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Na+/Ca2+ exchange activity in neonatal rabbit ventricular myocytes

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1Cardiac Membrane Research Laboratory, Simon Fraser University, Burnaby, British Columbia; 2Cardiovascular Sciences, BC Research Institute for Children’s and Women’s Health, Vancouver, British Columbia, Canada; and 3Laboratorio de Fisiología Celular, Servei de Cardiología, Hospital de Sant Pau, Barcelona, Spain

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Huang, Jingbo, Leif Hove-Madsen, and Glen F. Tibbits. Na+/Ca2+ exchange activity in neonatal rabbit ventricular myocytes. Am J Physiol Cell Physiol 288: C195–C203, 2005. First published August 16, 2004; doi:10.1152/ajpcell.00183.2004.—Much less is known about the contributions of the Na+/Ca2+ exchanger (NCX) and sarcoplasmic reticulum (SR) Ca2+ pump to cell relaxation in neonatal compared with adult mammalian ventricular myocytes. Based on both biochemical and molecular studies, there is evidence of a much higher density of NCX at birth that subsequently decreases during the next 2 wk of development. It has been hypothesized, therefore, that NCX plays a relatively more important role for cytosolic Ca2+ decline in neonates as well as, perhaps, a role in excitation-contraction coupling in reverse mode. We isolated neonatal ventricular myocytes from rabbits in four different age groups: 3, 6, 10, and 20 days of age. Using an amphotericin-perforated patch-clamp technique in fluo-3-loaded myocytes, we measured the caffeine-induced inward NCX current (I_{NCX}) and the Ca2+ transient. We found that the integral of I_{NCX}, an indicator of SR Ca2+ content, was greatest in myocytes from younger age groups when normalized by cell surface area and that it decreased with age. The velocity of Ca2+ extrusion by NCX (V_{NCX}) was linear with [Ca2+]i and did not indicate saturation kinetics until [Ca2+]i reached 1–3 μM for each age group. There was a significantly greater time delay between the peaks of I_{NCX} and the Ca2+ transient in myocytes from the youngest age groups. This observation could be related to structural differences in the subsarcolemmal microdomains as a function of age.

ontogeny of cardiac excitation-contraction coupling; sodium/calcium exchanger; cytosolic calcium concentration; subsarcolemmal calcium concentration; sarcoplasmic reticulum calcium content

IT IS COMMONLY ACCEPTED that the relatively small amount of Ca2+ that enters via the activation of L-type Ca2+ channels triggers Ca2+ release from the sarcoplasmic reticulum (SR) in a process known as Ca2+ -induced Ca2+ release in adult mammalian myocytes (12, 20, 32). The resultant rapid rise in intracellular Ca2+ concentration ([Ca2+]i) in the cytosol allows Ca2+ to bind to the thin myofilament protein troponin C, causing cell contraction. During relaxation, the prominent mechanisms involved in the decline of [Ca2+]i and responsible, therefore, for mechanical relaxation are the SR Ca2+ pump (SERCA2A) and the sarcolemmal Na+/Ca2+ exchanger (NCX). The sarcolemmal Ca2+/ATPase and mitochondrial Ca2+ uniporter contribute to a much lesser degree to the decline of [Ca2+]i, and collectively have been referred to as the slow removal system. The relative contributions of these transporters have been investigated extensively in adult mammalian ventricular myocytes and show species variation (5–8, 33, 43). In adult rabbits, values of 28, 70, and 2% have been attributed to the NCX, SERCA2A, and slow removal system, respectively, toward the decline of [Ca2+]i (5).

However, much less is known about the roles of these Ca2+ transporters during cardiac development. Several studies have indicated that NCX mRNA and protein levels as well as peak NCX current (I_{NCX}) were two to six times higher at birth compared with adult values (2, 3, 14, 24). Therefore, it has been suggested that the function of NCX in neonate cardiomyocytes is relatively more important than that in adults. For example, in a few recent studies (3, 6, 60), investigators using field stimulation and analysis of the decay of the Ca2+ transient have shown that NCX plays a relatively more substantial role in extrusion of Ca2+ in intact immature ventricular myocytes. However, it is relatively difficult to address the quantitative impact on the function of a living neonate myocyte. The rate of NCX transport depends on the subsarcolemmal [Na+] and [Ca2+], which are difficult, if not impossible, to directly measure. Furthermore, these values are influenced to a large degree by the spatial architecture of the dyadic junction between the transverse (T) tubules and the junctional SR. Recently, a considerable amount of effort has been focused on the “fuzzy space” in the adult cardiomyocyte. However, with an absence of T tubules in the neonate cardiomyocytes (up to ~10 days of age), the nature of the fuzzy space is even more poorly understood in the immature heart.

In this study, using the whole cell perforated patch-clamp technique and [Ca2+]i measurements, we attempted to analyze the relationship between NCX activity and subsarcolemmal [Ca2+] in the immature heart and to determine how it changes during ontogeny.

METHODS

Isolation of ventricular myocytes. Ventricular myocytes were isolated from the hearts of New Zealand White rabbits (of either sex) from four distinct age groups [3 (3d), 6 (6d), 10 (10d), and 20 (20d) days postpartum] by using a method previously described (50). In brief, after the rabbits were intraperitoneally injected with pentobarbital sodium (65 mg/kg body wt) and heparin (15 mg/kg body wt), the hearts were rapidly excised in 4°C Ca2+-free solution. The hearts were perfused in the Langendorff mode, first with collagenase (Yakult, Tokyo, Japan) and then with protease (Sigma), at age-appropriate concentrations and perfusion speeds. The ventricles were subsequently removed and chopped into small pieces in the storage solution and washed twice using the storage solution.

Because the hearts from the different age groups varied considerably in terms of their weight, collagen content, and other characteristics, different protocols had to be developed to isolate Ca2+-tolerant myocytes from the different age groups. These differences included

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Table 1. Parameters for neonate myocyte isolation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Age, days</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase concentration, mg/ml</td>
<td></td>
<td>0.06</td>
<td>0.10</td>
<td>0.15</td>
<td>0.15–0.20</td>
</tr>
<tr>
<td>Collagenase volume, ml</td>
<td></td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10–20</td>
</tr>
<tr>
<td>Protease concentration, mg/ml</td>
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<td>0.07</td>
<td>0.10</td>
<td>0.10</td>
</tr>
<tr>
<td>Protease volume, ml</td>
<td></td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>15.0</td>
</tr>
<tr>
<td>Perfusion speed, ml/s</td>
<td></td>
<td>1.8</td>
<td>1.8</td>
<td>1.8</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Variations in the concentration and volume of collagenase, the concentration and volume of protease, and the perfusion pump speed. The details of these differences are shown in Table 1.

Both collagenase and protease solutions contained 1% bovine serum albumin (BSA). The Ca$^{2+}$-free solution contained (in mM) 100 NaCl, 10 KCl, 1.2 KH$_2$PO$_4$, 5 MgSO$_4$, 50 tauroine, 20 glucose, and 10 HEPES and was adjusted with NaOH to pH 7.2 at room temperature. The storage solution contained (in mM) 120 Cs$_2$SO$_4$, 5 MgCl$_2$, 20 tauroine, 1 EGTA, 10 glucose, and 10 HEPES and was adjusted with KOH to pH 7.3–7.4 at room temperature.

Whole cell perforated-patch voltage clamp. Isolated myocytes were allowed to settle on the bottom of a recording chamber mounted on the stage of an inverted microscope (Nikon TE 200) and were superfused with the standard external solution. Ionic currents were measured using the whole cell perforated-patch voltage-clamp technique with an Axopatch 200B patch amplifier (Axon Instruments, Foster City, CA). Data acquisition and voltage protocols were controlled using pCLAMP software (version 8.0; Axon Instruments). Standard internal and external solutions were used to eliminate Na$^+$ and K$^+$ currents. The internal pipette solution contained (in mM) 110 CsCl, 5 Mg ATP, 1 MgCl$_2$, 20 tetraethylammonium (TEA), 0.025 EGTA, 5 sodium phosphocreatine, and 10 HEPES and was adjusted with CsOH to pH 7.1. The standard external solution contained (in mM) 115 NaCl, 20 CsCl, 1 MgCl$_2$, 1.8 CaCl$_2$, 5 sodium pyruvate, 10 glucose, and 10 HEPES and was adjusted with NaOH to pH 7.4. The perforated-patch configuration was used by including 300–400 µg/ml amphotericin B (water solubilized; Sigma) in the patch pipette. Patch pipettes were pulled from thin-walled filamented glass capillaries (ID 1.1 mm, OD 1.65 mm; World Precision Instruments, Sarasota, FL). The pipette resistance was 1.5–2.5 MΩ. The seal resistance was 2.0–20.0 GΩ, and access resistance decreased to <20 MΩ within 10–20 min after seal formation. Cells showing a sudden drop in access resistance were discarded. All measurements were determined at room temperature.

Experimental protocols. After seal formation, the cell was lifted from the bottom of the petri dish and placed in front of a quartz capillary tip (ID 200 µm) of a fast-switching perfusion device (QMM Micromanifold; ALA Scientific Instruments, Westbury, NY). A Marzhäuser MM-33 micromanipulator (Fine Science Tools, North Vancouver, BC, Canada) was used to position the quartz tip of the perfusion system within 100 µm of the cell. The gravity-fed solutions coming into the manifold were controlled by a custom-built rapid switching device that employed multiple Lee solenoids (model AA0501418H; Westbrook, CT), each of which was triggered by TTL pulses programmed within the pCLAMP experimental protocol. This allowed for the rapid application of the 10 mM caffeine that was added to the external bath solution used in the experiments for SR Ca$^{2+}$ release. In control experiments, either KB-R7943 (10–40 µM) or 4,4’-disothiocyanostilbene-2,2’-disulfonic acid (DIDS; 100 µM) was added to the caffeine solution to determine the ionic nature of the caffeine-induced current.

Measurement of [Ca$^{2+}$]$\text{_{i}}$. The [Ca$^{2+}$]$\text{_{i}}$ was measured with the fluorescent Ca$^{2+}$ indicator fluo-3 acetoxyethyl ester (fluo-3 AM; Molecular Probes, Eugene, OR). Cells were incubated with 5–10 µM fluo-3 AM for 30–40 min at room temperature and then washed once using the storage solution. Cells were kept in the storage solution for 1 h before experiments were started. Fluo-3 was excited at 488 nm, and fluorescence emission was measured using a 530-nm band-pass filter. The band-pass width was ±20 nm. We used a Fluorescence System Interface device (Ionoptix, Milton, MA) to record the output from photon-counting photomultiplier tubes. A sampling interval of 10 ms was used to achieve a reasonable signal-to-noise ratio. Fluorescence signals were captured unfiltered but displayed after filtering with a low-pass Butterworth filter with a cutoff frequency of 10 Hz. The [Ca$^{2+}$]$\text{_{i}}$ was calculated from the formula [Ca$^{2+}$]$\text{_{i}}$ = $(F_{\text{Fmax}} - F) / (F_{\text{Fmin}} - F)$. F, where F$_{\text{Fmin}}$ is the background fluorescence determined from a cell-free area and F$_{\text{Fmax}}$ is the fluorescence acquired after the cell was depolarized to +150 mV for 10–20 s to maximize [Ca$^{2+}$]$\text{_{i}}$ and kill the cell at the end of each experiment. Ka is the Ca$^{2+}$ dissociation constant with fluo-3, and a value of 400 nM was used for all age groups (34, 55). It is well recognized that the concentration of Ca$^{2+}$ indicator dyes such as fluo-3 can influence the results, particularly if the myocyte is overloaded with the dye and constitutes a significant source of cytosolic Ca$^{2+}$ buffering. Overloading of the cell by fluo-3 can introduce artifacts in both the kinetics and magnitude of the Ca$^{2+}$ transient and the subsequent contractile properties. In the present study, care was taken not to overload the cell by observing the Ca$^{2+}$ transient and contractile kinetics and amplitude in response to both step depolarizations and caffeine application. Cells in which the amplitude of the contraction was too small in response to these conditions were rejected. Typically, under control conditions, myocytes contracted by ~10% of their resting length when depolarized to +10 mV and by ~25% of their resting length when exposed to caffeine.

Data analysis. Data are presented as means ± SE. Curving fitting was carried out using the Origin 6.0 software (Microcal Software, Northampton, MA). Statistical significance of the results was tested using one-way ANOVA (SPSS 11.0) or Student’s t-test for paired or unpaired samples. Post hoc tests were performed using Tukey’s multiple comparisons. A P value <0.05 was considered to be significant.

RESULTS

Developmental changes in body and cell parameters. As expected and as shown in Table 2, body weight, cell length, and total cell membrane capacitance ($C_m$) (and therefore cell surface area) all increased significantly with age. There was a significant difference in body weight among all groups except for the 3d vs. 6d groups ($P < 0.05$ for 6d vs. 10d, $P < 0.001$ for other pairs). The $C_m$ of the 20d group was significantly different from that of all other groups ($P < 0.0001$), and there was a significant difference as well between the 10d and 3d groups ($P < 0.005$); however, there were no significant differ-

Table 2. Developmental changes in rabbit cardiac ventricular myocytes

<table>
<thead>
<tr>
<th>Age, days</th>
<th>3</th>
<th>6</th>
<th>10</th>
<th>20</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of rabbits</td>
<td>17</td>
<td>15</td>
<td>10</td>
<td>18</td>
</tr>
<tr>
<td>Weight, g</td>
<td>79.5±5.4†</td>
<td>106±2.8°</td>
<td>158.4±17.1*</td>
<td>340.9±15.1*</td>
</tr>
<tr>
<td>No. of myocytes</td>
<td>40</td>
<td>35</td>
<td>31</td>
<td>40</td>
</tr>
<tr>
<td>Length, µm</td>
<td>52.8±1.9*</td>
<td>67.8±3.5†</td>
<td>74.2±3.6</td>
<td>93.2±2.7</td>
</tr>
<tr>
<td>$C_m$, pf</td>
<td>13.3±0.6</td>
<td>14.8±0.6</td>
<td>17.1±0.6</td>
<td>28.8±0.9*</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$\text{_{i}}$, rest, nM</td>
<td>141±3</td>
<td>149±2</td>
<td>148±2</td>
<td>143±2</td>
</tr>
<tr>
<td>[Ca$^{2+}$]$\text{_{i}}$, peak, µM</td>
<td>1.2±0.1</td>
<td>1.1±0.1</td>
<td>1.7±0.3</td>
<td>1.7±0.2</td>
</tr>
</tbody>
</table>

$C_m$ membrane capacitance; [Ca$^{2+}$]$\text{_{i}}$, intracellular Ca$^{2+}$ concentration. †Significantly different from all other groups. ‡Significantly different from two other groups. ††Significantly different from one other group.
ences for the 6d vs. 3d and 6d vs. 10d groups. Cell length showed significant differences among all age groups except for the 6d vs. 10d groups. Resting [Ca^{2+}]i, and peak [Ca^{2+}]i, induced by caffeine application were not significantly different among groups even though there was a tendency toward a higher level of peak [Ca^{2+}]i in the older age groups.

SR Ca^{2+} loading, SR Ca^{2+} content, and velocity of Ca^{2+} extrusion of NCX. In these experiments, SR Ca^{2+} content was estimated using the integral of the caffeine-induced current. Figure 1A shows the experimental protocol. The first 10 mM caffeine application was used to clear the SR Ca^{2+} content. Afterward, 20 repetitive depolarizations (1 Hz) were used to reload the SR with Ca^{2+}. For each repeat, the cell was held at a potential of −80 mV, first depolarized to +40 mV for 200 ms, and then depolarized to +10 mV for 400 ms. Subsequently, a second application of 10 mM caffeine was used to evaluate the amount of SR Ca^{2+} reloading. Figure 1B shows representative traces of the experimental protocol in a myocyte from a 3d rabbit: the solid trace (top) is the [Ca^{2+}]i, calculated from the fluorescence transient, and the shaded trace (bottom) is the inward NCX current (I_{NCX}). With an NCX stoichiometry of 3:1 (Na^{+}:Ca^{2+}) (10, 11, 20, 41, 46), the integral of second caffeine-induced inward I_{NCX} was used to measure SR Ca^{2+} content with the implicit assumption that the contribution of the “slow Ca^{2+} removal system” was negligible. Figure 1C is a histogram of the SR Ca^{2+} content for each age group, normalized by membrane surface area. The highest value was observed in the 3d group and was more than twofold greater than in the 20d group. There were significant differences for the 3d vs. 10d (P < 0.05) and 3d vs. 20d groups (P < 0.001).

Because of the possibility that the caffeine-induced elevation in [Ca^{2+}]i could induce a Ca^{2+}-activated Cl− current (I_{CL,ca}), which might introduce artifacts in our determination of SR Ca^{2+} content, we included 100 μM DIDS with the caffeine in control experiments. In these experiments, we calculated Cl−-equilibrium to be approximately −5 mV, and thus, at a holding potential of −80 mV, if such a current were to be activated, it would result in a Cl− efflux or an inward I_{Cl} that potentially could overestimate our measured I_{SNCX} integral, which was used as an index of SR Ca^{2+} content. The addition of 100 μM DIDS did, in fact, reduce the integral of the caffeine-induced inward currents by a slight amount (7 ± 2 and 8 ± 3% in 3d and 20d rabbit cells, respectively; n = 6 for each group). This DIDS-sensitive current inactivated rapidly relative to the total current, and thus the time to peak inward DIDS-insensitive current was prolonged slightly compared with the total current. The time difference of the peaks between the caffeine-induced currents in the absence of DIDS and in the DIDS-insensitive group was 24.5 ± 2.7 and 287 ± 17 ms in 3d and 20d rabbit cells, respectively. These differences were not significant and were not included in the data analysis.

Time course between peaks of I_{NCX} and [Ca^{2+}]i. There was a significant time delay between the peak of I_{SNCX} and the peak of [Ca^{2+}]i, as shown in Fig. 2A and B (B shows the same data in A with an expanded time scale), in a 3d myocyte. The solid line represents the inverted I_{NCX}, and the shaded line reflects the [Ca^{2+}]i, calculated from Ca^{2+} transient induced by caffeine. In this representative experiment record, the peak of I_{SNCX} preceded the peak of [Ca^{2+}]i by ∼140 ms. In all instances, the rise of Ca^{2+} extrusion of NCX (V_{SNCX}) significantly preceded that of [Ca^{2+}]i. As shown in Fig. 2C, there was a significant decrease in the time delay between the two peaks as a function of age. For example, the mean time delay in the 3d group was 287 ± 64 ms, which was more than fourfold that observed in the 20d group (64 ± 13 ms), and the difference was highly significant (P < 0.0001). This substantially longer time delay observed in the 3d group would also result in a greater underestimation of NCX function when expressed as the slope of V_{SNCX} as a function of [Ca^{2+}]i. The time delay between peaks was highly correlated with SR Ca^{2+} content in younger age groups, especially the 3d group (R^2 = 0.87, 0.59, and 0.57 for the 3d, 6d, and 10d groups, respectively; P < 0.01). There was no significant correlation between time delay and SR Ca^{2+} content in the 20d group (R^2 = 0.32). In the SR Ca^{2+} content range of 25–40 amol/pF, the time delay was 183 ± 40 and 70 ± 17 ms for the 3d and 20d groups, respectively, and this difference was highly significant (P = 0.003; n = 20), despite the fact that the SR Ca^{2+} content was not significantly different (29.4 ± 1.3 and 27.5 ± 0.9 amol/pF in 3d and 20d groups, respectively).

V_{SNCX} as a function of [Ca^{2+}]i. Figure 3A shows an entire representative linear regression fitting of V_{SNCX} as a function of [Ca^{2+}]i, taken from the caffeine-induced Ca^{2+} transient in a 20d rabbit ventricular myocyte. The open and shaded circles in
However, the \([Ca^{2+}]\) measured by fluo-3 reflects largely the bulk-phase cytosolic \([Ca^{2+}]\) \(([Ca^{2+}]_{\text{SM}})\). It is therefore likely that the \(V_{\text{NCX}}\) driven by the \([Ca^{2+}]_{\text{SM}}\) in fact, was much larger than one would predict from the \([Ca^{2+}]_{\text{SM}}\) measured during the caffeine-induced contracture. If one assumes that the linear relationship between \(V_{\text{NCX}}\) and \([Ca^{2+}]\) is maintained in the subsarcolemmal space as well and that the slope of the linear regression is the same as that measured during caffeine application (caffeine slope), then one can estimate the \([Ca^{2+}]_{\text{SM}}\).

For example, in Fig. 3A, for the given \(V_{\text{NCX}}\) as indicated by the arrow \((\sim 20 \text{ amol} \cdot \text{s}^{-1} \cdot \text{pF}^{-1})\), the \([Ca^{2+}]_{\text{SM}}\) was calculated to be \(-2 \mu M\), whereas \([Ca^{2+}]\) was determined to be \(-0.5 \mu M\). From this relationship and as shown in Fig. 3C, the peaks of

![Figure 2](image_url)

Fig. 2. Time courses of both \(I_{\text{NCX}}\) and \([Ca^{2+}]\). A: the solid line represents \(I_{\text{NCX}}\) (inverted), and the shaded line represents the \([Ca^{2+}]\) in a 3d myocyte. In this representative trace, the peak \(I_{\text{NCX}}\) precedes the peak \([Ca^{2+}]\) by \(-140 \text{ ms}\). B: expanded time scale showing same data in A. C: histogram showing the time delay between the peaks of \(I_{\text{NCX}}\) and \([Ca^{2+}]\) for each age group. There is a more than fourfold difference in values for the 3d and 20d groups \((*P < 0.001)\), which is highly significant.

![Figure 3](image_url)

Fig. 3. \(V_{\text{NCX}}\) as a function of \([Ca^{2+}]\), and the estimation of subsarcolemmal \([Ca^{2+}]\) \(([Ca^{2+}]_{\text{SM}})\). A: an entire representative fitting trace between \(V_{\text{NCX}}\) and \([Ca^{2+}]\), taken from caffeine-induced \(Ca^{2+}\) transient in a 20d myocyte. Open and shaded circles represent the fitting traces taken from before and after the peak of the \(Ca^{2+}\) transient, respectively. The solid line represents the caffeine-induced linear regression slope (caffeine slope) of the fitting trace taken from the peak of the \(Ca^{2+}\) transient. The dotted line shows the magnitude of the \(NCX\) as indicated by the arrow \((\sim 20 \text{ amol} \cdot \text{s}^{-1} \cdot \text{pF}^{-1})\). B: histogram of the slope of \(V_{\text{NCX}}\) vs. \([Ca^{2+}]\) for each age group. There is a significant difference in values for the 20d group \((**P < 0.001)\) compared with each of the other groups. There is a significant difference in values for the 3d group \((**P < 0.05)\) compared with the 10d group. C: the peak of the inferred \([Ca^{2+}]_{\text{SM}}\) calculated using the caffeine slope and the peak of \(I_{\text{NCX}}\) for each age group. Statistically, there are no significant differences among values for the groups.
[Ca^{2+}]_{SM}$ calculated from the peak of $V_{NCX}$ and its caffeine slope were $1.95 \pm 0.25, 1.95 \pm 0.34, 2.04 \pm 0.42$, and $1.44 \pm 0.14 \mu M$ for the 3d, 6d, 10d, and 20d groups, respectively, and were not statistically different from each other.

$V_{NCX}$ in the presence of blockers of SR Ca^{2+} store function.

The driving forces for NCX during the contracture and relaxation periods are likely to be quite different even for a given $[Ca^{2+}]_i$. During the caffeine-induced contracture, one would predict that $[Na^{+}]_{SM}$ might increase as a consequence of Ca^{2+} extrusion by NCX. The effect of this would be to make the NCX reversal potential more negative and reduce the driving force for normal mode NCX activity. During normal twitches it is unlikely that $[Na^{+}]_{SM}$ accumulates to the same degree, if at all. Thus the slope of $V_{NCX}$ vs. $[Ca^{2+}]_i$ measured after the peak of the Ca^{2+} transient probably underestimates NCX function under physiological conditions.

To avoid this, we measured $V_{NCX}$ using the blockers of SR Ca^{2+} store function (SR blockers) cyclopiazonic acid (CPA; 25 $\mu M$), a blocker of SERCA2A, and ryanodine (Ry; 10 $\mu M$), which locks the ryanodine receptor (RyR) in a subconducting open state, in the 3d and 20d groups only ($n = 7$).

Figure 4A shows the protocol and representative traces of membrane current and fluo-3 fluorescence of a myocyte from a 20d rabbit. In the presence of SR Ca^{2+} blockers, the cell was depolarized to various voltages (+30 mV in the representative trace from a 20d myocyte shown in Fig. 4A) for 3s, which was intended to make the distribution of Ca^{2+} homogeneous between the subsarcolemmal and bulk cytosolic compartments by the end of depolarization. It was assumed, therefore, that at this point $[Ca^{2+}]_i$ equaled $[Ca^{2+}]_{SM}$. Neither caffeine-induced currents nor Ca^{2+} transients in response to both caffeine applications indicated that the SR Ca^{2+} store function was successfully blocked by those inhibitors. We assumed, in this case, that normal mode NCX activity (shown as a tail current represented by the shaded trace in Fig. 4A) induced by repolarizing the cell to $-80$ mV was entirely responsible for the Ca^{2+} transient decline (solid trace) and cell relaxation. Therefore, the relationship between the tail $I_{NCX}$ and $[Ca^{2+}]_{SM}$ (or $[Ca^{2+}]_i$) at each corresponding time (indicated by dotted lines) was investigated. Under these conditions, the relationship of $V_{NCX}$ with $[Ca^{2+}]_i$ was also linear. As speculated, the slope of $V_{NCX}$ vs. $[Ca^{2+}]_i$ dramatically increased compared with that observed in caffeine-induced contractures. Figure 4B clearly shows the slope of the linear regression determined in the presence of the SR blockers (blocker slope), and it was almost twice as great as the caffeine slope in both age groups (3d and 20d). There were

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**Fig. 4.** $V_{NCX}$ as a function of $[Ca^{2+}]_{SM}$. **A:** the protocol and a representative trace of this protocol (current, shaded trace; Ca^{2+} transient, solid trace) in a 20d myocyte. In the presence of 25 $\mu M$ cyclopiazonic acid (CPA) and 10 $\mu M$ ryanodine (Ry), the cell was depolarized to a given voltage (e.g., +30 mV in this experiment). Both caffeine applications were used to ensure the efficacy of the pharmacological block. $V_{NCX}$ was inferred by the tail $I_{NCX}$ induced by repolarizing the cell to $-80$ mV. The $[Ca^{2+}]_i$, at each corresponding time point (shown by dotted lines) was assumed to be the $[Ca^{2+}]_{SM}$ attributed to the Ca^{2+} homogeneous distribution between subsarcolemmal and bulk cytosolic compartments by the end of depolarization. There is a linear relationship between the $V_{NCX}$ and the $[Ca^{2+}]_{SM}$ as well, and the slope determined by linear regression in the presence of blockers is referred to as blocker slope. **B:** histogram of caffeine slope and blocker slope in 3d and 20d myocytes ($n = 7$). There is a significant difference between caffeine slope and blocker slope within each age group ($**P < 0.001$). There are significant differences for caffeine slope ($*P < 0.05$) and blocker slope ($**P < 0.01$), respectively, between the age groups.
significant differences between the caffeine slope and the blocker slope within each of the two age groups ($P < 0.001$). In addition, there were significant differences for caffeine slope ($P < 0.05$) and blocker slope ($P < 0.001$) between the 3d and 20d groups. The peaks of the $[\text{Ca}^{2+}]_i$, in caffeine-slope and blocker-slope measurements were $0.82 \pm 0.34$ and $0.87 \pm 0.24$ μM for the 3d group and $1.31 \pm 0.50$ and $0.99 \pm 0.30$ μM for the 20d group, respectively. Statistically, there were no significant differences in peak $[\text{Ca}^{2+}]_i$ between caffeine slope and blocker slope within each age group and no significant differences in caffeine slope or blocker slope between the two groups.

**DISCUSSION**

SR $\text{Ca}^{2+}$ content. The use of caffeine to release SR $\text{Ca}^{2+}$, resulting in the attendant inward $I_{\text{NCX}}$, is a tool that has been used frequently to determine SR $\text{Ca}^{2+}$ content in cardiac myocytes. The underlying assumption is that the caffeine-induced current is the sole or predominate current observed under these conditions. However, it is well known that elevated $[\text{Ca}^{2+}]_i$, can activate other channels, including those carrying K$^+$ and Cl$^-$. Given that Cs$^+$ was used to replace K$^+$ and 20 mM TEA was included in the pipette solutions, it is highly unlikely that a $\text{Ca}^{2+}$-induced K$^+$ current contaminated our results. $I_{\text{Cl,Ca}}$ have been demonstrated in rabbit (61) and mouse (59) ventricular myocytes. This current is blocked by both SITS and DIDS (for a recent review, see Ref. 28). In our experimental protocol, 100 μM DIDS reduced the integral of the caffeine-induced inward current slightly (~7%), but there were no developmental effects of this observation. We assume that the DIDS-sensitive current was $I_{\text{Cl,Ca}}$ and that it inactivated very rapidly relative to the total caffeine-induced current in myocytes from both 3d and 20d myocytes. Trafford et al. (54) found in adult ferret ventricular myocytes that caffeine induced both $I_{\text{NCX}}$ and $I_{\text{Cl,Ca}}$, but that the latter preceded $I_{\text{NCX}}$ by a significant amount, leading the authors to conclude that the $I_{\text{Cl,Ca}}$ channel “saw” a greater local increase in $[\text{Ca}^{2+}]_i$ as a result of greater proximity to RyR relative to that of NCX to RyR. Our data from immature rabbit myocytes over a range of developmental stages are virtually identical to the phenomenon observed in adult ferret myocytes, although the conclusion as to the microdomains of these transporters remains to be tested.

Further evidence that the caffeine-induced inward current is largely $I_{\text{NCX}}$ is our observation that the current was extremely sensitive to KB-R7943. We found that the decay of this current was best fit by a double exponential equation. For example, in 3d myocytes under control conditions, the fast and slow time constants of decay ($\tau$) were $198 \pm 18$ and $1,422 \pm 201$ ms, respectively ($n = 6$; data not shown). In the presence of 10 μM KB-R7943 (a dose thought to predominately block reverse mode $I_{\text{NCX}}$), the fast and slow $\tau$ were $405 \pm 237$ and $2,586 \pm 359$ ms, respectively, and were both significantly different from those of the control group ($P < 0.05$). The slowing of the caffeine-induced inward current by KB-R7943 was dose dependent, a dose of 40 μM virtually completely (>90%) blocked this current, and the $\text{Ca}^{2+}$ transient remained elevated for a prolonged time in all age groups (data not shown) under these conditions. Thus these observations support our premise that the caffeine-induced inward current was primarily (>90%) $I_{\text{NCX}}$ and that this parameter served as a reasonable estimation of SR $\text{Ca}^{2+}$ content in all age groups.

Contrary to the prevailing view in the literature, there was actually a greater amount of $\text{Ca}^{2+}$ stored in the SR, when normalized by cell surface area, in the younger age groups. The SR $\text{Ca}^{2+}$ content expressed in this manner was more than twofold larger in the 3d compared with the 20d group. It has been suggested that the neonatal mammalian ventricular myocytes have a relatively sparse and immature SR compared with that in adults on the basis of ultrastructural (26, 39, 40), biochemical (22, 40), and pharmacological studies (37, 38, 52). Therefore, it has been assumed that the SR in neonatal ventricular myocytes would not be able to store and release comparable amounts of $\text{Ca}^{2+}$ on a beat-to-beat basis compared with the adult. However, the observation that caffeine-induced $\text{Ca}^{2+}$ transients and contractures were robust and spatially homogeneous in neonate hearts was reported recently by others (3, 25, 35). Delbridge et al. (18) reported that in adult rabbit ventricular myocytes, the SR $\text{Ca}^{2+}$ content determined by caffeine-induced $I_{\text{NCX}}$ integral was $1,208 \pm 190$ amol; the mean steady-state SR $\text{Ca}^{2+}$ load was calculated to be $87 \pm 13$ μmol/l nonmitochondrial cytosolic volume. In the 20d group in the present study, the SR $\text{Ca}^{2+}$ content was 30 amol/pF.

Assuming a surface-to-volume ratio of 6.4 pF/pl nonmitochondrial cytosolic volume derived from the adult rabbit myocyte (47), the SR $\text{Ca}^{2+}$ content in the 20d group would be 180 μmol/l nonmitochondrial cytosolic volume, which is still twice that in steady state in adult myocytes. Balaguer et al. (3) reported that there were no significant differences in SR $\text{Ca}^{2+}$ load among the 1- to 2-day-old, 10- to 12-day-old, and adult rabbit groups when field stimulation was used. The different results could be due to the use of different methods. In our study, we used a whole cell perforated patch-clamp technique and were able to control the membrane potential, important for the study of NCX function. Because there is a higher caffeine-releasable $\text{Ca}^{2+}$ stored in the SR in the neonate compared with the adult, the question must be raised whether this $\text{Ca}^{2+}$ participates in excitation-contraction (E-C) coupling under physiological conditions. Haddock et al. (25) demonstrated that in neonatal rabbit ventricular myocytes, the $\text{Ca}^{2+}$ transient appeared to reflect mainly diffusion from the subsarcolemmal region to the myocyte center and did not seem to involve appreciable SR $\text{Ca}^{2+}$ release. Using blockers of L-type $\text{Ca}^{2+}$ channels and SR function, investigators in other studies (15, 16, 35, 51) have suggested that transsarcolemmal $\text{Ca}^{2+}$ fluxes play a larger role in the beat-to-beat regulation of cardiac contraction relative to the $\text{Ca}^{2+}$ released from SR in the developing mammalian heart. However, our laboratory group (50, 53) previously showed that in the neonate heart there is substantial colocalization of dihydropyridine receptor and RyR, albeit in the periphery of the cell, even in 3d myocytes. Indeed, in our experiments the $\text{Ca}^{2+}$ transient was reduced during twitches elicited by depolarization when SR blockers (CPA and Ry) were applied (data not shown), even in the 3d group. Therefore, we speculate that SR $\text{Ca}^{2+}$ release may be involved in E-C coupling in the neonate heart to a greater degree than previously realized but still less than that in the adult heart. Further experimentation is required to clarify this point.

**NCX function.** The linear relationship between $V_{\text{NCX}}$ and $[\text{Ca}^{2+}]_i$, after the peak of $[\text{Ca}^{2+}]_i$, observed in the present study...
has been reported in trout atrial myocytes (27) but is not consistent with other reports (5, 6, 11). In those studies, the investigators combined the integrated Ca\(^{2+}\) fluxes, Ca\(^{2+}\) passive buffering capacity, and Ca\(^{2+}\) transient and found a sigmoidal relationship between \(V_{NCX}\) and [Ca\(^{2+}\)]\(_i\) and they reported that \(V_{NCX}\) was saturated at \(\sim 600 \text{ nM [Ca}^{2+}\text{]}\). The maximum value of \(V_{NCX}\) (\(V_{\text{max}}\)) was found to be 46 and 27 \(\mu\text{mol-l cytosol}^{-1}\cdot\text{s}^{-1}\) for adult rabbits and rats, respectively.

In our study, \(V_{NCX}\) did not exhibit saturation even up to values of 1–3 \(\mu\text{M [Ca}^{2+}\text{]}\), and the peak of \(V_{NCX}\) (e.g., 26 \(\mu\text{mol-l cytosol}^{-1}\cdot\text{s}^{-1}\) in the 20d group, or 166 \(\mu\text{M-l cytosol}^{-1}\cdot\text{s}^{-1}\)) was much greater than the \(V_{\text{max}}\) reported in the previous studies from adults, but it is still much smaller than that reported in trout atrial myocytes (\(\sim 151 \mu\text{mol-l cytosol}^{-1}\cdot\text{s}^{-1}\)).

Rapid caffeine application indirectly disables the SR Ca\(^{2+}\) pump function by increasing RyR open probability. Therefore, the Ca\(^{2+}\) extrusion in the presence of caffeine is likely mediated by forward mode NCX, mitochondria Ca\(^{2+}\) uniporter, and Ca\(^{2+}\) pump on the sarcolemma. We assumed that NCX was the only contributor to the Ca\(^{2+}\) decline induced by application of caffeine because the slow removal system is thought to play a minor role in the Ca\(^{2+}\) decline compared with that of NCX (7, 8, 10, 33, 43). However, there have been some studies showing that in immature myocytes, the slow removal system may play a relatively more important role (4, 6, 36). In the present study, \(V_{NCX}\) was downregulated at least twofold from 3 to 20 days of age. This result is consistent with the two- to fourfold higher NCX density in newborn rabbits than in adult rabbits (1, 2, 14, 24, 31, 45, 57), based on immunoreactivity, mRNA and protein levels, and \(I_{NCX}\). Thus, we think that the higher \(V_{NCX}\) is due mainly to the higher NCX density in the neonate myocyte.

Balaguru et al. (3) reported that the time to 75% of cell relaxation for caffeine application in newborn rabbit myocytes (180 ± 20 ms) was not statistically different from that in adult rabbit myocytes (200 ± 30 ms). This discrepant result is possibly related to the fact that SR Ca\(^{2+}\) content was not significantly different between these age groups in their study. Bassani et al. (6) reported that the contribution of NCX to the relaxation declined from 24% to 5% from birth to adulthood in rat cardiomyocytes.

The slope of the linear regression of \(V_{NCX}\) as a function of [Ca\(^{2+}\)]\(_i\) determined in the presence of the SR blockers was almost twofold that measured after caffeine application. This result demonstrated that there was an underestimation of the slope determined using application of caffeine, which is likely due to subsarcolemmal Na\(^+\) accumulation (19, 23, 42, 58), a consequence of Ca\(^{2+}\) extrusion by NCX during the caffeine-induced contracture. In this contracture period, the relationship between NCX and Ca\(^{2+}\) is complicated by the fact that both Ca\(^{2+}\) and Na\(^+\) accumulate in the subsarcolemmal space. However, once the Ca\(^{2+}\) reaches a peak and starts to decline, it appears that \(V_{NCX}\) is linear with [Ca\(^{2+}\)]\(_{SM}\) even with the possibility that Na\(^+\) accumulated in this restricted microdomain. In vivo, the increased [Ca\(^{2+}\)]; and cell repolarization increase the driving force for forward mode NCX activity, which contributes to cell relaxation, a condition similar to that using the SR function blockers. Therefore, we believe that under physiological conditions, \(V_{NCX}\) is linear with Ca\(^{2+}\) and does not exhibit saturating kinetics even up to [Ca\(^{2+}\)]\(_{SM}\) levels of 1–3 \(\mu\text{M}\). On the basis of the higher capacity of NCX in forward mode in the neonate heart, we and others (30, 44) have speculated that NCX functions to a greater extent in reverse mode as well and may be an important source of Ca\(^{2+}\) in normal E-C coupling.

**Time delay of peaks of \(I_{NCX}\) and \([\text{Ca}^{2+}]_i\).** Trafford et al. (56) reported that there was a time hysteresis between the peak of \(I_{NCX}\) and Ca\(^{2+}\) transient induced by application of caffeine in adult rat ventricular myocytes. The time differential for this hysteresis was 133 ms, much longer than the 64 ms observed in the present study for the 20d group. This discrepancy could be due to species or age differences. In the 3d group there was a 286-ms time delay between the two peaks. Although the present study showed a positive correlation between SR Ca\(^{2+}\) content and the time delay, particularly in the youngest groups, age group time lag differences persisted even with the same SR Ca\(^{2+}\) load. The basis for this difference is not clear and appears to be counterintuitive, given the smaller diffusional distances in the myocytes from younger animals. We hypothesize that this significant discrepancy is likely based on developmental differences in the nature of the microdomain in which RyR and NCX exist. In a study by Haddock et al. (25), investigators using line scanning confocal microscopy found that in an adult rabbit ventricular cell, the Ca\(^{2+}\) transients were uniform across the width of the cell in response to electrical stimulation, whereas in the newborn, the Ca\(^{2+}\) transients were much greater at the periphery than at the cell center. However, in the same experiments, the Ca\(^{2+}\) distribution in response to caffeine appeared to be homogeneous in both age groups. The longer time delay and the higher \(V_{NCX}\) resulted in a significantly greater amount (50% of total in the 3d group and 17% of total in the 20d group) of Ca\(^{2+}\) already pumped out at the time of the peak of the Ca\(^{2+}\) transient (\(P < 0.005\)) in the 3d group. The Ca\(^{2+}\) left in the cytosol at that point in time was similar between the age groups (data not shown).

\([\text{Ca}^{2+}]_{SM}\). It is very difficult to directly measure [Ca\(^{2+}\)]\(_{SM}\) or [Ca\(^{2+}\)] in other restricted microdomains. In recent years some investigators have tried to predict the [Ca\(^{2+}\)]\(_{SM}\) based on \(I_{NCX}\) (56, 58). We used a similar approach and calculated the [Ca\(^{2+}\)]\(_{SM}\) based on the linear regression of \(V_{NCX}\) as a function of [Ca\(^{2+}\)]. As shown by these other groups in the adult myocyte and in our study of neonates, [Ca\(^{2+}\)]\(_{SM}\) is at least fourfold [Ca\(^{2+}\)].

**Limitations of study.** In our present study, we used the same fluo-3 Ca\(^{2+}\) dissociation constant (\(K_d\)) of 400 nM for all age groups. Because the \(K_d\) is protein dependent, it is not clear whether \(K_d\) would be differentially affected during development (9). In addition, we normalized \(V_{NCX}\) by cell surface (pF) instead of cell volume (pl). Because there is likely to be a higher surface-to-volume ratio in younger age groups (24, 26), the effect of this assumption would be to underestimate \(V_{NCX}\) in the younger age groups.

It should be pointed out that all of the experiments were performed in the presence of Cs\(^+\) and at 23°C, which is considerably below the physiological core temperature observed in rabbits (−38°C). K\(^+\) is commonly replaced by Cs\(^+\) in the solutions for experiments of this nature to reduce the contaminating effects of an outward K\(^+\) current. Despite the prevalence of this protocol, its potential effect cannot be ignored. Kawai et al. (29), for example, using saponin-skinned adult rat cardiomyocytes, demonstrated that the frequency of spontaneous SR Ca\(^{2+}\) release was significantly reduced when K\(^+\) was substituted with Cs\(^+\). This observation was not exam-
ined in the present study, but if we assume that there is no differential effect of Cs\(^+\) as a function of ontogeny, then our conclusions remain unchanged. Although the amplitude of both spontaneous and caffeine-induced SR Ca\(^{2+}\) release was only slightly affected by Cs\(^+\), the rate of release was slowed.

Although experiments on myocytes are frequently done at less than physiological temperatures to preserve myocyte function, one cannot ignore the impact of these experimental conditions on the derived results. We have determined that the Q\(_{10}\) of the cloned and expressed mammalian NCX1.1 (including both peak and steady state) is 2.4–2.6 (21). Use of a value of 2.5 would imply that the absolute value of V\(_{\text{NCX}}\) (in amol-s\(^{-1}\)-pF\(^{-1}\)) determined at 23°C would be approximately threefold less than that expected at physiological temperatures, all other things being equal. However, the higher I_NCX values expected at 37°C might also result in higher [Na\(^+\)]_SM affecting both the driving force and inactivation kinetics, thereby reducing the differences in V\(_{\text{NCX}}\) between the temperatures. Thus it is difficult to extrapolate. Because there is no evidence that isoforms other than NCX1.1 are expressed during ontogeny, we expect and assume that these are the same for all age groups. We believe, therefore, that although there might be quantitative differences if the experiments were conducted at higher temperatures, the conclusions nevertheless would remain the same.

In conclusion, in this study we found that there was a significantly greater SR Ca\(^{2+}\) content, when normalized per unit cell surface area, in younger age groups and that the SR can be rapidly reloaded with Ca\(^{2+}\). We also found that V\(_{\text{NCX}}\) is linear with [Ca\(^{2+}\)] during relaxation and did not exhibit any indication of saturation up to 1–3 \(\mu\)M [Ca\(^{2+}\)]. V\(_{\text{NCX}}\) decreased at least twofold in the period from 3 to 20 days of age, and this was likely due to a downregulation of NCX density during this period. Finally, there was a significantly greater time delay between the peak of I_NCX and the peak of [Ca\(^{2+}\)]\(_{\text{cyt}}\), in the myocytes from immature hearts, and this may be a reflection of developmental differences in subsarcolemmal microdomains. Although the subcellular localization of NCX relative to RyR has not been studied in detail in the developing heart, some inferences can be made from both the present and previous studies. Our group (49) previously found that the RyR are aligned along the Z lines at the earliest stages postpartum that were examined (3 days old) in a manner not different from that observed in adult myocytes. With respect to NCX, two previous studies (17, 25) demonstrated that NCX was distributed in the sarcolemma near the Z lines. Although colocalization of these proteins in adult rabbit myocytes is questionable (48), this possibility is supported to some degree by the data in the present study and deserves to be examined further using three-dimensional microscopy.

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