Minor contribution of cytosolic Ca\textsuperscript{2+} transients to the pacemaker rhythm in guinea pig sinoatrial node cells

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Submitted 4 August 2010; accepted in final form 12 October 2010

IT IS WELL ESTABLISHED that the spontaneous action potential (AP) can be reconstructed by incorporating ionic currents found in sinoatrial node (SAN) cells into a mathematical cell model without assuming the critical involvement of intracellular Ca\textsuperscript{2+} dynamics [the “membrane clock” (M) model; for examples, see Refs. 8, 9, 15, 22, 37, and 46]. Recently, a novel pacemaker model has been proposed in which the slow diastolic depolarization is largely dependent on inward Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange (NCX) current (I\textsubscript{NCX}) evoked by spontaneous local Ca\textsuperscript{2+} release from the sarcoplasmic reticulum (SR) [the “Ca\textsuperscript{2+} clock” (C) model] (29, 30). This C hypothesis was supported by experimental findings that the spontaneous rate was markedly depressed by applying ryanodine to inhibit SR function in atrial (17, 48) and SAN (4, 16, 35) cells and that the repetitive release of Ca\textsuperscript{2+} was observed under voltage-clamp conditions, accompanied by cyclic current fluctuations. However, the degree of pacemaker depression by ryanodine is quite variable, from complete cessation of the rhythm (28) to a null effect (24) (for a review, see Ref. 23). Also, the pacemaker depression induced by applying ryanodine for >5 min (chronic effect) might be due to secondary effects of modulating Ca\textsuperscript{2+}-mediated signal transduction, for example, the maintenance of L-type Ca\textsuperscript{2+} current (I\textsubscript{L}) activity through Ca\textsuperscript{2+}/calmodulin kinase II (CaMKII) or Ca\textsuperscript{2+}-activated adenyl cyclase (AC) (32, 42, 47) (see the DISCUSSION). Furthermore, repetitive release of Ca\textsuperscript{2+} was only observed in a limited number of voltage-clamp experiments in rabbit SAN cells (44). Thus, it remains a matter of debate as to whether or not this C mechanism is dominant in normal SAN primary cells.

The purpose of this study was to examine the contribution of cytosolic Ca\textsuperscript{2+} transients to the pacemaker rhythm (acute effects), both experimentally and theoretically, separately from the plausible chronic effects. Over short time periods (~30 s), we found that the spontaneous firing rate was little, if any, changed, in contrast to full blockade of contraction, and that both the AP duration (APD) and I\textsubscript{L} activity were significantly prolonged by the acute intracellular application of BAPTA through the patch electrode tip. These responses were well explained by assuming Ca\textsuperscript{2+} chelation in the proximity of the channel pore as well as in the bulk cytosol in the M model, whereas the C model predicted rapid disruption of the spontaneous activity by BAPTA. Therefore, it was suggested that the pacemaker rhythm in guinea pig SAN cells is not dependent on Ca\textsuperscript{2+} transients on a beat-to-beat basis, contrary to the mechanism hypothesized in the C model. Simulation analysis using both the M and C pacemaker models fully supported this view.

MATERIALS AND METHODS

Model analysis. Two models were used, the Maltsev-Lakatta (ML) model (30) and the Himeno et al. (HSMN) model (15), representing the C and M models, respectively. Both models were coded by simBio (38). The model schemas are shown in Fig. 1 and the abbreviations are shown in Table 1. In the ML model, Ca\textsuperscript{2+} concentrations were...
Fig. 1. Schematic diagram showing the intracellular fluid compartments for Ca\(^{2+}\) and Ca\(^{2+}\)-related ionic components in the Matsu-
sev-Lakata (ML) and Himeno et al. (HSMN) models. See Table 1 for abbreviations.

calculated separately for the global cytosol ([Ca\(^{2+}\)]\(_{\text{g}}\)) and submembrane space ([Ca\(^{2+}\)]\(_{\text{swm}}\)). Ca\(^{2+}\) influx through \(I_{\text{CaL}}\), T-type Ca\(^{2+}\) current (\(I_{\text{CaT}}\), and background Ca\(^{2+}\) current (\(I_{\text{CaB}}\)) diffuses into the global cytosol only through the subspace, and [Ca\(^{2+}\)]\(_{\text{m}}\), is pumped into the network SR (NSR) by sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) [flux or equivalent current of SR Ca\(^{2+}\) uptake (\(j_{\text{SRCa}}\)]. The Ca\(^{2+}\) concentration in the NSR then diffuses to the junctional SR (JSR) [Ca\(^{2+}\) diffusion flux or equivalent current from the NSR to JSR (\(j_{\text{JSR}}\)], and is released from the JSR to the subspace via ryanodine receptors (RyRs) [flux or equivalent current of SR Ca\(^{2+}\) release (\(j_{\text{SRCl}}\)]). The increase in [Ca\(^{2+}\)]\(_{\text{swm}}\) potentiates \(I_{\text{SCX}}\) to depolarize the membrane.

The ML Ca\(^{2+}\) cycling plays a key role in the Ca mechanism in the ML model. In contrast, the HSMN model does not assume a subspace, and all Ca\(^{2+}\) fluxes through the surface as well as SR membranes (\(I_{\text{CaL}}\), \(I_{\text{CaT}}\), \(I_{\text{SCX}}\), plasma membrane Ca\(^{2+}\)-ATPase current, equivalent current of SR Ca\(^{2+}\) release, equivalent current of Ca\(^{2+}\) leak from SR uptake sites, and equivalent current of SR Ca\(^{2+}\) uptake) are directly added to [Ca\(^{2+}\)]. To determine the Ca\(^{2+}\)-mediated inactivation of L-type Ca\(^{2+}\) channels (APPENDIX, Eqs. A2–A4), the local Ca\(^{2+}\) concentration near the channel pore ([Ca\(^{2+}\)]\(_{\text{im}}\)) was determined according to Shirokov model (10, 31, 37, 40), as follows:

\[
[Ca^{2+}]_{\text{cm}} = [Ca^{2+}]_{\text{i}} - f_s \times I_{\text{CaL}},
\]

where \(f_s\) is a factor to convert Ca\(^{2+}\) flux through the single channel to a local Ca\(^{2+}\) concentration on the Ca\(^{2+}\)-binding site for Ca\(^{2+}\)-mediated inactivation and is model adjusted. The activation of RyR channels via coupling with Ca\(^{2+}\) channels was also assumed as a function of \(I_{\text{CaL}}\) and [Ca\(^{2+}\)].

Lead potential (\(V_L\)) analysis (5) was used to estimate the quantitative contribution of individual currents to the ionic mechanisms underlying pacemaker depolarization or AP repolarization in the mathematical models. The contribution is shown as absolute values (\(c\)) in Fig. 6D and as relative values (\(r_c\)) in Fig. 3.

Whole cell voltage- and current-clamp experiments. All experiments were approved by and carried out according to guidelines laid down by the Shiga University of Medical Science Animal Experiment Committee and “Guidelines for the Care and Use of Laboratory Animals.”

SAN cells were isolated from guinea pigs (250–400 g) as previously described (41) and were used within 1 day of isolation. The single SAN pacemaker cells used in our experiments showed regular spontaneous contractions and faint striations (as shown in Fig. 2), which were similar to the characteristics of those used in experiments recording sustained inward current (\(I_{\text{Na}}\)) in guinea pigs (12, 41). Whole cell recordings were conducted with patch-clamp electrodes with tip resistances of 2–4 MΩ when filled with the standard pipette solution (for the composition, see Solutions and drugs). The input capacitance of the preparation varied over 34–61 pF. For the perforated patch-clamp recordings, amphotericin B [200 μg/mL dissolved in DMSO (<0.5%)] was added to the pipette solution. After gigaseal formation, AP amplitudes increased gradually as access resistance decreased to 20–40 MΩ. To switch from perforated-patch to ruptured-patch recording modes, stronger suction was transiently applied to the pipette interior. Experiments were conducted at 34 ± 1°C.

Abbreviations for the AP parameters are shown in Table 1. All measurements are expressed as means ± SE, and differences between mean values were assessed with ANOVA and Student’s t-test for paired data. \(P < 0.05\) was considered significant.

Monitoring contraction. Spontaneous movement of a cell was monitored using a charge-couple device camera mounted on the microscope. Image data, as in Fig. 2, were stored as QuickTime movies and converted into AVI files by TMPGEnc (http://www.tmpgenc.net/) for analysis using ImageJ software (National Institutes of Health). We selected a rectangular region of interest (~7 × 10 μm²) along the direction of the cell contraction, and a semiquantitative index of contraction was determined by the number of colored pixels within this region of interest.

Solutions and drugs. The composition of the bath solution was (in mM) 140 NaCl, 5.4 KCl, 1.8 CaCl₂, 0.5 MgCl₂, 0.33 NaH₂PO₄, 5.5 glucose, and 5.0 HEPES (pH adjusted to 7.4 with NaOH). The pipette solution contained (in mM) 110 K-aspartate, 20 KCl, 1 MgCl₂, 5 EGTA, 5 Mg-ATP, 5 creatine phosphate, and 5 HEPES (pH 7.2). To record \(I_{\text{CaL}},\) pipette K⁺ was replaced with Cs⁺ in the voltage-clamp experiments. The nominally Ca⁺-free solution used for cell isolation was prepared by omitting CaCl₂. BAPTA (10 mM) was added to the pipette solution, and ryanodine (30 μM) and thapsigargin (1 μM) were added to the external solution when required. The Kraftrühe solution for cell preservation contained (in mM) 70 K-glutamate, 30 KCl, 10 KH₂PO₄, 1 MgCl₂, 20 taurine, 0.3 EGTA, 10 glucose, and 10 HEPES (pH adjusted to 7.2 with KOH). Chemicals were obtained from Sigma-Aldrich.

RESULTS?

Effects of Ca\(^{2+}\) chelation predicted in the C model. To characterize the ionic mechanisms in the ML and HSMN models, we applied \(V_L\) analysis (5). The representation of the \(r_c\) values (Fig. 3) over the diastolic interval indicated that the two models shared a common feature, which corresponded to the M mechanism. Namely, the large positive \(r_c\) value of the
fast component of the delayed rectifier $K^+$ current ($I_{Kr}$) at the beginning of diastolic depolarization decreased with time and was substituted by other inward currents, such as $I_{CaL}$, $I_{Na}$, and $I_{CaT}$, as well as hyperpolarization-activated current ($I_h$), in several other SAN models before the massive activation of $I_{CaL}$. Conversely, the two models were clearly different in respect to $I_{Na}$, which had a much larger and positive contribution in the ML model than in the HSMN model except for the beginning of diastole, where the $r$ value in both models was negative (decelerating the diastolic depolarization), reflecting a progressive decay of the $Ca^{2+}$ transient. In the ML model, the $r$ value of $I_{Na}$ already reversed its sign at $\approx 40$ ms after the maximum diastolic potential (MDP) because of the gradual increase in $[Ca^{2+}]_{sub}$ through spontaneous $Ca^{2+}$ release from the SR. Thereafter, the $r$ value of $I_{Na}$ remained positive to drive the major part of diastolic depolarization (C mechanism). In the HSMN model, the $r$ value of $I_{Na}$ remained negative throughout almost all of the diastolic period, and the M mechanism brought membrane voltage ($V_m$) to the threshold potential independent of the C mechanism.

As expected from the large and positive $r_c$ value of $I_{Na}$, the chelation of intracellular $Ca^{2+}$ by application of 10 mM BAPTA stopped the spontaneous AP in the ML model (Fig. 4, top left). The underlying mechanisms were better demonstrated by using a moderate concentration of BAPTA. As shown in Fig. 4, top right, 0.5 mM BAPTA abolished the AP generation temporarily until its buffering capacity was saturated. Closer inspection revealed that a single $Ca^{2+}$ transient was evoked in $[Ca^{2+}]_{sub}$, even though $[Ca^{2+}]_{sub}$ was completely buffered, indicating that a massive SR $Ca^{2+}$ release into the subspace can overwhelm the $Ca^{2+}$-buffering capacity. The cessation of spontaneous activity, observed subsequently, was caused by the inhibition of $J_{SRCaRel}$ through depletion of both $Ca^{2+}$ concentration in the JSR and $[Ca^{2+}]_{sub}$. The SR $Ca^{2+}$ content, thereafter, gradually recovered through continuous $Ca^{2+}$ influx via $I_{CaL}$ (window current), allowing a progressive recovery of $J_{SRCaRel}$ and thereby inward $I_{Na}$ to trigger nearly full-sized APs $\approx 8$ s after the BAPTA application. On the other hand, spontaneous APs were hardly affected in the HSMN model, even when 10 mM BAPTA was applied (as shown later in Fig. 6B). Clearly, then, experimental responses to the intracellular injection of BAPTA will be crucial in examining the $Ca^{2+}$ clock in the generation of spontaneous APs.

Effects of BAPTA on spontaneous APs and $I_{CaL}$ inactivation in experiments. Figure 5A,a shows spontaneous APs and contractions recorded from an isolated guinea pig SAN cell. At 7 s (indicated by an arrow at the top), the patch membrane was ruptured by the application of strong suction to the pipette containing 10 mM BAPTA. As the cytosol was dialyzed with BAPTA, cell contractions disappeared within $\approx 10$ beats ($\approx 3$ s; Fig. 5A,b) reflecting chelation of bulk $[Ca^{2+}]$, whereas spontaneous APs continued (Fig. 5A,a) with a slight and delayed increase in cycle length (CL; 5.6% increase at 3 s after the rupture; Fig. 5A,c). Interestingly, APD at 50% repolarization ($APD_{50}$) was markedly prolonged over several tens of seconds (Fig. 5, A,d and B). Essentially the same responses were observed in all seven of the cells examined. Table 2 shows a comparison of the AP parameters before and 30 s after the

Table 1. Abbreviations used in the ML and HSMN models and experiments as well as AP parameters

<table>
<thead>
<tr>
<th>ML Model</th>
<th>HSMN Model</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>$I_{CaL}$, pA</td>
<td>$I_{CaL}$</td>
<td>L-type $Ca^{2+}$ current</td>
</tr>
<tr>
<td>$I_{CaT}$, pA</td>
<td>$I_{CaT}$</td>
<td>T-type $Ca^{2+}$ current</td>
</tr>
<tr>
<td>$I_{CaX}$, pA</td>
<td>$I_{CaX}$</td>
<td>Na+/Ca2+ exchanger current</td>
</tr>
<tr>
<td>$I_{CaR}$, pA</td>
<td>$I_{CaR}$</td>
<td>Background $Ca^{2+}$ current</td>
</tr>
<tr>
<td>$I_{CaMN}$</td>
<td>$I_{CaMN}$</td>
<td>Plasma membrane $Ca^{2+}$-ATPase current</td>
</tr>
<tr>
<td>$I_{Na}$, pA</td>
<td>$I_{Na}$</td>
<td>Sustained inward current</td>
</tr>
<tr>
<td>$I_{Kr}$, pA</td>
<td>$I_{Kr}$</td>
<td>Slow component of the delayed rectifier $K^+$ current</td>
</tr>
<tr>
<td>$I_{Kr}$</td>
<td>$I_{Kr}$</td>
<td>Fast component of the delayed rectifier $K^+$ current</td>
</tr>
<tr>
<td>$I_h$</td>
<td>$I_h$</td>
<td>Hyperpolarization-activated current</td>
</tr>
<tr>
<td>$I_{Ca}$, mM</td>
<td>$I_{Ca}$</td>
<td>$Ca^{2+}$ in the bulk cytosol</td>
</tr>
<tr>
<td>$I_{CaX}$, mM</td>
<td>$I_{CaX}$</td>
<td>$Ca^{2+}$ in the extracellular space</td>
</tr>
<tr>
<td>$I_{CaSAMP}$, mM</td>
<td>$I_{CaSAMP}$</td>
<td>$Ca^{2+}$ in the NSR or SR uptake sites</td>
</tr>
<tr>
<td>$I_{CaSR}$, mM</td>
<td>$I_{CaSR}$</td>
<td>$Ca^{2+}$ in the JSR or SR release sites</td>
</tr>
<tr>
<td>$I_{CaSR}$, mM</td>
<td>$I_{CaSR}$</td>
<td>$Ca^{2+}$ channels on the cytomembrane</td>
</tr>
<tr>
<td>$I_{CM}$</td>
<td>$I_{CM}$</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>$I_{CM}$</td>
<td>$I_{CM}$</td>
<td>Calmodulin</td>
</tr>
<tr>
<td>$I_{TC}$ and $I_{TMC}$</td>
<td>$I_{TC}$ and $I_{TMC}$</td>
<td>Tropinin ($Ca^{2+}$ and Mg$^{2+}$ sites for the ML model)</td>
</tr>
<tr>
<td>$I_{CC}$</td>
<td>$I_{CC}$</td>
<td>Calsoquestrin</td>
</tr>
<tr>
<td>$I_{SERCA}$</td>
<td>$I_{SERCA}$</td>
<td>SERCA sarcoplasmatic reticulum</td>
</tr>
<tr>
<td>$I_{Ca}$</td>
<td>$I_{Ca}$</td>
<td>$Ca^{2+}$-ATPase</td>
</tr>
<tr>
<td>$I_{Na}$, mM/ms</td>
<td>$I_{Na}$, mM/ms</td>
<td>Flux or equivalent current of SR $Ca^{2+}$ uptake</td>
</tr>
<tr>
<td>$I_{Na}$, mM/ms</td>
<td>$I_{Na}$, mM/ms</td>
<td>$Ca^{2+}$ diffusion flux or equivalent current from the NSR to JSR</td>
</tr>
<tr>
<td>$I_{SRCaRel}$, mM/ms</td>
<td>$I_{SRCaRel}$, mM/ms</td>
<td>Flux or equivalent current of SR $Ca^{2+}$ release</td>
</tr>
<tr>
<td>$I_{SRCaRel}$, mM/ms</td>
<td>$I_{SRCaRel}$, mM/ms</td>
<td>Equivalent current of $Ca^{2+}$ leak from SR uptake sites</td>
</tr>
</tbody>
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AP Parameters

| OSP, mV | Overshoot potential |
| MDP, mV | Maximum diastolic potential |
| DD, mV/s | Rate of diastolic depolarization |
| Vm, V/s | Maximum rate of rise |
| APD50, ms | AP duration at 50% repolarization |
| CL, ms | Cycle length |

5 μm

Fig. 2. An isolated sinoatrial node (SAN) cell attached with a patch-clamp electrode (shadow).
rupture of the perforated patch to allow BAPTA into the cytosol. Among the six AP parameters, only the prolongation of APD₅₀ was statistically significant. The slight prolongation of CL by \(64.47/137\times 32.83\) ms was largely attributable to the prolongation of APD₅₀ by \(38.72/206\times 8.1\) ms. Neither CL nor APD₅₀ were modified when the cells were dialyzed with the pipette solution containing no BAPTA (from \(434.9/261\times 56.97\) to \(452.18/272\times 51.55\) ms in CL and from \(113.5/209\times 10.25\) to \(117.60/219\times 7.54\) ms in APD₅₀, \(n=4\)).

The prolongation of APD₅₀ (Fig. 5B) might be due to inhibition of the \(\text{Ca}^{2+}\)-mediated inactivation of \(I_{\text{CaL}}\) induced by BAPTA. To confirm this hypothesis, we conducted voltage-clamp experiments using the following experimental protocol: after rupture of the patch membrane, the voltage clamp was switched on and clamp pulses to 0 mV from a holding potential of \(-40\) mV were applied every 5 s. Indeed, the rate of \(I_{\text{CaL}}\) inactivation was gradually decreased and reached a minimum \(-20\) s after rupture of the membrane (Fig. 5C).
the inactivation of \( I_{CaL} \) was observed in all five cells examined, concomitant with the prolongation of APD50 (Fig. 5, A, c and d). The peak amplitude and time constant of inactivation of \( I_{CaL} \) in this cell was increased to \( I_{m} = 11002 \) 265 pA and 14.0 ms (at 18 s) from \( I_{m} = 238 \) pA and 7.8 ms (at 3 s), respectively. The average time constant of inactivation of \( I_{CaL} \) was increased from 8.25 to 17.17 ms (at 3 s) to 17.17 ms (18 s) (\( n = 4 \)). If the pipette solution containing no BAPTA was used, no significant change was observed in the time constant [from 8.31 to 9.00 ms (18 s) (\( n = 4 \))].

Continuous AP with APD prolongation by BAPTA in the HSMN model. The findings described above appear to be consistent phenomenologically with the M mechanism. To substantiate this view and elucidate the underlying mechanisms, the effects of \( Ca^{2+} \) chelation were examined in two steps in the HSMN model. In the first simulation, only cytosolic \( Ca^{2+} \) was buffered with 10 mM BAPTA (\([Ca^{2+}]_{i} < 8.5 \) nM). The rate of spontaneous AP was slightly increased (CL and APD50 were slightly decreased by 9.1 and 6.7%, respectively; data not shown), largely due to removal of the negative contribution of \( I_{NCX} \) (Fig. 3). In the second step, the prolongation of AP observed experimentally was examined by taking advantage that the \( Ca^{2+} \)-mediated inactivation was explicitly calculated as a function of \([Ca^{2+}]_{cm} \) in the HSMN model. We found that the delayed \( I_{CaL} \) inactivation (Fig. 6 A) and prolongation of APD50 (Fig. 6 C, top) were well reproduced by reducing \( f_{s} \) from 0.18 to 0.09 in Eq. 1 (see also Eqs. A2–A4), reflecting partial \( Ca^{2+} \) chelation of \( Ca^{2+} \) influx through \( I_{CaL} \) channels near the channel mouth (11, 21, 34). Spontaneous rhythms were not changed largely by the BAPTA application, as shown in Fig. 6 B and Table 2. In this simulation, an increase in the amplitude of \( I_{CaL} \) at the overshoot potential (OSP) and

Table 2. Effects of BAPTA on AP parameters in the present experiments and in the simulation by the HSMN model

<table>
<thead>
<tr>
<th></th>
<th>OSP, mV</th>
<th>MDP, mV</th>
<th>DD, mV/s</th>
<th>( V_{\max} ), V/s</th>
<th>APD50, ms</th>
<th>CL, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiments (( n = 6 ))</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.17 ± 2.28</td>
<td>-65.22 ± 2.00</td>
<td>54.25 ± 3.06</td>
<td>9.64 ± 2.81</td>
<td>120.06 ± 20.47</td>
<td>409.88 ± 52.78</td>
</tr>
<tr>
<td>10 mM BAPTA 30 s after rupture</td>
<td>12.73 ± 3.28</td>
<td>-62.27 ± 2.67</td>
<td>59.31 ± 4.17</td>
<td>11.26 ± 3.84</td>
<td>158.78 ± 17.53*</td>
<td>474.35 ± 81.42</td>
</tr>
<tr>
<td>Simulation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>12.93</td>
<td>-64.81</td>
<td>84.03</td>
<td>4.33</td>
<td>86.65</td>
<td>455.00</td>
</tr>
<tr>
<td>10 mM BAPTA 30 s after application</td>
<td>18.84</td>
<td>-69.71</td>
<td>93.15</td>
<td>4.77</td>
<td>138.09</td>
<td>526.30</td>
</tr>
</tbody>
</table>

Values are means ± SE. \( V_{\max} \) values were corrected for a liquid junction potential of −10 mV. *\( P < 0.05 \).
the disappearance of $I_{NCX}$ at MDP led to a small jump in the amplitudes of AP after the abrupt application of BAPTA, as indicated by the arrow in Fig. 6B (for current amplitudes, see also Fig. 6C), although neither modification was significant in experiments. By comparing the variation of all $Ca^{2+}$-dependent currents before and during BAPTA application, it seemed that the increase in $I_{CaL}$ was the largest in amplitudes compared with modifications in other currents such as the slow component of the delayed rectifier $K^+$ current ($I_{Kr}$) and $I_{NCX}$, as shown in Fig. 6C. To evaluate the contribution of these currents to AP prolongation, $V_m$ analysis was applied to the repolarizing phase of the AP (Fig. 6D). In the control AP, $I_{CaL}$ contributed a fraction of about $0.5 \text{ mV/ms}$ to net $dV_m/dt$ of about $-0.7 \text{ mV/ms}$ through $Ca^{2+}$-mediated inactivation. In the presence of BAPTA, the contribution of $I_{CaL}$ was reduced to one-half ($-0.25 \text{ mV/ms}$) due to retarded $Ca^{2+}$-mediated inactivation. The contribution of $I_{Kr}$ was through removal of inactivation by the negative shift of $V_m$ but was independent of $[Ca^{2+}]_i$. We conclude that the prolongation of APD$_{50}$ in the presence of BAPTA was largely due to the relief of $I_{CaL}$ from $Ca^{2+}$-mediated inactivation.

**Experimental effects of blocking SR function on spontaneous AP.** Although ryanodine provides a potential clue to estimate the contributions of SR function, its chronotropic effects have been shown to be largely variable among different studies (23, 24, 28). Accordingly, inhibition of SR function by reducing the SR volume to 0.1% (22) or by reducing the SR pumping rate to zero (30) in mathematical models led to a slight (less than ±5%) modification of the firing rate in the HSMN and Kurata et al. (KHIS) models, whereas a marked deceleration by >40% with dysrhythmic excitations was simulated in the ML model (30).

These different model predictions were tested in experiments in which SR function was rapidly and efficiently blocked by the simultaneous application of high concentrations of ryanodine and thapsigargin. After spontaneous APs in the perforated patch-clamp mode were recorded, the normal bath solution was switched to a solution containing drugs. $V_m$ and $CL$ were recorded before and after the application of the drugs, and the contraction traces were used as indexes of the effectiveness of SR blockage (Fig. 7). Unlike the BAPTA experiments, contraction did not stop within a few beats after the drug application but was depressed progressively. At 150 s postapplication, the contraction disappeared, leaving the $CL$ of spontaneous AP almost intact (Fig. 7, top right). Similar responses were observed in two other cells. The amplitude of AP was gradually decreased after ~90 s of the pharmacological intervention (Fig. 6, top panel), and the spontaneous activity ceased after ~5 min. This delayed effect might be due to secondary depression of $I_{CaL}$ through depression of CaM-CaMKII (43) and/or $Ca^{2+}$-dependent AC activity sig-

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**Fig. 6. Simulated effects of BAPTA on $I_{CaL}$, AP, ionic currents, and $V_m$ analysis profiles in the HSMN model.**

- **A:** $I_{CaL}$ obtained 30 s after numerical integration superimposed on that obtained in the control. The amplitude of the current traces was normalized to the maximum amplitude obtained at the peak. Current traces in absolute value are shown in the inset.
- **B:** continuous AP generation was observed after BAPTA application (arrow).
- **C:** simulated AP and major $[Ca^{2+}]_i$-dependent currents were also compared before and after BAPTA application.
- **D:** results of $V_m$ analysis (arrow). The time profile of $c$ values during the repolarization phase before and after BAPTA application are shown with time-dependent changes of $V_l$ and $V_m$.

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nal transduction during the chronic inhibition of Ca\textsuperscript{2+} homoeostasis (32, 47).

Possibility of Ca\textsuperscript{2+} accumulation in the subsarcolemmal space in SAN cells. For the Ca\textsuperscript{2+} clock to be functional in a C model, the assumption of subspace seems to be critical. It is usually assumed that Ca\textsuperscript{2+} is accumulated in membrane-limited spaces, such as diadic or triadic spaces, in ventricular or skeletal myocytes. In contrast, histological studies have shown that there are few T-tubules observed, and SR and myofibrils are poorly developed and disorganized in central SAN cells (18, 19, 24, 33, 39). Alternatively, a possible explanation for the accumulation could be that the Ca\textsuperscript{2+} diffusion is slow enough to localize in subsarcolemmal space spatially and temporally, as if there is a functional barrier for Ca\textsuperscript{2+} diffusion to the myoplasm (22, 26). To examine this possibility, simulations were performed using a simple membrane and cytoplasmic space model without subcellular organelles that would interfere with the free diffusion of Ca\textsuperscript{2+} (Fig. 8A). From various values of diffusion coefficients for Ca\textsuperscript{2+} in cytoplasm in the literature, one of the smallest numbers (13 \(\mu\text{m}^2/\text{s}\)) was selected for the simulation (2). As shown in Fig. 8B, peak Ca\textsuperscript{2+} concentration at the channel pore diffused rapidly and disappeared within 1 ms after closure of the channel (see Eq. A6). A very shallow concentration gradient around 0.05 \(\text{M}\) was left after calculation of a couple of milliseconds. Due to this rapid flattening of the localized Ca\textsuperscript{2+} peak, the concentration gradient under...
the surface membrane was shallow, even when multiple channel events occurred sequentially (Fig. 8, C.a, b, and c).

**DISCUSSION**

The mathematical model provides a strong clue to the solution of a problem. Without any mathematical consideration, quantitative questions, such as the Ca\(^{2+}\) clock vs. membrane clock, would never be solved. For example, the Ca\(^{2+}\)-dependent mechanism, in a broad definition, may include not only the C mechanism, as revealed by \(V_L\) analysis (Fig. 3) in the present study, but also all cellular mechanisms related to membrane Ca\(^{2+}\) flux as well as variations in [Ca\(^{2+}\)]. For a constructive discussion, we need to clarify our question. The C mechanism defined by the ML model is unequivocal, and therefore this working hypothesis could clarify our question. The C mechanism defined by the ML model is not applicable to the pacemaker mechanism in the bulk Ca\(^{2+}\) space and the subsarcolemmal space of only 20 nm in depth was delayed by several minutes, even if a retardation of BAPTA in the cytosol is assumed. Indeed, no diffusion barrier is considered in the KHIS model for BAPTA. The deceleration of \(I_{Ca}\) inactivation induced by BAPTA in the present study (Fig. 4C) supports this rapid equilibration of BAPTA. The delayed time course of \(\sim 20\) s after disruption of the patch membrane (Fig. 3) might be attributed to limited diffusion through the electrode tip. If so, model prediction using the ML model might have been inappropriate to examine local Ca\(^{2+}\) control, although our primary objective of testing the relevance of the C mechanism assumed in the ML model was successfully achieved.

As to the case of diadic space limited by two adjacent sheets of lipid membrane [estimated to be 12–15 nm in width in the ventricular myocardium (14, 25)], the presence of subsarcolemmal cisternae was revealed in SAN cells under electron microscopy (1, 18). The colocalization of NCX1 and cardiac RyRs within this narrow space (28) may also help to protect the C mechanism against the intervention of Ca\(^{2+}\) chelation. Besides, the experimental finding (Fig. 4, A and C) that BAPTA blocked the Ca\(^{2+}\)-mediated inactivation of the \(I_{Ca}\) channel as quickly as depressing the contraction does not necessarily indicate diffusion of BAPTA into the diadic space. This is because the major population of \(I_{Ca}\) channels affected by BAPTA might have been expressed on the sarcolemmal region free from the diadic space. However, the experimental finding of Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) resistant to EGTA by Chen et al. (6) in SAN cells is pertinent to this issue. They observed that local Ca\(^{2+}\)-release events were smaller in size and shorter in duration and, moreover, that the amplitude of \(I_{NCX}\) potentiated by this local Ca\(^{2+}\) release was negligibly small during the application of 4 mM EGTA buffer compared with control conditions. This finding indicates that EGTA can diffuse into the diadic space to partially chelate Ca\(^{2+}\) transients. Furthermore, the persistent local cross-signaling between \(I_{Ca}\) channels and RyRs were mostly supported by refilling of the SR by SERCA, which was kept active with the free Ca\(^{2+}\) concentration adjusted to 150 nM in the 4 mM EGTA-Ca\(^{2+}\) buffer (see Fig. 7 in Ref. 6).

Irrespective of the above CICR mechanism resistant to Ca\(^{2+}\) chelation in the diadic space, however, the Ca\(^{2+}\) release through RyR channels accompanied with activation of inward \(I_{NCX}\) should be largely depressed in our experimental condition. This is because Ca\(^{2+}\) uptake into the SR by the major population of SERCA, exposed mainly to the bulk medium, was blocked by Ca\(^{2+}\) chelation in the cytosol, causing depletion of SR Ca\(^{2+}\) contents during the rapid BAPTA application. A relevant situation could be simulated by applying 10 mM BAPTA only in the bulk cytosolic space, leaving the subsarcolemmal space BAPTA free in the ML model, which resulted in the cessation of spontaneous APs in the HSMN model (not shown).

Taken together, the experimental as well as simulation results make it unlikely that inward \(I_{NCX}\) was activated during the diastole to support the C mechanism in our experimental condition of acute BAPTA application. The experimental finding that the spontaneous AP rhythm was unaffected when the bulk Ca\(^{2+}\) transient was depressed (as indicated by the cessation of the contraction) well support the conclusion of a negligible contribution of Ca\(^{2+}\) release to pacemaker depolarization.

To the best of our knowledge, this is the first study to test the rapid effects of cytosolic Ca\(^{2+}\) chelation on spontaneous AP generation and contraction by dialyzing isolated SAN cells with BAPTA. To date, previous studies (3, 20, 27, 28, 36, 43,
In the absence of intracellular 
Ca2⁺ signaling cascades. It has been suggested that [Ca2⁺], is involved in regulating Ical by enhancing inactivation and facilitation through calmodulin (49). In guinea pig ventricular myocytes, rundown of normal pacemaker activity would be disrupted after prolonged [Ca2⁺] reduction, for example, by incubation with BAPTA-AM or SR blockers.

APPENDIX

Equations to simulate the effects of BAPTA. In both the ML and HSMN models, BAPTA effects were introduced according to the following KHS model (22):

\[
df_{\text{BAPTA}}/dt = k_{\text{BAPTA}} \times [Ca^{2+}] \times (1 - f_{\text{BAPTA}}) - k_{\text{BAPTA}} \times f_{\text{BAPTA}}
\]

where \( f_{\text{BAPTA}} \) stands for the fraction of BAPTA bound to Ca2⁺ and the binding rate constants are \( k_{\text{BAPTA}} = 0.11938 \text{ ms} \) and \( k_{\text{BAPTA}} = 940 \text{ mM/ms} \).

In the HSMN model, it was assumed that the effect of Ical on [Ca2⁺] was reduced by 50% by BAPTA in Eq. 1. The transition rate for U to UCa is defined as follows:

\[
\text{rate}(U \rightarrow UCa) = k_{U,UCa} \times [Ca^{2+}] \times (1 - f(AP)) \times p(AP)
\]

The transition rate for C to CCA and other rate constants are defined as follows:

\[
\text{rate}(C \rightarrow CCA) = k_{C,CCA} \times \left( \frac{[Ca^{2+}]_{in}}{[ATP]} \times p(AP) \right) \times p(C)
\]

where \( k_{C,CCA} = 0.143 \), \( k_{U,UCa} = 0.0003 \), \( k_{U,CCa} = 0.35 \), \( k_{C,CCa} = 6.954 \text{ mM/ms} \), \( k_{U,CCa} = 0.0042 \), and \( k_{U,UCa} = k_{C,CCa} = k_{U,UCa} = k_{C,CCa} = k_{U,UCa} = k_{C,CCa} = k_{U,UCa} = k_{C,CCa} \) as in the Kyoto model (31, 37). The equation for the open probability \( p(O) \) of Ical is defined as follows:

\[
p(O) = p(AP) \times (p(U) + p(UCa)) \times y/\{1 + (1.4/[ATP])\}
\]

illustrates that Ical is sensitive to the intracellular ATP concentration ([ATP]) and becomes available when all of the voltage-dependent, Ca2⁺-dependent and ultra-slow (γ) gates of the channel are open.

The p(O) of ICa is determined by two gates, the voltage-dependent gate (γ₁) and the Ca2⁺-dependent gate (γ₂), as follows:

\[
p(O) = y₁^2 \times (0.9 \times y₂ + 0.1)
\]

\[
y₂ \text{ is composed of three states, as in the diagram, and the rate constants are defined as follows:}
\]

\[
\alpha_{y₂} = 4 \times [Ca^{2+}], \quad \beta_{y₂} = 0.000148
\]

\[
\alpha_{y₂} = 0.005, \quad \beta_{y₂} = 0.03
\]

Simulation of Ca2⁺ diffusion beneath the surface membrane. The temporal variation of [Ca2⁺] is calculated by Fick’s law, as follows:

\[
du/dt = -D \times \nabla^2 u + f
\]

where \( u \) is the amount of Ca2⁺, \( D \) is the diffusion coefficient of Ca2⁺ in the myoplasm and is set 13 \( \mu \text{m}^3/\text{s} \), \( \nabla^2 \) is the Laplace operator, and \( f \) is the external inflow. If we assume single current amplitude as 0.06 pA at −40 mV in the slow diastolic depolarization in the HSMN model, then inflow can be calculated as 0.06 \times 10^{-12}/\text{Faraday constant}(2.0 \text{ mol/s})$, and an open channel would be observed in every \( 2 \times 2 \mu \text{m}^2 \) surface membrane when the input capacitance of 32 pF was converted to whole cell membrane area (28). A diffusion space

TABLE 3. Effects of BAPTA on spontaneous firing rate in sinoatrial node cells from the literature and in the present experiments
of 2 × 2 × 2 μm³ in size was assumed with the reflecting boundary condition except for the membrane surface. The calculation was performed with a lattice spacing of 0.01 μm and a time step of 0.1 μs. The initial value of Ca²⁺ concentration was set to zero, and no sink of flux was assumed.

ACKNOWLEDGMENTS

The authors acknowledge insightful discussions with Dr. N. Inagaki and Dr. S. Fujimoto as well as members in the Biosimulation Project. The authors also thank Dr. T. Powell for helpful discussions and review of the manuscript.

GRANTS

This work was supported by the Innovation Cluster of the Ministry of Education, Culture, Sports, Science and Technology Japan, a Grant-In-Aid for Young Scientists from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y. Himeno), and the Ritsumeikan-Global Innovation Research Organization of Ritsumeikan University.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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