA/pF) remains somewhat smaller in newborn than in adult cells (14, 22). There is also a significant body of literature that the Na$^+$/Ca$^{2+}$ exchange current (as well as the mRNA and protein levels) is larger in newborn than in adult cells (4, 5, 8, 13) and that the Na$^+$/Ca$^{2+}$ exchange current may play a more prominent role in excitation-contraction coupling in newborn than in adult cells (20). Furthermore, intracellular Ca$^{2+}$ buffering power has been shown to be two to three times lower in newborn than in adult myocytes (6).

Our results indicate that, when the dialysis-patch technique is used, with either EGTA or BAPTA buffering intracellular Ca$^{2+}$, the time course of recovery from inactivation at −80 mV is the same for newborn and adult myocytes and remains unchanged when the density of $I_{Ca}$ is dramatically increased by application of forskolin for adult cells but is slowed by application of forskolin to the newborn cells. We suggest that the large increase in $I_{Ca}$ for the newborn cells may overcome the added buffer capacity of the EGTA or BAPTA with the forskolin application, particularly because the newborn cells have a significantly larger surface-to-volume ratio than adult cells and thus may experience a larger increase in intracellular [Ca$^{2+}$] for a similar increase in $I_{Ca}$ density. For $V_{cond}$ of −50 mV (vs. −80 mV) for the EGTA or BAPTA conditions, the recovery from inactivation is slower but remains the same for the newborn and adult cells except when forskolin is applied to newborn cells, where the same phenomenon of slowed recovery for newborn cells is seen as for $V_{cond}$ of −80 mV but now occurs with even slower recovery. In the earlier report from Wetzel et al. (32), they showed a slower recovery from inactivation for newborn than for adult cells; this study used an elevated [Ca$^{2+}$] of 10 mM, which may account for this difference.

The use of the perforated-patch technique removes the effect of the added Ca$^{2+}$ buffering of the EGTA or BAPTA with the dialysis-patch technique, forcing the cell to rely on its intrinsic buffer capacity. This uncovers an intrinsic difference between adult and newborn cells, with newborn cells having significantly slower recovery from inactivation under control conditions. In addition, the use of forskolin now slows the recovery from inactivation in both the adult and newborn cells. Furthermore, using the broken patch with no added Ca$^{2+}$ buffering and without augmenting $I_{Ca}$ also shows a developmental difference in the time course of recovery from inactivation, with newborns being slower than adults. These results suggest that the newborn may have an intrinsically lower Ca$^{2+}$ buffering capacity than the adults. The developmental differences in recovery from inactivation are shown at $V_{cond}$ of either −80 or −50 mV, with more exaggerated effects at the −50-mV level. The slowing of the recovery from inactivation with $V_{cond}$ of −50 mV compared with that found at $V_{cond}$ of −80 mV occurs with the perforated patch and with the dialysis patch with either EGTA or BAPTA. Although this may be partly due to a voltage-dependent change in the time constant for recovery of $I_{Ca}$, there may also be a significant contribution of slower clearing of intracellular Ca$^{2+}$ by the Na$^+$/Ca$^{2+}$ exchange pump due to the depolarization. The large inward current present during the conditioning pulse to either −80 or −50 mV for the newborn cells (and a much smaller inward current for the adult cells) under perforated-patch conditions either in control solution or in the forskolin solution may be attributed to Na$^+$/Ca$^{2+}$ exchange current, although we have not
further studied this current component. The larger amplitude of this current in newborn than in adult cells is consistent with the larger expression of Na+/Ca2+ exchange in the newborn cells (4, 5, 8, 13) and the decreased Ca2+ buffering capacity (without added intracellular Ca2+ buffering by EGTA or BAPTA) of newborn cells (6). Note that this current appears to saturate with respect to the amplitude of Ica, being at the same level and time course in the control and forskolin solutions but being decreased at −50 mV compared with −80 mV and appearing to be turned off very quickly with the depolarization to +10 mV. This is consistent with recent reports from Ginsburg and Bers (12), suggesting that increases in cAMP do not increase Na+/Ca2+ exchange pump activity in adult rabbit myocytes.

One limitation of our study is that the experiments were performed at room temperature to ensure stability of the cell recordings; thus, to extrapolate these results to a physiological temperature, would be uncertain. This study emphasizes the observation that intracellular dialysis with a Ca2+ buffer (whether it is EGTA or BAPTA) can alter the kinetics of Ica and, in this study, masked differences in recovery from inactivation of Ica between newborn and adult rabbit ventricular cells. The difference in recovery from inactivation with development was unmasked by using either no extrinsic Ca2+ buffering and the broken patch or using the perforated patch and the cell’s intrinsic Ca2+ buffering.

ACKNOWLEDGMENTS
Present address of T. Namiki: Chiba Prefectural Tohgane Hospital, Tohgane City, Chiba 283-8588, Japan.

GRANTS
This work was supported by National Heart, Lung, and Blood Institute Grant HL-077485 (R. W. Joyner).

REFERENCES