Ryanodine receptor function in newborn rat heart

Claudia G. Pérez, Julio A. Copello, Yanxia Li, Kimberly L. Karko, Leticia Gómez, Josefina Ramos-Franco, Michael Fill, Ariel L. Escobar, and Rafael Mejía-Alvarez. Ryanodine receptor function in newborn rat heart. Am J Physiol Heart Circ Physiol 288: H2527–H2540, 2005. First published December 30, 2004; doi:10.1152/ajpheart.00188.2004.—The role of ryanodine receptor (RyR) in cardiac excitation-contraction (E-C) coupling in newborns (NB) is not completely understood. To determine whether RyR functional properties change during development, we evaluated cellular distribution and functionality of sarcoplasmic reticulum (SR) in NB rats. Sarcomeric arrangement of immunostained SR Ca2+-ATPase (SERCA2a) and the presence of sizeable caffeine-induced Ca2+ transients demonstrated that functional SR exists in NB. E-C coupling properties were then defined in NB and compared with those in adult rats (AD). Ca2+ transients in NB reflected predominantly sarcromeral Ca2+ entry, whereas the RyR-mediated component was ~13%. Finally, the RyR density and functional properties at the single-channel level in NB were compared with those in AD. Ligand binding assays revealed that in NB, RyR density can be up to 36% of that found in AD, suggesting that some RyRs do not contribute to the Ca2+ transient. To test the hypothesis that RyR functional properties change during development, we incorporated single RyRs into lipid bilayers. Our results show that permeation and gating kinetics of NB RyRs are identical to those of AD. Also, endogenous ligands had similar effects on NB and AD RyRs: sigmoidal Ca2+ dependence, stronger Mg2+-induced inhibition at low cytoplasmic Ca2+ concentrations, comparable ATP-activating potency, and caffeine sensitivity. These observations indicate that NB rat heart contains fully functional RyRs and that the smaller contribution of RyR-mediated Ca2+ release to the intracellular Ca2+ transient in NB is not due to different single RyR channel properties or to the absence of functional intracellular Ca2+ stores.

Ca2+ release; sarcoplasmic reticulum; excitation-contraction coupling

IN ADULT (AD) HEART MUSCLE, contraction is initiated by a sudden increase of myoplasmic free Ca2+ concentration ([Ca2+]i), generated mainly by Ca2+ release from the sarcoplasmic reticulum (SR). This SR Ca2+ flux is mediated by ryanodine receptors (RyRs) that open in response to a small trigger Ca2+ stimulus provided by voltage-dependent Ca2+ channels in the sarcolemma. This process of RyR activation is known as Ca2+-induced Ca2+ release (CICR) (11, 13). The relative contribution of CICR to the intracellular Ca2+ transient in mammalian heart changes during postnatal development (2; for a review, see Ref. 50). In newborns (NB), the extent of RyR-mediated CICR is small, and the Ca2+ required for contractile activation arises almost entirely from sarcromeral Ca2+ influx (12, 25, 49). In AD, however, the sarcomembrall Ca2+ influx is relatively small and constitutes the trigger for RyR-mediated CICR. In the mature heart, nearly all the Ca2+ required for contractile activation is provided by RyR-mediated CICR. This dramatic transient occurs during the first few weeks of postnatal life, coinciding with the maturation of the transverse tubule (T tube) and SR membrane systems (12, 16, 37, 46). Although the absence or poor development of T tubes and SR in NB heart muscle (16, 37) may imply that RyR-mediated intracellular Ca2+ signaling is not essential for cardiac differentiation/development, several lines of evidence indicate that RyR function could be significant even in the early stages of cardiac muscle development. For instance, it has been reported that mRNA of the cardiac isoform of the RyR channel (RyR2) is present in fetal cardiac muscle (6, 17). In addition, ryanodine binding studies have reported substantial amounts of the cardiac isoform of the RyR (RyR2) protein expressed as early as day 1 of postnatal life (22, 39, 49, 53). Functional studies of cardiac excitation-contraction (E-C) coupling in NB also suggest an important role of RyR in the immature heart. Those studies show that parameters such as systolic tension, global intracellular Ca2+ transient amplitude, and spontaneous intracellular Ca2+ release events are sensitive to ryanodine to an unexpectedly large extent (21, 31, 46, 49). Similarly, in NB cultured myocytes, stretch-activated Ca2+ transients are greatly reduced (70–100%) when RyRs are blocked (30, 42). Nevertheless, other functional studies report negligible levels of RyR-mediated CICR despite the existence of functional SR Ca2+ stores in NB myocytes (18). Likewise, we (12) recently reported that in the intact NB whole heart, the ryanodine-sensitive component of the Ca2+ transient was not larger than 12%. Thus there is no consensus regarding the role that RyR channels play in NB cardiac E-C coupling. One factor contributing to the uncertainty is that the salient studies have utilized different methodologies, preparations, and species. Another factor relates to the technical difficulties associated with obtaining acutely isolated myocytes and/or with the very small size of the hearts. Finally, the lack of single RyR channel studies establishing whether RyR function is the same in NB and AD cells have contributed to this uncertainty as well.

In this work, developmental changes in the cellular distribution of the SR were defined by immunostaining SR Ca2+-ATPase (SERCA2a) in NB and AD cells. Intracellular Ca2+ transients were examined using confocal microscopy in acutely isolated NB rat ventricular myocytes. The density of RyR protein was defined with ryanodine binding assays in both SR...
microsomes and whole heart homogenate from AD and NB rat cardiac muscle. The single-channel functional properties of NB rat RyR were also determined for the first time in planar lipid bilayer studies and were compared with those of AD. Our results indicate that in acutely isolated myocytes from the NB rat heart, the contribution of RyR-mediated CICR to the intracellular Ca\(^{2+}\) transient is negligible. This is interesting, because there are sizeable amounts of RyR protein present in the NB cell, and at least some of this protein forms RyR channels that release Ca\(^{2+}\) from intracellular stores when stimulated by caffeine. Furthermore, single-channel studies demonstrate that RyR channels in AD and NB cells have nearly identical permeation properties, gating kinetics, and ligand regulation characteristics. Thus the NB cells have functional Ca\(^{2+}\) stores containing working AD-like RyR channels, yet these stores do not contribute substantially to the intracellular Ca\(^{2+}\) transient during E-C coupling.

**METHODS**

**Isolation of ventricular myocytes.** Ventricular myocytes were enzymatically isolated from hearts of NB (1–5 days old) and AD (8 wk old) Sprague-Dawley rats of either sex. Cell isolation was conducted in accordance with Institutional Animal Care and Use Committee guidelines. Rats were injected intraperitoneally with a mixture of pentobarbital sodium (10 mg/g body wt) and heparin (2.2 U/g body wt). After 10 min, the heart was quickly removed by thoracotomy and rinsed in normal Tyrode solution (in mM: 100 NaCl, 10 KCl, 4 MgSO\(_4\), 50 taurine, 20 glucose, 2 CaCl\(_2\), and 10 HEPES, pH 7.4 adjusted with NaOH) at 37°C. The aorta was cannulated and connected to a Langendorff perfusion apparatus. The heart was then perfused with 1.28 mg/ml collagenase I (Sigma) and 0.12 mg/ml protease XIV (Sigma) for AD and 0.5 mg/ml collagenase (Yakult) for NB; 1.28 mg/ml collagenase I (Sigma, St. Louis, MO) and 0.12 mg/ml protease XIV (Sigma) for AD. The heart was then perfused with Kрафтбріт (KB) medium (in mM: 85 KCl, 30 KH\(_2\)PO\(_4\), 5 MgSO\(_4\), 5 Na\(_2\)ATP, 5 pyruvate, 5 dL-β-hydroxybutyrate, 5 creatine, 20 taurine, 20 glucose, 50 g/l polyvinylpyrrolidone (PVP-40), and 1 EGTA, pH 7.2 adjusted with KOH) for 10 min. After washout of the enzyme, both ventricles were separated from the atria and then minced. Single myocytes were obtained by gentle dispersion with a wide-bore pipette and then filtered through a cell strainer (40-μm Nylon). The entire isolation procedure was carried out with solutions equilibrated with 100% O\(_2\) at 37°C. Isolated cells were incubated for at least 30 min in KB medium at 4°C, placed in normal Tyrode solution, and kept at room temperature (RT; 21–23°C) until used. This procedure consistently yielded healthy and Ca\(^{2+}\)-tolerant NB and AD myocytes. Only quiescent myocytes with clearly visible striations were used for fluorescence experiments.

**Immunofluorescence labeling.** Double immunofluorescence labeling was conducted on ventricular myocytes from NB (2 days old) and AD rat hearts. Mouse and rabbit monoclonal antibodies directed against SERCA2a (Affinity Bioreagents, CO) and α-actinin (Sigma), respectively, were used. Acutely isolated myocytes were fixed for 10 min in 3% buffered paraformaldehyde solution, washed with PBS twice, and permeabilized for 10 min with a PBS solution containing 0.1% Triton X-100. The cells were incubated overnight with the primary antibodies, both at a dilution of 1:250, and were exposed for 2 h to fluorophore-conjugated secondary antibodies (Texas red antirat mouse IgG for SERCA2a and Alexa 488 anti-rabbit IgG for α-actinin; Molecular Probes, Eugene, OR) at a dilution 1:200. To determine nonspecific labeling, we also conducted control experiments without the primary antibody (data not shown). SERCA2a and α-actinin immunoreactivity was determined with a Radiance 2000 MP laser scanning confocal microscope (Bio-Rad, Hercules, CA) equipped with a ×40 oil immersion objective lens (Plan Fluor, numerical aperture = 1.3; Nikon). The excitation light was generated using two laser sources: green helium-neon (543 nm) and argon ion (488 nm). Emitted fluorescence was sequentially collected by a pair of photomultiplier tubes in bands between 515–530 nm (for Alexa 488) and 600–650 nm (for Texas red). A Kalman averaging filter (N = 3) was used to reduce the noise. Images (1,024 × 1,024 pixels) were acquired in XY frame-scan mode. Zoom values of 4.6 and 10 (pixel sizes of 60 and 30 nm) were used for NB and AD, respectively. A theoretical maximal resolution of ~230 nm was estimated for these conditions (numerical aperture and excitation wavelengths). Thus our sampling rates were at least twofold greater than the Nyquist rate (~115 nm).

**Image analysis.** Data were analyzed from 8-bit/pixel RGB false-color images. Alexa 488 labeling was displayed on the red channel, and Texas red signal was shown on the green channel. To estimate the degree of SERCA2a/α-actinin colocalization, we conducted a standard particle analysis with ImageJ 1.31 software (NIH). Background fluorescence of each image (pixels with fluorescence intensity below a threshold value of 25%) was subtracted. Only particles larger than 3 pixels and smaller than 1,000 pixels were considered. Distribution of the sampled particles was arrayed in squared matrices (Microsoft Excel 2002; Redmond, WA), and the interparticle distance was measured. The distance to the nearest neighboring particle was determined for each particle and plotted in a frequency distribution histogram. The extent of colocalization was estimated from the corresponding cumulative probability histogram. The statistical significance (P) of the developmental differences of SERCA2a/α-actinin colocalization and the maximal difference (D) were determined using the Kolmogorov-Smirnov test.

**Intracellular [Ca\(^{2+}\)] \(\text{m}\) measurements.** Single myocytes were incubated in 20 μM fluo-4 AM (Molecular Probes) at RT. Adequate fluorescence levels were obtained after loading periods of ~30 min for AD cells and ~45 min for NB cells. Signs of cell damage (i.e., contracture, cytoplasmic granules, or membrane blebs) appeared when longer incubation times were used. The incubation period was ended by rinsing the cells with dye-free normal Tyrode solution at RT for 15 min. Loaded myocytes were then placed on the stage of an inverted laser scanning confocal microscope (LSM 410; Carl Zeiss) equipped with a ×40 oil-immersion objective lens (Plan-Neofluar, numerical aperture = 1.3; Zeiss). Fluo-4 was excited with the 488-nm line of an argon laser. Emitted fluorescence was collected through a 515-nm long-pass emission filter. Fluo-4 images were recorded in line-scan mode with 512 pixels per line at 250 Hz. Action potentials (APs) were triggered by field stimulation with the use of platinum electrodes embedded in the experimental chamber. All AP-stimulated intracellular Ca\(^{2+}\) transients were recorded in normal Tyrode solution at RT. Only myocytes that exhibited vigorous contractility in response to field stimulation were used in this study. Ca\(^{2+}\) transient image files were analyzed with ImageJ 1.3 software (NIH).

**SR microsomes preparation.** SR microsomes were obtained from NB (1 day old) and AD (200 g) rat ventricles as previously described (33). Briefly, rats were anesthetized with intraperitoneal injection of pentobarbital sodium (10 mg/g body wt). Hearts were quickly removed by thoracotomy and rinsed in Ca\(^{2+}\)-free Tyrode solution. After the atria and all visible vascular tissues were trimmed off, the ventricles were immersed in a solution containing protease inhibitors (in μM: 0.1 aprotinin, 500 benzamidine, 1 leupeptin, 1 pepstatin A, and 200 PMSF) at 4°C. These protease inhibitors were used throughout the entire microsome preparation procedure. The ventricles were homogenized (3 times for 30 s) with a Polytron homogenizer at low speed in ice-cold saline solution (in mM: 154 NaCl and 10 Trizma maleate, pH 6.8). The resulting homogenate was then centrifuged for 20 min at 4,000 g. The 4,000-g supernatant was filtered through cheesecloth and spun again for 20 min at 8,000 g. The 8,000-g supernatant was spun in an ultracentrifuge at 100,000 g for 40 min. The 100,000-g pellet, which contained SR microsomes, was resus-
pended in a storage solution (in mM: 154 NaCl, 10 Trizma maleate, and 300 sucrose, pH 6.8), flash frozen, and stored at −80°C until used. The protein concentration of all NB and AD SR microsome preparations was determined using the Lowry method.

**Ryanodine binding.** Ryanodine binding was quantified in SR microsome preparations from NB (1 day old) and AD hearts and from AD skeletal muscle. The AD skeletal muscle was used as a positive control. The binding assays were carried out by incubating the SR microsomes (1 mg of total protein) in 150 μl of reaction mixture containing 100 mM KCl, 20 mM HEPES, and 60 nM [3H]ryanodine (56.9 Ci/mmol). In specific, reaction and dihydropropyridine binding was measured in NB and AD ventricle (whole tissue) homogenate. The protein concentration in the whole tissue homogenate was 10 mg/ml. In these assays, the 150-μl reaction mixture contained either 1 M KCl, 100 μM free Ca2+, 20 mM HEPES, and 0–100 nM [3H]ryanodine or 25 mM Tris, 10 mM Na-HEPES, 1 mM EDTA, 1.1 MgCl2, and 15 nM [3H]PN200-110. All the binding assays were conducted in the presence of protease inhibitors (0.1 mg/ml leupeptin and 0.1 mg/ml aprotinin). Nonspecific binding was measured using either 37 μM cold ryanodine or 16.6 μM cold nifedipine. The incubation lasted for 90 min and was carried out at 37°C. To define the Ca2+ dependence of ryanodine binding, we repeated the assay at different free [Ca2+]i (10−7 to 10−3 M). The cardiac Ca2+ dependence data were fitted with Eq. 1.

\[ y = \frac{B_{\text{max}}}{1 + \left( \frac{K_a}{[Ca^{2+}]^{n}} \right)^{m}} \]  

where \( B_{\text{max}} \) corresponds to the maximal number of binding sites, \( K_a \) represents half-maximal activation, and \( n_H \) is the Hill coefficient. The skeletal Ca2+ dependence data were fitted with Eq. 2.

\[ y = \frac{B_{\text{max}}}{1 + \left( \frac{[Ca^{2+}]}{K_i} \right)^{n_H} + \left( \frac{1}{K_i} \right)^{m_H}} \]

where \( K_i \) represents half-maximal inactivation and \( n_H \) is the Hill coefficient of the inactivation component of the equation.

**Planar lipid bilayers.** Single SR Ca2+ release channels were reconstituted into artificial planar lipid bilayers by fusing SR microsomes as previously described (33). Briefly, planar bilayers were formed across a 150-μm-diameter aperture in a Delrin partition. Bilayer-forming solution contained a mixture of phosphatidylethanolamine and phosphatidylcholine (7:3, 50 mg/ml decane; Avanti Polar Lipids, Pelham, AL). SR microsomes were added to one side of the bilayer (cis, cytosolic) (51). Membrane potential was controlled in the cis compartment by using a conventional patch-clamp amplifier (Axopatch 200B; Axon Instruments, Burlingame, CA), while the other side of the bilayer (trans) was connected to the ground of the amplifier. The current signal was digitized at a rate of 10 kHz with a 12-bit analog-to-digital/digital-to-analog converter (Digidata 1200; Axon Instruments), filtered with a Bessel filter at 2 kHz, and stored for later analysis. The recording solutions contained 20 mM HEPES-Tris (pH 7.4), 10 μM added Ca2+, and the charge carrier. The charge carrier was either Cs+ [50/250 mM (cis/trans) CsCH3SO3] or Ca2+ [2–30 mM trans CaCH3SO3]. Free [Ca2+]i in the cis chamber was adjusted to various levels (0.1 μM to 1 mM) and was buffered with either EGTA (see Fig. 8) or HEDTA (see Fig. 9). Free [Ca2+]i in the recording solutions was measured off-line with custom-made Ca2+ electrodes (1).

**Single-channel analysis.** Unitary current amplitude was measured by fitting Gaussian functions to the total amplitude histograms (i.e., difference between mean current of the closed and open peaks). Open probability (\( P_o \)) values were determined either from the relative areas of the Gaussian distributions fit to total amplitude histograms or from idealized records obtained using the 50% amplitude criteria. Open (O) and closed (C) times were obtained from idealized records and plotted as histograms. Modal gating behavior, studied in bilayers containing only one channel, was evaluated from dependency plots constructed as two-dimensional dwell-time distributions. A detailed description of how dependency plots were constructed can be found in supplemental data for this article, which may be found at http://ajpheart.physiology.org/cgi/content/full/00188.2004/DC1.

Data acquisition, unitary current measurements, statistical analysis, and data processing were performed using commercially available software packages (pCLAMP version 8.0; Axon Instruments, Union City, CA; Microsoft Excel 2002; and Microlab Origin version 7.0; Northampton, MA). Gating kinetics analysis was conducted with custom-made routines written in the LabView version 7.0 programming language (National Instruments, Austin, TX). Averaged data points represent means ± SE. Statistical significance was determined with two-sample paired t-tests. The data presented were obtained from 60 isolated myocytes (10 AD and 50 NB), 9 SR microsome preparations (2 AD and 7 NB), and 97 bilayer incorporations (52 AD and 45 NB). Preliminary versions of this work were previously presented at Annual Biophysical Society Meetings.

**RESULTS**

**SERCA2a shows sarcomeric distribution in NB ventricular myocytes.** In NB ventricular myocytes, a poorly developed SR has been associated with the absence of a well-formed T-tubular system. For this reason, we examined the level of maturity and structural organization of the SR in relation to the sarcomeric arrangement of the cytoskeleton in NB rat. To this end, SERCA2a and α-actinin were immunostained in double-labeling experiments conducted on acutely isolated myocytes from NB (2 days old) and AD rat hearts. Our results indicate that in NB myocytes, the fluorescence signal arising from SERCA2a (Fig. 1A, left) was already strong and well defined, consistent with a high level of SR organization. In addition, this arrangement exhibited a distribution oriented transversely to the longitudinal axis of the cell. Regular spacing of ~2 μm between fluorescent bands was evident, corresponding well with the sarcomeric length observed with α-actinin staining in the same NB myocyte (Fig. 1A, middle) and in AD myocytes (Fig. 1B, middle). The colocalized pixels (Fig. 1A and B, right, in white) indicate that SERCA2a and α-actinin are closely associated in a sarcomeric arrangement. The high degree of colocalization indicates that this transversely striated pattern most likely arises from the Z lines and the junctional SR. The exclusion of SERCA2a and α-actinin from the nuclear region of the cell further indicates the sarcomeric distribution of the SR in NB myocytes. In AD myocytes, a clear correlation between SR distribution (Fig. 1B, left) and the sarcomere (Fig. 1B, middle) was also evident as a transversely striated pattern. However, a second pattern of axially oriented stripes was evident only in AD. Because this pattern did not colocalize with α-actinin, it could be attributed to the longitudinal free SR. Quantification of the distance between fluorescent particles revealed a significant reduction in colocalization with development (Fig. 1C). This could further indicate the emergence of a more prominent longitudinal SR in AD.

**Ca2+ transients in single ventricular myocytes.** The general attributes of AP-induced intracellular Ca2+ transients (n > 50) were first defined in acutely isolated ventricular myocytes from the NB rat (Fig. 2). The Ca2+ transients were recorded in fluo-4-loaded myocytes acutely dissociated from NB and AD rat hearts. The two-dimensional line-scan image shown in Fig.
A, top, reveals an inhomogeneous pattern of intracellular \([Ca^{2+}]\) distribution upon electrical stimulation in a 5-day-old NB myocyte. After 200 ms of field stimulation, a ring of increased fluorescence in the subsarcolemmal region was evident, whereas in the center of the cell the fluorescence increase was relatively small. The fluorescence intensity plot along the line marked with the asterisk shows a U-shaped distribution of \([Ca^{2+}]\) across the cell, with larger fluorescence intensity at the edges of the cell. A three-dimensional (3-D) reconstruction image (Fig. 2A, bottom) also reveals the heterogeneous distribution of intracellular \([Ca^{2+}]\).

Developmental changes of the \([Ca^{2+}]\) transient kinetics were also defined by measuring the rising (time to peak) and decaying phases in NB and AD ventricular myocytes. Normalized \([Ca^{2+}]\) transients from a 5-day-old NB and AD are shown in Fig. 2C. The time to peak of the NB \([Ca^{2+}]\) transient was substantially longer (>2-fold) than that of the AD cell. However, the time constant of the decay was similar to that of the AD cell. Data pooled from 1-day-old NB and AD cells are summarized graphically in Fig. 2D. Time to peak became significantly shorter with age, whereas the time constant of the \([Ca^{2+}]\) transient decay did not exhibit significant differences between the NB and AD cells.

Because previous studies have demonstrated the existence of RyRs in NB cells, it is possible that at this age, RyR-mediated \([Ca^{2+}]\) release makes an important contribution to the \([Ca^{2+}]\) transient. To estimate this component, we recorded \([Ca^{2+}]\) transients in AD and NB cells in the absence (control) and in the presence of a high dose (1 or 10 \(\mu M\)) of ryanodine (Fig. 3A). The ryanodine application had two different effects: reduction of the peak amplitude of the \([Ca^{2+}]\) transient and elevation of the resting \([Ca^{2+}]\). In AD cells, peak amplitude was reduced ~70% and the resting \([Ca^{2+}]\) level was substantially elevated. However, in 1-day-old NB, peak amplitude was reduced ~13% and the resting \([Ca^{2+}]\) level was only slightly elevated. This implies that the contribution of RyR-mediated \([Ca^{2+}]\) release to the \([Ca^{2+}]\) transient in NB cells is small. This could result from the absence of functional RyR-mediated SR \([Ca^{2+}]\) stores in NB cells. To directly test this possibility, we
applied caffeine to NB cells. Figure 3B shows results of a representative experiment conducted on a 2-day-old NB ventricular myocyte. Figure 3B, top, shows a longitudinal “x-t” line scan, and the trace (Fig. 3B, bottom) shows the corresponding averaged fluorescence intensity. To ensure adequate SR Ca$^{2+}$ load, we first elicited AP-induced Ca$^{2+}$ transients at a frequency of 0.5 Hz. After a 4-s quiescent period, 5 mM caffeine was rapidly applied to the cell. As a result, a transient increase of intracellular [Ca$^{2+}$] was observed. This transient was slightly larger and slower compared with the electrically stimulated Ca$^{2+}$ transient in a 4-day-old NB myocyte. 

Fluorescence intensity measured at the center (C; shaded curve) and subsarcolemma (SS; solid curve) is shown at bottom. C: developmental changes of Ca$^{2+}$ transient kinetics. Averaged fluorescence values (10 traces) from AD and NB (5 days old) were normalized and overlapped. Time to peak in AD was 125.2 ms, whereas that in NB was 268 ms. Ca$^{2+}$ transient decay ($\tau$) was fitted with a single-exponential function (blue curves) for both AD ($\tau = 261.8$ ms) and NB ($\tau = 271.3$ ms). D: pooled data illustrate developmental changes in the Ca$^{2+}$ transient kinetics. *Statistically different ($P < 0.05$) from 1-day-old NB.

Ryanodine binding in NB and AD rat heart. An alternative explanation for the minor contribution of RyR-mediated SR Ca$^{2+}$ release to the intracellular Ca$^{2+}$ transient in NB cells is that RyR density early after birth may be very low. To test this possibility, we determined specific ryanodine binding to the SR microsomal fraction from NB (1 day old) and AD rat ventricles (Fig. 4, A and B). The SR microsomes isolated from NB muscle contained ~64% lower ryanodine binding site density ($B_{\text{max}}$) compared with SR microsomes isolated from AD muscle. In contrast, the ryanodine binding affinity of these two preparations was similar ($K_d = 25–32$ nM). As expected for the cardiac RyR isoform, the Ca$^{2+}$ dependence of ryanodine binding in both NB and AD SR microsomes was sigmoidal between 100 nM and 1 mM Ca$^{2+}$ (Fig. 4C) (15, 34). $K_a$ for both data sets occurred between 2 and 3 $\mu$M with a $n_H$ of ~2. The reduced $B_{\text{max}}$ in the NB case is also evident in these data. As a positive reference, the characteristic bell-shaped Ca$^{2+}$ dependence of the skeletal RyR isoform (7) also is shown (Fig. 4C). The similarity of [Ca$^{2+}$] dependence parameters ($K_a$ and $n_H$) of the NB and AD cardiac binding data suggests that the RyRs in these preparations have similar functional attributes (i.e., Ca$^{2+}$ sensitivity) but simply exist at lower density in NB cells. 

To evaluate the developmental changes in RyR density relative to the entire cell content, we also measured ryanodine binding in crude homogenate from NB and AD rat ventricles (Fig. 4D). Because the RyR is both physically and functionally associated with the L-type Ca$^{2+}$ channel also known as the dihydropyridine receptor (DHPR), DHPR density and DHPR-to-RyR density ratio also were determined in NB and AD hearts (Fig. 4D). The NB ventricles contained a lower density of both RyR and DHPR compared with the AD tissue (~74% and 58% lower, respectively). In contrast, the DHPR-to-RyR density ratio was 40% larger in NB than in AD, which could reflect an increase in the Ca$^{2+}$ signaling gain of the system.
Reconstitution of single NB RyR Ca$^{2+}$ release channels. Representative single RyR channel activity reconstituted from NB and AD SR microsomes is shown in Fig. 5. These recordings were obtained at $-10$ mV in a 0.01/10 mM (cis/trans) Ca$^{2+}$ gradient. The NB and AD channels were spontaneously active in the presence of 10 μM Ca$^{2+}$ (Fig. 5, top). In both cases, the corresponding total amplitude histograms indicate that the main current amplitude was ~3 pA. In both channels, control gating was characterized by frequent brief openings with few events lasting more than a few milliseconds. The permeation and gating of both the NB and AD channels were clearly modified by the addition of ryanodine (Fig. 5, bottom). Ryanodine induced long-duration opening events to a subconductionate level, which is the classic action of ryanodine on a single RyR channel (41). Thus these results positively identify these channels as RyR channels and, more importantly, confirm that the NB SR microsome preparations contain functional RyR channels. The next step was to determine whether the functional properties of single NB and AD RyR channels are similar.

Permeation properties of the NB RyR channel. The permeation properties of the NB and AD RyR channels were defined with Cs$^{+}$ and Ca$^{2+}$ as charge carrier (Fig. 6). Stationary single-channel activity was recorded at different holding potentials in the presence of either a Cs$^{+}$ (Fig. 6A) or Ca$^{2+}$ gradient (Fig. 6B). The slope conductance ($\gamma$) of the corresponding current-voltage relationship for NB RyR channels with Cs$^{+}$ or Ca$^{2+}$ as charge carrier was ~420 and ~100 pS, respectively (Fig. 6, A and B, right). There were no significant differences in either $\gamma_{Cs}$ or $\gamma_{Ca}$ between the NB and AD RyR channels. The relative selectivity of the NB and AD channels for Ca$^{2+}$ and Cs$^{+}$ was determined from the apparent reversal potential ($E_{rev}$) during a 3-s voltage ramp (from $-50$ to $+70$ mV) in asymmetric ionic conditions. Single-channel activity was recorded in the presence of equal Ca$^{2+}$ and Cs$^{+}$ concentrations (30 mM) on opposite sides of the membrane. Two representative traces are shown in Fig. 6C. $E_{rev}$ (+47 and +49 mV), indicated by the arrows, was defined as the point at which the unitary current crossed the zero level (horizontal line). At potentials negative to $E_{rev}$, opening events (downward deflections) represent net Ca$^{2+}$ current, whereas at potentials positive to $E_{rev}$, opening events (upward deflections) result from net Cs$^{+}$ currents. From these $E_{rev}$ data, the Ca$^{2+}$/Cs$^{+}$ permeability ratio ($P_{Ca}/P_{Cs}$) was calculated using the following form of the Goldman-Hodgkin-Katz equation:

$$\frac{P_{Ca}}{P_{Cs}} = \left( \frac{[Cs^+]_{cis}}{[Ca^{2+}]_{trans}} \right) \cdot e^{2F_{rev}/RT}$$

where $F$ is Faraday’s constant, $R$ is the gas constant, $T$ is temperature, and $[Cs^+]_{cis}/[Ca^{2+}]_{trans}$ equals 1. The calculated $P_{Ca}/P_{Cs}$ values of the NB and AD RyR channels were similar with values of ~10, indicating that both channels were ~10-fold more selective for Ca$^{2+}$. Thus the NB preparations not only contain functional RyR channels, but these channels also have permeation properties identical to those of their AD counterparts.

Modal gating kinetics of NB RyR channel. The RyR-mediated Ca$^{2+}$ mobilization depends not only on the amplitude of unitary Ca$^{2+}$ current amplitude but also on the duration of RyR openings. The RyR is known to gate in modal fashion (43, 54), which means that the channel spontaneously shifts between periods of low and high $P_o$. To investigate whether gating kinetics of NB RyR were different from those of AD, we...
studied modal gating behavior under stationary conditions (Fig. 7). To this end, we collected long steady-state recordings of NB and AD RyR channel activity at 0 mV in the presence of a 0.01/30 mM (cis/trans) Ca\(^{2+}\) gradient. Selected NB and AD single RyR channel records illustrating the different gating modes are shown (Fig. 7, A and B, left). Some periods of low \(p_{o}\) (short openings and long closures) are marked by dotted lines, whereas some other periods of high \(p_{o}\) (long openings and short closures) are marked by red lines. To quantify this gating behavior, we constructed open and shut 3-D dwell-time dependency plots by assuming that either all events (open and shut) are independent or adjacent events (open-shut) are linked. The difference between the random and linked histograms reveals the correlation between particular open and shut dwell times. The NB and AD 3-D difference plots (Fig. 7, A and B, right) also are shown to have distinct peaks and valleys. The peaks indicate positive correlations between particular classes of open and shut times. One peak indicates that short openings and long closures are positively correlated. This corresponds to the low-\(p_{o}\) mode. The other peak indicates that long openings and short closures also are positively correlated. This, of course, corresponds to the high-\(p_{o}\) mode. Valleys indicate regions of negative correlation (low likelihood of occurrence). The similarity between the 3-D difference plot for the NB and AD channels is clear, indicating that under these experimental conditions, the NB and AD channels exhibit comparable modal gating kinetics. Thus the NB preparations not only contain functional RyR channels, but their gating is like that of the AD channel.

**Regulation of NB RyR channels by endogenous ligands.** A key aspect of single RyR channel regulation is its cytosolic Ca\(^{2+}\) sensitivity (8). In the present study, the cytosolic Ca\(^{2+}\) sensitivity of NB and AD channels was compared under steady-state experimental conditions. Figure 8A shows representative single-channel recordings obtained at three different cytoplasmic [Ca\(^{2+}\)] (1 \(\mu\)M, 100 \(\mu\)M, and 1 mM). In these studies, the charge carrier was a monovalent cation (Cs\(^{+}\)) to prevent potential gating effects in the cytoplasmic side induced by Ca\(^{2+}\) going thorough the pore. In addition, the use of Cs\(^{+}\) maximized the signal-to-noise ratio while minimizing potential currents through SR K\(^{+}\) channels (10, 19). In the presence of 1 \(\mu\)M Ca\(^{2+}\), the activity of both NB and AD channels was low, consisting primarily of brief openings separated by long closures. In both cases, channel activity increased substantially when the cytoplasmic [Ca\(^{2+}\)] was elevated. To better quantify the Ca\(^{2+}\) sensitivity of these channels, their average \(P_{o}\) (\(n = 4\) for each age) at several different [Ca\(^{2+}\)] was determined, normalized, and plotted as a function of cytoplasmic [Ca\(^{2+}\)] (Fig. 8B). The averaged data were fitted with the following

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**Fig. 4.** Developmental changes in specific \([\text{H}]\)ryanodine binding. A: nonlinear \([\text{H}]\)yanodine binding \((n = 3)\) to SR microsomes from NB (diamonds) and AD (circles). B: Scatchard analysis of the data shown in A. Ryanodine binding site density \((B_{\text{max}})\) was 0.49 pmol/mg protein in NB and 1.35 pmol/mg protein in AD, and the apparent ryanodine binding affinity \((K_{d})\) was 32 ± 5 nM in NB and 25 ± 4 nM in AD. C: [Ca\(^{2+}\)] dependence of specific \([\text{H}]\)yanodine binding to SR microsomes obtained from NB heart (1 day old; diamonds), AD heart (circles), and AD skeletal muscle (triangles). Smooth curves were generated using Eq. 1 for heart and Eq. 2 for skeletal muscle. בארים were 0.46, 0.78, and 0.92 pmol/mg protein for NB heart, AD heart, and AD skeletal muscle, respectively. Half-maximal activation \((K_{a})\) was 2.9, 2.0, and 3.4 \(\mu\)M for NB heart, AD heart, and AD skeletal muscle, respectively. Half-maximal inactivation \((K_{i})\) for skeletal muscle was 22 \(\mu\)M. The Hill coefficient \((n_{H})\) was 2, 1.7, and 1.8 for NB heart, AD heart, and AD skeletal muscle, respectively. Values for DHPR-to-RyR ratio \((\text{right axis})\) were obtained from the ratio of \([\text{H}]\)nifedipine/[\text{H}]ryanodine to the whole heart homogenate. DHPR-to-RyR binding density ratio was 0.25 and 0.15 for NB and AD, respectively.

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**Fig. 5.** Developmental changes in specific \([\text{H}]\)nifedipine binding. \(B_{\text{max}}\) was 4 fmol/mg wet wt in NB and AD, respectively. *Statistically different \((P < 0.05)\) from AD. Nifedipine binding density was 8.7 ± 1 and 20.5 ± 0.8 fmol/mg in NB and AD, respectively. Values for DHPR-to-RyR ratio \((\text{right axis})\) were obtained from the ratio of \([\text{H}]\)nifedipine/[\text{H}]ryanodine binding to the whole heart homogenate. DHPR-to-RyR binding density ratio was 0.25 and 0.15 for NB and AD, respectively.
equation, which contains both activation and inactivation components:

$$P_o = P_o^{\text{max}} \cdot \left( \frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + K_{\text{act}}} \right) \cdot \left( 1 - \frac{[\text{Ca}^{2+}]}{[\text{Ca}^{2+}] + K_{\text{inact}}} \right)$$  (4)

The parameter $P_o^{\text{max}}$ represents the maximal $P_o$, $K_{\text{act}}$ corresponds to the activation midpoint, $a$ is the activation coefficient, $K_{\text{inact}}$ represents the inactivation midpoint, and $i$ is the inactivation coefficient. The best fit was obtained for NB when $K_{\text{act}} = 1.6 \, \mu\text{M}$, $a = 5.5$, $K_{\text{inact}} = 6.3 \, \text{mM}$, and $i = 2.2$; and for AD when $K_{\text{act}} = 3.2 \, \mu\text{M}$, $a = 2.3$, $K_{\text{inact}} = 5 \, \text{mM}$, and $i = 2.3$. Thus the NB and AD RyR channel activity exhibited very similar cytosolic Ca$^{2+}$ sensitivity.

Cytoplasmic ATP is another important endogenous activator of the RyR channel. Figure 9A shows its effect in NB and AD RyR channels in the presence of a low cytosolic [Ca$^{2+}$]. The single RyR channel recordings shown are 10- and 5-s segments obtained from NB and AD, respectively, in each of the sequential (left to right) experimental conditions indicated. The bar values ($n = 4$) below each recording indicate the $P_o$ in each experimental condition. In control conditions (100 nM cytosolic Ca$^{2+}$), the NB and AD RyR channels exhibited a very low level of activity, consistent with the data presented in Fig. 8B.

Additions of progressively larger cytosolic concentrations of ATP (0.4 to 2 mM) activated the channels to $P_o$ values of $-0.4$. This effect was fully reversible when ATP was removed. Addition of caffeine substantially activated the channels as well. Thus there were no appreciable differences in the responses of NB and AD RyR channels to the ATP or caffeine challenges.

The inhibitory action of cytosolic Mg$^{2+}$ also was defined for both the NB and AD RyR channels (Fig. 10). In this study, single RyR channel activity was recorded at 0 mV in the presence of 5 μM cytosolic Ca$^{2+}$ with different cytosolic concentrations of Mg$^{2+}$ (0.1 to 2 mM). Representative NB and AD single-channel recordings are shown (Fig. 10, left). Single RyR channel activity decreased as the cytosolic Mg$^{2+}$ concentration was raised. The presence of cytosolic Mg$^{2+}$ reduced both the $P_o$ and unitary current of the channels. The reduction in current amplitude occurred because Mg$^{2+}$, being permeable, can compete with charge carrier for occupancy of the pore (33). The reduction in $P_o$ occurred because Mg$^{2+}$ directly competes with Ca$^{2+}$ for the activation site of the channel (28, 38). To test this interpretation, we repeated the Mg$^{2+}$-induced inhibition experiments at a higher cytosolic [Ca$^{2+}$] (100 μM). Under these conditions, cytosolic Mg$^{2+}$ still reduced $P_o$, but...
Fig. 6. Single-channel permeation properties of NB and AD RyR. A: Cs\(^+-\) conductance. Single-channel activity from NB (left) and AD (middle) SR microsomes recorded in 50/250 (cis/trans) mM Ca(CH\(_3\)SO\(_3\))\(_2\) and symmetrical 10 \(\mu\)M Ca\(^{2+}\) at the indicated membrane potentials. Opening events are shown as downward deflections. Zero-current level corresponds to the closed state (C) and is indicated by horizontal dotted lines. A current-voltage relationship (right) was constructed with pooled data from 4 (AD; circles) and 3 (NB; diamonds) experiments. Ca\(^{2+}\) conductance values obtained by linear regression are 426 \pm 35 and 413 \pm 22 pS for NB and AD, respectively. B: Ca\(^{2+}\) conductance. Single-channel activity from NB (left) and AD (middle) SR microsomes recorded at indicated potentials. Unitary current was recorded in 0.01/30 (cis/trans) mM Ca(CH\(_3\)SO\(_3\))\(_2\) and symmetrical 20 mM HEPES. A current-voltage relationship (right) was constructed with pooled data from 3 (AD; circles) and 4 (NB; diamonds) experiments. Ca\(^{2+}\) conductance values obtained by linear regression are 102 \pm 15 and 98 \pm 18 pS for NB and AD, respectively. C: Ca\(^{2+}\)/Cs\(^+-\) selectivity. Single RyR channel activity was recorded from NB and AD SR microsomes with a 2-s voltage ramp (from -50 to +70 mV, 0 mV holding potential). The 
trans solution contained 30 mM Ca(CH\(_3\)SO\(_3\))\(_2\) and 20 mM HEPES (pH 7.4), whereas the cis solution contained 30 mM CaCH\(_3\)SO\(_3\), 20 mM HEPES, and 10 \(\mu\)M CaCl\(_2\) (pH 7.4). Cation selectivity was estimated from the apparent reversal potential (\(E_R\), indicated by arrows). Zero-current level (closed state) is indicated by horizontal dotted lines. The permeability ratio (\(P_{Ca}/P_{Cs}\)), calculated using Eq. 3, has a value of 9.3 for NB and 10.6 for AD.

higher concentrations were required. These data are summarized in dose-response plots (Fig. 10, right). These plots were constructed from pooled data from four experiments at each [Ca\(^{2+}\)]. The \(P_o\) values were normalized to the maximal value obtained before Mg\(^{2+}\) addition. The smooth curves were generated using the following equation:

\[
P_o = \frac{1}{1 + \left(\frac{[\text{Mg}^{2+}]}{K_d}\right)^n} \tag{5}
\]

The parameter \(K_d\) corresponds to the IC\(_{50}\). In the presence of 100 \(\mu\)M cytoplasmic Ca\(^{2+}\), the Mg\(^{2+}\) affinity was substantially reduced. In the case of the NB (Fig. 10A), the \(K_d\) increase was sixfold (from 0.26 to 1.6 mM). A comparable result was obtained for the AD RyR channel (Fig. 10B).

**DISCUSSION**

In this study, two novel contributions were presented. First, the developmental changes of the intracellular Ca\(^{2+}\) transient, particularly of the RyR-mediated fraction, were defined in acutely isolated ventricular myocytes from the NB rat heart. The originality of this part of the study is that most previous works focusing on this topic have been conducted either in different species [rabbit (18)] or with different experimental preparations [multicellular (12, 49), primary cultures (21, 31), and fetal cells (46)]. A second unique aspect is the multidisciplinary experimental approach used in this study that includes scanning confocal microscopy of the SR cellular distribution and RyR-mediated Ca\(^{2+}\) release, radiolabeled ryanodine and dihydropyridine (DHP) binding, and single RyR channel recording to directly define, for the first time, the functional profile of NB RyR. Our results indicate that the RyR-mediated CICR contribution to the AP-triggered intracellular Ca\(^{2+}\) transient in NB cells is negligible. This is interesting because the NB cells have functional intracellular Ca\(^{2+}\) stores that contain working RyR channels. Furthermore, our single-channel studies revealed that these NB RyR channels have similar permeation, gating kinetics, and regulatory properties compared with AD RyR channels. Therefore, our results indicate that other mechanisms must exist to explain why in NB cells these channels do not contribute more substantially to the AP-triggered intracellular Ca\(^{2+}\) transient. Possible answers are discussed below.

It is possible that RyR channel density in NB cells is just too low to make a substantial contribution to the AP-triggered intracellular Ca\(^{2+}\) transient. One way to evaluate this possibility is to compare RyR density and the fraction of RyR-mediated CICR in NB and AD cells. The RyR-mediated CICR contribution in AD cells represents 70% of the AP-triggered...
Ca\(^{2+}\) transient (see Fig. 3). In NB cells, however, it represents only 13% of the AP-triggered Ca\(^{2+}\) transient. If RyR contribution is directly proportional to the RyR density, the expected RyR density in NB should be \(~18\%\) of that present in the AD [according to a simple linear relationship of (70\% CICR/100\% RyR density) in AD = (13\% CICR/\sim18\% RyR density) in NB]. However, ryanodine binding data from Fig. 4A indicate that the actual RyR protein density in SR microsomes from NB cells is 36\%, a value twofold larger than the simplistic prediction. Several factors should be considered, however, before any conclusion is drawn. Our binding data from SR microsomal preparations involved normalization to total SR protein. Because this total may also change during development, RyR and DHPR protein density was determined in crude homogenates of NB and AD ventricular tissue (Fig. 4D). Our results in crude homogenate were normalized to milligrams of wet tissue. If we assume a total protein concentration of 109 mg/ml cell volume and a ventricular density of 1.06 g/ml (3), our results would indicate a density of ryanodine binding sites of 337 and 1,306 fmol/mg protein in NB and AD, respectively. Similarly, the density of DHP binding sites would correspond to 84 and 190 fmol/mg protein in NB and AD, respectively. Our values correspond well with those reported previously for RyR and DHPR density (14, 23, 35, 39). In addition, our value for the RyR-to-DHPR ratio in AD tissue of 6.4 also agrees with the values of 6.9 and 7.3 in rat ventricle reported by Wibo et al. (53) and Bers and Stiffel (4), respectively. Together, our binding data showed that the RyR protein density in NB was \sim25\% of that present in the adult. Therefore, our results indicate that some RyRs do not effectively contribute to the AP-triggered Ca\(^{2+}\) transient.

This idea was recently addressed by Sedarat et al. (45), who evaluated the level of coimmunolocalization of DHPRs and RyRs in NB rabbit heart myocytes of different ages. Their results indicate that in 3-day-old myocytes, the fraction of RyRs colocalized with DHPRs is no larger than 10\%. Thus our observation that RyR-mediated CICR represents 13\% of the AP-triggered Ca\(^{2+}\) transient is consistent with that fraction.

Although in the AD rat, only 37\% of RyRs colocalize with DHPRs (44), the large ryanodine-sensitive component of the Ca\(^{2+}\) transient indicates that in the mature heart, even the uncoupled RyRs are efficiently recruited by CICR during an AP. In NB, however, only a fraction of RyRs appears to participate in the E-C coupling. This raises an interesting question: What is the functional role of the rest of the RyRs in NB cells? The “other” RyRs simply may be in the pipeline for future insertion into the developing E-C coupling apparatus, or they may be mediating intracellular Ca\(^{2+}\) signaling unrelated to E-C coupling.

Thus the lower RyR channel density in NB cells likely is not the only reason for the small RyR-mediated CICR contribution to the AP-triggered intracellular Ca\(^{2+}\) transient. The arguments...
presented above and published previously have assumed that the RyR channels in NB and AD cells are functionally equivalent entities. The validity of this assumption was tested in our study.

To our knowledge, this study is the first in which NB RyR functional properties have been defined at the single-channel level. Unitary conductance (in \(Cs^+\) and \(Ca^{2+}\)), ion selectivity (\(P_{Cs}/P_{Ca}\)), and stationary modal gating kinetics, as well as the channel’s sensitivity to exogenous (caffeine and ryanodine) and endogenous (\(Ca^{2+}\), ATP, and \(Mg^{2+}\)) ligands, were carefully defined in this work. No substantial differences were detected during development. The steady-state cytosolic \(Ca^{2+}\) sensitivity of the NB RyR channel was nearly identical to that found in AD and corresponded to the RyR type 2 isoform (7, 8, 29). Thus the single-channel results presented clearly validate the assumption that the RyR channels in NB and AD cells are functionally equivalent entities and are most likely arising from the same molecular isoform (i.e., type 2). Consequently, changes in single-channel properties cannot be proposed as a mechanism to explain the developmental differences in RyR contribution to the AP-triggered intracellular \(Ca^{2+}\) transient.

Alternatively, it is possible that the signaling environment around the RyR channels in the NB cells will affect their contribution to the AP-triggered intracellular \(Ca^{2+}\) transient. In AD cells, most RyR channels are associated to DHPRs, whereas in NB cells, because of the absence of T tubules, the RyRs are either coupled to the sarcolemma in the periphery or uncoupled, forming corbular SR (45). For this reason, the \(Ca^{2+}\) transient reflects primarily the \(Ca^{2+}\) influx across the sarcolemma. Our results (Fig. 2, A and B) are consistent with this view; that is, \(Ca^{2+}\) signal starts in the cell’s periphery and then propagates to the center, where it activates uncoupled RyRs.
less effectively because of slower kinetics (2, 13). This pattern of AP-triggered Ca\textsuperscript{2+} entry also occurs in AD atrial myocytes (5, 20, 26) and Purkinje cells (9), where a well-developed T-tubular system is absent.

Other mechanisms by which the local signaling environment could screen or attenuate the RyR function in NB involve cross talk with inositol 1,4,5-trisphosphate receptors [Ins(1,4,5)P\textsubscript{3}Rs]. This interaction could be favored because of the relatively large Ins(1,4,5)P\textsubscript{3}R density found in the immature heart (40). In this regard, it has been reported in cultured cells from NB rat that Ins(1,4,5)P\textsubscript{3}R-mediated Ca\textsuperscript{2+} release can reduce the contribution of RyRs to the intracellular Ca\textsuperscript{2+} signaling due to Ca\textsuperscript{2+} depletion of the SR (21a). Although our work did not directly address this question, the sizeable caffeine-sensitive Ca\textsuperscript{2+} release observed in NB myocytes (Fig. 2B) makes this mechanism unlikely.

In conclusion, our study shows that there is a marked difference between AD and NB cells in the contribution of RyR-mediated CICR to the AP-triggered intracellular Ca\textsuperscript{2+} transients. The CICR component in NB is very small and cannot be attributed to the absence of functional intracellular Ca\textsuperscript{2+} stores or to differential single RyR channel properties. Instead, our results suggest that the small contribution of CICR to the AP-triggered Ca\textsuperscript{2+} transients in NB cells arise, at least partially, from the lower density of RyRs. Other contributing factors not investigated in the present study may include the cellular RyR distribution in NB cells and the relative distance

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**Fig. 10.** Modulation of NB and AD RyR channel activity: Mg\textsuperscript{2+} inhibition. A: single-channel recordings (left) show the effect of different Mg\textsuperscript{2+} concentrations on NB RyR channel activity recorded at a holding potential of 0 mV in the presence of either 5 or 100 \muM free Ca\textsuperscript{2+} on the cytoplasmic (cis) side of the channel and 50/250 (cis/trans) mM CsCH\textsubscript{3}SO\textsubscript{3} as charge carrier. A dose-response curve (right) of pooled data from 4 different channel experiments shows the inhibitory effect of Mg\textsuperscript{2+} in the presence of 5 (diamonds) and 100 \muM (circles) cytoplasmic (cis) Ca\textsuperscript{2+}. Smooth curves were generated using Eq. 5. The best fit was obtained at 5 \muM Ca\textsuperscript{2+} when \textit{K}_{d} = 256 \muM and \textit{n}_{H} = 2.5. The best fit was obtained at 100 \muM Ca\textsuperscript{2+} when \textit{K}_{d} = 1.6 \muM and \textit{n}_{H} = 1.6. B: single-channel recordings (left) show the effect of different Mg\textsuperscript{2+} concentrations on AD RyR channel activity recorded at a holding potential of 0 mV in the presence of either 5 or 100 \muM free Ca\textsuperscript{2+} on the cytoplasmic (cis) side of the channel. A dose-response curve (right) of pooled data from 3 different channel experiments shows the inhibitory effect of Mg\textsuperscript{2+} in the presence of 5 (diamonds) or 100 \muM (circles) cytoplasmic (cis) Ca\textsuperscript{2+}. The best fit was obtained at 5 \muM Ca\textsuperscript{2+} when \textit{K}_{d} = 180 \muM and \textit{n}_{H} = 4.6. The best fit was obtained at 100 \muM Ca\textsuperscript{2+} when \textit{K}_{d} = 2.4 \muM and \textit{n}_{H} = 3.3.
between these Ca\(^{2+}\) release channels and the sarcolemmal Ca\(^{2+}\) trigger.

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