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

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Huang J, Hove-Madsen L, Tibbits GF. SR Ca\(^{2+}\) refilling upon depletion and SR Ca\(^{2+}\) uptake rates during development in rabbit ventricular myocytes. Am J Physiol Cell Physiol 293: C1906–C1915, 2007. First published October 10, 2007; doi:10.1152/ajpcell.00241.2007.—While it has been reported that a sparse sarcoplasmic reticulum (SR) and a low SR Ca\(^{2+}\) pump density exist at birth, we and others have recently shown that significant amounts of Ca\(^{2+}\) are stored in the neonatal rabbit heart SR. Here we try to determine developmental changes in SR Ca\(^{2+}\) loading mechanisms and Ca\(^{2+}\) pump efficacy in rabbit ventricular myocytes. SR Ca\(^{2+}\) loading (load\(_{SR}\)) and k\(_{0.5}\) (Ca\(^{2+}\) concentration at half-maximal SR Ca\(^{2+}\) uptake) were higher and lower, respectively, in younger age groups. Inhibition of the L-type calcium current (k\(_{Ca}\)) with 15 \(\mu\)M nifedipine dramatically reduced load\(_{SR}\) in older but not in younger age groups. In contrast, subsequent inhibition of the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX) with 10 \(\mu\)M KB-R7943 strongly reduced load\(_{SR}\) in the younger but not the older age groups. Accordingly, the time integral of the inward NCX current (tail \(_{NCX}\)) elicited on repolarization was highly sensitive to nifedipine in the older groups and sensitive to KB-R7943 in the younger groups. Interestingly, slow SR loading took place in the presence of both nifedipine and KB-R7943 in all age groups, although it was less prominent in the older groups. We conclude that the SR loading capacity at the earliest postnatal stages is at least as large as that of adult myocytes. However, reverse-mode NCX plays a prominent role in SR Ca\(^{2+}\) loading at early postnatal stages while k\(_{Ca}\) is the main source of SR Ca\(^{2+}\) loading at late postnatal and adult stages.

It is well documented that a relatively small amount of Ca\(^{2+}\) that 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\(^{2+}\) released from the SR and transmembrane Ca\(^{2+}\) influx provide ~70% and 30% of the total Ca\(^{2+}\) during cell contraction, respectively (8, 17). Accordingly, ~28% of the Ca\(^{2+}\) removed from the cytosol during cell relaxation is extruded by the Na\(^{+}\)/Ca\(^{2+}\) exchanger (NCX), and the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)\(_{2a}\) pump transports ~70% of total Ca\(^{2+}\) back into the SR (3). The sarcolemmal Ca\(^{2+}\)-ATPase (plasma membrane Ca\(^{2+}\)-ATPase, PMCA) and mitochondrional Ca\(^{2+}\) uniporter, collectively referred to as the slow Ca\(^{2+}\) removal system, remove the remaining ~2% (2).

Ultrastructural data from the neonatal heart have led to the suggestion that the SR is relatively sparse at birth and develops gradually from neonate to adult through the first few weeks of life (11, 25). Several studies have also indicated that SERCA mRNA and protein expression levels are ~50% of adult levels at birth and gradually increase to adult levels by postpartum day 15 (9, 11), leading further support to the notion that SERCA\(_{2a}\) function is less important in neonates than in adults (5, 10). Consequently, it has been assumed that the SR in neonatal ventricular myocytes is unable to store amounts of Ca\(^{2+}\) comparable to those of adult myocytes. However, recent studies have challenged this assumption. Observations such as robust and spatially homogeneous caffeine (Caf)-induced Ca\(^{2+}\) transients and contractures have been reported in neonate hearts (1, 10, 16). Moreover, integration of the Caf-induced inward NCX current (\(_{NCX}\)) in neonatal rabbit ventricular myocytes revealed that SR Ca\(^{2+}\) loading (load\(_{SR}\)) at rest was significantly larger in early neonatal than in late neonatal and adult ventricular myocytes (14), suggesting that store-operated Ca\(^{2+}\) entry (SOCE) may contribute significantly to load\(_{SR}\) in the neonate rabbit heart. The aim of the present study was therefore to elucidate the cellular mechanisms contributing to refilling of the SR on a beat-to-beat basis during postnatal development.

To achieve this, we used a whole cell perforated patch-clamp technique and Ca\(^{2+}\) transient measurements combined with pharmacological manipulation of the L-type Ca\(^{2+}\) channel and the NCX, the main sarcolemmal Ca\(^{2+}\) sources in the adult mammalian ventricular myocytes (3, 12, 14). Our results show that the SR Ca\(^{2+}\) pump is capable of efficiently loading the SR with Ca\(^{2+}\) even at the earliest neonatal stage, but that the principal Ca\(^{2+}\) sources contributing to load\(_{SR}\) change 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⁺]) at left in contrast to the Caf-induced Ca²⁺ transient (Caf [Ca²⁺]) 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 NCX: tail I NCX at left and Caf I NCX 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.
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+}], = K_d(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. \Delta F is the incremental fluorescence measured from baseline or the background fluorescence in the presence of a cell. K_d 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 load_{SR} and as a consequence frequently generated spontaneous release. Therefore, these data were not included in the analysis. The time integral of the I_{NCX} (f_{NCX}) induced by the second Caf application was used as a measure of load_{SR} during the preceding 3 s depolarization (12). The average V_{SR} (amol/s/pF) was obtained by dividing load_{SR} (f_{NCX}, amol/pF) by the duration of the loading period (3 s).

Data analysis. Data are presented as means \pm 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 \leq 0.05 was taken to be significant.

RESULTS

Depolarization dependence of Ca\textsuperscript{2+} transient, tail I_{NCX}, 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_{Ca}, and Fig. 1D shows the inward I_{NCX} on repolarization (tail I_{NCX}) and during the second Caf application (Caf I_{NCX}) in a 3d myocyte. The corresponding time integrals of tail I_{NCX} (f_{tail I_{NCX}}) and Caf I_{NCX} (f_{Caf I_{NCX}}) are shown in Fig. 1E. Peak I_{Ca}, 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 I_{NCX} 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 f_{tail I_{NCX}} from each individual f_{tail I_{NCX}} 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 (R\textsuperscript{2} 0.98). The peak [Ca\textsuperscript{2+}], increased with subsequent depolarizations and reached steady state near the 10th depo-
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 Cafi [Ca\(^{2+}\)]\(_i\) at right with the same scale), inward \(I_{\text{NCX}}\) (tail \(I_{\text{NCX}}\) at left and Cafi \(I_{\text{NCX}}\) at right with different scales), and time integral of corresponding \(I_{\text{NCX}}\) (tail \(I_{\text{NCX}}\) at left and Cafi \(I_{\text{NCX}}\) 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 (\(\Delta F\))]/difference in background fluorescence between absence and presence of cell (F\(_0\)); 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. \(n = 12\).
lization. The response was comparable for all age groups, which was neither proportional to cumulative Ca\(^{2+}\) entry nor to load\(_{SR}\). Figure 2C compares the cumulative net Ca\(^{2+}\) 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\(^{2+}\) entry in all age groups, suggesting that an additional source of load\(_{SR}\) exists.

Age-dependent difference in nifedipine-insensitive Ca\(^{2+}\) transients and sarcolemmal Ca\(^{2+}\) 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 \(\mu\)M Nif, a selective inhibitor of L-type Ca\(^{2+}\) 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\(^{2+}\) 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\(^{2+}\) transients were abolished. Figure 3, C and D, show the cumulative net Ca\(^{2+}\) entry and the \([Ca^{2+}]_i\) amplitude in the presence of Nif for the different age groups. Note that Nif abolished sarcolemmal Ca\(^{2+}\) entry and Ca\(^{2+}\) transients in older but not in younger age groups.

Age-dependent difference in nifedipine and KB-R7943-insensitive SR Ca\(^{2+}\) 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). \([Ca^{2+}]_i\) (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 \(\int Caf\) \(I_{NCX}\) at right with different scales) are shown from top to bottom, respectively. The remaining depolarization-dependent Ca\(^{2+}\) 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). \(n = 12\).

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 \(\int Caf\) \(I_{NCX}\) at right with different scales) are shown from top to bottom, respectively. The remaining depolarization-dependent Ca\(^{2+}\) 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). \(n = 12\).
presence of both 15 μM Nif and 10 μM KB-R7943 (KB-R). [Ca\(^{2+}\)]\(_i\), inward I\(\text{SCX}\) (tail I\(\text{SCX}\) and Caf I\(\text{SCX}\)), and their time integrals (I\(\text{SCX}\) and Caf I\(\text{SCX}\)) are shown from top to bottom, respectively, in Fig. 4, A and B. The depolarization-dependent Ca\(^{2+}\) transient and tail I\(\text{SCX}\) 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\(\text{SCX}\) 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 load\(\text{SR}\) source exists and is insensitive to Nif and KB-R. Figure 4C shows that Nif- and KB-R-insensitive load\(\text{SR}\) 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 load\(\text{SR}\) as shown in Fig. 4C. The relative contributions (Con = 1) of Nif-sensitive, KB-R-sensitive, and Nif+KB-R-insensitive load\(\text{SR}\) are shown in Fig. 5D for the different age groups. The calculation of the contribution of Nif+KB-R-insensitive load\(\text{SR}\) is predicated on two observations: 1) steady state in control solution was achieved at ~10th depolarization (~50 s) and 2) Nif+KB-R-insensitive load\(\text{SR}\) is linear with time for up to 100 s (13). Therefore the contribution of Nif+KB-R-insensitive load\(\text{SR}\) relative to Con is derived from the assumption that its loading is the half-value of Nif+KB-R-insensitive load\(\text{SR}\) (~100 s). In accordance with Fig. 5B, Nif-sensitive load\(\text{SR}\) significantly increased with age, while KB-R-sensitive load\(\text{SR}\) and Nif+KB-R-insensitive load\(\text{SR}\) significantly decreased with age.

Age-dependent changes in \(k_{0.5}\) of SR Ca\(^{2+}\) uptake. Since age-dependent changes in load\(\text{SR}\) 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\(^{2+}\)]\(_{\text{PS}}\)) was used as the [Ca\(^{2+}\)]\(_i\) corresponding to its average V\(\text{SR}\). Figure 6C shows the average Ca\(^{2+}\) transient traces and its corresponding second Caf-induced I\(\text{NCX}\) 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\(\text{NCX}\) is greater in 3d than 56d cells.
despite the fact that the amplitudes of the Ca\(^{2+}\) transients were comparable in the two groups. The representative average VSR as a function of \([\text{Ca}^{2+}]_{\text{PS}}\) 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: 

\[
V_{\text{SR}} = V_{\text{max}} \cdot [\text{Ca}^{2+}]_{\text{PS}}^{nH}/(K_{0.5} + [\text{Ca}^{2+}]_{\text{PS}}^{nH}) \quad (R^2 = 0.98)
\]

to obtain the asymptotic value of VSR (Vpeak), \(K_{0.5}\) (the value of \([\text{Ca}^{2+}]_{\text{PS}}\) at half of Vpeak) 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 Vpeak 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\(_{\text{SR}}\) in neonatal and adult hearts. In accordance with our previous report (12), load\(_{\text{SR}}\) after SR Ca\(^{2+}\) depletion was significantly greater in the neonatal compared with the adult heart when normalized per unit membrane capacitance. Moreover, our
results show that NCX plays a prominent role in loadSR in the 3d heart, whereas \(I_{Ca}\) is the main sarcolemmal \(Ca^{2+}\) source for loadSR in late neonatal and adult rabbit ventricular myocytes.

### Age-dependent changes in relative contributions of \(I_{Ca}\) and reverse-mode NCX

The present study shows that 10 consecutive depolarizations are sufficient to fully reload the SR after transient exposure to 10 mM Caf. This was true for all rabbit eupative depolarizations are sufficient to fully reload the SR after transient exposure to 10 mM Caf. 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 \(I_{Ca}\) and a decreased tail \(I_{NCX}\) 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 \(I_{Ca}\) is the main pathway for sarcolemmal \(Ca^{2+}\) entry in the adult rabbit heart (8, 17), and in agreement with this notion, we find that loadSR as well as the \(Ca^{2+}\) 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 \(Ca^{2+}\) transient were insensitive to Nif and 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 \(Ca^{2+}\) refilling at early neonatal stages. Moreover, total sarcolemmal \(Ca^{2+}\) efflux estimated via tail \(I_{NCX}\) at steady state in 3d hearts was more than threefold the \(Ca^{2+}\) transient in nifedipine in older age groups (Fig. 3, C, and Fig. 5). In contrast, in the early developmental stages, loadSR as well as the \(Ca^{2+}\) transient were insensitive to Nif but were sensitive to subsequent addition of KB-R (Fig. 4A and Fig. 5).

### Age-dependent changes in \(Nif + KB-R\)-insensitive \(SR Ca^{2+}\) loading

Interestingly, a considerable amount of loadSR was observed at all age stages despite the fact that the \(Ca^{2+}\) 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 \(Ca^{2+}\) refilling significantly decreased with age. The average rate of sarcolemmal \(Ca^{2+}\) influx induced by depolarization was 1.1 and 0.5 pC-pF\(^{-1}\)-s\(^{-1}\) for 3d and 56d myocytes, respectively, which is 15- to 50-fold greater than our previous estimate of the average \(Ca^{2+}\) influx rate via SOCE during the first 10 s after \(SR Ca^{2+}\) depletion (13). Thus, although SOCE may contribute to SR refilling after \(Ca^{2+}\) 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%) for \(SR Ca^{2+}\) 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.

### Cellular mechanisms underlying loadSR provide important insight into the intact working heart

With an increase in the number of depolarizations, \([Ca^{2+}]_{i}\) 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 \(Ca^{2+}\) influx via depolarization compared with isolated muscle (31), the developmental changes in the sources of \(Ca^{2+}\) 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 \(Ca^{2+}\) 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.

### \(SR Ca^{2+}\) pump activity during development

Our findings that 10 consecutive depolarizations were sufficient to reload the SR independently of the age stage although loadSR (normalized by membrane capacitance) was more than threefold greater in 3d than in 56d myocytes (Fig. 2C) suggest that at the early developmental stages there is an increase in J) SERCA2a \(V_{max}\), affinity of SERCA2a for calcium, and/or 3) \([Ca^{2+}]_{i}\) in the microdomain in which SERCA2a resides. Our estimates of \(V_{peak}\) (12.8 amol \(Ca^{2+}\)-pF\(^{-1}\)-s\(^{-1}\) or 82 \(\mu\)M-litter cytosol \(-1\)-s\(^{-1}\)) and \(k_{0.5}\) (0.38 \(\mu\)M) in the 56d group agree well with values reported by Bassani et al. (2) in intact myocytes from adult rabbits. The \(n_{H}\) 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 \(V_{peak}\), as measured in our study, is not different between the age groups. Studies of SR vesicles have shown that the maximum SR \(Ca^{2+}\) 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 \(Ca^{2+}\) uptake

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<th>Age, days</th>
<th>3</th>
<th>10</th>
<th>20</th>
<th>56</th>
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<td>No. of myocytes</td>
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<tr>
<td>(k_{0.5}), (\mu)M</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>
</tr>
<tr>
<td>(V_{peak}), 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>(n_{H})</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. \(k_{0.5}\), \(Ca^{2+}\) concentration at half-maximal sarcoplasmic reticulum (SR) \(Ca^{2+}\) uptake; \(V_{peak}\), asymptotic value of SR uptake rate; \(n_{H}\), slope of the sigmoid curve. *Significantly different from 20-day and 56-day groups (\(p < 0.05\)). Differences in \(V_{peak}\) and \(n_{H}\) 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\(^{2+}\)]\(_{\text{m}}\), measurement 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, \(\beta\)-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 \(\mu\)M and an \(n_{H}\) of 1.4, which compare favorably to the \(k_{0.5}\) of 0.4 \(\mu\)M and \(n_{H}\) 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/pF 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.

GRANTS

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REFERENCES