Subcellular $[\text{Ca}^{2+}]_i$ Gradients During Excitation-Contraction Coupling in Newborn Rabbit Ventricular Myocytes

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Abstract—The central role of T-tubule and sarcoplasmic reticulum (SR) diadic junctions in excitation-contraction (EC) coupling in adult (AD) ventricular myocytes suggests that their absence in newborn (NB) cells may manifest as an altered EC coupling phenotype. We used confocal microscopy to compare fluo-3 $[\text{Ca}^{2+}]_i$, transients in the subsarcolemmal space and cell center of field-stimulated NB and AD rabbit ventricular myocytes. Peak systolic $[\text{Ca}^{2+}]_i$, occurred sooner and was higher in the subsarcolemmal space compared with the cell center in NB myocytes. In AD myocytes, $[\text{Ca}^{2+}]_i$ rose and declined with similar profiles at the cell center and subsarcolemmal space. Disabling the SR (10 $\mu\text{mol/L}$ thapsigargin) slowed the rate of rise and decline of $\text{Ca}^{2+}$ in AD myocytes but did not alter $\text{Ca}^{2+}$ transient kinetics in NB myocytes. In contrast to adults, localized SR $[\text{Ca}^{2+}]_i$ release events (“$\text{Ca}^{2+}$ sparks”) occurred predominantly at the cell periphery of NB myocytes. Immunolabeling experiments demonstrated overlapping distributions of the Na$^+$/Ca$^{2+}$ exchanger and ryanodine receptors (RyR2) in AD myocytes. In contrast, RyR2s were spatially separated from the sarcolemma in NB myocytes. Confocal sarcolemmal imaging of di-8-ANEPPS–treated myocytes confirmed an extensive T-tubule network in AD cells, and that T-tubules are absent in NB myocytes. A mathematical model of subcellular $\text{Ca}^{2+}$ dynamics predicts that $\text{Ca}^{2+}$ flux via the Na$^+$/Ca$^{2+}$ exchanger during an action potential can account for the subsarcolemmal $\text{Ca}^{2+}$ gradients in NB myocytes. Spatial separation of sarcolemmal $\text{Ca}^{2+}$ entry from SR $\text{Ca}^{2+}$ release channels may minimize the role of SR $\text{Ca}^{2+}$ release during normal EC coupling in NB ventricular myocytes.

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Key Words: $\text{Ca}^{2+}$ development ■ T-tubule ■ excitation-contraction coupling ■ modeling

In adult (AD) ventricular muscle, transsarcolemmal $\text{Ca}^{2+}$ entry through voltage-gated $\text{Ca}^{2+}$ channels triggers the release of sarcoplasmic reticulum (SR) $\text{Ca}^{2+}$ stores. These whole-cell transients can be described by the spatial and temporal summation of local $\text{Ca}^{2+}$ transients (Ca$^{2+}$ “sparks”) that arise from $\text{Ca}^{2+}$ release from single, or clusters of, SR $\text{Ca}^{2+}$ release channels at T-tubule–SR junctions. In newborn (NB) rabbit ventricular myocytes, the T-tubule network is largely absent, the SR is relatively sparse, and L-type $\text{Ca}^{2+}$ channel expression is diminished. Thus, it is thought that the immature SR assumes a minor role in excitation-contraction (EC) coupling. NB myocytes have a relatively large surface area-to-volume ratio, their myofilaments are located subsarcolemmally, cytosolic $\text{Ca}^{2+}$ buffering is lower, and Na$^+$/Ca$^{2+}$ exchanger (NCX1) expression and activity are enhanced. This combination of features is suited to the utilization of transsarcolemmal $\text{Ca}^{2+}$ flux as an alternative mechanism to deliver $\text{Ca}^{2+}$ to (and from) the myofilaments. Indeed, at birth, EC coupling can occur independently of SR $\text{Ca}^{2+}$ release by utilizing $\text{Ca}^{2+}$ entry via outward Na$^+$/Ca$^{2+}$ exchange to directly activate the contractile proteins. In common with NB rabbit ventricular myocytes, atrial myocytes have a sparse T-tubular network. However, in this cell type, close associations between the SR and the sarcolemma facilitate a systolic rise in $[\text{Ca}^{2+}]_i$, function in a manner homologous to diadic T-tubular–SR junctions in ventricular cells. Systolic elevations of $[\text{Ca}^{2+}]_i$, occur first in the subsarcolemmal space (SS) that subsequently triggers $\text{Ca}^{2+}$ release from spatially and functionally distinct $\text{Ca}^{2+}$ stores in the cell center (CC). More recently, Hüser et al and Berlin reported that, in contrast to an apparently uniform rise in whole-cell $[\text{Ca}^{2+}]_i$, in field-stimulated AD ventricular cells, regional $\text{Ca}^{2+}$ gradients occur in cat and guinea pig atrial myocytes. These data, combined with the observation that coupling between L-type $\text{Ca}^{2+}$ current and SR release channels occurs at the T-tubules in mature ventricular cells, suggest that regional differences in $[\text{Ca}^{2+}]_i$, may occur in immature ventricular myocytes and that the postnatal acquisition of T-tubules may be of central importance in the postnatal transition to a mature pattern of EC coupling. We have used a combination of confocal $\text{Ca}^{2+}$ and T-tubule
imaging, fluorescent immunolabeling, and mathematical modeling to characterize the postnatal transition to AD, SR-dependent EC coupling phenotype. We report that significant whole-cell [Ca\textsuperscript{2+}] gradients occur during EC coupling in immature myocytes and that this phenotype may be attributable to developmental differences in both SR function and the EC coupling microarchitecture.

**Materials and Methods**

**Animals**

All animals were cared for in compliance with the recommendations from the Declaration of Helsinki and the NIH Guiding Principles in the Care and Use of Animals.

**Cell Isolation and Solutions**

Ventricular myocytes were isolated from the hearts of 1- to 14-day-old and AD (>150-day-old) New Zealand White rabbits using a collagenase-based digestion technique described previously. Ventricular myocytes were stored at room temperature (22 to 25°C) until used. All experiments were performed within 10 hours of cell isolation.

**Confocal Ca\textsuperscript{2+} Imaging**

Ventricular myocytes were loaded (NB, 10 minutes; AD, 30 minutes, 22°C) with the acetoxyethyl ester of the Ca\textsuperscript{2+} fluorophore, fluo-3 (1 μmol/L; Molecular Probes, Inc). Cells were subsequently washed (30 minutes) in Tyrode’s solution to allow sufficient time for intracellular de-esterification of the dye. Fluo-3-loaded cells were allowed to settle on a coverslip mounted on the stage of an inverted microscope (Nikon Diaphot 300) equipped with a ×60 objective (Nikon Fluor, oil immersion; numerical aperture, 1.4). The microscope was attached to a confocal laser-scanning unit (Molecular Dynamics Multimicroprobe 2001). Myocytes were electrically stimulated (0.5 Hz, 1- to 5-ms square wave pulses, voltage 20% above threshold amplitude) through parallel platinum field electrodes. The composition of the extracellular normal Tyrode’s solution was as follows (in mmol/L): NaCl 137, KCl 5.4, HEPES 10, MgCl\textsubscript{2} 1, CaCl\textsubscript{2} 1.8, and NaH\textsubscript{2}PO\textsubscript{4} 0.33 adjusted to pH 7.4 at 34°C with NaOH. In each experiment, a vertical scan through the entire cell facilitated the positioning of the confocal z-plane in the CC for all subsequent line-scan recordings. Fluo-3 fluorescence was excited with the 488-nm line of an argon laser and emitted fluorescence >510 nm detected. Images were acquired using a Silicon Graphics Indy workstation running Imagespace software (Molecular Dynamics). Additional postacquisition analysis was performed using NIH Image software (ScionCorp).

**Intracellular calcium images were calibrated according to the following:**

\[
\left[\text{Ca}^{2+}\right]_i = \frac{K_x \times R}{(K_d/\left[\text{Ca}_{\text{total}}\right] - R + 1)}
\]

where \(R\) is the normalized fluorescence (\(F/F_{\text{baseline}}\)), and \(K_x\) is the dissociation constant of the Ca\textsuperscript{2+}- fluo-3 complex.\textsuperscript{13} We used a \(K_d\) for fluo-3 in the cytoplasmic environment of muscle cells of 1.1 μmol/L.\textsuperscript{14}

The relaxation phase of Ca\textsuperscript{2+} transients recorded at the SS and CC was fitted to a single exponential function to obtain values for the relaxation time constant (\(t_{\text{rel}}\)). The initial rate of rise of fluo-3 transients (dF/dt) was calculated by fitting the upstroke to a linear function and expressed as change in fluorescence intensity per ms.

**Detection of Unitary SR Ca\textsuperscript{2+} Release Events (Ca\textsuperscript{2+} Sparks)**

Fluorescent imaging of Ca\textsuperscript{2+} sparks was performed in fluo-3–loaded NB and AD rabbit ventricular myocytes using a confocal system (LSM 410, Carl Zeiss) equipped with an Argon ion laser (model 2014 series, 25 mW; Uniphase) and ×40 oil objective (Zeiss Plan-NeoFluarc; numerical aperture, 1.3), as described previously.\textsuperscript{15} A single line across the entire cell width was scanned repetitively (250 Hz), and composite line-scan images were constructed by stacking 512 lines horizontally with time running from left to right. Myocytes were superfused with normal Tyrode’s solution (+1 μmol/L isoproterenol) and field stimulated (1 Hz). When Ca\textsuperscript{2+} transients reached steady state (1 to 2 minutes), the stimulation was stopped and Ca\textsuperscript{2+} spark frequency quantified at the subsarcolemmal and CC during a subsequent 20-second period of rest. All images were background subtracted using IDL software (Research Systems Inc).

In some experiments, the spatial and temporal profile of [Ca\textsuperscript{2+}]i in response to caffeine-induced SR Ca\textsuperscript{2+} release was assessed. After achieving steady state (1 to 2 minutes), the electrical stimulus was stopped and a Na\textsubscript{0}/Ca\textsubscript{0}/10 mmol/L EGTA solution containing caffeine (10 mmol/L) was rapidly applied for 5 to 10 seconds to activate SR Ca\textsuperscript{2+} release and prevent net SR Ca\textsuperscript{2+} reuptake. The time course of the caffeine-induced Ca\textsuperscript{2+} transients at the cell edges and CC were assessed in terms of time to peak (ms) and peak background-corrected fluorescence (F/Fo).

**Determination of Diastolic [Ca\textsuperscript{2+}]i**

NB and AD myocytes were loaded (NB, 10 minutes; AD, 20 minutes; 22°C) with the acetoxyethyl ester of indo-1 (10 μmol/L+0.02% [wt/vol] pluronic F-127) as described previously. Quiescent and field-stimulated (0.5 Hz) myocytes were superfused with Tyrode’s solution, and diastolic fluorescence was measured at 405 and 485 nm (excitation, 360 nm) using a DeltaRamp high-speed multiwavelength illuminator (Photon Technology International Inc). Auto-fluorescence and background fluorescence were subtracted from both emission signals. [Ca\textsuperscript{2+}]i, was calculated according to the following formula:

\[
\frac{[\text{Ca}^{2+}]}{R} = \frac{K_x \times R}{(K_d/\left[\text{Ca}_{\text{total}}\right] - R + 1)}
\]

\(R_{\text{max}}\) and \(R_{\text{min}}\) were determined using saturating (20 mmol/L) and nominally Ca\textsuperscript{2+}-free (10 mmol/L EGTA) solutions, respectively, in the presence of butanedione monoxime (50 mmol/L) and the nonfluorescent ionophore, 4-bromo-A 23187 (10 mmol/L). In separate experiments using aqueous solutions, butanedione monoxime did not alter the \(K_d\) or \(\beta\) of indo-1 (data not shown).

**Measurement of Electrically and Caffeine-Evoked Cell Shortening**

Shortening amplitudes recorded during the rapid application of caffeine were used as an index of SR Ca\textsuperscript{2+} content in thapsigargin (TG)-treated myocytes.\textsuperscript{3} Cell shortening of NB and AD myocytes was quantified at room temperature (22°C to 25°C) using a video-edge detection system (Crescent Electronics) as described previously.\textsuperscript{3,8} Shortening was measured (in μm) at one end and expressed as a percentage of resting cell length.

**T-Tubule Imaging**

Myocytes from each age group were incubated in di-8-ANEPPS (5 μmol/L), a nonpermeant, plasma membrane-selective fluorescent dye (Molecular Probes, Inc).\textsuperscript{10,11} Cells were incubated for 5 minutes at 22°C irrespective of their age and subsequently washed for 10 minutes in Tyrode’s solution. To detect the presence of T-tubules, the dye was excited with the 488-nm line of an argon laser and emitted fluorescence >510 nm recorded in image-scan mode.

**Indirect Immunofluorescent Labeling of Na\textsuperscript{+}-Ca\textsuperscript{2+} Exchange and Ryanodine Receptor (RYR2) Proteins**

Fluorescent immunolabeling was used to assess expression patterns of cardiac isoforms of the NCX1 and RyR2 proteins in rabbit ventricular myocytes. Freshly isolated myocytes were adhered to poly-L-lysine–coated coverslips, where they were fixed and permeabilized by immersion for 10 minutes in 3.7% formaldehyde+0.2% Triton X-100 in PBS. After washing 3 times in PBS, slides were blocked by incubation (10 minutes) in 5% rabbit serum (Jackson ImmunoResearch, West Grove, PA) to minimize nonspecific anti-
body adsorption. After removing the blocking buffer, cells were coincubated with primary monoclonal antibodies (1.0 to 10 μg/mL in blocking buffer; 60 minutes at 22°C) raised against the cardiac isoforms of the NCX1 (mouse anti-NCX1; IgM, Affinity Bioreagents) and RYR2 (mouse anti-RyR2; IgG, Affinity Bioreagents). After being washed three times in PBS, cells were incubated sequentially (45 to 60 minutes, 22°C) with appropriate fluorochrome-conjugated secondary antibodies (FITC-conjugated anti-mouse IgG and Texas Red-conjugated anti-mouse IgM; Jackson ImmunoResearch Laboratories). In preparation for microscopy, cells were washed three times in PBS and mounted in antifade agent (0.1% p-phenylenediamine in PBS adjusted to pH 8, 50% glycerol). Appropriate control experiments included the incubation of myocytes with either primary or secondary antibodies alone (data not shown). Dual-channel optical sections were obtained on the day of sample preparation using confocal microscopy (Molecular Dynamics Multiprobe 2001) and displayed simultaneously in pseudocolor to indicate the subcellular location of each antigen.

Mathematical Modeling of Subsarcolemmal Ca2+ Transients in NB Ventricular Myocytes

We have adapted our model of Ca2+ signaling in AD ventricular myocytes18 to describe the spatial and temporal changes in subcellular Ca2+ in neonatal rabbit ventricular myocytes. This model incorporates key morphological and electrophysiological features of NB rabbit ventricular myocytes, including the following: (1) their geometric approximation to a simple cylinder of physiologically relevant dimensions4 (length=55 μm; radius=4 μm), (2) enhanced Na+-Ca2+ exchange current density (8 times larger), (3) elimination of SR Ca2+ release flux, (4) Ca2+ diffusion, (5) rapid Ca2+ buffering, and (6) a Ca2+ sink (possibly SR, mitochondria, or additional slow Ca2+ buffers). By assuming a spatially homogenous interior, radial symmetry was imposed, yielding a 1-dimensional system when solved in cylindrical coordinates described by the following:

\[
\frac{\partial [Ca^{2+}]}{\partial t} = \beta_i \left( D_{Ca} \left[ \frac{\partial^2 [Ca^{2+}]}{\partial r^2} + \frac{1}{r} \frac{\partial [Ca^{2+}]}{\partial r} \right] + J_{Ca} + J_{sink} \right)
\]

where \( t \) is time, \( r \) is the distance from the center of the cell, \( \beta_i \) is the fractional buffering of Ca2+ in the myoplasm by Ca2+ binding proteins using the rapid buffering approximation,\(^{17} \) \( k_{opt} \) is myo or ssl to denote myoplasmic or subsarcolemmal buffering (see below), \( J_{Ca} \) is Ca2+ flux across the sarcolemma, and \( J_{sink} \) is the contribution from Ca2+ sources and sinks within the myocyte. It was assumed that buffering in the neonatal cardiac myocyte is less than that of the adult.\(^{6} \) Hence, the total amount of the stationary Ca2+ buffers in the cell was \( [M_{total}]=100 \text{μmol/L} \) with dissociation constant \( K_d=0.96 \text{μmol/L} \), which is slightly lower than the value of 123 μmol/L measured in AD rat cardiac myocytes.\(^{18} \) It was also assumed that there was 10 μmol/L mobile buffer (\( [M_{mobile}] \) with dissociation constant \( K_d=1.1 \text{μmol/L} \). The rapid buffering approximation assumes that the buffers in the myoplasm are in equilibrium and is described by the equation below:

\[
\beta_{myo} = \left( 1 + \frac{[M_{mobile}]/K_d}{1+[Ca^{2+}]/K_d} \right)^{-1}
\]

Buffering in the SS by the negatively charged groups in the sarcolemma is also described by the following rapid buffering approximation\(^{15} \):

\[
\beta_{ssl} = \left( 1 + \frac{[SL_{mobile}]/K_d}{1+[Ca^{2+}]/K_d} \right)^{-1}
\]

where \([SL_{mobile}]\) is the total amount of subsarcolemmal Ca2+ buffer and \( K_d \) is the Ca2+ dissociation constant of the subsarcolemmal buffer. The diffusion constant for Ca2+ moving into the cell (\( D_{Ca}=132 \text{μm}^2/\text{s} \)) was calculated using the value measured (225 μm/s) in cytoplasmic extracts scaled for diffusion in the transverse direction. The scaling was derived by comparing wave propagation velocities in the transverse and longitudinal directions and the relation that the wave velocity is proportional to the square root of the diffusion constant in excitable medium.\(^{22} \)

The sum of the Ca2+ fluxes \( J_{Ca} \) is described by the following:

\[
J_{Ca} = \begin{cases} 
0 & 0 \leq \text{r}\leq 4 \text{ μm} \\
\frac{I_{Ca,b}A_{g}}{F_{VSSL}} + \frac{I_{Ca,b}A_{g}}{2F_{VSSL}} & \text{r} = 4 \text{ μm}
\end{cases}
\]

where \( F \) is Faraday’s constant, \( A_{g} \) is the capacitance surface area, and \( V_{SSL} \) is the volume of SS. The outer 0.01 μm of the myocyte was assumed to constitute the SS, of which 68% is available as myoplasm.\(^{23} \) The Na+-Ca2+ exchange current (\( I_{Na,Ca} \)) and the background Ca2+ (\( I_{Ca,b} \)) currents were as described for an AD guinea pig ventricular myocyte model.\(^{16} \) The Na+-Ca2+ exchange current (\( I_{Na,Ca} \)) is described by the following:

\[
I_{Na,Ca} = \frac{1}{K_{Na,Ca}} + \frac{[Na^{+}]}{K_{Na,Ca}^{(4)} + [Ca^{2+}]} + 1 + k_{Na,e}^{(4)} e^{-V_{mem}/RT} \times \left( e^{V_{mem}/RT} [Na^{+}]_{SSL} [Ca^{2+}]_{SSL} - e^{-V_{mem}/RT} [Na^{+}]^{(4)} [Ca^{2+}]_{SSL} \right)
\]

where \( V \) is the membrane potential, \( R \) is the ideal gas constant, \( T \) is the absolute temperature, \( \eta \) controls the voltage dependence of Na+-Ca2+ exchange, \( K_{Na,Ca} \) is the Ca2+ half-saturation constant, \( K_{Na} \) is the Na+ half-saturation constant, and \( k_{Na,e} \) is the Na+-Ca2+ exchange saturation factor at very negative potentials. The background Ca2+ current is described by the following:

\[
I_{Ca,b} = \frac{K_{Ca,Ca}}{V_{mem} - V_{Ca,b}}
\]

The sink flux can represent uptake and a leak from any source such as the SR, mitochondria,\(^{24} \) or some other slowly buffering pool. It is described by the following:

\[
J_{sink} = -v_{sink}[Ca^{2+}]_{SSL}^{(2)} + \frac{v_{sink}([Ca^{2+}]_{SSL}^{(2)} - [Ca^{2+}])}{k_{opt}^{(2)} + [Ca^{2+}]_{SSL}^{(2)}}
\]

where \( v_{sink} \) is the maximum uptake rate for the sink, \( k_{opt} \) is \( [Ca^{2+}] \) for half-maximal uptake, and \( V_{Ca,b} \) is the leak rate out of the sink. The Ca2+ balance for the sink is as follows:

\[
\frac{\partial [Ca^{2+}]_{sink}}{\partial t} = \beta_{sink} \frac{V_{myo}}{V_{sink}}
\]

Numerical Methods

The model was solved using the Cranck-Nicholson method\(^{25} \) in FORTRAN on a Silicon Graphics Indy Workstation. Diastolic [Ca2+] was set uniformly at 244 μmol/mL, as measured in this study. An action potential waveform acquired from a 1-day-old NB rabbit ventricular myocyte under current-clamp conditions was used as the voltage protocol in the simulation. The data were output as an ASCII array with a step size of 0.01 μm and a time step of 0.001 ms and were plotted in 3 dimensions (Origin 5.0, MicroCal). The channel parameter values are shown in Table 3. Initially, [Ca2+] and [Ca2+]_sink were allowed to reach equilibrium values by running the simulation with the cell at a holding potential of -74.3 mV.

Statistical Analysis

Data are expressed mean±SEM. Data were compared using 1-way ANOVA and, when appropriate, Student-Newman-Keuls or Fisher’s Exact test with SigmaStat statistical analysis software (Jandel Scientific). Statistical significance was accepted when \( P<0.05 \).
Results

Postnatal Changes in Diastolic \( [\text{Ca}^{2+}]_i \)

The pseudocalibration of fluo-3 signals into \( [\text{Ca}^{2+}]_i \) requires an estimate of diastolic \( [\text{Ca}^{2+}]_i \).\(^{13}\) Whereas a value of 100 nmol/L is commonly used for quiescent AD myocytes, our experiments also included the use of NB myocytes for which diastolic free \( [\text{Ca}^{2+}]_i \) is unknown. Therefore, we measured diastolic free \( [\text{Ca}^{2+}]_i \) in indo-1–loaded quiescent and paced (0.5 Hz) NB and AD myocytes for subsequent use in the pseudocalibration of confocal \( [\text{Ca}^{2+}]_i \) images. In quiescent myocytes, diastolic \( [\text{Ca}^{2+}]_i \) was significantly higher in NBs compared with AD myocytes (NB, 174 ± 24; AD, 100 ± 5 nmol/L, n = 12 to 14; \( P < 0.05 \)). In both age groups, diastolic \( [\text{Ca}^{2+}]_i \), was raised significantly during steady-state field stimulation (NB, 244 ± 55; AD, 160 ± 21 nmol/L, n = 7 to 10; \( P < 0.05 \)). In light of these data, we used values of 244 and 160 nmol/L for diastolic \( [\text{Ca}^{2+}]_i \) to calibrate fluo-3 signals acquired from field-stimulated NB and AD myocytes, respectively. Estimates of diastolic \( [\text{Ca}^{2+}]_i \), in TG-treated myocytes were not obtained and, therefore, data are expressed as background-corrected fluorescence intensity.

Calcium Gradients in AD Myocytes

Spatial patterns of whole-cell \( [\text{Ca}^{2+}]_i \), were investigated in single field-stimulated AD ventricular myocytes. Figure 1A shows the outline of an AD cell and the position of the line that was scanned repetitively (100 Hz). The line-scan image in Figure 1B illustrates that systolic elevations in \( [\text{Ca}^{2+}]_i \) occurred simultaneously across the entire width (in the central \( z \)-plane) of the myocyte after each depolarizing pulse. This is further highlighted by the local \( [\text{Ca}^{2+}]_i \), transients at the SS and CC (Figure 1C). In both regions, \( [\text{Ca}^{2+}]_i \) rose rapidly with superimposable upstrokes (d\( F \)/d\( T \), SS, 2.1 ± 0.4; CC, 2.2 ± 0.3; n = 5; Table 1) and subsequently declined with similar time constants (\( \tau_{\text{relax}} \), SS, 154 ± 13; CC, 148 ± 17 ms; n = 5; Table 1). Furthermore, peak systolic \( [\text{Ca}^{2+}]_i \), was similar between these 2 subcellular locations (SS, 940 ± 76; CC,
1002±70 nmol/L, n=9). Finally, the uniform appearance of the 3-dimensional surface plot of the line-scan image (Figure 1D) is further indication of a simultaneous elevation of $[\text{Ca}^{2+}]_i$ at the SS and CC.

**Calcium Gradients in NB Myocytes**

Identical experiments were performed to assess spatial patterns of $[\text{Ca}^{2+}]_i$ in single NB ventricular myocytes. These cells were consistently narrower and shorter than AD myocytes, which contributes to their significantly greater surface area-to-volume ratio. The cell outline and the position at which the scan line was positioned in a NB cell are shown in Figure 2A. In contrast to AD myocytes, the composite line-scan image in Figure 2B illustrates regional differences in $[\text{Ca}^{2+}]_i$ after each electrical stimulus. During the initial phase of each transient, $\text{Ca}^{2+}$ increased significantly in the SS.

| TABLE 1. Effect of Disabling SR Function on Fluo-3 $[\text{Ca}^{2+}]_i$ Transients in NB and AD Ventricular Myocytes |
|-------------------------------------------------|-------------------------------------------------|
| Control | Thapsigargin (10 µmol/L) |
| Systolic $[\text{Ca}^{2+}]_i$, nmol/L | Rate of Rise of $[\text{Ca}^{2+}]_i$ Transient Upstroke | $\tau_{\text{relax}}, \text{ms}$ | Rate of Rise of $[\text{Ca}^{2+}]_i$ Transient Upstroke | $\tau_{\text{relax}}, \text{ms}$ |
| AD | | | | |
| CC | 1002±70 | 2.2±0.3 | 148±17 | 0.37±0.04* | 446±28* |
| SS | 940±76 | 2.1±0.4 | 154±13 | 0.42±0.06* | 432±28* |
| NB | | | | | |
| CC | 549±23 | 0.27±0.04† | 515±26† | 0.31±0.04† | 473±17† |
| SS | 1128±65 | 0.96±0.1 | 307±26 | 0.82±0.1 | 274±19 |

Time constants ($\tau_{\text{relax}}$) of the relaxation phase of $\text{Ca}^{2+}$ transients (in ms) were calculated by fitting to a single exponential function. The rates of rise of the $\text{Ca}^{2+}$ transient upstroke are given in change in fluorescence intensity per millisecond. Data are mean±SEM; n=5 to 6.

*P<0.05 vs control; †P<0.05 vs SS.

**Figure 2.** Regional $[\text{Ca}^{2+}]_i$ in NB myocytes. A, Outline of a NB (1-day-old) myocyte and the position of the scan line. B, A composite line-scan image acquired during electrical stimuli illustrating spatial and temporal differences in the evolution of $[\text{Ca}^{2+}]_i$ between the SS and CC. C, $[\text{Ca}^{2+}]_i$ transients recorded at the SS (red line) and CC (black line). $[\text{Ca}^{2+}]_i$ at the subsarcolemma increased before and more rapidly than in the CC. D, 3-dimensional reconstruction of the line-scan image in panel B.
followed by a smaller rise in the CC. This is further highlighted in the local Ca\(^{2+}\) transients obtained at the SS, which had a more rapid upstroke than at the CC (dF/dT, SS, 0.96±0.1; CC, 0.27±0.04 F units/ms; n=6). Furthermore, peak systolic [Ca\(^{2+}\)] was significantly higher in the SS compared with the CC (SS, 1128±65; CC, 549±23 nmol/L, n=13) and declined more rapidly during relaxation (t\(_{relax}\), SS, 307±16; CC, 515±26 ms; n=6). These features are further emphasized in the 3-dimensional surface plot of the line-scan image in which the characteristic U-shaped temporal and spatial profiles of [Ca\(^{2+}\)] are indicative of an enhanced elevation of [Ca\(^{2+}\)] in the SS compared with the CC.

**Calcium Gradients in TG-Treated Myocytes**

We performed experiments to assess the contribution of SR Ca\(^{2+}\) stores to the Ca\(^{2+}\) transients and patterns of subcellular Ca\(^{2+}\) distribution observed in NB and AD myocytes. We compared Ca\(^{2+}\) gradients in field-stimulated myocytes that were untreated or exposed to TG (10 \(\mu\)mol/L) to disable SR Ca\(^{2+}\) reuptake. In AD myocytes, TG treatment prolonged the time course of the [Ca\(^{2+}\)] transient to a similar degree at both the SS and CC (Figure 3A and 3B, Table 1). However, the spatial pattern of the Ca\(^{2+}\) transient remained unchanged (Figure 3B). In contrast, in NB myocytes, TG did not affect the time course of [Ca\(^{2+}\)] transients at either the SS or the CC (Figure 3C and 3D and Table 1).

To confirm that TG disabled the SR in both NB and AD myocytes, we measured the contractile response of myocytes to the rapid application of caffeine (10 mmol/L). As we have previously reported, steady-state, electrically induced contractions were of larger amplitude in AD compared with NB myocytes (AD, 7.1±1%; NB, 3.9±0.2; n=5 to 8; Table 2). In both age groups, the rapid application of caffeine elicited contractions of greater magnitude than the corresponding electrically evoked twitches (AD, 10.4±0.3%; NB, 5.7±0.6; n=5 to 7; Table 2). In electrically stimulated NB and AD

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**Figure 3.** Role of SR calcium in regional [Ca\(^{2+}\)]. A, Line-scan image in an untreated control AD myocyte. White arrows indicate electrical stimuli. The upstroke of regional [Ca\(^{2+}\)] transients plotted at the SS (red line) and CC (black line) were superimposable and declined with similar time constants. B, Line-scan image in a TG-treated AD myocyte (10 \(\mu\)mol/L). [Ca\(^{2+}\)] transients at the subsarcolemma and CC were prolonged and decayed with a slower time course but remained superimposable. C, Line scan of an untreated NB myocyte and associated depolarization-induced [Ca\(^{2+}\)] transients illustrating regional differences in [Ca\(^{2+}\)]. D, Line-scan image of a TG-treated NB myocyte during depolarizing electrical stimuli. Regional differences in [Ca\(^{2+}\)] transients at the subsarcolemma and CC were unaffected by TG treatment.
myocytes that had been previously treated with TG, the rapid application of caffeine did not elicit a contraction, consistent with the depletion of releasable SR Ca\textsuperscript{2+} stores. In some experiments, the application of a second caffeine pulse confirmed that in control myocytes the initial application of caffeine released all available Ca\textsuperscript{2+} from the SR in each age group (data not shown).

**Caffeine-Induced Ca\textsuperscript{2+} Gradients in NB and AD Myocytes**

We used confocal Ca\textsuperscript{2+} imaging to assess the spatial and temporal evolution of subcellular Ca\textsuperscript{2+} transients induced by the rapid application of caffeine (10 mmol/L) in fluo-3–loaded NB and AD myocytes. Line-scan images confirmed our previous finding that caffeine elicits the release of substantial SR Ca\textsuperscript{2+} stores to evoke a Ca\textsuperscript{2+} transient and contraction in both NB and AD myocytes (Figure 4). In NB myocytes, the uniform nature of the fluo-3 signal during the rapid application of caffeine (Figure 4C) was consistent with caffeine-induced SR Ca\textsuperscript{2+} release occurring simultaneously at the edges of the cell and CC (time to peak, cell edges, 62.4 ± 5.6 and 63.4 ± 7.3 ms; CC, 67 ± 5.9 ms; n=8; P<0.05). Similar uniform responses to caffeine were observed in AD myocytes (time

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<td>10.4±0.3*</td>
<td>5.4±0.3†</td>
<td>0</td>
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<tr>
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<td>5.7±0.6*</td>
<td>3.9±0.5</td>
<td>0*</td>
<td>4.0±0.4</td>
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The amplitude of electrical stimulus– and caffeine-induced contractions (% diastolic cell length) was measured in non–fluo-3–loaded myocytes. Concentrations of TG and ryanodine were 10 μmol/L and 1 μmol/L, respectively. Data are mean±SEM; n=5 to 8.

*P<0.05 vs respective electrically induced twitch; †P<0.05 vs control.

**Figure 4.** Subcellular location of Ca\textsuperscript{2+} sparks in NB myocytes. A, Ca\textsuperscript{2+} sparks recorded in a fluo-3–loaded NB ventricular myocyte during a 20-s period of rest (immediately after steady-state pacing at 1 Hz). Ca\textsuperscript{2+} sparks were observed predominantly at the SS and rarely in the CC. For clarity, the SS (red) and CC (black) traces are offset. B and C, Rapid application of caffeine (10 mmol/L) immediately after the cessation of electrical pacing elicited a uniform rise in [Ca\textsuperscript{2+}] in both AD (B) and NB (C) myocytes. In each age group, line plots illustrate the superimposable profiles of the caffeine-induced transients at the cell edges (red line) and CC (black line).
to peak, cell edges $31.6 \pm 1.6$ and $28.6 \pm 3.3$ ms; CC, $30.6 \pm 1$ ms; $n = 4$; $P < 0.05$; Figure 4B).

**Unitary SR Ca$^{2+}$ Release Events (Ca$^{2+}$ Sparks)**

Ca$^{2+}$ sparks are local elevations of [Ca$^{2+}$], arising either as a result of spontaneous SR Ca$^{2+}$ release or in response to Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels, and are thought to represent the elementary events underlying cardiac EC coupling.26,27 We therefore undertook experiments to determine the spatial distribution of Ca$^{2+}$ sparks in NB and AD rabbit ventricular myocytes. In NB myocytes, Ca$^{2+}$ sparks were predominantly observed in close proximity to the sarcolemmal membrane (Figure 4A). In each of the 25 myocytes studied, we observed Ca$^{2+}$ sparks at cell periphery. However, Ca$^{2+}$ sparks were detected in the CC in only 4 of 25 cells ($P < 0.05$; Fisher exact test). On average there were $6.3 \pm 0.7$ sparks per 20 seconds detected at the cell periphery ($n = 25$). In the few neonatal cells that did exhibit centrally located Ca$^{2+}$ sparks, spark frequency at the CC was only $1.25 \pm 0.3$ sparks per 20 seconds ($P < 0.05$; $n = 4$). Considering the smaller number of cells showing central Ca$^{2+}$ sparks (4 of 25), the overall likelihood of central sparks was $\approx 30$ times lower than sparks near the sarcolemma. In 5 AD myocytes studied, $7.4 \pm 1.5$ sparks/cell per 20 seconds were distributed across the entire width of the cell. In one of these cells, simultaneous SL labeling with di-8-ANEPPS confirmed that each of these sparks coincided with the z-lines.12

**Postnatal T-Tubule Development**

To characterize the postnatal acquisition of the T-tubule network, we used confocal microscopy to visualize sarcolemmal membrane topography in myocytes incubated in the membrane-selective fluorescent probe di-8-ANEPPS.10 Confocal images of di-8-ANEPPS–treated AD ventricular myocytes revealed punctate patterns of SL labeling. These markings were spaced at regular intervals (corresponding to the resting sarcomere length of $\approx 1.9 \mu m$; Figure 5A) consistent with the labeling of T-tubule invaginations. In juvenile (10- to 14-day-old) myocytes, a sparse, irregular, underdeveloped, and inhomogeneous T-tubular network was present (Figure 5B). In contrast, there was no visible punctate staining of NB (1- to 5-day) myocytes, such that their SL appeared to be noninvaginated, consistent with the absence of T-tubule invaginations in the NB rabbit (Figure 5C).

**Indirect Fluorescent Immunolocalization of NCX1 and RYR2 Proteins**

The confocal images in Figure 6 illustrate the subcellular location of NCX1 and RyR2 proteins at 3 stages of rabbit myocyte development. In NB myocytes, intense, uniform NCX1 labeling (pseudocolored red) was observed exclu-
sively at the peripheral sarcolemma, further illustrating the absence of T-tubules at birth (see Figure 5C). In contrast, RyR2 labeling (pseudocolored green) was mainly in the cell interior. The 2 fluorescent signals appeared to originate from spatially distinct domains, consistent with separation of the sarcolemma and SR at birth. In juvenile myocytes, intense peripheral NCX1 and intracellular RyR2 labeling were also observed. However, an additional narrow subsarcolemmal band of yellow (indicative of a colocalization of the Texas Red– and FITC-derived fluorescent signals) was observed, suggesting the presence of increased colocalization of SR sites with the sarcolemma. In contrast, AD myocytes displayed significant overlap of NCX1 and RyR2 antigens at the z-lines consistent with T-tubule maturation and the formation of diadic junctions.

Mathematical Modeling of Subsarcolemmal Ca\(^{2+}\) in NB Myocytes

We adapted our AD guinea pig myocyte model\(^{16}\) to develop a preliminary mathematical model of Ca\(^{2+}\) fluxes in NB rabbit ventricular myocytes. A native action potential obtained from a 1-day-old myocyte was used as the command potential in the simulation. Values for the other parameters used in the model are listed in Table 3. Figure 7B illustrates the predicted spatial and temporal changes in [Ca\(^{2+}\)] across the 8-\(\mu\)m diameter of a cylinder approximating the geometry of a NB myocyte. Notable features of the simulated Ca\(^{2+}\) transients are that, in common with our experimental data obtained in native NB myocytes (Figure 2D), a rapid rise in systolic [Ca\(^{2+}\)] is predicted to occur at the cell periphery before a slower and smaller elevation at the CC. Predicted [Ca\(^{2+}\)] transients at the subsarcolemma and CC are shown in Figure 7C, which again illustrate the temporal and spatial differences in [Ca\(^{2+}\)]. Although this model could be further refined, it emphasizes that the smaller, slower [Ca\(^{2+}\)] transients observed experimentally at the CC (see Figure 2) can be explained theoretically by diffusion of Ca\(^{2+}\) that has entered from the surface membrane. Furthermore, this preliminary mathematical simulation supports the concept that transsarcolemmal Ca\(^{2+}\) fluxes generated by the NCX1 are the principal pathways for contraction and relaxation of NB ventricular myocytes.

Discussion

We have used confocal Ca\(^{2+}\) imaging, fluorescent immunolabeling, and mathematical modeling to study the spatial and temporal patterns of Ca\(^{2+}\) distribution in NB and AD ventricular myocytes. Our data demonstrate that subcellular gradi-
ents of [Ca\(^{2+}\)], occur in NB myocytes, in contrast to the uniform elevation and decline of whole-cell [Ca\(^{2+}\)], observed in AD myocytes. Confocal imaging of the sarcolemmal membrane revealed that the time course of the postnatal acquisition of a mature T-tubular network coincided with the transition to a mature pattern of depolarization-induced changes in [Ca\(^{2+}\)]. These results support the concept that the postnatal transition to a mature EC coupling phenotype requires the coordinate development of a spatially integrated EC coupling microarchitecture.

Evidence for the Existence of Releasable SR Ca\(^{2+}\) Stores in NB Myocytes

Age-related differences in contractile function in mammalian hearts have been attributed to a diminished involvement of the SR in EC coupling at birth.\(^{28,29}\) However, we\(^{3}\) and others\(^{30}\) have demonstrated that the amplitude of caffeine-induced SR calcium release is quite robust in neonatal ventricular myocytes. These data suggest that the SR is primed with Ca\(^{2+}\) in NB ventricular myocytes, but, paradoxically, Ca\(^{2+}\) is not released in response to electrical stimuli. Immunolabeling of RyR2 receptors reveals that SR Ca\(^{2+}\) release channels are present, even at birth. Furthermore, the response to a rapid application of caffeine in NB myocytes confirms the functionality of these channels.

Although functional RyR2 receptors are present at birth, results from our coimmunolabeling studies suggest that a spatial disconnect exists between the sarcolemma and the interior SR. Whether these internal RyRs are diffusely distributed or begin to align along z-lines,\(^{31,32}\) they are not associated with T-tubules. Additional studies are necessary to more precisely define the developmental staging of the coassembly and subcellular positioning of key proteins involved in EC coupling.

A paucity or immaturity of T-tubular diadic junctions between L-type calcium channels and SR calcium release channels in immature cells may functionally isolate sarcolemmal Ca\(^{2+}\) entry from triggering SR Ca\(^{2+}\) release. Presumably,
the depolarization-induced rise in [Ca\(^{2+}\)] at the Ca\(^{2+}\) sensor of the RyR2 is insufficient to trigger SR Ca\(^{2+}\) release in NB myocytes. The efficacy of TG in depleting SR Ca\(^{2+}\) and their sensitivity to caffeine supports the concept of functional isolation rather than a fundamental inability of NB cells to release their Ca\(^{2+}\) stores.

Evidence Against the Involvement of SR Ca\(^{2+}\) Flux in EC Coupling in NB Myocytes

Several lines of evidence support a minimal role for SR Ca\(^{2+}\) in EC coupling at birth in the rabbit. In AD but not NB myocytes, SR Ca\(^{2+}\) depletion prolonged and reduced the amplitude of whole-cell Ca\(^{2+}\) transients. Furthermore, inhibition of SR Ca\(^{2+}\) transport with TG in NB myocytes did not alter subcellular [Ca\(^{2+}\)] gradients, Ca\(^{2+}\) transients, or contraction amplitudes. This is consistent with transsarcolemmal Ca\(^{2+}\) entry rather than SR Ca\(^{2+}\) release providing the predominant source of activator Ca\(^{2+}\) for contraction. The lack of spatial Ca\(^{2+}\) gradients in AD myocytes, even after TG treatment, implies that Ca\(^{2+}\) entry across the surface of the T-tubule membrane is able to create a relatively synchronous and uniform rise in whole-cell [Ca\(^{2+}\)]. The time constant of [Ca\(^{2+}\)] decline was also spatially uniform in AD myocytes, with or without TG. In contrast, in NB cells, superficial [Ca\(^{2+}\)] decline is faster than at the center (even with TG). This reflects the superficial location of the main functional Ca\(^{2+}\) removal mechanism (Na\(^{+}\)-Ca\(^{2+}\) exchange). The SR Ca\(^{2+}\) pump may be very slow, because if there is no SR Ca\(^{2+}\) release, the intra–SR [Ca\(^{2+}\)] will limit net SR Ca\(^{2+}\) uptake.\(^{33}\)

Developmental Acquisition of T-Tubules and a Mature EC Coupling Phenotype

Confocal sarcolemmal imaging illustrates that T-tubules are absent at birth and are acquired during the first 3 weeks of life in rabbits. While T-tubule development may occur at an earlier developmental stage in humans,\(^ {34}\) the molecular mechanism(s) and developmental regulation of T-tubule formation in both cardiac and skeletal muscle of all species remain unclear.\(^ {35}\) However, there is little doubt that the T-tubular network plays a central role in EC coupling in mature ventricular myocytes.

Peripheral Location of Ca\(^{2+}\) Sparks in NB Ventricular Myocytes

The local control theory of EC coupling proposes that SR Ca\(^{2+}\) release is triggered by a high local [Ca\(^{2+}\)], established between L-type Ca\(^{2+}\) channels in the T-tubules and juxtaposed RyR2(s) in the junctional SR.\(^ {36}\) In support of this, di-8-ANNEPS labeling has illustrated that Ca\(^{2+}\) sparks originate at diadic junctions across the entire width of the cell along the T-tubules in AD ventricular myocytes.\(^ {12}\) In contrast,
we have demonstrated that sparks originate predominantly in the SS of NB myocytes. Interestingly, our immunolabeling data indicate that RyRs are also present in the interior of NB myocytes, suggesting that their absence is not a factor in determining the lack of sparks in the CC. This is further supported by our observation that caffeine elicited a uniform rise in [Ca\(^{2+}\)], throughout NB myocytes.

One intriguing possible explanation for the preferential subsarcolemmal Ca\(^{2+}\) spark location together with the apparent absence of Ca\(^{2+}\)-induced Ca\(^{2+}\) release during the twitch is that Ca\(^{2+}\) sparks may result from the local clustering of RyRs\(^{37}\) that may be present at the periphery of NB cells (but not often in the center). However, if these RyR clusters are not localized in tight enough proximity to L-type Ca\(^{2+}\) channels in mature diads, Ca\(^{2+}\)-induced Ca\(^{2+}\) release during EC coupling may not occur. It is also possible that NB RyR have altered sensitivity to activating Ca\(^{2+}\)/(R. Mejía-Alvarez, personal communication, February 1999), which could contribute to this same functional scenario (without requiring a spatial disconnect of diads). It is also conceivable that Ca\(^{2+}\) sparks are seen preferentially at the periphery, because the restricted space between the SR and SL membranes allows unitary RyR openings to be more likely to become regenerative in a cluster (versus RyRs located more toward the cell interior). Distinguishing the validity of these or other explanations will require additional work.

### Mathematical Modeling of Ca\(^{2+}\) Gradients in NB Myocytes

On the basis of previous studies, we have proposed that the NCX1 is the predominant Ca\(^{2+}\) transport pathway for contraction and relaxation in NB rabbit ventricular myocytes. Our preliminary mathematical model has shown that without incorporating SR Ca\(^{2+}\) release (or even \(I_{Ca}\)), transsarcolemmal Ca\(^{2+}\) fluxes via the NCX1 are sufficient to account for the Ca\(^{2+}\) transients observed experimentally in NB myocytes. Furthermore, our theoretical simulation predicts that subcellular [Ca\(^{2+}\)], gradients will be generated by Na\(^{+}\)-Ca\(^{2+}\) exchange in NB cells. The predicted gradients were comparable with those that we measured experimentally. The fact that the experimentally determined [Ca\(^{2+}\)], gradients were unchanged after SR Ca\(^{2+}\) depletion and inhibition of SR Ca\(^{2+}\) reuptake underscores the central contribution of transsarcolemmal Ca\(^{2+}\) fluxes to contraction and relaxation in neonatal ventricular myocytes.

In summary, our data provide compelling evidence in support of a predominantly SR-independent EC coupling mechanism in NB rabbit ventricular myocytes. Transsarcolemmal Ca\(^{2+}\) fluxes appear to be sufficient for direct delivery of Ca\(^{2+}\) to, and removal from, the contractile proteins. Although this EC coupling mechanism results in substantial [Ca\(^{2+}\)], gradients, diffusional limitations do not prevent contraction and relaxation. We propose that as myocytes grow postnatally, the declining surface-to-area volume ratio results in insurmountable diffusional barriers. Consequently, the fundamental processes of EC coupling must change to provide adequate Ca\(^{2+}\) delivery to the centrally located myofilaments. This is achieved by the postnatal development of T-tubules and tight diadic coupling of Ca\(^{2+}\) entry to SR Ca\(^{2+}\) release, thereby promoting spatially uniform whole-cell Ca\(^{2+}\) transients.

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### References


Subcellular $[Ca^{2+}]_i$ Gradients During Excitation-Contraction Coupling in Newborn Rabbit Ventricular Myocytes

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