SR Ca\(^{2+}\) refilling upon depletion and SR Ca\(^{2+}\) uptake rates during development in rabbit ventricular myocytes

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IT IS WELL DOCUMENTED that a relatively small amount of Ca\(^{2+}\) enters through L-type Ca\(^{2+}\) channels during the action potential triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) in a process known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR) in adult mammalian myocytes (6, 21). In adult rabbit ventricular myocytes, it has been reported that the Ca\(^{2+}\) pump is capable of efficiently loading the SR even at the earliest neonatal stage, but that the principal Ca\(^{2+}\) sources contributing to load\(\text{SR}\) during ontogeny.

MATERIALS AND METHODS

Isolation of ventricular myocytes. Animals were cared for in accordance with the principles established by the Canadian Council on Animal Care (CCAC). The Simon Fraser University Animal Care Committee approved the use of animals and the experimental protocol used in this study in accordance with CCAC regulations. Ventricular myocytes were isolated from the hearts of New Zealand White rabbits (of either sex) from four distinct age groups, 3 (3d), 10 (10d), 20 (20d), and 56 (56d) days postpartum, by methods previously described (12, 13, 17).

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Whole cell perforated-patch voltage clamp. Whole cell amphoterin-perforated voltage-clamp technique was used at room temperature as described previously (12, 13, 17). The internal pipette solution contained (in mM) 110 CsCl, 5 MgATP, 1 MgCl₂, 20 tetraethylammonium, 5 Na₂ phosphocreatine, and 10 HEPES, pH 7.1 (adjusted with CsOH). The standard external solution contained (in mM) 130 NaCl, 5 CsCl, 1 MgCl₂, 2.0 CaCl₂, 5 Na-pyruvate, 10 glucose, and 10 HEPES, pH 7.4 (adjusted with NaOH). All drugs were purchased from Sigma (St. Louis, MO). Because nifedipine (Nif) is very sensitive to light, particular precautions were taken. A fresh working solution of 15 μM Nif was made by diluting a fresh 15 mM stock solution (dissolved in DMSO), resulting in a final DMSO concentration of 0.1%. The entire Nif delivery pathway including the micro-manifold was light tight.

Fig. 1. General protocol and representative traces from a 3-day-old (3d) myocyte. A: general experimental protocol. The sarcoplasmic reticulum (SR) Ca²⁺ was cleared by the 1st application of caffeine (Caf) and then followed by a train of 20 repetitive depolarizations at 0.2 Hz. The 2nd Caf (dark gray bar) was applied to evaluate the SR Ca²⁺ content. B: representative traces of Ca²⁺ transients (intracellular Ca²⁺ concentration, [Ca²⁺]ᵢ), the depolarization-induced Ca²⁺ transient at left in contrast to the Caf-induced Ca²⁺ transient (Caf [Ca²⁺]ᵢ) at right with the same scale. C: representative traces of L-type Ca²⁺ current (I_{Ca}). D: tail Na⁺/Ca²⁺ exchanger (NCX) current (I_{NCX}); tail I_{NCX} at left and Caf I_{NCX} at right with a different scale. E: time integral of the corresponding I_{SCX}; [tail I_{SCX} at left and Caf I_{SCX} at right with a different scale]. F and G: net Ca²⁺ entry and cumulative net Ca²⁺ entry, respectively, as a function of depolarization. The depolarization dependence was observed in Ca²⁺ transient, tail I_{NCX}, and net Ca²⁺ entry; steady state (SS) was achieved at ~10th depolarization.

SR Ca²⁺ UPTAKE RATES IN NEONATE CARDIOMYOCYTES

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Measurement of Ca\textsuperscript{2+} fluorescence. Intracellular Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]) was measured with the fluorescent Ca\textsuperscript{2+} indicator fluo-3 AM as described previously (12, 13, 17). The [Ca\textsuperscript{2+}] was calculated from the formula [Ca\textsuperscript{2+}] = Kd(F - F_{min})/(F_{max} - F), where F_{min} is the background fluorescence determined from a cell-free area and F_{max} is the fluorescence acquired after the cell was depolarized to +150 mV for 10–20 s to maximize [Ca\textsuperscript{2+}], and kill the cell at the end of each experiment. F_{0} was taken as the difference between the background fluorescence determined in the absence and presence of a cell in the area of measurement. ΔF is the incremental fluorescence measured from baseline or the background fluorescence in the presence of a cell. Kd is the fluo-3 Ca\textsuperscript{2+} dissociation constant, and a value of 400 nM was used for all age groups (20).

General protocol. Figure 1A shows the SR Ca\textsuperscript{2+} loading experimental protocol applied in Fig. 1, B and C, as well as Figs. 3 and 4. The SR Ca\textsuperscript{2+} was first cleared by a brief Caf application. A train of 20 repetitive depolarizations at 0.2 Hz (first depolarized to −40 mV for 50 ms in order to inactivate Na\textsuperscript{+} channels and T-type Ca\textsuperscript{2+} channels, and then depolarized to +10 mV for 400 ms, at a holding potential of −80 mV) was then initiated 5 s after Caf removal. Immediately after the 20th depolarization, Caf was applied again to evaluate the SR Ca\textsuperscript{2+} content.

Figure 6A shows the experimental protocol used to measure the SR Ca\textsuperscript{2+} uptake rate (V_{SR}) and [Ca\textsuperscript{2+}]. The first Caf application was used to clear the SR Ca\textsuperscript{2+} content, and the cell was then depolarized to various voltages (from −30 mV to +50 mV, in increments of 20 mV) for 3 s to load the SR. Long depolarizations were used to induce a relative spatial and temporal homogeneity of [Ca\textsuperscript{2+}]. Pulses longer than 3 s were also tried, but these frequently resulted in cell death, particularly in the younger age groups. More positive depolarizations were also tried, but these often resulted in saturation of loadSR and as a consequence frequently generated spontaneous release. Therefore, these data were not included in the analysis. The time integral of the lnC\textsubscript{X} (I_{lnC\textsubscript{X}}) induced by the second Caf application was used as a measure of loadSR during the preceding 3-s depolarization (12). The average V_{SR} (amol·s\textsuperscript{-1}·pF\textsuperscript{-1}) was obtained by dividing loadSR (I_{lnC\textsubscript{X}}, amol/pF) by the duration of the loading period (3 s).

Data analysis. Data are presented as means ± SE. Statistical significance of the results was tested with a one-way ANOVA (SPSS 11.0) or Student’s t-test for paired or unpaired samples. Post hoc tests were taken with Tukey multiple comparisons. A P value of ≤0.05 was taken to be significant.

RESULTS

Depolarization dependence of Ca\textsuperscript{2+} transient, tail lnC\textsubscript{X}, as well as sarcolemmal Ca\textsuperscript{2+} entry in a 3d myocyte. Figure 1B shows representative Ca\textsuperscript{2+} transient recordings ([Ca\textsuperscript{2+}], including the second Caf-induced Ca\textsuperscript{2+} transient, Caf [Ca\textsuperscript{2+}], Fig. 1C shows the corresponding I_{C\textsubscript{X}}, and Fig. 1D shows the inward lnC\textsubscript{X} on repolarization (tail lnC\textsubscript{X}) and during the second Caf application (Caf lnC\textsubscript{X}) in a 3d myocyte. The corresponding time integrals of tail lnC\textsubscript{X} (tail lnC\textsubscript{X}) and Caf lnC\textsubscript{X} (Caf lnC\textsubscript{X}) are shown in Fig. 1E. Peak I_{C\textsubscript{X}} in the 3d group did not show a significant decrease in magnitude with depolarization number, although this phenomenon was observed in older age groups (data not shown). Both the Ca\textsuperscript{2+} transient and tail lnC\textsubscript{X} showed a depolarization-dependent increase that reached a steady state near the 10th depolarization. Assuming that sarcolemmal Ca\textsuperscript{2+} entry and extrusion are equal in magnitude at steady state, subtraction of the average value of the last 5 tail lnC\textsubscript{X} from each individual f_{tail lnC\textsubscript{X}} should give the net Ca\textsuperscript{2+} entry during each depolarization. Figure 1F shows the net Ca\textsuperscript{2+} entry for each depolarization, and the cumulative net Ca\textsuperscript{2+} entry during the 20 depolarizations is shown in Fig. 1G.

Age-dependent differences in sarcolemmal Ca\textsuperscript{2+} entry, Ca\textsuperscript{2+} transients, and SR Ca\textsuperscript{2+} loading. Figure 2A shows that the cumulative net Ca\textsuperscript{2+} entry significantly decreased with age while the number of depolarizations required to achieve steady state was comparable in all age groups. Figure 2B shows the maximum amplitude of [Ca\textsuperscript{2+}], as a function of number of depolarizations. The data were well fit with a Boltzmann function (R2 = 0.98). The peak [Ca\textsuperscript{2+}], increased with subsequent depolarizations and reached steady state near the 10th depol-
Fig. 3. Age-dependent differences in nifedipine (Nif)-sensitive Ca\(^{2+}\) transients and sarcolemmal Ca\(^{2+}\) entry. A and B: representative traces from a 3d myocyte (A) and a 56d myocyte (B) in the presence of Nif; the [Ca\(^{2+}\)]\(i\) (depolarization-induced [Ca\(^{2+}\)]\(i\) at left and Caf [Ca\(^{2+}\)]\(i\) at right with the same scale), inward \(I_{\text{NCX}}\) (tail \(I_{\text{NCX}}\) at left and Caf \(I_{\text{NCX}}\) at right with different scales), and time integral of corresponding \(I_{\text{NCX}}\) (tail \(I_{\text{NCX}}\) at left and Caf \(I_{\text{NCX}}\) at right with different scales) are shown from top to bottom, respectively. The depolarization-dependent Ca\(^{2+}\) transient and the tail \(I_{\text{NCX}}\) were abolished by the addition of Nif in 56d but not in 3d myocytes. C and D: cumulative net Ca\(^{2+}\) entry normalized by cell membrane capacitance (pC/pF; C) and amplitude of [Ca\(^{2+}\)]\(i\) (incremental fluorescence (ΔF)/difference in background fluorescence between absence and presence of cell (F₀); D) as a function of depolarization in the presence of Nif in 3d, 10d, 20d, and 56d myocytes. Nif significantly abolished sarcolemmal Ca\(^{2+}\) entry as well as the Ca\(^{2+}\) transient in older age groups but not in younger age groups. \(n = 12\).
larization. The response was comparable for all age groups, which was neither proportional to cumulative Ca$^{2+}$/H$_{11001}$ entry nor to load$_{SR}$. Figure 2C compares the cumulative net Ca$^{2+}$/H$_{11001}$ entry and the load$_{SR}$ (load$_{SR}$-Con) as a function of age. Both decreased significantly with age, but load$_{SR}$ was larger than cumulative Ca$^{2+}$/H$_{11001}$ entry in all age groups, suggesting that an additional source of load$_{SR}$ exists.

**Age-dependent difference in nifedipine-insensitive Ca$^{2+}$/H$_{11001}$ transients and sarcolemmal Ca$^{2+}$/H$_{11001}$ entry.** Figure 3, A and B, are representative traces from a 3d myocyte and a 56d myocyte, respectively, using the same protocol as shown in Fig. 1A but in the presence of 15 μM Nif, a selective inhibitor of L-type Ca$^{2+}$/H$_{11001}$ channels that completely blocked $I_{Ca}$ in all age groups (data not shown). [Ca$^{2+}$]$_i$, inward $I_{NCX}$ (tail $I_{NCX}$ and Caf $I_{NCX}$), and their time integrals ($\int I_{NCX}$ and $\int$ Caf $I_{NCX}$) are shown from top to bottom, respectively, in Fig. 3, A and B. The depolarization-dependent Ca$^{2+}$/H$_{11001}$ transient and the tail $I_{NCX}$ were abolished by the addition of Nif in 56d but not in 3d myocytes. Both Caf [Ca$^{2+}$]$_i$ and Caf $I_{NCX}$ were still observed in 56d myocytes, although tail $I_{NCX}$ and Ca$^{2+}$/H$_{11001}$ transients were abolished. Figure 3, C and D, show the cumulative net Ca$^{2+}$/H$_{11001}$ entry and the [Ca$^{2+}$]$_i$ amplitude in the presence of Nif for the different age groups. Note that Nif abolished sarcolemmal Ca$^{2+}$/H$_{11001}$ entry and Ca$^{2+}$/H$_{11001}$ transients in older but not in younger age groups.

**Age-dependent difference in nifedipine and KB-R7943-insensitive SR Ca$^{2+}$/H$_{11001}$ loading.** Figure 4, A and B, show representative traces from a 3d myocyte and a 56d myocyte in the presence of Nif and KB-R7943 (KB-R). 

**Fig. 4.** Age-dependent differences in load$_{SR}$ in the presence of Nif and KB-R7943 (KB-R). A and B: representative traces from a 3d myocyte (A) and a 56d myocyte (B) in the presence of Nif+KB-R. [Ca$^{2+}$]$_i$ (depolarization induced at left and Caf [Ca$^{2+}$]$_i$ at right with the same scale), $I_{NCX}$ (tail $I_{NCX}$ at left and Caf $I_{NCX}$ at right with different scales), and time integral of $I_{NCX}$ ($\int I_{NCX}$ at left and Caf $\int I_{NCX}$ at right with different scales) are shown from top to bottom, respectively. The remaining depolarization-dependent Ca$^{2+}$/H$_{11001}$ transient and the tail $I_{NCX}$ observed in 3d myocytes in the presence of Nif were abolished by the addition of KB-R. C: load$_{SR}$ in the presence of both Nif and KB-R (NIF+KB-R insensitive) as a function of age, which significantly decreased with age (***$P < 0.005$ for both 3d and 10d vs. 56d and **$P < 0.01$ for 3d vs. 20d), $n = 12$. 

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presence of both 15 μM Nif and 10 μM KB-R-7943 (KB-R). [Ca^{2+}]_{i}, inward NCX (tail NCX and CaF NCX), and their time integrals (ΔI_{NCX} and ∆Caf I_{NCX}) are shown from top to bottom, respectively, in Fig. 4, A and B. The depolarization-dependent Ca^{2+} transient and tail I_{NCX} observed in 3d myocytes with Nif were abolished by the subsequent addition of KB-R. However, a significant Caf-induced Ca^{2+} transient and a considerable amount of Caf I_{NCX} were still observed in both 3d and 56d myocytes, although the values were significantly greater for 3d than for 56d myocytes, confirming that an additional loadSR source exists and is insensitive to Nif and KB-R. Figure 4C shows that Nif- and KB-R-insensitive loadSR was significantly smaller in older compared with younger age groups.

**Age-dependent changes in relative contributions of different sarcolemmal Ca^{2+} sources.** Figure 5A shows the cumulative Ca^{2+} entry in control and Nif conditions for the different age groups (there is no cumulative Ca^{2+} entry in the presence of NIF+KB-R). The relative contributions of Nif-sensitive and KB-R-sensitive (but Nif insensitive) Ca^{2+} entry are shown in Fig. 5B. KB-R-sensitive but Nif-insensitive Ca^{2+} entry was dominant at the earliest developmental stage and significantly decreased with age; in contrast, Nif-sensitive Ca^{2+} entry significantly increased with age and became dominant at the latest developmental stage examined. Figure 5C shows the corresponding control, Nif, and Nif+KB-R or Nif+KB-R-insensitive loadSR as shown in Fig. 4C. The relative contributions (Con = 1) of Nif-sensitive, KB-R-sensitive, and Nif+KB-R-insensitive loadSR are shown in Fig. 5D for the different age groups. The calculation of the contribution of Nif+KB-R-insensitive loadSR is predicated on two observations: 1) steady state in control solution was achieved at ~10th depolarization (~50 s) and 2) Nif+KB-R-insensitive loadSR is linear with time for up to 100 s (13). Therefore the contribution of Nif+KB-R-insensitive loadSR relative to Con is derived from the assumption that its loading is the half-value of Nif+KB-R-insensitive loadSR (~100 s). In accordance with Fig. 5B, Nif-sensitive loadSR significantly increased with age, while KB-R-sensitive loadSR and Nif+KB-R-insensitive loadSR significantly decreased with age.

**Age-dependent changes in k_{0.5} of SR Ca^{2+} uptake.** Since age-dependent changes in loadSR may be due to changes in the SERCA2a Ca^{2+} affinity, we estimated this parameter at the different postnatal stages by measuring the SR Ca^{2+} uptake rate as a function of bulk phase [Ca^{2+}]_{i}. Figure 6B shows representative traces of the Ca^{2+} transient and membrane current elicited by the protocol shown in Fig. 6A (on depolarization to +30 mV) in a 3d cell. The magnitude of the Ca^{2+} transient after it reached a plateau state ([Ca^{2+}]_{PS}) was used as the [Ca^{2+}]_{i}; corresponding to its average V_{SR}. Figure 6C shows the average Ca^{2+} transient traces and its corresponding second Caf-induced I_{NCX} from five each of 3d and 56d cells at depolarization steps from −30 mV to +50 mV. It is clear that the second Caf-induced I_{NCX} is greater in 3d than 56d cells.
despite the fact that the amplitudes of the Ca\(^{2+}\)/H\(_{{11001}}\) transients were comparable in the two groups. The representative average VSR as a function of [Ca\(^{2+}\)/H\(_{{11001}}\)]\(_{{PS}}\) derived from Fig. 6C (including data point of VSR at −80 mV) is shown in Fig. 6D. Data points were fit with the Hill equation: \(V_{SR} = V_{max} \cdot [Ca^{2+}]_{PS}^{nH} / (K_{0.5} + [Ca^{2+}]_{PS}^{nH})\) \((R^2 \approx 0.98)\) to obtain the asymptotic value of VSR (V\(_{peak}\), k\(_{0.5}\) (the value of [Ca\(^{2+}\)]\(_{{PS}}\) at half of V\(_{peak}\)) and Hill coefficient n\(_{H}\) (the slope of the sigmoid curve). From Fig. 6D, it is clear that k\(_{0.5}\) is smaller in 3d than 56d cells; k\(_{0.5}\) increased significantly after 10 days of age. In contrast, there were no significant differences in either V\(_{peak}\) or n\(_{H}\) between groups (Table 1).

**DISCUSSION**

It has been suggested that neonatal mammalian ventricular myocytes have a relatively sparse and immature SR compared with that in adults (11, 25), but recent studies indicate that a prominent SR Ca\(^{2+}\) accumulation can take place in neonatal myocytes (12, 13, 21). Here we have attempted to identify, quantify, and compare the mechanisms contributing to load\(_{SR}\) in neonatal and adult hearts. In accordance with our previous report (12), load\(_{SR}\) after SR Ca\(^{2+}\) depletion was significantly greater in the neonatal compared with the adult heart when normalized per unit membrane capacitance. Moreover, our
The early developmental stage is still too small (5% of total ventricular myocytes examined independent of the age stage). This was true for all rabbit corative depolarizations are sufficient to fully reload the SR after transient exposure to 10 mM Ca2+. The present study shows that 10 consec-
tive depolarizations were sufficient to reload the SR after transient exposure to 10 mM Ca2+. This was true for all rabbit
ventricular myocytes examined independent of the age stage. In the older age groups, the calcium reloading was primarily
due to an enhanced ICa and a decreased tail INcx during the first 10 depolarizations. This is in accordance with previous reports
on adult rat and ferret hearts (28) and is also consistent with a previous study from our laboratory (14). It is generally agreed
that the ICa is the main pathway for sarcolemmal Ca2+ entry in the adult rabbit heart (8, 17), and in agreement with this notion,
we find that loadSR as well as the Ca2+ transient were sensitive to nifedipine in older age groups (Fig. 3, C and D, and Fig. 5).
In contrast, in the early developmental stages, loadSR as well as the Ca2+ transient were insensitive to Nif but were sensitive to
subsequent addition of KB-R (Fig. 4A and Fig. 5). The findings indicate that reverse-mode NCX plays an important role in both
excitation-contraction (E-C) coupling and SR Ca2+ refill-
ing at early neonatal stages. Moreover, total sarcolemmal Ca2+
efflux estimated via tail INCX at steady state in 3d hearts was about twice the magnitude of that in 56d hearts (0.4 vs. 0.2
pC/pF), and the first [Ca2+] transient in younger age groups was larger than that in 56d hearts and sensitive to KB-R (Fig.
2B and Fig. 3D), confirming previous findings of a more prominent role of reverse-mode NCX in neonatal E-C coupling
(14, 30).

It should be noted that the slow Ca2+ removal system has been suggested to play a greater role in neonatal compared with
adult heart (3, 16, 29). In rat ventricular myocytes, the slow Ca2+ removal system has been reported to be approximately
two times greater in immature than in adult cardiac myocytes (3). Therefore, it is possible that the contributions of sarcolemmal
Ca2+ efflux and SR Ca2+ content determined by the integral of INCX are underestimated in younger age groups.
However, the contribution of the slow Ca2+ removal system in the early developmental stage is still too small (5% of total
Ca2+) to be considered physiologically significant (3).

Age-dependent changes in Nif+KB-R-insensitive SR Ca2+
loading. Interestingly, a considerable amount of loadSR was
observed at all age stages despite the fact that the Ca2+ transient was abolished by the addition of Nif and KB-R (Fig.
4). This is in accordance with observations of Trafford et al. (28) in quiescent adult ferret and rat myocytes and a previous
study from our laboratory (13) showing that this phenomenon can likely be ascribed to SOCE in rabbit cardiac myocytes. In
agreement with this study, we observed that the contribution of SOCE to total SR Ca2+ refilling significantly decreased with
age. The average rate of sarcolemmal Ca2+ influx induced by depolarization was 1.1 and 0.5 pC/pF-s1 for 3d and 56d
myocytes, respectively, which is 15- to 50-fold greater than our previous estimate of the average Ca2+ influx rate via SOCE
during the first 10 s after SR Ca2+ depletion (13). Thus, although SOCE may contribute to SR refilling after Ca2+
depletion, this mechanism is unlikely to make a significant contribution to E-C coupling on a beat-to-beat basis even in the
neonate myocyte. The remarkable loadSR via SOCE (40%) after SR Ca2+ depletion does, however, suggest that it might
contribute to regulate loadSR at the earliest developmental stages. Our previous results indicated that SOCE loadSR was
intimately modulated by NCX (13), which we suggested resulted from the SOCE influx pathway and NCX being in the
same microdomain. More recent evidence from two different laboratories has strongly supported the concept that TRP-C3
channels and NCX colocalize in heart tissue (7, 8). Although not the focus of this study, it is tempting to speculate that
TRP-C3 channels may contribute to the observed SOCE.

The cellular mechanisms underlying loadSR provide important insight into the intact working heart. With an increase in
the number of depolarizations, [Ca2+] and loadSR dramatically increase, providing the bases for the phenomenon of the
positive force-frequency relationship observed in rabbit ventricular muscle strip and intact heart (19, 31) preparations.
Although the intact heart might be less dependent on Ca2+-
influnx via depolarization compared with isolated muscle (31), the developmental changes in the sources of Ca2+ for SR
refilling in an intact heart are likely to be similar to those observed for the single myocyte, and these observations are
clinically significant. For example, a prolonged whole heart arrest during open-heart surgery in the newborn will likely be
more prone to cause SR Ca2+ overload and arrhythmogenesis as a result of a greater SOCE; therefore, a different approach
for arresting the newborn heart during cardiopulmonary bypass may be beneficial.

SR Ca2+ pump activity during development. Our findings that 10 consecutive depolarizations were sufficient to reload
the SR independently of the age stage although loadSR (nor-
malized by membrane capacitance) was more than threefold
greater in 3d than in 5d6 myocytes (Fig. 2C) suggest that at
the early developmental stages there is an increase in J) SERCA2a
Vmax, 2) affinity of SERCA2a for calcium, and/or 3) [Ca2+]i
in the microdomain in which SERCA2a resides. Our esti-
mates of Vpeak (12.8 amol Ca2+/pF-s1 or 82 nM-liter
amol cytosol-s1) and k0,5 (0.38 μM) in the 56d group agree well
with values reported by Bassani et al. (2) in intact myocytes
from adult rabbits. The nH value observed from our data is
close to the theoretical maximum of 2, but smaller than that
observed by other groups (2) using intact cardiomyocytes, which might be explained by differences in the measurement
and analysis techniques. The data shown in Table 1 indicate that Vpeak, as measured in our study, is not different between
the age groups. Studies of SR vesicles have shown that the
maximum SR Ca2+ uptake rates increase during development
in a variety of species (23, 24) and are consistent with previous
observations of sparse SR and a lower density of SERCA2a in

Table 1. Parameters measured in SR Ca2+ uptake

<table>
<thead>
<tr>
<th>Age, days</th>
<th>3</th>
<th>10</th>
<th>20</th>
<th>56</th>
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<tr>
<td>No. of myocytes</td>
<td>25</td>
<td>25</td>
<td>25</td>
<td>25</td>
</tr>
<tr>
<td>k0.5, μM</td>
<td>0.25±0.01*</td>
<td>0.26±0.04</td>
<td>0.32±0.02</td>
<td>0.38±0.04</td>
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<tr>
<td>Vpeak, amol·s-1·pF-1</td>
<td>12.44±0.82</td>
<td>10.49±1.22</td>
<td>11.62±0.79</td>
<td>12.79±1.03</td>
</tr>
<tr>
<td>nH</td>
<td>1.90±0.13</td>
<td>1.75±0.12</td>
<td>2.31±0.16</td>
<td>2.2±0.23</td>
</tr>
</tbody>
</table>

Values are means ± SE. k0.5, Ca2+ concentration at half-maximal sarco-
plasmic reticulum (SR) Ca2+ uptake; Vpeak, asymptotic value of SR uptake rate; nH, slope of the sigmoid curve. *Significantly different from 20-day and 56-day groups (P < 0.05). Differences in Vpeak and nH within age groups did not reach a level of significance.
neonates (25, 27). The discrepancies between the results of the present study and those presented above are probably related to differences in technique. The advantages of in vitro preparations such as microsomal vesicles include control over “cytosolic” [Ca\(^{2+}\)] measurements of initial rates for a more accurate determination of \(V_{\text{max}}\) and affinity, as well as regulation of intravesicular [Ca\(^{2+}\)] with oxalate or similar compounds. However, the major disadvantage is that the SERCA2a has been removed from its native environment with the attendant potential loss of regulatory cofactors [e.g., G, proteins, β-receptors, SR-associated phosphatases, and phospholamban (PLB)] and destruction of the microdomain in which SERCA2a resides. Despite these differences, however, it is useful to compare our data in the 56d intact myocyte with data from SR vesicles from the adult rabbit heart. In one such a study by Xu and Narayan (33) in which uptake was determined at 37°C, they found a \(V_{\text{max}}\) of 489 nmol Ca\(^{2+}\)-mg protein\(^{-1}\)-min\(^{-1}\), which is comparable to that determined by others in different species (10, 15). In addition, they found a \(k_0.5\) value of 0.6 μM and an \(n_{\text{H}}\) of 1.4, which compare favorably to the \(k_0.5\) of 0.4 μM and \(n_{\text{H}}\) of 2.2 determined in the present study. To convert the microsomal \(V_{\text{max}}\) (in nmol Ca\(^{2+}\)-mg protein\(^{-1}\)-min\(^{-1}\)) to units comparable to the \(V_{\text{peak}}\) (in amol Ca\(^{2+}\)-pF\(^{-1}\)-s\(^{-1}\)) observed in the present study, we used the following assumptions: 10-fold microsomal purification factor, 120 mg homogenate protein/g wet wt, 2.43 g wet wt/ml cytosol, 4.58 pF/pl cytosol (4, 26), and a \(Q_{10}\) of 2 to derive a value of 12.1 amol Ca\(^{2+}\)-pF\(^{-1}\)-s\(^{-1}\) in vesicles vs. 12.8 amol Ca\(^{2+}\)-pF\(^{-1}\)-s\(^{-1}\) determined in intact myocytes in the present study. Because we cannot unequivocally state that our \(V_{\text{SR}}\) reflects initial rates because of the complexity of the preparation, we chose to refer to the maximal \(V_{\text{SR}}\) in our study as \(V_{\text{peak}}\) instead of \(V_{\text{max}}\). However, the fact that the \(V_{\text{peak}}\) values are comparable to the \(V_{\text{max}}\) determined under the simpler and more controlled in vitro conditions serves to allay many of these concerns.

Consistent with the notion that affinity of SERCA2a for Ca\(^{2+}\) was altered, the \(k_0.5\) for SR loading significantly increased with age (Table 1). However, studies using electrical field stimulation of immature ventricular myocytes have suggested a diminished contribution of the SR Ca\(^{2+}\) pump to cytosolic Ca\(^{2+}\) removal (1, 3, 34), which would appear to contradict our findings. On the other hand, another study using electrical field stimulation recently demonstrated that a functional SR is present long before birth in a linear heart tube (22), and the different results are likely due to the specific experimental conditions and/or species differences.

Indeed, one possible explanation for a higher SR Ca\(^{2+}\) affinity without a change of \(V_{\text{max}}\) in neonate rabbit ventricular myocytes may be a lower PLB expression relative to that of SERCA2a (9, 18). In agreement with this notion, it has been demonstrated that PLB-deficient myocytes exhibit a higher SR Ca\(^{2+}\) load (6), and it was recently demonstrated that the degree of PLB phosphorylation per SERCA2a was greater in the fetus and newborn compared with adult (32). It has been reported that the SR Ca\(^{2+}\) depletion prompts the phosphorylation of PLB to stimulate store refilling (5). An alternative explanation to the apparently higher Ca\(^{2+}\) affinity of the SR Ca\(^{2+}\) pump in neonate myocytes is that there are age-dependent differences in the subsarcolemmal microdomain (12, 13) resulting in a higher [Ca\(^{2+}\)] in the immediate vicinity of the SERCA2a in the neonate heart for a given level of bulk phase [Ca\(^{2+}\)]. Indeed, we recently showed that a narrow cleft (20 nm) between the sarcolemma and SR observed in both 3d and 56d myocytes was delimited by a threefold longer SR in 3d than 56d myocytes (300 vs. 100 nm), resulting in a much more restricted microdomain in the early developmental stages. It is therefore possible that an apparent greater Ca\(^{2+}\) affinity of the SR Ca\(^{2+}\) pump observed in neonate myocytes may at least partly result from measurement of \(V_{\text{SR}}\) as a function of the average bulk [Ca\(^{2+}\)].

**Conclusions.** In conclusion, there is a switch in the sarcolemmal calcium fluxes contributing to SR Ca\(^{2+}\) refilling from a predominance of NCX and SOCE at the earliest stage to a predominance of \(I_{\text{Ca}}\) at later stages. Moreover, the number of depolarizations required to achieve steady state did not vary during development, although steady-state load\(_{\text{SR}}\) was threefold larger in the neonatal heart. This may be explained by either a higher Ca\(^{2+}\) affinity of the SERCA2a in neonatal myocytes or a higher local [Ca\(^{2+}\)] around SERCA2a for a given level of [Ca\(^{2+}\)] in the bulk phase. Together, this suggests that age-dependent downregulation of SR calcium sequestration and increased dependence on calcium entry through L-type calcium channels during the first 20 days postpartum in rabbit ventricular myocytes is due to a selective downregulation of NCX-dependent SR Ca\(^{2+}\) refilling.

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**REFERENCES**