Developmental Changes in Cardiac Myocyte Calcium Regulation

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Developmental changes in the contributions of transsarcolemmal Ca\(^{2+}\) influx and Ca\(^{2+}\) release from intracellular storage sites to myocardial contraction were evaluated in isolated ventricular myocytes from neonatal (aged 1–7 days) and adult (aged 8–10 weeks) New Zealand White rabbits. Contractions ceased in one beat when extracellular Ca\(^{2+}\) was decreased from 1 mM to micromolar levels using a rapid perfusion technique. On reperfusion with 1 mM Ca\(^{2+}\), recovery of control contraction amplitude occurred after significantly fewer beats in neonatal myocytes compared with adult myocytes, and after 1 minute compared with 5 minutes of reduced Ca\(^{2+}\). After 15 minutes of perfusion with either 1 or 10 \(\mu\)M ryanodine, contraction amplitude decreased in both age groups, but the decrease was significantly greater in adults than in neonates. These experiments indicate that isolated ventricular myocytes may be used in the study of developmental changes in intracellular Ca\(^{2+}\) regulation. Results suggest that cardiac contraction in neonates is relatively more dependent on transsarcolemmal Ca\(^{2+}\) influx. Furthermore, although Ca\(^{2+}\) release from intracellular storage sites is present in both neonates and adults, its role in cardiac contraction is more significant in adults. (Circulation Research 1990;67:574–579)

Contractility and contractile reserve are decreased in the neonate as compared with the adult.\(^1\)–\(^4\) Myocardial contractility is related to intracellular Ca\(^{2+}\) concentration, and the mechanisms for increasing intracellular Ca\(^{2+}\) include transsarcolemmal Ca\(^{2+}\) influx and Ca\(^{2+}\) release from intracellular storage sites, in particular, the sarcoplasmic reticulum (SR).\(^5\)–\(^7\) Calcium-45 uptake in intact cardiac myocytes has been shown to be distributed in “rapidly” and “slowly” exchangeable compartments,\(^8\) proposed to correlate with transsarcolemmal Ca\(^{2+}\) influx and SR Ca\(^{2+}\), respectively.\(^8,9\)

In the present study, we compared the characteristics of freshly isolated myocytes from 1–7-day-old neonatal rabbits with those from 8–10-week-old adult rabbits. Using techniques previously developed for measuring contraction amplitude and the rapid exchange of extracellular perfusate,\(^9\) we studied developmental alterations in the “rapidly” and “slowly” exchangeable compartments of Ca\(^{2+}\) and their relative contributions to contraction. We investigated the contributions of transsarcolemmal Ca\(^{2+}\) influx and SR Ca\(^{2+}\) by observing the rate of recovery to control contraction amplitude after periods of reduced extracellular Ca\(^{2+}\). We studied SR Ca\(^{2+}\) release by observing the decrease in contraction amplitude after the administration of 1 and 10 \(\mu\)M ryanodine, an alkaloid known to alter Ca\(^{2+}\) release from the SR.\(^10,11\)

Materials and Methods

Neonatal and adult cardiac myocytes were prepared by a technique similar to that described by Mitra and Morad.\(^12\) Notable differences included lower enzyme concentrations and longer digestion times. The methods used in the measurement of contraction amplitude and rapid exchange of extracellular fluid were developed by Rich et al.\(^9\)

Cell Isolation

Neonates. Neonatal myocytes were obtained from 1–7-day-old New Zealand White rabbits weighing 75–125 g. The rabbits were anticoagulated with 1,000 units/kg sodium heparin and anesthetized with sodium pentobarbital (50 mg/kg i.p.). The excised heart was placed in 3.5 mM Ca\(^{2+}\) Tyrode’s containing (mM) NaCl 136, KCl 5.4, NaH\(_2\)PO\(_4\) 0.3, MgCl\(_2\) 1.0, HEPES 10, d-mannitol 4, and thiamine HCl 0.6, and supplemented with 0.18 g% glucose and 0.022 g% pyruvic acid. After spontaneous beating had cleared the ventricles of blood, the aorta was cannulated, and the heart was perfused for 3 minutes at 2.2 ml/min.
with Ca\textsuperscript{2+}-free Tyrode’s using a peristaltic pump (Minipulse, Gilson International, Middleton, Wis.). The heart was then perfused for 6–8 minutes at the same rate with 25 ml of recirculated, Ca\textsuperscript{2+}-free Tyrode’s solution containing 1.0 mg/ml collagenase Type I (Sigma Chemical Co., St. Louis) and 0.075 mg/ml protease Type XIV (Sigma). After enzymatic digestion, the heart was perfused for 4 minutes with 0.1 mM Ca\textsuperscript{2+} Tyrode’s to remove residual enzyme, and the cells were harvested by gently teasing the tissue with forceps.

\textit{Adults.} Adult myocytes were obtained from 8–10-week-old rabbits weighing 2.0–2.5 kg. The procedure for cell isolation was similar to that used in neonatal rabbits with the following exceptions: sodium heparin (150 units/kg) and sodium pentobarbital (50 mg/kg) were administered intravenously, the enzyme perfusion rate was 56 ml/min, and total enzyme volume was 150 ml. After being harvested, adult myocytes were filtered, allowed to pellet at 1g, and washed with 0.1 mM Ca\textsuperscript{2+} Tyrode’s.

Solutions were prepared with deionized water passed through a four-element purifier (Nanopure D2798, Barstead/Thermolyne Corp., Dubuque, Iowa) to achieve a resistivity greater than 15 MΩ, pH was adjusted to 7.4, and then solutions were filtered through a 0.2-μm filter (Acrodisc, Gelman Sciences, Ann Arbor, Mich.). In addition, solutions for cell isolation were warmed to 37° C and bubbled with 100% oxygen.

Cells were determined to be healthy and were selected for study based on several criteria. First, cells contracted consistently in response to pacing. Cells with aftercontractions, alternating amplitudes of contraction, or more than a 20% interbeat variation at steady state before or after experimental manipulation were eliminated from the study. Second, selected cells were free from intracellular granules or membrane discontinuities. Third, cells perfused with reduced extracellular Ca\textsuperscript{2+} remained viable throughout both the 1- and 5-minute exposures. Finally, cells that appeared to contract along their longitudinal axes were selected to allow accurate measurements of cell shortening. Acutely isolated myocytes remained responsive to pacing and suitable for study 4–8 hours after preparation. Both neonatal and adult myocytes were cylindrical and had discrete I and A bands (Figure 1).

\textbf{Cell Perfusion and Measurement of Contraction Amplitude}

Cells adhered to culture dishes previously incubated with 5% defined fetal calf serum (Hyclone Laboratories, Inc., Logan, Utah).\textsuperscript{9} A selected cell was simultaneously paced and perfused by a directed stream from one of two bottles of solution entering the dish through the common arm of a Y connector. Solution changes were controlled by a miniature solenoid valve (model LFAA-12-016,18H, The Lee Co., Westbrook, Conn.), triggered to activate after a selected pacing stimulus. Solution entering the dish was directed across the cell surface and removed by a suction cannula positioned opposite the solution inlet. Adequate flow rates and suction were maintained to allow a complete change of solution within 300 msec.\textsuperscript{9}

The image of the contracting cell was reproduced with a high-resolution CCD video camera (WV-BL202, Panasonic, Osaka, Japan) on a video monitor with a final magnification ×2,000. Changes in cell length were detected using a photocell mounted in apposition to the monitor screen and were recorded on a chart recorder (Brush 440, Gould Instrument Co., Cleveland).

\textbf{Experimental Procedure}

Control measurements of the change in cell length with contraction (contraction amplitude) were obtained at 23° C, with the ventricular myocytes bathed in 1.0 mM Ca\textsuperscript{2+} Tyrode’s and paced with platinum electrodes at 0.2 Hz, 1.5–2 times threshold, with a 5-msec pulse duration. The effect of reduced extracellular Ca\textsuperscript{2+} on adult and neonatal myocytes was assessed by exposing the cells to Ca\textsuperscript{2+}-free Tyrode’s (measured at 6–8 μM Ca\textsuperscript{2+} by a Ca\textsuperscript{2+}-ion electrode, Orion Research Inc., Cambridge, Mass.) for 1 minute, then again for 5 minutes after an intervening 3–5-minute recovery period. The response of neonatal and adult myocytes to ryanodine at 23° C was evaluated by measuring the changes in contraction amplitude at 1-minute intervals after 1 and 10 μM ryanodine (lot 86-2049-1; AgriSystems International, Wind Gap, Pa.).
**NEONATE**

![Representative tracings from neonatal and adult myocytes subjected to 5 minutes of reduced Ca\(^{2+}\) perfusate. Contractions ceased within one beat after perfusion with Ca\(^{2+}\)-free Tyrode's solution (0 Ca\(^{2+}\)). On reperfusion with 1 mM Ca\(^{2+}\), neonates required fewer beats than adults to return to control amplitude. Signal-to-noise ratio was smaller in neonates secondary to decreased cell size and a decreased baseline contraction amplitude.](image)

**ADULT**

![Representative tracings from neonatal and adult myocytes subjected to 5 minutes of reduced Ca\(^{2+}\) perfusate. Contractions ceased within one beat after perfusion with Ca\(^{2+}\)-free Tyrode's solution (0 Ca\(^{2+}\)). On reperfusion with 1 mM Ca\(^{2+}\), neonates required fewer beats than adults to return to control amplitude. Signal-to-noise ratio was smaller in neonates secondary to decreased cell size and a decreased baseline contraction amplitude.](image)

**FIGURE 2.**

Data Analysis

A computer-aided repeated-measures analysis of variance (BMDP-85, BMDP Statistical Software, Inc., Los Angeles) was performed in the reduced extracellular Ca\(^{2+}\) studies with one between-group factor (age) and one within-group factor (time). The age factor contained two levels (neonates and adults), as did the time factor (1 and 5 minutes of reduced Ca\(^{2+}\)). Statistical analysis was performed using as dependent variables 1) the number of beats for return to preintervention amplitude (plateau value within 100±20% of control) and 2) the amplitude of the first contraction after return to 1 mM Ca\(^{2+}\). A nonpaired Student's t test was used to identify significant differences between neonates and adults at each minute of ryanodine exposure.

**Results**

**Reduced Extracellular Ca\(^{2+}\)**

In all cases, contractions ceased in one beat when the cardiac myocytes were perfused with Ca\(^{2+}\)-free Tyrode's (Figure 2). On return to 1 mM Ca\(^{2+}\), adult myocytes (n=9) returned to peak contraction amplitude in 6.3±0.87 (mean±SEM) beats after 1 minute and 11.2±1.24 beats after 5 minutes of reduced Ca\(^{2+}\) (Figure 3). Neonatal myocytes (n=9) required fewer beats to recover after both 1 minute (1.9±0.43 beats) and 5 minutes of reduced Ca\(^{2+}\) (4.8±0.52 beats). Thus, in both instances, neonates required significantly fewer beats to recover than adults (p<0.001), and both neonates and adults recovered significantly faster after 1 minute as compared with 5 minutes of reduced extracellular Ca\(^{2+}\) (p<0.001).

When the amplitude of the first contraction after the return to 1 mM Ca\(^{2+}\) was evaluated, the amplitude of the first contraction in adults was significantly smaller than in neonates after both 1 and 5 minutes of reduced Ca\(^{2+}\) (p<0.001), and within the neonatal age group, the amplitude of the first contraction decreased significantly between 1 and 5 minutes of reduced Ca\(^{2+}\) perfusate (p<0.001) (Figure 3).

**Ryanodine**

A decrease in contraction amplitude occurred after perfusion with 1 and 10 μM ryanodine in adult (n=10) and neonatal (n=10) myocytes (Figure 4). In 10 μM ryanodine, there was a significantly larger decrease in adults versus neonates throughout the 15-minute perfusion period (p<0.001). In 1 μM ryanodine, adults showed a significantly smaller response than neonates after 2 and 3 minutes.
(p<0.05) but a larger response after 14 and 15 minutes (p<0.05).

Discussion

The techniques presented here have been previously applied to the study of ventricular myocytes from adult rabbits and neonatal and adult rats.9 "Rapidly" and "slowly" exchangeable Ca2+ pools have been proposed to correlate with transsarcolemmal Ca2+ influx and SR Ca2+, respectively.8,9 Based on this proposal, the findings of the present study are consistent with previous findings suggesting that myocardial contraction in the neonate is relatively dependent on transsarcolemmal Ca2+ influx, and in the adult is relatively dependent on SR Ca2+ release.

In the present study, recovery of control contraction amplitude occurred more rapidly in neonates than adults after both 1 and 5 minutes of exposure to reduced Ca2+. Assuming that the fast Ca2+ pool correlates with transsarcolemmal Ca2+ influx, this may be interpreted as evidence that the neonate is relatively more dependent on transsarcolemmal Ca2+ influx compared with the adult, perhaps compensating for an immature SR. The increased number of beats for return to control amplitude after 5 minutes as compared with 1 minute of reduced Ca2+ in both age groups may reflect the increased depletion of the slow Ca2+ pool (SR Ca2+) resulting from a longer period of perfusion with reduced extracellular Ca2+.

Thus, a component of SR Ca2+ is necessarily present in the neonate even if its contribution to cell contraction is less than in the adult.

Although this interpretation is consistent with previous studies demonstrating decreased volume and function of neonatal SR,13-21 our results might also be explained by a decreased rate of Ca2+ loss from intracellular stores in neonates as compared with adults when perfused with reduced extracellular Ca2+. Although this alternative explanation is considered unlikely in light of evidence that immature SR loses Ca2+ passively at a greater rate than mature SR,20 a decreased rate of Ca2+ loss from intracellular stores might result from differences in action potential duration or in the rate of Ca2+ rest decay. An increase in action potential duration at the same pacing rate would result in a net decrease in the membrane potential and decreased Ca2+ efflux via the sodium-calcium exchanger. Similarly, a slower rate of Ca2+ rest decay22,23 would result in decreased efflux of intracellular Ca2+. Unpublished measurements from this lab confirm that neonatal myocytes exhibit electrical activity when paced in reduced Ca2+. However, they demonstrate a 25% decrease rather than increase in action potential duration. Furthermore, the rate of Ca2+ rest decay has been shown previously to be accelerated in neonates compared with adults (see Figure 5 of Reference 17).

Experiments performed in ryanodine lend further support to the theory that SR Ca2+ contributes less to cell contraction in neonates as compared with adults. In both 1 and 10 μM ryanodine, contraction amplitude decreased significantly less in neonatal myocytes than in adult myocytes after 15 minutes. Previous studies have shown that ryanodine acts with relative specificity on the SR, by inhibiting the increase in net cytoplasmic Ca2+ through inhibition of SR Ca2+ release,11,24 by enhanced release of Ca2+ into the cytoplasm through an "SR Ca2+-release channel" followed by Ca2+ release into the extracellular space,10,25 or by direct efflux of SR Ca2+ into the extracellular space during cell relaxation.10 Further evidence that ryanodine acts on the SR comes from measurements of changes in extracellular Ca2+ using double-barreled Ca2+-sensitive electrodes,23,26 as well as isolation and characterization of the ryanodine receptor from canine myocardium.27,28

The effects of ryanodine have been shown to be dependent on drug concentration24,25,29,30 and
Consistent responses to ryanodine were obtained by Hilgemann et al.\(^29\) only when the myocytes were incubated in the drug at 37°C before study at room temperature. Furthermore, enzyme activity and purity varies with individual lots of ryanodine. Analysis of the lot of ryanodine used in the present study revealed increased purity and a 50\% increase in enzyme activity per milligram compared with more recent preparations (direct correspondence with AgriSystems). Our experiments were performed at 23°C, and the myocytes were not incubated in the drug before study. However, the response of our myocytes to ryanodine was consistent, perhaps because our myocytes were continuously perfused at rapid rates with fresh ryanodine solution. Because ryanodine washes out very slowly, it was not possible to demonstrate a return to control amplitude after discontinuing ryanodine.

Because the adult has a more fully developed SR than the neonate based on structural and functional correlation,\(^14\)–\(^21\) electrophysiological data,\(^13,31,32\) and biochemical characterization,\(^20,24,33–37\) the greater response to ryanodine in adults is not unexpected. A decreased initial response to 1 µM ryanodine was noted in adult rabbits in this study as well as in others (see Figure 11 of Reference 9). This age and dose-specific finding might be related to a decreased rate of drug diffusion across the adult cell membrane (e.g., secondary to a decreased surface-to-volume ratio) or an age-specific effect on the inward Ca\(^{2+}\) current caused by ryanodine.\(^31,38\)

In summary, significant functional differences exist in the “rapidly” and “slowly” exchangeable compartments of activator Ca\(^{2+}\) in the neonatal versus adult rabbit myocardium. Based on the results of the present study, we make the following conclusions: 1) isolated ventricular myocytes may be used in the study of developmental changes in intracellular Ca\(^{2+}\) regulation, 2) neonates show a relative independence on the rapidly exchangeable component of activator Ca\(^{2+}\) (which has been proposed to correlate with transsarcomembranous Ca\(^{2+}\) influx), and 3) a slowly exchangeable component of activator Ca\(^{2+}\) (which has been proposed to correlate with SR Ca\(^{2+}\)) is present in neonates as well as in adults but plays a more significant role in adults.

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