Ionic basis of ryanodine's negative chronotropic effect on pacemaker cells isolated from the sinoatrial node

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Ryanodine, a compound that reduces by twofold the conductance of Ca\(^{2+}\)-release channels in the sarcoplasmic reticulum (SR) (25), slows the final phase of diastolic depolarization and, therefore, pacemaker activity in a number of cardiac preparations: strips of dilated human right atrium (7), subsidiary pacemaker cells of cat right atrium (26), pacemaker cells of cat (18) and guinea pig (24) sinoatrial (SA) nodes, and pacemaker cells isolated from cat right atrium (32) and rabbit SA node (17). The present study was designed to test the hypothesis that ryanodine's negative chronotropic effect on rabbit SA node cells is due to a reduction of inward currents that are modulated by intracellular Ca\(^{2+}\) (Ca\(^{2+}\)). The fluorescent indicator indo 1 was used to monitor Ca\(^{2+}\), and the perforated-patch configuration of the whole cell patch-clamp technique (15) was employed to record spontaneous electrical activity and ionic currents in single pacemaker cells after 2 or 3 days in culture. Indo 1 allows continuous detection of two emission wavelengths and their ratio, thereby avoiding the switching time required for dual-excitation fluorescence indicators like fura 2. The perforated-patch technique avoids complete dialysis of the cytoplasm by the patch pipette, thereby preventing the loss of compounds that are essential for automaticity (19, 21). 1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)-acetoxyethyl ester (AM), a rapidly acting calcium chelator, was used to test whether reduced intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(i\)) plays a role in the inhibitory effects of ryanodine. Our results confirm that ryanodine inhibits the release of Ca\(^{2+}\) from the SR of rabbit SA node pacemaker cells and suggest that reductions of inward Na\(^+\)/Ca\(^{2+}\) exchange current (I\(_{Na,Ca}\)) and T-type Ca\(^{2+}\) current (I\(_{Ca,T}\)) account for the negative chronotropic effect of ryanodine.

METHODS

Cell isolation and culture. Our method for isolating SA node cells is from the hearts of male New Zealand White rabbits (1.0–1.5 kg) has been described in detail (19, 21). The cells were plated on glass coverslips (number 0) in 35-mm plastic dishes that contained a culture medium (19, 21) and were stored in an incubator (95% air-5% CO\(_2\)) at 37°C. Measurement of intracellular Ca\(^{2+}\). Cultured SA node cells were incubated with 25 µM indo 1-AM for 10–15 min at room temperature and then washed with Tyrode solution for at least 15 min. This concentration and loading period were adopted to optimize the signal-to-noise ratio for indo 1 fluorescence without excessive buffering of Ca\(^{2+}\). A Photon Technology International (PTI, South Brunswick, NJ) filter-based detection system was used to record indo 1 fluorescence simultaneously at 405 and 485 nm during epifluorescence illumination at 365 nm. Fluorescence was measured as photons per second, and PTI's FELIX software was used to acquire the fluorescence ratio (F\(_{405}/F_{485}\)) and electrophysiological data simultaneously. Fluorescence was measured from a rectangular area roughly the size of the cell under study. The background fluorescence, recorded from a cell-free field of the same size, was subtracted from each of the two signals before the fluorescence ratio was calculated. To minimize photobleaching of indo 1, we exposed the cells to 365-nm light only during the recording period.

Electrophysiological recordings. A model 3900A patch-clamp amplifier (Dagan, Minneapolis, MN) and ruptured-patch or perforated-patch whole cell recordings (19, 21) were used to record ionic currents or spontaneous electrical activity in isolated pacemaker cells after 2 or 3 days in vitro. Cells were superfused with Tyrode solution containing (in mM) 130 NaCl, 5.4 KCl, 1.8 CaCl\(_2\), 0.6 MgCl\(_2\), 0.6 NaH\(_2\)PO\(_4\), 18 NaHCO\(_3\), and 5.5 dextrose, pH adjusted to 7.4 with the addition of 95% O\(_2\) or 95% air and 5% CO\(_2\). The pipette solution for perforated-patch recordings with nystatin contained impermeant divalent cations to minimize the Donnan potential [in mM: 75 K\(_2\)SO\(_4\), 55 KCl, 7 MgCl\(_2\), 10 dextrose, and 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES)] and was titrated to pH 7.2 with KOH. To isolate Ca\(^{2+}\) currents, the modified Tyrode solution contained the following blockers (in mM): 0.01–0.03 tetrodotoxin, 2 CsCl, 4 4-aminopyridine, and 2 BaCl\(_2\), and the pipette solution contained Cs\(^+\) instead of K\(^+\). Because amphotericin B can...
achieve a lower access resistance than nystatin, current tail measurements and some Ca\(^{2+}\) current measurements employed amphotericin B in a pipette solution that contained (in mM) 5 NaCl, 125 CsCl, 5 MgATP, 10 dextrose, 10 ethylene glycol-bis(\(\beta\)-aminoethyl ether)-N\(,\)N\(,\)N\(,\)N\(-\)tetraacetic acid (EGTA), and 10 HEPES and was titrated to pH 7.2 with CsOH. This solution also could be used for ruptured-patch recordings in the same cell. In some experiments, the ruptured-patch technique was used to promote rundown of L-type Ca\(^{2+}\) current (\(I_{\text{Ca,L}}\)) so as to isolate \(I_{\text{Ca,T}}\). In those measurements, MgATP and HEPES were omitted and the pipette solution contained (in mM) 140 CsCl, 10 NaCl, and 10 dextrose and was titrated to pH 7.2 with CsOH. The calculated corrections for liquid-junction potentials between the modified Tyrode and nystatin or amphotericin B pipette solutions were \(-2.9\) and \(-5.2\) mV, respectively (19, 21).

Because these potentials were unchanged by the addition of ryanodine or BAPTA, we did not make such corrections. A stock solution of nystatin (50 mg/ml in dimethyl sulfoxide (DMSO)) or amphotericin B (30 mg/ml in DMSO) was diluted in the appropriate pipette solution and ultrasonicated. The tip of the pipette was filled with antibiotic-free solution and the rest of the pipette with nystatin (300 µg/ml)- or amphotericin B (400 µg/ml)-containing solution. Pipettes were made on a model P80/PC puller (Sutter Instrument, Novato, CA); their resistances ranged from 1 to 5 M\(\Omega\). After a pipette-membrane seal had formed (resistance, 10–20 G\(\Omega\)), we waited 10–15 min for patch perforation before making the electrophysiological measurements. Series resistance compensation was maximized to minimize the time constant for decay of the capacitive transient.

Solutions. The cell isolation solutions and culture medium have been described (19, 21). The indo 1-AM loading solution was made by adding 25 µg indo 1-AM (Texas Fluorescence Laboratory, Austin, TX), 25 µl DMSO, 45 µl fetal calf serum (GIBCO, Grand Island, NY), and 2.2 µl of 25% (wt/wt) Pluronic F-127 (Sigma Chemical, St. Louis, MO) in DMSO to 1 ml of a HEPES-buffered balanced salt solution (19, 21) that contained 1.8 mM CaCl\(_2\). The final concentration of indo 1-AM was 25 µM. Drug-containing solutions were prepared by appropriate dilution of the stock solutions except for caffeine, which was added as a powder directly to the Tyrode solution. BAPTA was loaded into the cells by adding 25 µM BAPTA-AM (Texas Fluorescence Lab) to the Tyrode solution. In each experiment, the isolated pacemaker cells were superfused with Tyrode solution at a rate of 1 ml/min. A control system (model TC-1, Cell Micro Controls, Virginia Beach, VA) was employed to maintain the temperature within ±0.5°C of 34 or 35°C.

Data analysis. Beat rates were calculated from the total period for 10 consecutive action potentials, and pCLAMP 6 software (Axon Instruments, Foster City, CA) was used to measure the characteristics of at least 3 action potentials; then these were averaged. Slopes of the initial and final phases of diastolic depolarization (DD\(_1\) and DD\(_2\), respectively) were obtained from linear fits (Fig. 1C), and the “takeoff” potential (TP) was approximated by the intersection of linear fits of the action potential upstroke and DD\(_2\). Hyperpolarization-activated inward current (\(I_{\text{f}}\)) was measured as the difference between the inward current at the end of 300-ms voltage steps, a duration that approximates diastole, and the instantaneous or “background” inward current (\(I_{\text{bg}}\)) at the onset of the step. Ca\(^{2+}\) currents were measured as the difference between the peak of the transient inward current and the current at the end of 300-ms voltage steps, which was assumed to be leakage current. Currents were normalized by...
DD2, which decreased from 35.1 to 19.5 mV/s (dashed
slower firing rate might be explained by the slower
means with
was used for statistical analyses, and differences between
significantly different. The fact that DD2, but not DD1, was
reduced by ryanodine is consistent with the microelec-
trode recordings of Rubenstein and Lipsius (18, 26) in
cat SA node and subsidiary pacemaker tissue and of
Rigg and Terrar (24) in guinea pig SA node tissue.
However, in those studies, a significant depolarization
of the maximum diastolic potential (MDP) and changes
in the action potential overshoot (OS) were also seen. In
the present study, ryanodine simultaneously reduced
the peak-to-peak amplitude of action potential-induced
Ca^{2+} transients by 19 ± 3% (P < 0.01) and increased
their time constant for decay by 51 ± 5% (P < 0.01).
Some slowing of BR and reduction of the Ca^{2+} transient
could be seen after just 2 min of exposure to ryanodine,
and a maximal effect was reached in <8 min. Thus the
results described below were obtained after 8–10 min
of exposure.
Blockade of SR Ca^{2+} release by ryanodine. To confirm
that the actions of ryanodine were due, in part, to its
effects on the SR, we used caffeine to release Ca^{2+}
from the SR and then tested whether ryanodine could block
this release. Because caffeine enhances I_{Ca,L} in rabbit
SA node tissue (27), it was necessary to block this
current before the addition of caffeine to eliminate its
contribution to the Ca^{2+} transient. Exposure of pacem-
aker cells to Ni^{2+}, which inhibits both I_{Ca,L} and I_{Ca,T}
in this preparation (21), rapidly blocked the action potential-
induced Ca^{2+} transients, and Ca^{2+} fell below the
diastolic level (Fig. 2A). Addition of 10 mM caffeine
produced a transient increase in Ca^{2+}, but a second
addition had no effect. This indicates that 10 mM
cafeine depleted the Ca^{2+} stores. With Ni^{2+} and
caffeine washout, there was complete recovery of the Ca^{2+}
transients. Pretreatment of another pacemaker cell
with 10 µM ryanodine prevented the caffeine-induced
Ca^{2+} transient, and pacemaker activity became arrhythm-
ic with Ni^{2+} and caffeine washout (Fig. 2B).

### RESULTS

**Effect of ryanodine on spontaneous electrical activity and Ca^{2+} transients.** During simultaneous recordings of electrical activity and the indo 1 fluorescence ratio, the addition of 10 µM ryanodine slowed the firing rate and reduced the amplitude of action potential-induced Ca^{2+} transients. For example, in one pacemaker cell, the beat rate (BR) decreased from 86 to 67 beats/min, and the time constant for its decay increased from 126 to 176 ms after 10 min (Fig. 1, A and B). The slower firing rate might be explained by the slower DD2, which decreased from 35.1 to 19.5 mV/s (dashed curve, Fig. 1C), and by depolarization of the TP from −51 to −42 mV (arrows, Fig. 1C). The effects of 10 µM ryanodine on the electrical activity of 15 cultured pacemaker cells are summarized in Table 1. Reductions of BR (30 ± 3%), maximum upstroke velocity (dV/dt_{max}, 12 ± 3%), DD2 (37 ± 3%), and TP (11 ± 2%) were all significant (P < 0.01). Results obtained in 10 freshly isolated pacemaker cells (not shown) were not significantly different. The fact that DD2, but not DD1, was reduced by ryanodine is consistent with the microelec-
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Rigg and Terrar (24) in guinea pig SA node tissue.
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ic with Ni^{2+} and caffeine washout (Fig. 2B).
Similar results were obtained in another 11 pacemaker cells.

Effect of ryanodine on hyperpolarization-activated and background inward currents. Because the slowing of spontaneous firing induced by ryanodine coincided with a slower DD2 (Fig. 1C), we tested the hypothesis that exposure to ryanodine leads to a decrement of \( I_f \) and \( I_{bg} \) inward currents that might contribute to this phase of the pacemaker potential. To activate these currents, we applied 300-ms voltage steps from a holding potential of \(-50\) mV to potentials between \(-50\) and \(-100\) mV. The records before and 10 min after exposure to \( 10 \mu M \) ryanodine show that ryanodine did not alter \( I_f \) or \( I_{bg} \) (Fig. 3A). In fact, no effect was seen after 2, 5, 10, or 15 min of exposure to ryanodine. In eight pacemaker cells, the current-voltage (I-V) relationships for \( I_f \) and \( I_{bg} \) before and 10 min after addition of ryanodine were not significantly different (Fig. 3B).

Effect of ryanodine on T- and L-type \( Ca^{2+} \) currents. Because the slowing of spontaneous firing induced by ryanodine coincided with a slower DD2 and depolarization of the TP (Fig. 1C), we tested the hypothesis that exposure to ryanodine leads to a decrement of \( I_{Ca,T} \) and \( I_{Ca,L} \), inward currents likely to contribute to these two phases of the electrical activity (8, 13). As demonstrated previously in SA node pacemaker cells, \( I_{Ca,L} \) and \( I_{Ca,T} \) can be separated by the holding potential (8, 13). Both currents could be elicited from a holding potential of \(-80\) mV, whereas only \( I_{Ca,L} \) could be activated from a holding potential of \(-40\) mV. In the present study, total \( Ca^{2+} \) current (\( I_{Ca} = I_{Ca,L} + I_{Ca,T} \)) was reduced after just 2 min of exposure to \( 10 \mu M \) ryanodine, however, as recorded in ventricular myocytes (1, 20, 23) and freshly isolated rod-shaped rabbit SA node cells (28), the amplitude of \( I_{Ca,L} \) was not changed significantly. Longer exposures (up to 15 min) also did not alter \( I_{Ca,L} \) significantly, ruling out "rundown" of \( I_{Ca,L} \) as the cause of the decline of \( I_{Ca} \). Figure 4A illustrates the effects of ryanodine on \( I_{Ca} \) and \( I_{Ca,L} \) (traces labeled R) in a representative pacemaker cell. Figure 4B shows the mean I-V relationships for eight pacemaker cells before and 10 min after addition of \( 10 \mu M \) ryanodine. The perforated-patch technique (with nystatin) was employed to minimize rundown of \( I_{Ca,L} \). Ryanodine reduced \( I_{Ca} \) significantly at \(-30\), \(-20\), and \(-10\) mV (\( P < 0.05 \)). In contrast, it had no significant effect on \( I_{Ca,T} \) at any potential. Significant changes in \( I_{Ca} \) were observed at \(-30\), \(-20\), \(-10\), 0, and \( 10 \mu M \) (\( P < 0.05 \)) in another 10 cells when the patch pipette contained amphotericin B to reduce the series resistance (data not shown). The absence of an effect of ryanodine on the amplitude of \( I_{Ca,L} \) suggests that the attenuation of \( I_{Ca} \) is due to a reduction of \( I_{Ca,T} \). This could explain the reductions of both DD2 and TP, because \( I_{Ca,T} \) is activated at those potentials (8, 13).

Further evidence that ryanodine reduces \( I_{Ca,T} \) is presented in Fig. 5. Because we could not find a concentration of \( Ni^{2+}, Cd^{2+}, \) or \( La^{3+} \) that would block \( I_{Ca,L} \) completely without also reducing \( I_{Ca,T} \) and because the dihydropyridines are not selective at diastolic potentials (4), we promoted rundown of \( I_{Ca,L} \) by using the ruptured-patch technique and omitting ATP and HEPES from the pipette solution (see METHODS for details). After \( I_{Ca,L} \) had run down almost completely (Fig. 5A), exposing the pacemaker cells to \( 10 \mu M \) ryanodine led to a small reduction of \( I_{Ca} \) (Fig. 5B) that could be attributed almost exclusively to \( I_{Ca,T} \). The mean I-V relationships of five cells exposed to such rundown conditions are shown in Fig. 5C. The differences at \(-30\), \(-20\), \(-10\), 0, and \( 10 \mu M \) were all significant (\( P < 0.05 \)).

Effect of BAPTA-AM on spontaneous electrical activity and \( Ca^{2+} \) transients. To test the hypothesis that a reduction of the amplitude of \( Ca^{2+} \) transients was responsible for ryanodine's slowing of DD2 and BR and depolarization of the TP, we exposed 14 pacemaker cells to BAPTA-AM, a rapidly acting \( Ca^{2+} \) chelator. During simultaneous recordings of electrical activity and the indo 1 fluorescence ratio, action potential-induced \( Ca^{2+} \) transients were eliminated 3–5 min after the addition
of 25 µM BAPTA-AM, whereas the spontaneous electrical activity continued (Fig. 6). BAPTA, like ryanodine, had no effect on DD1, yet it slowed the DD2 and BR, reduced the dV/dt max, and depolarized the TP (P < 0.01; Table 2). However, unlike ryanodine, BAPTA prolonged the duration of the action potential measured at −20 mV (Dur; P < 0.05) and depolarized the MDP (P < 0.01). These results suggest that the effects of ryanodine on DD2, BR, dV/dt max, and TP might arise, in part, from the reduction of [Ca2+]i that accompanies ryanodine’s inhibition of SR Ca2+ release. The additional effects of BAPTA on the Dur and MDP might be explained by the likelihood that BAPTA can reduce [Ca2+] in the cytoplasm to a greater degree than can ryanodine (see DISCUSSION).

Effect of ryanodine on Ca2+ currents in BAPTA-AM-loaded cells. To determine whether the reduction of Ica was due to changes in [Ca2+]i or to ryanodine itself, we first reduced [Ca2+]i with BAPTA and then added ryanodine. After the pacemaker cells had been exposed to 25 µM BAPTA-AM for 10 min, the amplitudes of Ica and Ica,L were unchanged (Fig. 7). This was confirmed by the mean I-V relationships for 8 cells in absence and presence of 10 µM ryanodine (after 10 min). For Ica (top, HP = −80 mV), mean values at −30, −20, and −10 mV are significantly different. For Ica,L (bottom, HP = −40 mV), no changes are significantly different.

Fig. 4. Effect of ryanodine on Ca2+ currents (Ica). A: Ica elicited by depolarizing voltage steps to potentials indicated from HP of −80 mV (left) and −40 mV (right); before (C) and 10 min after (R) addition of 10 µM ryanodine. B: mean I-V relationships for 8 cells in absence and presence of 10 µM ryanodine (after 10 min). For Ica (top, HP = −80 mV), mean values at −30, −20, and −10 mV are significantly different. For Ica,L (bottom, HP = −40 mV), no changes are significantly different.

Fig. 5. Effect of ryanodine on T-type Ca2+ current. A: Ica,L elicited from an HP of −40 mV after rundown. B: total Ca2+ current elicited by depolarizing voltage steps from an HP of −80 mV to potentials indicated before (left) and 10 min after addition of 10 µM ryanodine (right). C: mean I-V relationships for Ica (HP = −80) in absence and presence of 10 µM ryanodine (n = 5). Changes at −30, −20, −10, 0, and 10 mV are all significant (P < 0.05).
changed, just as we had seen in the absence of BAPTA (Fig. 7). After the pacemaker cells had been exposed to ryanodine for 10 min, the reduction of $I_{\text{Ca}}$ was significant at $\pm 30$, $\pm 20$, and $\pm 10$ mV ($P < 0.05$); however, there was no significant effect on $I_{\text{Ca,L}}$ (Fig. 8B). These results suggest that ryanodine's reduction of $I_{\text{Ca}}$ might be due to a direct effect of ryanodine on T-type Ca$^{2+}$ channels, independent of changes in [Ca$^{2+}$]$_i$.

Effects of BAPTA and ryanodine on slow inward current tails. This set of experiments was designed to test the hypothesis that ryanodine also reduces inward $I_{\text{Na/Ca}}$ in SA node pacemaker cells. By decreasing the amplitude of Ca$^{2+}$ transients, ryanodine would be expected to diminish the extrusion of Ca$^{2+}$ via the Na$^+$/Ca$^{2+}$ exchanger and thereby reduce the net influx of Na$^+$, i.e., $I_{\text{Na/Ca}}$. After repolarization of the membrane potential after a 5-ms test pulse to $\pm 20$ mV, a slow decay of inward current could be seen (Fig. 9). The amplitude of this slow inward current tail seemed to depend on the amplitude of $I_{\text{Ca,L}}$ elicited during the test pulse, and the tail could be eliminated if $I_{\text{Ca,L}}$ was completely inactivated (at a holding potential of 0 mV).

**Table 2. Effect of BAPTA-AM on electrophysiological characteristics of cultured rabbit sinoatrial node cells**

<table>
<thead>
<tr>
<th></th>
<th>MDP, mV</th>
<th>OS, mV</th>
<th>dV/dt$_{\text{max}}$, V/s</th>
<th>Dur, ms</th>
<th>DD$_1$, mV/s</th>
<th>DD$_2$, mV/s</th>
<th>TP, mV</th>
<th>BR, beats/min</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>$-71 \pm 2$</td>
<td>$27 \pm 2$</td>
<td>$4.5 \pm 0.2$</td>
<td>$221 \pm 11$</td>
<td>$54 \pm 5$</td>
<td>$36 \pm 3$</td>
<td>$-55 \pm 2$</td>
<td>$94 \pm 5$</td>
</tr>
<tr>
<td>BAPTA</td>
<td>$-64 \pm 2$</td>
<td>$25 \pm 2$</td>
<td>$3.5 \pm 0.2$</td>
<td>$266 \pm 20$</td>
<td>$59 \pm 4$</td>
<td>$25 \pm 2$</td>
<td>$-46 \pm 2$</td>
<td>$65 \pm 5$</td>
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<tr>
<td>$P$</td>
<td>$&lt;0.01$</td>
<td>NS</td>
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<td>$&lt;0.05$</td>
<td>NS</td>
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Values are means ± SE. Fourteen cells were exposed to 25 µM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA)-acetoxymethyl ester (AM) for 10 min. MDP, OS and TP were not corrected for a pipette-to-bath liquid-junction potential of $-2.9$ mV.

**Fig. 7. Effect of ryanodine on Ca$^{2+}$ currents in presence of BAPTA.** Ca$^{2+}$ currents were elicited by depolarizing voltage steps from HP of $-80$ mV ($I_{\text{Ca}}$) and $-40$ mV ($I_{\text{Ca,L}}$) under control conditions (left), 8 min after addition of 25 µM BAPTA-AM (middle), and 10 min after addition of 10 µM ryanodine (right).
Although this slow decay could be interpreted as an \( I_{Ca,T} \) tail (4), this is highly unlikely, because \( I_{Ca,T} \) would have been completely inactivated at the employed holding potentials of \(-40, -20, \) and 0 mV (8, 13). Addition of 25 \( \mu \)M BAPTA-AM blocked the tails completely in seven SA node pacemaker cells; moreover, BAPTA slowed inactivation but did not alter the amplitude of \( I_{Ca,L} \) (Fig. 9A). In nine pacemaker cells, exposure to 10 \( \mu \)M ryanodine also blocked the slow tails and slowed the inactivation of \( I_{Ca,L} \) (Fig. 9B). When the holding potential was \(-40 \) mV, ryanodine reduced the peak of the tail from \(-173 \pm 22 \) to \(-59 \pm 13 \) pA \((P < 0.01)\) and decreased the time constant of its decay from \(13 \pm 3 \) to \(7 \pm 1 \) ms \((P < 0.05)\).

Effects of lower concentrations of ryanodine. To identify the contribution of each process, a reduction of \( I_{Ca,T} \) and blockade of SR \( Ca^{2+} \) release, to the negative chronotropic effect of ryanodine, we used lower concentrations of ryanodine and looked for a dose-dependent difference in its effects. Unfortunately, we could not find such a difference. At concentrations of 5 and 2 \( \mu \)M (6 and 9 cells, respectively), ryanodine continued to slow pacemaker activity, reduce \( I_{Ca,T} \), partially block inward current tails, and block caffeine-induced \( Ca^{2+} \) transients. At a concentration of 1 \( \mu \)M, ryanodine's effects on \( I_{Ca,T} \) and SR \( Ca^{2+} \) release still could not be separated. Ryanodine reduced \( I_{Ca,T} \) in three of four cells, and it blocked caffeine-induced \( Ca^{2+} \) transients in four of four cells. We considered 1 \( \mu \)M ryanodine to be the “threshold” dose, because this concentration slowed pacemaker activity in only two of five cells.

**DISCUSSION**

Previously, we showed that ryanodine reduces the amplitude of action potential-induced \( Ca^{2+} \) transients and slows the firing of cultured SA node pacemaker cells (17). The latter effect could be due to changes in any of the currents that contribute to pacemaker activity. However, the present study was designed to test the hypothesis that ryanodine's negative chronotropic effect is due to a reduction of inward currents that contribute to pacemaker activity and can be modulated by \( Ca^{2+} \). Such currents include the following: 1) \( I_{f} \), which may (12) or may not (31) depend on \([Ca^{2+}]_{i}\); 2) time-independent \( I_{bg} \) or “sustained” inward currents carried by \( Na^{+}\) (10, 14); 3) \( I_{NaCa} \), which depends on both the \( Na^{+} \) and \( Ca^{2+} \) electrochemical gradients (11, 32); and 4) \( I_{Ca,L} \), which also depend on the \( Ca^{2+} \) gradient (5, 8, 13).

With the use of 10 \( \mu \)M ryanodine, a concentration sufficient to block SR \( Ca^{2+} \) release (Fig. 2), we found no significant effect on either \( I_{f} \) or \( I_{bg} \) (Fig. 3). In contrast, 10 \( \mu \)M ryanodine did reduce \( I_{f} \) significantly in freshly isolated rod-shaped rabbit SA node cells (28). We have no explanation for this discrepancy. In our cultured SA node pacemaker cells, 10 \( \mu \)M ryanodine reduced \( I_{Ca} \) significantly at \(-30, -20, \) and \(-10 \) mV (Fig. 4B). Even though they were not statistically significant, reductions of \( I_{Ca} \) were also observed at \(-50 \) and \(-40 \) mV (Fig. 4A), potentials at which \( I_{Ca,T} \) is activated (8, 13) and the takeoff potential is seen (Fig. 1C, Table 1). In rabbit SA node cells, the role of \( I_{Ca,T} \) is to accelerate DD2 and maintain the action potential threshold at more negative potentials (5, 13). For example, blockade of \( I_{Ca,T} \) by 40 \( \mu \)M \( Ni^{2+} \) prolonged DD2 without changing DD1 (13), just as we have seen in our own experiments with ryanodine (Fig. 1C). Nevertheless, other mechanisms might also play a role, because ryanodine reduced but did not block \( I_{Ca,T} \) (Fig. 4). With the use of two different recording procedures, the perforated-patch technique (with nystatin in 8 cells and amphotericin B in 10 cells) and the ruptured-patch technique (to promote rundown of \( I_{Ca,L} \) in 5 cells), we observed a consistent decline in the amplitude of \( I_{Ca} \) after addition of ryanodine. This decline cannot be explained by rundown of \( I_{Ca,L} \), because \( I_{Ca,L} \) was recorded about the same time as \( I_{Ca} \) and was unaffected by ryanodine (Figs. 4, A and B). Given that \( I_{Ca} = I_{Ca,L} + I_{Ca,T} \), these results suggest that ryanodine slows pacemaker activity, in part, by reduc-
The rundown experiment (Fig. 5) provides additional support for this hypothesis, because, in the virtual absence of $I_{Ca, L}$, $I_{Ca}$ was reduced significantly by ryanodine.

Studies of ventricular myocytes (1, 20, 23) and SA node pacemaker cells (28) suggest that ryanodine, at concentrations between 1 and 10 µM, does not act directly on L-type Ca$^{2+}$ channels; instead, its effects are mediated by changes in $[Ca^{2+}]_{i}$. In fact, privileged cross signaling between ryanodine receptors and L-type Ca$^{2+}$ channels has been suggested to occur in adult rat ventricular myocytes (3, 29). Previously, we showed that $I_{Ca, L}$ inactivation is dependent on $[Ca^{2+}]_{i}$ in SA node pacemaker cells (22), and those observations are supported by the present results in which BAPTA slowed the inactivation of $I_{Ca, L}$ (Figs. 7 and 9A). However, little slowing of $I_{Ca, L}$ inactivation was observed after the addition of ryanodine (Fig. 4A), suggesting that its reduction of $[Ca^{2+}]_{i}$ was small. This was confirmed by the modest decrease (19%) in the amplitude of action potential-induced Ca$^{2+}$ transients after the addition of ryanodine (Fig. 1B). A similar discrepancy can be seen when one compares the effects of ryanodine and BAPTA on the MDP and Dur of the action potential in Tables 1 and 2. Ryanodine (10 µM) had no significant effect, whereas BAPTA depolarized the MDP and increased the Dur significantly. Interestingly, in freshly isolated rod-shaped rabbit SA node cells, 1 µM ryanodine not only slowed pacemaker activity but also increased the OS and Dur significantly; however, it had no effect on the MDP (28). In contrast, 1 or 2 µM ryanodine depolarized the MDP and changed the OS significantly in cat subsidiary (26) and guinea pig SA node (24) pacemaker cells. Such differences might be explained by the relative changes in $[Ca^{2+}]_{i}$ produced by ryanodine or by the relative abundance of SR in cat, guinea pig, and rabbit pacemaker cells. In the present study, it is possible that ryanodine had a direct effect on $I_{Ca,T}$, independent of the changes in $[Ca^{2+}]_{i}$, considering that ryanodine reduced $I_{Ca}$ regardless of whether BAPTA was present (Figs. 7 and 8B) or not (Fig. 4B).

In our cultured SA node pacemaker cells, we observed a relatively slow inward current tail that consistently followed depolarizing voltage steps that activated $I_{Ca,L}$ (see Controls, Fig. 9). A similar current tail can be seen when one compares the effects of ryanodine and BAPTA on the MDP and Dur of the action potential in Tables 1 and 2. Ryanodine (10 µM) had no significant effect, whereas BAPTA depolarized the MDP and increased the Dur significantly. Interestingly, in freshly isolated rod-shaped rabbit SA node cells, 1 µM ryanodine not only slowed pacemaker activity but also increased the OS and Dur significantly; however, it had no effect on the MDP (28). In contrast, 1 or 2 µM ryanodine depolarized the MDP and changed the OS significantly in cat subsidiary (26) and guinea pig SA node (24) pacemaker cells. Such differences might be explained by the relative changes in $[Ca^{2+}]_{i}$ produced by ryanodine or by the relative abundance of SR in cat, guinea pig, and rabbit pacemaker cells. In the present study, it is possible that ryanodine had a direct effect on $I_{Ca,T}$, independent of the changes in $[Ca^{2+}]_{i}$, considering that ryanodine reduced $I_{Ca}$ regardless of whether BAPTA was present (Figs. 7 and 8B) or not (Fig. 4B). Although BAPTA blocked the Ca$^{2+}$ transients (Fig. 6), and slowed the inactivation of $I_{Ca,L}$ (Figs. 7 and 9), it had no effect on the duration of either $I_{Ca}$ or $I_{Ca,L}$ (Figs. 9B and 8A). Therefore, it is possible that BAPTA failed to reach the T-type Ca$^{2+}$ channels. Thus we cannot rule out the possibility that ryanodine's reduction of $I_{Ca,T}$ was due to its attenuation of the Ca$^{2+}$ transients (Fig. 1B). In fact, a reduction of $[Ca^{2+}]_{i}$ has been shown to decrease $I_{Ca,T}$ in canine ventricular and Purkinje cells (30).

In our cultured SA node pacemaker cells, we observed a relatively slow inward current tail that consistently followed depolarizing voltage steps that activated $I_{Ca,L}$ (see Controls, Fig. 9). A similar current tail, recorded in atrial myocytes and pacemaker cells, has been attributed to the Na$^{+}$/Ca$^{2+}$ exchanger (2, 6, 9, 32). BAPTA's blockade of such tails (Fig. 9A) is consistent with previous results, in which cat latent pacemaker cells were dialyzed with EGTA (32), and supports the hypothesis that this current depends on $[Ca^{2+}]_{i}$. In agreement with some but not all previous studies of rabbit atrial myocytes (6, 9) and cat latent pacemaker cells (32), ryanodine inhibited slow inward current tails in our rabbit SA node pacemaker cells (Fig. 9B). Additional results in cat latent pacemaker cells (32) demonstrated that $I_{Na/Ca}$ can contribute significantly to the generation of diastolic depolarization, particularly DD2. Nevertheless, the role of $I_{Na/Ca}$ in rabbit SA node pacemaker activity remains to be clarified. Although ryanodine (10 µM) had just a small effect on the...
amplitude of action potential-induced Ca\(^{2+}\) transients that were measured by indo 1 in the cytoplasm (Fig. 1), the slow inward current tails were abolished by the same concentration of ryanodine (Fig. 9B). This suggests that the whole cell spatially averaged [Ca\(^{2+}\)], measured by indo 1, is an unreliable indicator of the Ca\(^{2+}\) that is released from the SR and is most effective in activating Na\(^+/Ca\(^{2+}\) exchange (16).

In summary, ryanodine's negative chronotropic effect is derived, in part, from a reduction of I\(_{Ca-T}\). This reduction can be explained by the attenuation of action potential-induced Ca\(^{2+}\) transients or by a direct effect of ryanodine on T-type Ca\(^{2+}\) channels. Although it is not known whether I\(_{NaCa}\) contributes to the diastolic depolarization of rabbit SA node cells, it is known to contribute to D\(_{Di}\) in cat latent pacemaker cells (32), and it generates inward current tails (2, 6, 9, 32) much like those we recorded (Fig. 9). Thus, on the basis primarily of theory and results in other preparations, it is possible that, after inhibition of SR Ca\(^{2+}\) release by ryanodine, the smaller action potential-induced Ca\(^{2+}\) transients could have reduced I\(_{NaCa}\) and, therefore, the beat rate.

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REFERENCES