Ontogeny of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in rabbit ventricular myocytes

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Huang J, Hove-Madsen L, Tibbits GF. Ontogeny of Ca\(^{2+}\)-induced Ca\(^{2+}\) release in rabbit ventricular myocytes. Am J Physiol Cell Physiol 294: C516–C525, 2008. First published December 19, 2007; doi:10.1152/ajpcell.00417.2007.—It is commonly accepted that L-type Ca\(^{2+}\) channels; sarcoplasmic reticulum (SR) Ca\(^{2+}\) depletion with caffeine. Therefore, the present study investigated the developmental changes of CICR in rabbit ventricular myocytes at 3, 10, 20, and 56 days of age. We found that the inhibitory effect of the L-type Ca\(^{2+}\) current (\(I_{Ca}\)) inhibitor nifedipine (Nif; 15 \(\mu\)M) caused an increasingly larger reduction of Ca\(^{2+}\) transients on depolarization in older age groups [from \(\sim 15\%\) in 3-day-old (3d) myocytes to \(\sim 90\%\) in 56-day-old (56d) myocytes]. The remaining Ca\(^{2+}\) transient in the presence of Nif in younger age groups was eliminated by the inhibition of Na\(^{+}/Ca^{2+}\) exchanger (NCX) with the subsequent addition of 10 \(\mu\)M KB-R7943 (KB-R). Furthermore, Ca\(^{2+}\) transients were significantly reduced in magnitude after the depletion of SR Ca\(^{2+}\) with caffeine in all age groups, although the effect was significantly greater in the older age groups (from \(\sim 40\%\) in 3d myocytes up to \(\sim 70\%\) in 56d myocytes). This SR Ca\(^{2+}\)-sensitive Ca\(^{2+}\) transient in the earliest developmental stage was insensitive to Nif but was sensitive to the subsequent addition of KB-R, indicating the presence of NCX-mediated CICR that decreased significantly with age (from \(\sim 37\%\) in 3d myocytes to \(\sim 0.5\%\) in 56d myocytes). In contrast, the \(I_{Ca}\)-mediated CICR increased significantly with age (from \(\sim 10\%\) in 3d myocytes to \(\sim 70\%\) in 56d myocytes). The CICR gain as estimated by the integral of the CICR Ca\(^{2+}\) transient divided by the integral of its Ca\(^{2+}\) transient trigger was smaller when mediated by NCX (\(\sim 1.0\) for 3d myocytes) than when mediated by \(I_{Ca}\) (\(\sim 3.0\) for 56d myocytes). We conclude that the lower-efficiency NCX-mediated CICR is a predominant mode of CICR in the earliest developmental stages that gradually decreases as the more efficient L-type Ca\(^{2+}\) channel-mediated CICR increases in prominence with ontogeny.

It is well documented that in the adult mammalian heart, a relatively small Ca\(^{2+}\) influx via L-type Ca\(^{2+}\) channels triggers Ca\(^{2+}\) release from the sarcoplasmic reticulum (SR) in a process known as Ca\(^{2+}\)-induced Ca\(^{2+}\) release (CICR). CICR is predicted on a functional coupling of the L-type Ca\(^{2+}\) channel, the ryanodine receptor (RyR), and well-developed T-tubules (5, 12, 32). In contrast, it is commonly accepted in neonates that there is no appreciable CICR due to the sparse SR and lack of T-tubules (27, 37) and that transsarcannelmal Ca\(^{2+}\) influx is responsible for excitation-contraction (E-C) coupling in the earliest developmental stages. Transsarcomelmal Ca\(^{2+}\) currents and the contribution of Ca\(^{2+}\) released from the SR have been extensively investigated in adult mammalian ventricular myocytes and show species variation (11, 42, 53). It has been reported that in adult rabbit ventricular myocytes the Ca\(^{2+}\) released from SR and transmembrane Ca\(^{2+}\) influx provide \(\sim 70\%\) and \(30\%\), respectively, of total Ca\(^{2+}\) in the steady state (11, 18). However, we have observed in 3-day-old (3d) cardiomyocytes that the magnitude of the Ca\(^{2+}\) transients were reduced by about \(40\%\) after the depletion of SR Ca\(^{2+}\) (19). Furthermore, there have been several reports that the neonate ventricular myocytes have a greater amount of Ca\(^{2+}\) stored in the SR than previously realized (19, 34). Recently, it was demonstrated that RyR isolated from neonate hearts have gating properties in planar lipid bilayers similar to that from adults (41). The high expression and activity of Na\(^{+}/Ca^{2+}\) exchanger (NCX) and a possible colocalization of NCX and RyR in the sarcolemma near the Z-lines provide the theoretical possibility of NCX-mediated CICR (6, 19). Given these findings, it is clear that the role of CICR in E-C coupling in the neonatal heart required reexamination.

In the present study, using the whole cell-perforated patch-clamp technique and Ca\(^{2+}\) transient measurements in rabbit ventricular myocytes, we demonstrated the presence of reverse-mode NCX-mediated CICR in the early developmental stages. With ontogeny this mechanism of CICR gradually disappeared and the more efficient L-type Ca\(^{2+}\) channel-mediated CICR became dominant.

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 the 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 (19–21).

Whole cell-perforated patch voltage clamp. Whole cell amphotericin-perforated voltage-clamp technique was used as described previously (19–21). The internal pipette solution contained (in mM) 110 CsCl, 5 MgATP, 1 MgCl\(_2\), 20 tetraethylammonium, 5 Na\(_2\) phosphocreatine, and 10 HEPES, and pH was adjusted to 7.1 with CsOH. The standard external solution contained (in mM) 130 NaCl, 5 CsCl, 1 MgCl\(_2\), 2.0 CaCl\(_2\), 5 Na-pyruvate, 10 glucose, and 10 HEPES, and pH was adjusted to 7.4 with NaOH.

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Measurement of Ca\(^{2+}\) fluorescence. The cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)_\text{cyt}]) was measured with the fluorescent Ca\(^{2+}\) indicator fluo-3 AM as described previously (19–21). \(F_0\) was the difference in the background fluorescence determined in the absence and presence of a cell in the area of measurement. \(\Delta F\) was the increment measured from baseline or the background fluorescence in the presence of a cell in the area of measurement.

**Experimental protocol.** A train of 20 repetitive depolarizations at 0.2 Hz from a holding potential of \(-80\) mV to 10 mV (cells were first depolarized to \(-40\) mV for 50 ms to inactivate Na\(^{+}\) and T-type Ca\(^{2+}\) channels and were then depolarized to 10 mV for 400 ms) was used to achieve a steady-state (data not shown). Then, the calcium transient elicited by the last of the 20 repetitive depolarizations (the steady state) was compared with the calcium transient elicited by a depolarization with a depleted SR Ca\(^{2+}\). The SR Ca\(^{2+}\)-depleted state was achieved by an 8-s, 10 mM rapid caffeine application (Caf). Figure 1A shows this experimental protocol schematically. The depolarization immediately after the Caf application and in the continuing (3 s) presence of Caf was considered as depolarization in the SR depleted state. The duration of the Caf application was limited to 11 s to prevent a possible increase in [cAMP] that could potentially result from longer exposures (54) and confound the results. Two inhibitors, 15 \(\mu\)M nifedipine (Nif), an L-type Ca\(^{2+}\) channel blocker, and 10 \(\mu\)M KB-R7943 (KB-R), a blocker primarily of NCX reverse mode under these conditions and to a lesser degree of L-type Ca\(^{2+}\) channel, were used sequentially throughout the study (2). In total, six distinct solutions were applied, as follows: control solution (Con), Nif, and Nif+KB-R in the steady state, and Caf, Caf+Nif, and Caf+Nif+KB-R in the SR depleted state. Figure 1B shows representative Ca\(^{2+}\) transients for 3d myocytes (left) and 56d myocytes (right) elicited by this protocol in the presence of three pairs of solutions. Figure 1, B1–B3, shows (from top to bottom) the calcium transients elicited by the experimental protocol in control conditions (B1), with Nif (B2), and with Nif+KB-R (B3).

All of the drugs were purchased from Sigma Chemical (St. Louis, MO). A fresh working solution of 15 \(\mu\)M Nif was made by diluting a fresh 15 mM stock solution (dissolved in DMSO). The entire Nif delivery pathway including the micromanifold was light tight to protect Nif from photolysis.

**Data analysis.** Data are presented as means ± SE. Statistical significance of the results was tested using 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. \(P \leq 0.05\) was taken to be significant.

**RESULTS**

**SR Ca\(^{2+}\) release contributes to the Ca\(^{2+}\) transient even in the younger age groups.** Figure 2A (top) shows the superimposition of depolarization-induced Ca\(^{2+}\) transients in the presence of control solution under steady-state conditions and in the presence of Caf in an SR Ca\(^{2+}\)-depleted state (shown in the

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**Fig. 1.** A: schema of the experimental protocol: the depolarization with sarcoplasmic reticulum (SR) Ca\(^{2+}\) in steady-state is compared with the depolarization at SR Ca\(^{2+}\) in a depleted-state with different solutions. In total, 6 distinct solutions were applied: control (Con), nifedipine (Nif), and Nif+KB-R7943 (KB-R), a blocker primarily of NCX reverse mode under these conditions and to a lesser degree of L-type Ca\(^{2+}\) channel, were used sequentially throughout the study (2). In total, six distinct solutions were applied, as follows: control solution (Con), Nif, and Nif+KB-R in the steady state, and Caf, Caf+Nif, and Caf+Nif+KB-R in the SR depleted state. Figure 1B shows representative Ca\(^{2+}\) transients for 3d myocytes (left) and 56d myocytes (right) elicited by this protocol in the presence of three pairs of solutions. Figure 1, B1–B3, shows (from top to bottom) the calcium transients elicited by the experimental protocol in control conditions (B1), with Nif (B2), and with Nif+KB-R (B3).
protocol illustrated in Fig. 1). The difference between representative Caf and Con Ca²⁺ transients defined as Caf-sensitive Ca²⁺ transients is shown in Fig. 2A (bottom). A significant Caf-sensitive Ca²⁺ transient was observed in 3d myocytes, although it was smaller than in 56d myocytes. Figure 2B shows the amplitudes of the calcium transient integrals in Caf and the Caf-sensitive Ca²⁺ transient (normalized to the corresponding Con transient) as a function of age. The Caf Ca²⁺ transients significantly decreased with age, and inversely Caf-sensitive Ca²⁺ transients increased with age (***P < 0.0001 between those age groups with the exception of 3d vs. 10d and 20d vs. 56d), n = 22.

Inhibition of ICa abolishes the Ca²⁺ transient in older but not younger age groups. Figure 3A1 shows the superimposition of representative depolarization-induced Ca²⁺ transients in Con, Nif, and Nif+KB-R containing solutions under steady-state conditions. As expected, Nif completely abolished the L-type Ca²⁺ channel current (ICa) in all age groups (data not shown) as well as the Ca²⁺ transient in 56d myocytes. However, a large Ca²⁺ transient was retained in 3d myocytes in the presence of Nif. The Nif-sensitive Ca²⁺ transient was obtained by subtracting the Nif Ca²⁺ transient from its corresponding Con as shown in Fig. 3A2. The magnitude of the Nif-sensitive Ca²⁺ transient was much greater in 56d myocytes than in 3d myocytes. Subsequent addition of 10 µM KB-R abolished the Nif-insensitive Ca²⁺ transient in 3d myocytes, and the KB-R-sensitive Ca²⁺ transient is shown in Fig. 3A3. Figure 3B shows the time integrals of the KB-R-sensitive and Nif-sensitive Ca²⁺ transients normalized to the Con transient as a function of age. As shown, the relative importance of KB-R-sensitive Ca²⁺ transients significantly decreased with age, whereas Nif-sensitive Ca²⁺ transients significantly increased with age.

ICa-mediated CICR increased with age. Figure 4A1 shows superimposition of representative depolarization-induced Ca²⁺ transients in Caf- and Caf+Nif-containing solutions, i.e., in an SR Ca²⁺-depleted state. Subtraction of the two Ca²⁺ transients represents Ca²⁺ influx via ICa (ICa Ca²⁺ transient), which is shown in Fig. 4A2. There was a much greater ICa Ca²⁺ transient in 56d myocytes (30% of Con) than in 3d myocytes. The Nif-sensitive Ca²⁺ transient is shown in Fig. 4A3 (the same as in Fig. 3A2), and subtraction of the ICa Ca²⁺ transient from Nif-sensitive Ca²⁺ transient is referred to as the ICa-mediated CICR (ICa-CICR) Ca²⁺ transient, which is shown in Fig. 4A4. The ICa-CICR Ca²⁺ transient was much greater in 56d myocytes (70% of Con) than in 3d myocytes (6% of Con). Figure 4B shows the relative contribution of ICa and ICa-CICR Ca²⁺ transients normalized to Con (in Fig. 1B1) as a function of age. Both ICa and ICa-CICR Ca²⁺ transients significantly increased with age.

**Fig. 2.** SR Ca²⁺-dependent Ca²⁺ transient. A: representative Ca²⁺ transients from 3d (left) and 56d (right) myocytes. Superimpositions of the Ca²⁺ transients on depolarizations in Con (black) and Caf (gray) with an expanded time scale are shown in top panel, and Caf-sensitive Ca²⁺ transients are shown in bottom panel. B: bar graph of the integral of Caf (black) and the Caf-sensitive Ca²⁺ transient on depolarization (gray) (Con value taken as unity) as a function of age. Caf Ca²⁺ transients significantly decreased with age, and inversely Caf-sensitive Ca²⁺ transients increased with age (***P < 0.0001 between those age groups with the exception of 3d vs. 10d and 20d vs. 56d), n = 22.
Reverse-mode NCX-mediated CICR is present at the earliest developmental stages and decreases with age. Figure 5A1 shows the superimposition of representative depolarization-induced Ca\(^{2+}\) transients in solutions with Caf\(^{2+}\)+Nif and Caf\(^{2+}\)+Nif+KB-R (using the protocol shown in Fig. 1), i.e., in an SR Ca\(^{2+}\)-depleted state. The Caf\(^{2+}\)+Nif Ca\(^{2+}\) transients were observed in 3d myocytes but not in 56d myocytes, and the former were abolished by the subsequent addition of 10 \(\mu\)M KB-R.

The derived KB-R-sensitive Ca\(^{2+}\) transient recorded in an SR Ca\(^{2+}\)-depleted state likely reflects the transmembrane Ca\(^{2+}\) influx via reverse-mode NCX (NCX Ca\(^{2+}\) transient), which is shown in Fig. 5A2. The KB-R-sensitive Ca\(^{2+}\) transients at steady state are shown in Fig. 5A3, and subtraction of the NCX Ca\(^{2+}\) transient from the KB-R-sensitive Ca\(^{2+}\) transient, therefore, is interpreted as a reflection of the reverse-mode NCX-mediated CICR (NCX-CICR Ca\(^{2+}\) transient), which is shown in Fig. 5A4. Figure 5B shows the contributions of NCX and NCX-CICR Ca\(^{2+}\) transients relative to the Con transient (Fig. 1B1). Both NCX and NCX-mediated CICR Ca\(^{2+}\) transients were greatest in 3d myocytes and significantly decreased with age. The CICR gain as shown in Fig. 5C was estimated by the ratio of the integral of CICR Ca\(^{2+}\) transient and the integral of the corresponding trigger Ca\(^{2+}\) transient, either NCX or \(I_{Ca}\). I\(_{Ca}\)-CICR gain increased with age. In contrast, the NCX-CICR gain decreased with age.

\(I_{Ca}\)-CICR in older age groups is more efficient than NCX-CICR in younger age groups. Figure 6A shows the superimposition of Ca\(^{2+}\) transients of \(I_{Ca}\)-CICR vs. NCX-CICR. The upstroke of the Ca\(^{2+}\) transient in 56d myocytes preceded that in 3d myocytes significantly. Time to peak (TTP; indicated by arrows in Fig. 6A) represents the time from the starting point to the peak of the bulk phase Ca\(^{2+}\) transient, which were \(-150\) and \(360\) ms for 56d and 3d myocytes, respectively, in the representative traces. TTPs for Con, KB-R-sensitive, and Nif-sensitive Ca\(^{2+}\) transients as a function of age are shown in Fig. 6B. TTP significantly decreased with age in Con Ca\(^{2+}\) transients. Differences in TTPs of KB-R-sensitive Ca\(^{2+}\) transients significantly decreased with age, and Nif-sensitive Ca\(^{2+}\) transients significantly increased with age (*\(P < 0.01\) for 3d vs. 10d and 20d vs. 56d; ***\(P < 0.0001\) for other pairs). \(n = 15\).
DISCUSSION

SR Ca\textsuperscript{2+} release contributes to E-C coupling even at the earliest developmental stage. It is well known that I\textsubscript{Ca}-induced Ca\textsuperscript{2+} release plays a crucial role in E-C coupling in adult mammalian myocytes (5, 12, 32). The contributions of Ca\textsuperscript{2+} released from the SR and the transsarcolemmal influx have been extensively investigated in adult mammalian ventricular myocytes and exhibit species variation (11, 42, 53). Values of 70% and 30% of total Ca\textsuperscript{2+} at the steady state, respectively, have been reported in adult rabbit ventricular myocytes (11, 18). In the neonatal heart, due to lack of T-tubule in the first 2 wk of life and reportedly sparse SR, it has been assumed that the SR is not able to store and release comparable amounts of Ca\textsuperscript{2+} on a beat-to-beat basis as that of the adult (27, 37). However, the prevailing view has been challenged by recent findings of robust and spatially homogeneous Caf-induced Ca\textsuperscript{2+} transients and contractures in neonate hearts (19, 34). Indeed, the amount of Ca\textsuperscript{2+} stored in the SR was greater in the younger age groups (threefold greater in 3d than in the 56d) when normalized per unit of membrane capacitance (19). The RyR2 receptors appear in well-organized arrays in the cytoplasm of myocytes, even in 3d rabbits, using immunolabeling (10, 46). In addition, the gating properties in planar lipid bilayers (41) and pharmacological properties (52) of RyR2 in neonates are similar to those in adults, and the observations of spontaneous Ca\textsuperscript{2+} oscillations in early-stage embryonic stem cell-derived cardiomyocytes (55) strongly imply that the RyR is capable of releasing Ca\textsuperscript{2+} at the earliest developmental stages. In the present study, the contribution of a Caf-sensitive Ca\textsuperscript{2+} transient either measured by its integral (Fig. 2C) or peak value (supplementary data Fig. 2A; the online version of this article contains supplemental data) was significant as early as 3d (40% of total Ca\textsuperscript{2+}) and amounted to 70% of total Ca\textsuperscript{2+} in 56d myocytes, indicating that the SR Ca\textsuperscript{2+} plays an appreciable role in E-C coupling, even at the earliest stage in rabbit cardiomyocytes. Moreover, a combination of 25 \textmu M cyclopiazonic acid and 10 \textmu M ryanodine reduced the total Ca\textsuperscript{2+} transient by 45% in 3d myocytes (see supplementary data Fig. 1). These findings are apparently contrary to those of Haddock et al. (16), who reported that the Ca\textsuperscript{2+} transient triggered by field stimulation shows a gradient from the sub-sarcolemmal region to the cell center in neonatal rabbit ventricular myocytes that was not inhibited by the addition of thapsigargin, a blocker of the SR Ca\textsuperscript{2+} pump, suggesting that there was no appreciable SR Ca\textsuperscript{2+} release. In contrast, Seki et al. (48) recently observed a significant reduction in the

Fig. 4. I\textsubscript{Ca}-mediated Ca\textsuperscript{2+}-induced Ca\textsuperscript{2+} release (CICR) increased with development. A: representative Ca\textsuperscript{2+} transient traces in 3d (left) and 56d (right) myocytes on depolarization. A1: the superimposition of Ca\textsuperscript{2+} transients of Caf (black) and Caf+Nif (gray). A2: the derived I\textsubscript{Ca} Ca\textsuperscript{2+} transients. A3: the Nif-sensitive Ca\textsuperscript{2+} transient. A4: the derived I\textsubscript{Ca}-mediated CICR Ca\textsuperscript{2+} transients. B: the bar graph of integrals of both I\textsubscript{Ca} (light gray) and I\textsubscript{Ca}-mediated CICR (I\textsubscript{Ca}-CICR; dark gray) Ca\textsuperscript{2+} transient on depolarization as a function of age. I\textsubscript{Ca}-CICR Ca\textsuperscript{2+} transient significantly increased with age (***P < 0.0001 for either 3d or 10d vs. either 20d or 56d); differences in the I\textsubscript{Ca} Ca\textsuperscript{2+} transient did not reach a level of significance except for 3d vs. 56d (†P < 0.05). n = 12.
magnitude of the Ca\(^{2+}\) transient in fetal rat myocytes by thapsigargin in both the subsarcolemma and the cell center. These apparent differences may result from differences in methodology and/or animal species used.

\(I_{\text{Ca}}\) and \(I_{\text{Ca-CICR}}\) play an increasingly important role with development. Here we report that Nif reduces the Ca\(^{2+}\) transient by more than 90\% in 56d myocytes (Fig. 3A) and the transsarcolemmal Ca\(^{2+}\) influx is mainly \(I_{\text{Ca}}\) dependent (\(I_{\text{Ca}}\) was \(\sim\)30\%), whereas the \(I_{\text{Ca-CICR}}\) was attributed to \(\sim\)70\% of total Ca\(^{2+}\) transient (Fig. 4B), which was also supported by the measurement of the peak of the Ca\(^{2+}\) transient (Fig. 2, B and D). This is consistent with the results reported in the adult rabbit heart by others (11, 18). In contrast, Nif only reduced the Ca\(^{2+}\) transient by \(\sim\)20\% in 3d myocytes (Fig. 3), and the contributions of \(I_{\text{Ca}}\) (\(\sim\)15\%) and \(I_{\text{Ca-CICR}}\) (\(\sim\)5\%) were much smaller than those in 56d myocytes. Thus, \(I_{\text{Ca}}\) does not play a major role in neonatal rabbit E-C coupling, and this is also consistent with other reports (21, 22, 24, 25, 58). The increasing functional importance of \(I_{\text{Ca}}\) and \(I_{\text{Ca-CICR}}\) with development is commensurate with the increasing density of \(I_{\text{Ca}}\) and the higher degree of colocalization between the L-type Ca\(^{2+}\) channel and RyR found in previous studies (10, 46, 47).

\(NCX\) and \(NCX-CICR\) play an important role in the early developmental stages. The role of NCX and NCX-CICR has been extensively investigated in adult ventricular myocytes of several species (7, 29–31, 39, 50, 56, 57). Although NCX-CICR has been observed under certain experimental conditions (39), it is generally agreed that the role of reverse-mode NCX is negligible under physiological condition in most mammalian species (7, 33, 50), with guinea pig ventricular myocytes (29) and possibly some other species as the exception. NCX-CICR in the neonate has not been investigated as much as in the adult cell, presumably due to the general consensus of nonfunctional SR in the neonatal heart (28, 37). In the present study, we found that the percentage of the total Ca\(^{2+}\) transient attributable to NCX and NCX-CICR was \(\sim\)43\% and \(\sim\)37\%, respectively, in 3d myocytes (Figs. 4 and 5), indicating that NCX and NCX-CICR play a significant role in E-C coupling in the earliest developmental stage. It is also supported by the measurement of the peak value of the Ca\(^{2+}\) transient (supplementary data Fig. 2C). The significant portion of the Ca\(^{2+}\) influx via reverse-mode NCX in the early developmental stages in the present study is consistent with other reports (15, 58) and is in accordance with the higher expression and activity of NCX in the neonatal heart (6, 19, 43). The presence of NCX-CICR in the early developmental stages is likely to relate to the greater NCX expression level, longer action potential, and reduced Ca\(^{2+}\) contribution from \(I_{\text{Ca}}\) and \(I_{\text{Ca-CICR}}\). Other important factors may be the microdomain geometry where NCX and RyR2 reside as well as the proximity between the two proteins. As expected (data not shown) and as reported in our previous studies, both the cell surface area as determined...
Fig. 6. I_{Ca-CICR} in older age groups is more efficient than NCX-CICR in the younger age groups. A: the superimposition of Ca^{2+} transients of I_{Ca-CICR} in 56d myocytes vs. NCX-CICR in 3d myocytes on depolarization. Time to peak is indicated by arrow. B: the bar graph of TTPs for Con (black), KB-R-sensitive (white), and Nif-sensitive (gray) Ca^{2+} transients as a function of age. TTP significantly decreased with age in Con group (*P < 0.05 for 3d vs. 10d and 20d vs. 56d; ‡‡‡P < 0.0001 for other pairs). However, the TTPs of KB-R-sensitive and Nif-sensitive Ca^{2+} transients did not reach a significant level except that of Nif-sensitive Ca^{2+} transient between 3d and 56d myocytes (‡P < 0.05). n = 22 C: the bar graphs of the CICR efficiencies as a function of age. The efficiencies of NCX-CICR (gray) significantly decreased with age. (‡‡P < 0.01 for 3d vs. 10d, †††P < 0.0001 either 3d or 10d vs. either 20d or 56d). Conversely, the efficiency of I_{Ca-CICR} (black) significantly increased with age (***P < 0.0001 for either 3d or 10d vs. either 20d or 56d), n = 12.

The efficiency and gain of CICR. The Ca^{2+} transient produced by I_{Ca-CICR} in the older age groups exhibits a rapid upstroke compared with that produced by NCX-CICR in the younger age groups (TTP 110 ms in 56d vs. 380 ms in 3d) (Fig. 6). As a result, the efficiencies to induce CICR (AF/s) were much greater for I_{Ca} than for NCX (~7.5 for I_{Ca-CICR} in 56d vs. ~1.0 for NCX-CICR in 3d). The value for adult cells is comparable to that reported by others in adult guinea pig ventricular myocytes (efficiency 1.9 for NCX-CICR and 8.8 for I_{Ca-CICR} and a TTP of 120 ms) (50). Moreover, a slow upstroke was unmasked in adult cells after inhibition of I_{Ca} or the depletion of SR Ca^{2+}, indicating that I_{Ca}-CICR through a mature T-tubular network is required for fast and synchronized Ca^{2+} release (8, 47). In contrast, the lack of T-tubules in neonatal myocytes and a 100–1000× slower Ca^{2+} unitary influx rate through NCX (3, 17) (as compared with I_{Ca}) results in a lower efficiency of NCX-induced CICR and a slower upstroke of the Ca^{2+} transient.

Another parameter closely related to the efficiency to induce CICR is the CICR gain. This parameter represents the amplification of CICR and is classically calculated as the SR Ca^{2+} release divided by the Ca^{2+} trigger signal producing it. Different studies have reported CICR gain in different species, ranging from ~16 at a membrane potential of 0 mV, using peak flux rates in rat ventricle (59), to a gain of 3–8 in rabbit for fractional release measurements with a varied SR Ca^{2+} load (49). In both studies, the SR Ca^{2+} release fraction was derived from a mathematical integration of the Ca^{2+} transient and other Ca^{2+} flux parameters, such as SR Ca^{2+} pump uptake, I_{Ca}, SR Ca^{2+} leak, Ca^{2+} buffering, and sarcolemma SR Ca^{2+} load (49).
pump. Since there is limited knowledge about the developmental change in these parameters, we are unable to infer the SR Ca\(^{2+}\) release from the Ca\(^{2+}\) transient. Therefore, we chose to estimate CICR gain in the present study as the ratio of the cytosolic Ca\(^{2+}\) transient produced by SR Ca\(^{2+}\) release and the Ca\(^{2+}\) transient produced by its trigger, without consideration of potential differential Ca\(^{2+}\) buffering, etc. The CICR gain was also measured by the Ca\(^{2+}\) transient peak value of CICR divided by the peak value of its corresponding trigger, and it showed a similar result (supplementary data Fig. 2E). Consequently, the smaller gain for I\(_{\text{Ca}}\)-CICR in the 56d myocytes in the present study as compared with values by Wier et al. (59) and Shannon et al. (49) is likely due to different means of calculating gain.

Independent of the method used to calculate the CICR gain, the calculation is expected to depend on the influx rate of the Ca\(^{2+}\) trigger, the physical distance of the trigger and the RyR (1, 51), and the nature of the microdomain, as well as the SR Ca\(^{2+}\) content (14, 49). A larger SR Ca\(^{2+}\) load as well as a more restricted microdomain observed in the early developmental stage (19, 20) may be expected to favor a larger NCX-CICR gain in 3d myocytes. However, we observed a smaller NCX-CICR gain in 3d myocytes (~1) as compared with I\(_{\text{Ca}}\)-CICR gain in 56d myocytes (~3), suggesting that the larger Ca\(^{2+}\) influx rate for I\(_{\text{Ca}}\) (3, 17) and colocalization of the trigger and the RyR are more important determinants of CICR gain. Indeed, we observed increased I\(_{\text{Ca}}\)-CICR gain and decreased NCX-CICR gain with age (Fig. 5C), which is in accordance with an increased colocalization of the dihydropyridine receptor and the RyR (46, 47) and a decreased colocalization of the NCX and the RyR (10).

Considerations on the model and possible limitations. In the present study, we used 15 μM Nif and 10 μM KB-R, sequentially, to differentiate between the roles of L-type Ca\(^{2+}\) channels and reverse-mode NCX to elicit CICR. Although these compounds at the concentrations used in the present study may also affect some other ion channels, these effects are not expected to affect the conclusions of the study. Thus, Nif has been reported to affect potassium channels (13, 23), but K\(^+\) currents were eliminated by replacement with Cs\(^+\) and tetraethyl ammonium, making the presence of K\(^+\) currents unlikely. Cs\(^+\), on the other hand, has been reported to decrease the frequency of spontaneous Ca\(^{2+}\) release from the SR in skinned adult rat cardiac myocytes (26) and to prolong the rise time and decay of SR Ca\(^{2+}\) release without affecting its amplitude. Although we did not examine the effect of Cs\(^+\) on SR Ca\(^{2+}\) release, similar RyR gating properties have been reported in neonates and adults (41), and therefore it was assumed that there are no differential effects of Cs\(^+\) as a function of age.

Although the inhibitory effect of KB-R7943 on NCX is well established, this compound also affects other ion channels. In our study, its inhibitory effect on the L-type Ca\(^{2+}\) channel (2, 19) and its dependence on [Na\(^+\)] (38) could be of some concern. However, in the present study, KB-R is always used subsequent to L-type Ca\(^{2+}\) channel inhibition with Nif, and we have previously shown that 10 μM KB-R acts primarily as an inhibitor of reverse-mode NCX activity under our experimental conditions (2, 19). KB-R has also been reported to inhibit inositol 1,4,5-trisphosphate receptor (IP\(_3\))-mediated Ca\(^{2+}\) release from the endoplasmic reticulum in intact nonexcitable HeLa cells (45). However, it is generally accepted that IP\(_3\)R does not play a major role in cardiac E-C coupling in single isolated ventricular myocytes postpartum due to its very low concentration and the kinetics of IP\(_3\)-mediated SR Ca\(^{2+}\) release (35, 36, 44).

Finally, calcium buffering of the Ca\(^{2+}\) indicator fluo-3 AM may affect the kinetics of the calcium transients, and it has been reported that the intrinsic Ca\(^{2+}\) buffering capacity is two to three times lower in neonatal ventricular myocytes derived from ventricular homogenates and permeabilized ventricular myocytes (4). However, the present study is based on comparisons of pharmacological manipulations within the same cell where the intrinsic Ca\(^{2+}\) buffering capacity or buffering by fluo-3 is likely to remain constant.

Conclusions. In the present study, we report that SR Ca\(^{2+}\) release is an important component of E-C coupling (~40%), even at the earliest neonatal stage, and that it plays an increasingly important role with ontogeny. In the early developmental stage, SR Ca\(^{2+}\) release was predominantly triggered by trans-subasrolemmal Ca\(^{2+}\) influx via reverse-mode NCX producing a slow rise of the calcium transient peaking ~380 ms after depolarization. In contrast, predominance of high-gain, high-efficiency I\(_{\text{Ca}}\)-induced CICR at later age stages produced a rapidly rising calcium transient peaking at ~110 ms. We conclude that the less efficient peripheral NCX-mediated CICR is the predominant mode in the earliest developmental stages, which gradually disappears as the more efficient L-type Ca\(^{2+}\) channel-induced CICR increases in prominence with development.

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