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Blood Calcium Levels in Immature Rats: Influence of Extracellular Calcium Concentration on Myocardial Calcium Handling

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Abstract: Calcium ions play an important role in several cell functions, from fertilization to cell death. The cytosolic Ca^{2+} concentration is much lower than the extracellular concentration ($[\text{Ca}^{2+}]_o$). The latter may markedly affect Ca^{2+} fluxes across the cell membrane and thus the cellular Ca^{2+} load. Thus, when working with preparations *in vitro*, it is important to keep $[\text{Ca}^{2+}]_o$ close to the *in vivo* value. In this study, we determined the calcemia in immature rats, for which values are currently unavailable, and investigated how supraphysiological $[\text{Ca}^{2+}]_o$ affects myocardial Ca^{2+} handling. Blood ionized $[\text{Ca}^{2+}]$ was similar in neonatal (2–5 days old) and adults Wistar rats (1.28 ± 0.03 and 1.31 ± 0.03 mmol/l; $n=6$ and 5 , respectively, $P>0.37$), and lower than the $[\text{Ca}^{2+}]_o$ range often used in experiments with neonatal myocardial preparations. Cytosolic Ca^{2+} transients, measured with indo-1 in neonatal ventricular myocytes, were enhanced by an increase in $[\text{Ca}^{2+}]_o$ from 1.2 to 2 mM, which also increased the Ca^{2+} content in the sarcoplasmic reticulum (SR), and changed the pattern of competition between the main transporters that remove Ca^{2+} from the cytosol (SR Ca^{2+} -ATPase and $\text{Na}^+/\text{Ca}^{2+}$ exchanger). These observations stress the importance of using physiological $[\text{Ca}^{2+}]_o$ values for reliability of results. It is expected that the present calcemia data, reported for the first time in immature rats, may contribute to the refinement of *in vitro* experiments with neonatal rat preparations.

Key words : calcemia, excitation-contraction coupling, myocardium, neonatal rat

Introduction

Calcium ions are cytotoxic when present at a high concentration in the intracellular medium. It is believed that early in evolution, cells have developed mechanisms to maintain the intracellular free Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) at very low levels (100 – 200 nM), which are approximately 4 orders of magnitude lower than the extracellular concentration ($[\text{Ca}^{2+}]_o$) in vertebrates [11].

Consequently, a high transmembrane electrochemical gradient is established, which favors Ca^{2+} influx through Ca^{2+} channels and transporters. From fertilization to apoptosis, Ca^{2+} plays a key role in the signaling of several processes: an increase in $[\text{Ca}^{2+}]_i$ due to transmembrane influx and/or release from intracellular stores is involved in regulation of cell proliferation, secretion, transcription, energetic metabolism, electrical activity, and contraction development [9].

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Transmembrane Ca^{2+} fluxes are highly dependent on the electrochemical gradient of the ion across the membrane and consequently on $[\text{Ca}^{2+}]_o$. On the other hand, the Ca^{2+} content of intracellular compartments is expected to be affected by the availability of the ion, which depends on the balance of transmembrane inward and outward fluxes. Thus, to better reproduce the *in vivo* conditions, the $[\text{Ca}^{2+}]_o$ used in *in vitro* studies should be close to that of the extracellular compartment.

Comparison of the blood/serum $[\text{Ca}^{2+}]$ in immature and adult mammals has shown variable results, i.e., greater, lower and similar values to those in adults have been observed in neonates, depending on the species [8, 17, 18, 21, 30]. Nevertheless, information on the values of blood/serum ionized $[\text{Ca}^{2+}]$ in immature rats is not currently available in the literature, although preparations from neonatal rodents, especially cardiomyocytes, are widely used for several kinds of studies (e.g., biochemistry, biophysics, genetics, physiology, pharmacology, bioengineering). As a result, variable $[\text{Ca}^{2+}]_o$ values have been used *in vitro*, which, if unrealistic, may lead to functional alterations and compromise the reliability of the results and conclusions.

The aims of the present study were: a) to determine the ionized $[\text{Ca}^{2+}]$ in the blood of neonatal rats; and b) to analyze the impact of exposing immature cardiomyocytes to a supraphysiological $[\text{Ca}^{2+}]_o$ value, often used in experiments with this preparation, on electrically-evoked Ca^{2+} transients and on the sarcoplasmic reticulum (SR) Ca^{2+} content.

Materials and Methods

Determination of the blood concentration of ionized Ca^{2+}

Specific pathogen-free Wistar Hannover rats (UnibHan:Wistar strain; colony originally established at CEMIB/UNICAMP with rats acquired from the Zentrallinstitut für Versuchstierzucht, Hannover, Germany, in 1987) bred under protection barrier system conditions at CEMIB/UNICAMP (an ICLAS monitoring/reference center) were used; these included adult males (3–4 months old, $n=5$) not submitted to previous fasting, and neonates of both sexes (2–5 days old, $n=11$) from different litters. The latter remained with the dams until the experiment to minimize fast-induced changes in blood pH and other parameters. Adult animals were maintained at $22 \pm 2^\circ\text{C}$, under a 12:12 h dark-light cycle (lights on at 7 AM), with free access to water and pelleted chow

(Nuvilab CR-1, Nuvital, Brazil).

All blood samples were collected at 2–4 PM to minimize interference of diurnal variations [20]. Adult rats were anesthetized with ketamine plus xylazine (50 and 10 mg/kg, respectively, i.p.), whereas the anesthesia procedure chosen for neonates was halothane inhalation, as injectable anesthetics have been shown to cause greater mortality and to be less effective for short-term surgical anesthesia in neonatal rodents than inhalant anesthesia [12]. Blood was collected by cardiac puncture into dry, lithium heparin-containing syringes (PICO50, Radiometer, Copenhagen, Denmark), mixed gently, and briefly stored at 4°C . In most cases, blood samples from 2 neonates were pooled. After collection, animals were sacrificed by carbon dioxide inhalation. Ionized $[\text{Ca}^{2+}]$ in the whole blood was measured with a Radiometer ABL800 Flex analyzer within less than 1 h after collection. The protocol for care and use of the animals was approved by the institutional Committee of Ethics in Animal Research (CEUA/IB/UNICAMP, protocol number 2228-1).

Experiments with ventricular myocytes isolated from immature rats

Ventricular myocytes were isolated from 5-day-old Wistar rats ($n=4$) by coronary perfusion with collagenase 1 (0.5 mg/ml, Worthington Biochem, Lakewood, NJ, USA), according to Bassani and Bassani [4]. Cells were used within 6 h after isolation.

For $[\text{Ca}^{2+}]_i$ measurement, myocytes were incubated with indo-1 AM (5 μM ; Molecular Probes, Eugene, OR, USA) for 10 min, and then perfused for 30 min for de-esterification of the dye. Cells were electrically stimulated at 1 Hz under perfusion with modified Tyrode's solution (TS; composition in mM: 140 NaCl, 6 KCl, MgCl_2 1, HEPES 5, glucose 11, pH 7.4) containing 1.2 mM CaCl_2 . Two types of caffeine-evoked Ca^{2+} transients were obtained in the absence of electrical stimulation [2, 5]: a) the first type was evoked by switching perfusion to TS containing 10 mM caffeine (Caf-TS) after a 5 s rest; and b) in the second type, cells were perfused with Na^+ -, Ca^{2+} -free TS solution (equimolar replacement of NaCl and CaCl_2 with LiCl and EGTA, respectively) for 20 s, followed by switching to the same solution containing 10 mM caffeine (Caf-00). Cells were electrically stimulated for 2 min prior to caffeine application. Afterward, the same protocol was repeated during perfusion with TS containing 2 mM CaCl_2 . The caffeine solution

used to evoke Caf-TS also contained 2 mM CaCl_2 .

Indo-1 was excited at 360 nm. After fluorescence background subtraction, the ratio of emission at 410 and 485 nm was converted to $[\text{Ca}^{2+}]_i$ according to Bassani and Bassani [4].

The rate of $[\text{Ca}^{2+}]_i$ decline (k) during electrically evoked twitches, Caf-TS and Caf-00 was determined by exponential fitting of the decaying phase of the Ca^{2+} transient. The SR Ca^{2+} content was estimated as the difference in total $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_T$), i.e., the sum of free and bound $[\text{Ca}^{2+}]$ at the peak and immediately before the Caf-00 transient [10], and expressed as $\mu\text{mol Ca}^{2+}$ per liter of non-mitochondrial cell water. For $[\text{Ca}^{2+}]_T$ estimation, Ca^{2+} binding to both indo-1 and endogenous passive Ca^{2+} buffers was taken into account, assuming [indo-1] as 20 μM , and considering the endogenous Ca^{2+} buffering parameters determined in the myocardium of neonatal rats [7].

From the $[\text{Ca}^{2+}]_i$ data, as well as from the estimated $[\text{Ca}^{2+}]_T$ and k values of the different types of transients, the empirical maximum velocity of Ca^{2+} transport ($V_{\max}$) was determined for the different transporters, i.e., SR Ca^{2+} -ATPase (SERCA), $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX), and the combination of the slow systems (i.e., sarcolemmal Ca^{2+} -ATPase and mitochondrial Ca^{2+} uniporter), as described by Bassani *et al.* [2], using the equation

$$d[\text{Ca}^{2+}]_T/d[\text{Ca}^{2+}]_i = V_{\max}/\{(1 + K_m)^n\},$$

where K_m and n represent the Michaelis-Menten constant and the Hill coefficient, respectively.

Data are presented as means accompanied by the respective standard error and/or 95% confidence interval (CI95%). Blood ionized $[\text{Ca}^{2+}]$ values in adults and neonates were compared with the Student's t -test for unpaired samples. The Student's t -test for paired samples was used for comparison of variables of the *in vitro* experiments. $P < 0.05$ was considered to indicate significance.

Results

As shown in Fig. 1, blood ionized $[\text{Ca}^{2+}]$ in neonatal rats was 1.28 ± 0.03 mmol/l (CI95%: 1.21–1.35 mmol/l; $n=6$), which was not statistically different ($P > 0.35$) from that in adult rats (1.31 ± 0.03 mmol/l; CI95%: 1.24–1.39 mmol/l; $n=5$).

The amplitude of Ca^{2+} transients recorded from neonatal rat ventricular myocytes paced at 1 Hz was enhanced by 70% after $[\text{Ca}^{2+}]_o$ was raised from 1.2 to 2

Fig. 1. Ionized Ca^{2+} concentration in the blood of neonatal (2–5 days of age) and adult (3–4 month old) Wistar rats. The horizontal lines represent the mean values in each group.

Fig. 2. Ca^{2+} transients evoked by electric stimulation at 1 Hz (twitch) and by application of 10 mM caffeine dissolved in Tyrode's solution with or without Na^+ and Ca^{2+} (Caf-TS and Caf-00, respectively), and recorded from a 5-day-old rat ventricular myocyte in the presence of 1.2 and 2 mM extracellular $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_o$).

mM ($P < 0.001$; Fig. 2), and the k value of $[\text{Ca}^{2+}]_i$ decline was significantly increased ($P < 0.05$). A small but significant increase in diastolic $[\text{Ca}^{2+}]_i$ was also observed ($P < 0.001$). In addition, raising $[\text{Ca}^{2+}]_o$ to 2 mM resulted in a 23% increase in the SR Ca^{2+} content ($P < 0.001$) (Table 1).

The $[\text{Ca}^{2+}]_i$ elevation induced by caffeine is attributed to release of the SR Ca^{2+} content due to the opening of

Table 1. Amplitude and rate constants of decline (k) of Ca^{2+} transients evoked by electric stimulation (twitch) and by application of 10 mM caffeine dissolved in Tyrode's solution with or without Na^+ and Ca^{2+} (Caf-TS and Caf-00, respectively), as well as the sarcoplasmic reticulum (SR) Ca^{2+} content, in isolated ventricular myocytes from 5-day-old rats exposed to different extracellular Ca^{2+} concentrations ($[\text{Ca}^{2+}]_o$)

	1.2 mM $[\text{Ca}^{2+}]_o$	2.0 mM $[\text{Ca}^{2+}]_o$
Twitch		
Diastolic $[\text{Ca}^{2+}]_i$ (μM)	0.226 ± 0.006	$0.246 \pm 0.007^*$
Amplitude (μM)	0.313 ± 0.022	$0.528 \pm 0.036^*$
k (s^{-1})	4.440 ± 0.200	$5.174 \pm 0.379^*$
Caf-TS		
Amplitude (μM)	0.724 ± 0.040	$0.982 \pm 0.079^*$
k (s^{-1})	0.767 ± 0.061	$0.476 \pm 0.032^*$
Caf-00		
Amplitude (μM)	0.878 ± 0.046	$1.569 \pm 0.160^*$
k (s^{-1})	0.104 ± 0.019	0.085 ± 0.016
SR Ca^{2+} content ($\mu\text{mol/l}$ cell water)	76.5 ± 2.4	$94.5 \pm 2.8^*$

Number of studied cells = 18. * $P < 0.05$ vs. 1.2 $[\text{Ca}^{2+}]_o$ (Student's t -test for paired samples).

the SR Ca^{2+} release channels by the alkaloid [24]. The amplitude of both types of caffeine-induced transients was augmented at 2 vs. 1.2 mM $[\text{Ca}^{2+}]_o$ ($P < 0.01$; Fig. 2). In contrast with the acceleration of $[\text{Ca}^{2+}]_i$ decline during twitches, the k value for Caf-TS was significantly decreased at the higher $[\text{Ca}^{2+}]_o$ ($P < 0.01$; Table 1), although the amplitude of the transient was greater. The $[\text{Ca}^{2+}]_i$ decline during Caf-00 was slowed in some cells, but the difference in k values did not attain statistical significance ($P > 0.08$).

Figure 3 shows the empirical $V_{\max}$ values estimated for SERCA, NCX and the slow $[\text{Ca}^{2+}]_o$ transporters. Whereas $[\text{Ca}^{2+}]_o$ did not exert a significant influence on the $V_{\max}$ of the latter ($P > 0.05$), increasing $[\text{Ca}^{2+}]_o$ from 1.2 to 2 mM reduced NCX $V_{\max}$ by 40% ($P < 0.001$), but enhanced SERCA $V_{\max}$ ($P < 0.01$; Student's t -test for paired samples).

Discussion

To our knowledge, this is the first report of blood ionized $[\text{Ca}^{2+}]$ values in rats under 1 week of age. The neonatal values determined in this study were comparable to those measured not only in adult rats from different strains (1.2–1.4 mmol/l [16, 26, 28]; present results), but also in healthy, full-term human newborns and in adult humans (reference interval: 1.1–1.4 mmol/l [1, 18, 21]).

Fig. 3. Empirical values of the maximum velocity of Ca^{2+} transport ($V_{\max}$) of the sarcoplasmic reticulum Ca^{2+} ATPase (SERCA; right ordinate axis), as well as of $\text{Na}^+/\text{Ca}^{2+}$ exchanger (NCX) in the Ca^{2+} extrusion mode and the slow Ca^{2+} transporters (slow) (right ordinate axis), determined in neonatal rat ventricular myocytes exposed to 1.2 and 2 mM extracellular $[\text{Ca}^{2+}]$. Data are expressed as the mean and SEM ($n=18$). * $P < 0.01$ vs 1.2 mM $[\text{Ca}^{2+}]$ (Student's t -test for paired samples).

Specifically regarding the immature myocardium, important developmental differences have been described in the contribution of Ca^{2+} fluxes across the sarcolemma and the SR membrane. Whereas in the adult rat ventricle it is estimated that approximately 90% of the Ca^{2+} cycling takes place between the SR and the cytosol, in neonates, SR Ca^{2+} release contributes less Ca^{2+} to contraction, which relies more on the transsarcolemmal Ca^{2+} flux than in adults [4, 13, 32]. In experiments with myocardial preparations from developing rats (as well as humans), it is common to use $[\text{Ca}^{2+}]_o$ values higher (1.8–2.0 mmol/l) than the blood ionized $[\text{Ca}^{2+}]$ reported here. High $[\text{Ca}^{2+}]_o$ is expected to favor systolic Ca^{2+} influx, while lessening Ca^{2+} efflux, by increasing the driving force for Ca^{2+} flux through channels, decreasing the driving force for Ca^{2+} efflux via the NCX, and enhancing the energy barrier imposed on the sarcolemmal Ca^{2+} ATPase. These changes would result in a greater cell Ca^{2+} load, which might modify the contribution of the intracellular and extracellular Ca^{2+} sources for contraction activation.

The present results of $[\text{Ca}^{2+}]_i$ measurement in neonatal myocytes confirm such predictions. In the mammalian myocardium, Ca^{2+} release from the SR, triggered by Ca^{2+} influx during the action potential, is considered the most important mechanism of excitation-contraction coupling. In the immature heart, however, the colocalization of sarcolemmal and SR Ca^{2+} channels (which allows efficient coupling of Ca^{2+} influx to release) is less prominent, and the SR Ca^{2+} release mechanism itself is less

sensitive to Ca^{2+} than in adults [14, 25, 32]. Accordingly, we observed that not only the $[\text{Ca}^{2+}]_o$ transient amplitude, but also the SR Ca^{2+} content at a physiological $[\text{Ca}^{2+}]_o$ were considerably lower in neonatal cells than in adult myocytes under similar experimental conditions (see 4 and 10 for comparison). However, both the transient amplitude and SR Ca^{2+} load were markedly enhanced at 2 mM $[\text{Ca}^{2+}]_o$.

The increase in the SR Ca^{2+} content, as well as the expected greater Ca^{2+} influx via voltage-dependent Ca^{2+} channels during excitation (due to the greater transmembrane Ca^{2+} electrochemical gradient), are probably responsible for the large increase of the twitch Ca^{2+} transient amplitude at 2 mM $[\text{Ca}^{2+}]_o$, as both factors were shown to enhance the contribution of SR Ca^{2+} release to excitation-contraction coupling [3]. It should be stressed that due to the paucity of passive Ca^{2+} binding sites in immature rat myocardium [7], even relatively small changes in Ca^{2+} fluxes have a large impact on $[\text{Ca}^{2+}]_i$ and thus on the several cellular processes affected by it.

Two main factors should contribute to increase the SR Ca^{2+} content at 2 mM $[\text{Ca}^{2+}]_o$. One of them would be the greater availability of cytosolic Ca^{2+} to be taken up by SERCA not only because of the greater Ca^{2+} influx but also due to less Ca^{2+} extrusion by the NCX, the main sarcolemmal transporter that removes Ca^{2+} from the cytosol. This may be particularly important in the neonatal myocardium, where NCX expression is higher and the exchanger plays a greater role in cytosolic Ca^{2+} clearance than in the adult [4, 27, 29]. We estimate that increasing $[\text{Ca}^{2+}]_o$ to 2 mM would cause a 10 mV negative shift of the NCX reversal potential, which should decrease diastolic Ca^{2+} efflux and possibly favor transport in the Ca^{2+} influx mode during sarcolemmal depolarization. Accordingly, our present results show that $[\text{Ca}^{2+}]_i$ decline during Caf-TS was significantly slower after $[\text{Ca}^{2+}]_o$ was raised. During this type of transient, cytosolic Ca^{2+} clearance relies mainly on the NCX and, to a lesser extent, on slow transporters [2, 5]. However, $[\text{Ca}^{2+}]_i$ decay due to the slow transporters alone (Caf-00, during which the NCX is thermodynamically inhibited) was not significantly changed. Thus, one may conclude that the slower $[\text{Ca}^{2+}]_i$ decline at Cf-TS is attributable mostly to a decrease in the rate of Ca^{2+} efflux mediated by the NCX. Accordingly, the empirical $V_{\max}$ value of the slow transporters was not affected by augmenting $[\text{Ca}^{2+}]_o$ to 2 mM, whereas that of the NCX was significantly decreased, which confirms the inhibition of cyto-

solic Ca^{2+} removal by the latter, but not by the slow systems. It is interesting to observe that shifting the NCX reversal potential in the negative direction by either decreasing $[\text{Na}^+]_o$ [15] or increasing $[\text{Ca}^{2+}]_o$ (present study) markedly enhances the SR Ca^{2+} load in the neonatal ventricle, but not in adult myocardium [15].

The other mechanism that is likely to contribute to greater SR Ca^{2+} accumulation at 2 mM $[\text{Ca}^{2+}]_o$ is stimulation of SERCA activity. This can be inferred from the significant abbreviation of $[\text{Ca}^{2+}]_i$ decline during electrically stimulated twitches, in spite of the marked prolongation of $[\text{Ca}^{2+}]_i$ decay during Caf-TS, a transient in which net SR Ca^{2+} uptake may be considered negligible. Accordingly, the difference between k_{twitch} and $k_{\text{Caf-TS}}$, which may be used as an approximation of the rate constant of $[\text{Ca}^{2+}]_i$ decay attributable to the SR Ca^{2+} uptake [22], was increased from 3.67 ± 0.20 to 4.70 ± 0.38 ($P < 0.02$) by raising $[\text{Ca}^{2+}]_o$ to 2 mM. Enhanced SERCA activity would also explain the relatively small increase in diastolic $[\text{Ca}^{2+}]_i$ ($< 10\%$) at 2 mM $[\text{Ca}^{2+}]_o$, despite the apparent decrease in NCX-mediated Ca^{2+} efflux. Still, acceleration of $[\text{Ca}^{2+}]_i$ decline during the twitch might have resulted simply from an increase in the SERCA velocity of transport due to the higher substrate concentration (i.e., Ca^{2+}), as the transients were augmented at 2 mM $[\text{Ca}^{2+}]_o$. To assess whether this was the case or else if the enzyme was actually stimulated, we determined the empirical SERCA $V_{\max}$, which is a constant, independent of $[\text{Ca}^{2+}]_i$. The significant increase in this constant in the presence of 2 mM $[\text{Ca}^{2+}]_o$ allows the conclusion that acceleration of the twitch $[\text{Ca}^{2+}]_i$ decline is attributable to SERCA stimulation, rather than to the increase in $[\text{Ca}^{2+}]_i$ itself.

A possible mechanism underlying SERCA stimulation is enhanced phosphorylation of phospholamban, a negative regulator of SERCA, by the Ca^{2+} /calmodulin-dependent kinase II (CaMKII) [19]. As the temporal average of $[\text{Ca}^{2+}]_i$ should be increased at 2 mM $[\text{Ca}^{2+}]_o$, greater activity of this enzyme in the autonomous state would be expected [19]. CaMKII was shown to play an important role in the SERCA-dependent $[\text{Ca}^{2+}]_i$ decline in adult rat cardiomyocytes paced at 1 Hz and to affect the empirical SERCA $V_{\max}$ [6, 23]. On the other hand, evidence is available that CaMKII is active and able to phosphorylate phospholamban and other proteins associated with the SR in the mammalian myocardium even before birth [31].

In general, the changes in myocardial Ca^{2+} transients

brought about by exposure to an unphysiologically high $[Ca^{2+}]_o$ indicate a shift in the pattern of Ca^{2+} handling and competition between SR (i.e., SERCA) and sarcolemmal (i.e., NCX) transporters toward that observed in the adult cells, in which Ca^{2+} cycling between the SR and the cytosol is more prominent. This is particularly important when one has in mind the widespread use of cells from neonatal rats (mostly cultured cardiac cells) in several kinds of experiments, from studies on gene regulation to drug tests. It is expected that the present results may contribute to the refinement of such experiments by the use of a more realistic ionic composition of the extracellular medium, which should lead to more reliable results and conclusions. Considering the importance of $[Ca^{2+}]_i$ for a myriad of cellular processes, alterations in cellular Ca^{2+} balance in the presence of unrealistic $[Ca^{2+}]_o$ values may affect results of experiments with other tissue and cell types, in addition to the myocardium.

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References

1. Baird, G.S., Rainey, P.M., Wener, M., and Chandler, W. 2009. Reducing routine ionized calcium measurement. *Clin. Chem.* 55: 533–540. [Medline] [CrossRef]
2. Bassani, J.W.M., Bassani, R.A., and Bers, D.M. 1994. Relaxation in rabbit and rat cardiac cells: species-dependent differences in cellular mechanisms. *J. Physiol.* 476: 279–293. [Medline]
3. Bassani, J.W.M., Yuan, W., and Bers, D.M. 1995. Fractional SR Ca release is regulated by trigger Ca and SR Ca content in cardiac myocytes. *Am. J. Physiol.* 268: C1313–C1319. [Medline]
4. Bassani, R.A. and Bassani, J.W.M. 2002. Contribution of Ca^{2+} transporters to relaxation in intact ventricular myocytes from developing rats. *Am. J. Physiol. Heart Circ. Physiol.* 282: H2406–H2413. [Medline]
5. Bassani, R.A., Bassani, J.W.M., and Bers, D.M. 1992. Mitochondrial and sarcolemmal Ca^{2+} transport reduce $[Ca^{2+}]_i$ during caffeine contractions in rabbit cardiac myocytes. *J. Physiol.* 453: 591–608. [Medline]
6. Bassani, R.A., Mattiazzi, A., and Bers, D.M. 1995. CaMKII is responsible for activity-dependent acceleration of relaxation in rat ventricular myocytes. *Am. J. Physiol.* 268: H703–H712. [Medline]
7. Bassani, R.A., Shannon, T.R., and Bers, D.M. 1998. Passive Ca^{2+} binding in ventricular myocardium of neonatal and adult rats. *Cell Calcium* 23: 433–442. [Medline] [CrossRef]
8. Berlin, D. and Aroch, I. 2009. Concentrations of ionized and total magnesium and calcium in healthy horses: effects of age, pregnancy, lactation, pH and sample type. *Vet. J.* 181: 305–311. [Medline] [CrossRef]
9. Berridge, M.J., Lipp, P., and Bootman, M.D. 2000. The versatility and universality of calcium signalling. *Nat. Rev. Mol. Cell Biol.* 1: 11–21. [Medline] [CrossRef]
10. Carvalho, B.M.R., Bassani, R.A., Franchini, K.G., and Bassani, J.W.M. 2006. Enhanced calcium mobilization in rat ventricular myocytes during the onset of pressure overload-induced hypertrophy. *Am. J. Physiol. Heart Circ. Physiol.* 291: H1803–H1813. [Medline] [CrossRef]
11. Case, R.M., Eisner, D., Gurney, A., Jones, Q., Muallem, S., and Verkhratsky, A. 2007. Evolution of calcium homeostasis: from birth of the first cell to an omnipresent signaling system. *Cell Calcium* 42: 345–350. [Medline] [CrossRef]
12. Danneman, P.J. and Mandrell, T.D. 1997. Evaluation of five agents/methods for anesthesia of neonatal rats. *Lab. Anim. Sci.* 47: 386–395. [Medline]
13. Escobar, A.L., Ribeiro-Costa, R., Villaba-Galea, C., Zoghbi, M.H., Pérez, C.G., and Mejía-Alvarez, R. 2004. Developmental changes of intracellular Ca^{2+} transients in beating rat hearts. *Am. J. Physiol. Heart Circ. Physiol.* 286: H971–H978. [Medline] [CrossRef]
14. Fabiato, A. 1982. Calcium release in skinned cardiac cells: variations with species, tissues, and development. *Fed. Proc.* 41: 2238–2244. [Medline]
15. Ferraz, S.A., Bassani, J.W.M., and Bassani, R.A. 2001. Rest-dependence of twitch amplitude and sarcoplasmic reticulum calcium content in the developing rat myocardium. *J. Mol. Cell. Cardiol.* 33: 711–722. [Medline] [CrossRef]
16. Fukuda, S., Tsuchikura, S., and Iida, H. 2004. Age-related changes in blood pressure, hematological values, concentrations of serum biochemical constituents and weights of organs in the SHR/Izm, SHRSP/Izm and WKY/Izm. *Exp. Anim.* 53: 67–72. [Medline] [CrossRef]
17. Levy, J.K., Crawford, P.C., and Werner, L.L. 2006. Effect of age on reference intervals of serum biochemical values in kittens. *J. Am. Vet. Med. Assoc.* 228: 1033–1037. [Medline] [CrossRef]
18. Loughhead, J.L., Mimouni, F., and Tsang, R.C. 1988. Serum ionized calcium concentrations in normal neonates. *Am. J. Dis. Child.* 142: 516–518. [Medline]
19. Maier, L.S. 2009. Role of CaMKII for signaling and regulation in the heart. *Front. Biosci.* 14: 486–496. [Medline] [CrossRef]
20. Milhaud, G., Perault-Staub, A., and Staub, J. 1972. Diurnal variation of plasma calcium and calcitonin function in the rat. *J. Physiol.* 222: 559–567. [Medline]
21. Nelson, N., Finnström, O., and Larsson, L. 1987. Neonatal reference values for ionized calcium, phosphate and magnesium: selection of reference population by optimality criteria. *Scand. J. Clin. Lab. Invest.* 47: 111–117. [Medline] [CrossRef]

22. Piacentino III, V., Weber, C.R., Chen, X., Weisser-Thomas, J., Margulies, K.B., Bers, D.M., and Houser, S.R. 2003. Cellular basis for abnormal calcium transients in failing human ventricular myocytes. *Circ. Res.* 92: 651–658. [[Medline](#)] [[CrossRef](#)]
23. Picht, E., DeSantiago, J., Huke, S., Kaetzel, M.A., Dedman, J.R., and Bers, D.M. 2007. CaMKII inhibition targeted to the sarcoplasmic reticulum inhibits frequency-dependent acceleration of relaxation and Ca^{2+} current facilitation. *J. Mol. Cell. Cardiol.* 42: 196–205. [[Medline](#)] [[CrossRef](#)]
24. Sitsapesan, R. and Williams, A.J. 1990. Mechanisms of caffeine activation of single calcium-release channels of sheep cardiac sarcoplasmic reticulum. *J. Physiol.* 423: 425–439. [[Medline](#)]
25. Snopko, R.M., Ramos-Franco, J., Di Maio, A., Karko, K.L., Manley, C., Piedras-Rentería, E., and Mejía-Alvarez, R. 2008. Ca^{2+} sparks and cellular distribution of ryanodine receptors in developing cardiomyocytes from rat. *J. Mol. Cell. Cardiol.* 44: 1032–1044. [[Medline](#)] [[CrossRef](#)]
26. Tordoff, M.G., Hughes, R.L., and Pilchack, D.M. 1998. Calcium intake by rats: influence of parathyroid hormone, calcitonin, and 1,25-dihydroxyvitamin D. *Am. J. Physiol.* 274: R214–R231. [[Medline](#)]
27. Vetter, R., Studer, R., Reinecke, H., Kólar, F., Ošťádalová, I., and Drexler, H. 1995. Reciprocal changes in post-natal expression of the sarcolemmal Na^{+} - Ca^{2+} exchanger and SERCA2 in rat heart. *J. Mol. Cell. Cardiol.* 27: 1689–1701. [[Medline](#)] [[CrossRef](#)]
28. Wang, W., Lewin, E., and Olgaard, K. 2002. Role of calcitonin in the rapid minute-to-minute regulation of plasma Ca^{2+} homeostasis in the rat. *Eur. J. Clin. Invest.* 32: 674–681. [[Medline](#)] [[CrossRef](#)]
29. Wiegerinck, R.F., Cojoc, A., Zeidenweber, C., Ding, G., Shen, M., Joyner, R.W., Fernandez, J.D., Kanter, K., Rm Kirshbom, P.M., Kogon, B.E., and Wagner, M.B. 2009. Force frequency relationship of the human ventricle increases during early postnatal development. *Pediatr. Res.* 65: 414–419. [[Medline](#)] [[CrossRef](#)]
30. Wolford, S.T., Schroer, R.A., Gohs, F.X., Gallo, P.P., Falk, H.B., and Dente, A.R. 1988. Effect of age on serum chemistry profile, electrophoresis and thyroid hormones in beagle dogs two weeks to one year of age. *Vet. Clin. Pathol.* 17: 35–42. [[Medline](#)] [[CrossRef](#)]
31. Xu, A., Hawkins, C., and Naranayan, N. 1997. Ontogeny of sarcoplasmic reticulum protein phosphorylation by Ca^{2+} -calmodulin-dependent protein kinase. *J. Mol. Cell. Cardiol.* 29: 405–418. [[Medline](#)] [[CrossRef](#)]
32. Ziman, A.P., Gómez-Viquez, N.L., Bloch, R.J., and Lederer, W.J. 2010. Excitation-contraction coupling changes during postnatal cardiac development. *J. Mol. Cell. Cardiol.* 48: 379–386. [[Medline](#)] [[CrossRef](#)]