Contribution of sarcolemmal calcium current to total cellular calcium in postnatally developing rat heart

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Abstract

Objective: The aim was to determine the contribution of sarcolemmal Ca influx through L-type Ca current to total cellular Ca in newborn and postnatally developing rat heart. Methods: Whole cell voltage clamp was used to study L-type Ca current in freshly isolated ventricular cells of 1, 6, 10, 15, 30-day-old and adult (120–150 days) rats. Amplitude, current density, inactivation rate and time integral of Ca current were determined at the experimental temperature of 36°C. Width, length, surface area, volume and surface-to-volume ratio of the isolated cells were also determined. Using the time integral of Ca current and volume of the myocytes the increment in total cellular Ca was calculated for the six developmental stages. Results: The amplitude of Ca current increased strongly during the postnatal maturation from 277 ± 25 pA at day 1 to 1961 ± 98 pA in the adult rat (P < 0.001). This increase was due to an almost proportional increase in cell size; accordingly the density of Ca current remained fairly constant, being 16.9 ± 1.8, 17.0 ± 1.8, 22.8 ± 1.4, 21.2 ± 1.5, 16.2 ± 2.0 and 15.9 ± 0.7 pA/pF for 1, 6, 10, 15, 30-day-old and adult rats, respectively. Charge transfer by Cd-sensitive Ca current during 200 ms voltage pulse from −45 mV to 0 mV increased from 7.18 ± 1.00 pC at day one to 24.80 ± 1.80 pC in the adult rat heart (P < 0.001). When normalized to the capacitive surface area of the myocytes the charge transfer by L-type Ca current was more than double in newborns (0.429 ± 0.074 pC/pF) as compared to the adults (0.188 ± 0.016 pC/pF) (P < 0.01). The difference is explained by slower inactivation rate of Ca current in newborn than adult rats. Time constant of the fast component was 5.92 ± 0.62 ms and 4.5 ± 0.4 ms (P < 0.05) for 1-day-old and adult rat, respectively. Time constant of the slow component decreased from 27.7 ± 2.0 to 21.7 ± 3.0, although the difference was not statistically significant (P = 0.26). The increment in total cellular Ca due to Ca influx through Ca channels was 54 ± 9 μmol l−1 in 1-day-old rat and decreased steadily during postnatal maturation to 8 ± 1 μmol l−1 in the adult rat (P < 0.001). The change is partly due to faster inactivation of Ca current in adults but mainly as a consequence of decreasing surface-to-volume ratio of growing myocytes. Sarcolemmal surface area increases almost 9 times from 1.001 × 103 μm² at day 1 to 8.675 × 103 μm² for the adult rat, whereas increase in cell volume is about 28-fold from 0.991 × 103 to 27.74 × 103 μm³; accordingly surface-to-volume ratio decreases from 1.05 ± 0.02 at day 1 to 0.36 ± 0.01 in the adult rat (P < 0.01). Conclusions: Contribution of sarcolemmal Ca influx through L-type Ca channels is over 6 times higher in newborn than mature rats. Therefore, the strong dependence of neonatal rat heart on extracellular Ca for contractile activation is, at least partially, explained by greater contribution of L-type Ca current to intracellular Ca.

Keywords: Calcium channel, L-type; Age; Development; Calcium fluxes; Calcium, intercellular concentration; Cell size; Excitation-contraction coupling; Rat, ventricular myocytes

1. Introduction

Cardiac contraction is activated by transient increase in myoplasmic Ca brought about by the combination of sarcolemmal influx of external Ca and Ca release from the intracellular stores. Several lines of evidence suggest that the relative importance of sarcolemma (SL) and sarcoplasmic reticulum (SR) in the regulation of excitation-contraction coupling undergoes significant changes during postnatal development. In adult mammals relatively small...
Ca entry through Ca channels and/or Na–Ca exchanger is sufficient to trigger a larger release of Ca from SR [1–3]. In newborn animals SR is rather poorly developed [4–7] and contraction must be more directly dependent on sarcolemmal Ca influx. Adult type of excitation–contraction coupling develops gradually during the early postnatal maturation. Contractile studies suggest that recirculating fraction of activator Ca in 1-day-old rat heart is only 6% but reaches the value of adult rat (85%) already at the age of about 35 days [8]. Experiments with Ca channel blockers and substances which interfere with SR function also suggest that sarcolemmal Ca influx dominates in newborn animals, while Ca release from SR becomes increasingly important with advancing age [9–12]. Although several kinds of indirect evidence emphasize the importance of sarcolemmal mechanisms in contractile activation of newborn rat heart, pathway(s) and quantity of sarcolemmal Ca influx have not been totally elucidated. The aim of the present study was, therefore, to determine the contribution of sarcolemmal Ca current to total cellular Ca in neonatal and adult rat heart, and thereby evaluate the importance of sarcolemmal mechanisms in contractile activation during the early postnatal development. To reach these goals the time integral of L-type Ca current and volume of freshly isolated ventricular myocytes were measured at different stages of postnatal maturation of the rat heart. It is shown that Ca current density remains relatively constant throughout the postnatal maturation, but due to larger surface-to-volume ratio and slower inactivation of Ca current in neonatal myocytes the contribution of sarcolemmal Ca current to cellular Ca is much more significant in newborn than adult rat heart.

2. Methods

2.1. Isolation of myocytes

Wistar rats of both sexes between 1 day (0–24 h; newborn) and 4–5 months of age were used. Six age-groups were studied: 1, 6, 10, 15 and 30-day-old neonates and adult rats (120–150 days old). In all age-groups isolation of ventricular myocytes was accomplished by coronary perfusion of cannulated hearts with dissociating enzymes. Composition of the physiological solution used in perfusion was as follows (mM): NaCl 100, KCl 10, KH₂PO₄ 1.2, MgSO₄ 4, taurine 50, glucose 20 and HEPES 10 at pH 6.9 (KOH). Aorta of neonatal rats between 1 and 15 days of age was cannulated to a blunt syringe needle (Microlance 27G3/4, o.d. 0.4 mm). Oxygenated Ca-free physiological solution was pumped through the coronary vessels at the rate of 1 ml/min. After 3–5 min of Ca washout the perfusion was continued with the same solution but with added collagenase (0.7 mg/ml; Sigma Type I; collagen digestion activity 280 units/mg per 5 h) and protease (0.07 mg/ml; Sigma XIV; caseinase activity 5.5 units/mg per min) for an additional 3–5 min. During the perfusion the heart was immersed in warm (35°C) and oxygenated Ca-free physiological solution. When the epicardial layer began to dissociate from the myocardium enzyme perfusion was terminated. The heart was placed in KB solution (Kraft Brühe, power soup) [13] and cells were freed with gentle agitation. Cannulated heart of 30-day-old and adult rats was attached to the base of a 65 cm Langendorf column and sequentially perfused with Ca-free solution and enzymes for 5 and 10 min, respectively. For these age-groups collagenase concentration was raised to 1.2 mg/ml and protease concentration to 0.12 mg/ml. Dissociated cells were stored in KB (5°C) and used within 6 h from the isolation.

2.2. Measurement of Iᵦ Ca

Only spindle-shaped, cross-striated cells were used in experiments (Fig. 1). The whole-cell configuration of the patch-clamp technique [14] at 36°C was used for Ca current measurement. An aliquot of the KB medium containing myocytes was transferred to the experimental chamber positioned on top of an inverted microscope (Leica DMIL). After the myocytes had settled on the glass
bottom of the chamber, they were superfused with prewarmed physiological solution (mM): NaCl 150, CsCl 5.4, MgCl₂ 1.2, CaCl₂ 1.8, glucose 10 and HEPES 10 (pH 7.4 with CsOH). Patch pipettes were fabricated with a vertical two-stage puller (L/M-3P-A, List-Electronic, Germany) from borosilicate glass (Vitrex microhaematocrit tubing, Modulohm A/S, Denmark). Electrodes of 1–2 µm inner diameter were filled with isotonic solution composed (mM) of CsCl 130, MgATP 5, TEA 15, HEPES 10, MgCl₂ 1, oxaloacetate 5, succinate 5 and EGTA 0.02 (pH 7.2).

When immersed in the physiological solution the pipette resistance varied between 0.8 and 2 MΩ. The junction potential was eliminated by adjusting the pipette current to zero before attaching the cell. The immersion depth of the pipette was kept as shallow as possible and the pipette capacitance (4–5 pF) was compensated after formation of a gigahm seal. After the patch was ruptured the series resistance and membrane capacitance were iteratively adjusted by respective compensation circuits of the Axopatch-1D amplifier (with CV-4 1/100 headstage). For 1- to 15-day-old rats the capacitance of the sarcolemma was directly read from the dial of the capacitance compensation circuit of the amplifier. If the cell capacitance exceeded the range of the compensation circuit of the amplifier (100 pF; 3 cells out of 7 in 30-day-old animals and 14 cells out of 16 in the adults), the capacitance was measured by integration of the capacitive current for 5 mV hyperpolarising pulses from a holding potential of −65 mV. To attain the largest possible capacity compensation the biggest adult myocytes were rejected from the electrophysiological experiments. In the adult cells the uncompensated cell capacitance was on average 34 pF which does not reduce the speed of the clamp since the peak current was achieved within 3 ms from the beginning of the clamp (Fig. 2). To elicit Ca currents the cells were pulsed over a range from −40 to +60 mV for 200 ms from a holding potential of −45 mV at the frequency of 0.5 Hz. Steady-state inactivation curves showed that at this holding potential over 95% of the L-type Ca current is available for activation (data not shown). The amplitude of Ca current was determined as the difference between inward peak and steady-state current at the end of 200 ms pulse (visual estimate) [15] and in 55% of cells also as Cd-blockable current (Fig. 2). No leakage correction was implemented. Current tracings were filtered at 1 kHz with a four-pole Bessel filter, digitized (TL-1 DMA, Axon Instruments, Inc.), and stored on the hard disc of the computer for off-line analyses by pClamp 6.01 software (Axon Instruments, Inc.).

Charge transfer through L-type Ca channel was determined by the integration of Cd-sensitive Ca current for 200 ms voltage pulse from HP of −45 mV to the test potential of 0 mV. Only the inactivating part of the Ca current was integrated. The total increment in intracellular Ca was calculated from the time integral of the current and myocyte volume according to the following equation

\[ \Delta C_{\text{Ca}_{\text{in}}} = \int I_{C_{d}}(t) \, dt \cdot (z \cdot F \cdot V)^{-1} \]

where \( z \) is the equivalent charge of 2 carried by the Ca ions; \( F \) is the Faraday constant with a value 96 500 A · s/mol; \( \int I_{C_{d}}(t)/dt \) is the time integral of Cd-sensitive Ca current and \( V \) is the volume of the myocyte. The Ca-accessible cell volume was considered to be 65% of the whole myocyte volume for all developmental stages.

The inactivation time constants of \( I_{C_{d}} \) were fitted with a biexponential function using the non-iterative Chebyshev method of pClamp 6.01 software.

### 2.3. Determination of cell size

For the analysis of cell volume and sarcolemmal surface area magnified image of the cell was projected through a video camera onto the screen of a TV monitor. The contours of the cell were drawn on transparents for later determination of maximum length and average width of the myocyte. Cell width was determined at three levels; in the middle and half way from the mid point to the ends.

For quantification of plane surface area and myocyte volume, myocytes were considered to be right cylinders with an elliptical cross-section. The cell volume was calculated from the equation:

\[ V = \pi \cdot a \cdot b \cdot l \]

where \( a \) and \( b \) are shorter and longer radius of the ellipse, and \( l \) is the length of the cell. The shorter radius of the ellipse was set to be 1/3 of the longer radius and accordingly the radii of the ellipse could be derived from the measured width \( w \) of the cell \((b = w/2\) and \(a = w/6\)).

The surface area of the cell \((A_s)\) is the sum of the surface of the cylinder \((A_1)\) and its ends \((A_2)\):

\[ A_s = A_1 + 2A_2 \]
Perimeter \((P)\) of the ellipse was approximated from the equation [16]:

\[
P = 2\pi \sqrt{1/2(a^2 + b^2)}
\]

Surface area of the cylinder is then

\[
A_1 = P \times l
\]

and surface area of the ends

\[
A_2 = \pi \cdot a \cdot b
\]

2.4. Statistical analysis

Results from multiple experiments are expressed as means ± s.e. Differences between age-groups were assessed with one-way analysis of variance. Paired comparisons between two age-groups were accomplished after variance analysis with Student-Newman-Keuls test. Differences were considered significant when \(P < 0.05\).

### 3. Results

#### 3.1. Postnatal changes in Ca current

Our analysis was designed to measure the density of L-type Ca current at relatively physiological conditions. Therefore, experiments were conducted at physiological body temperature (36°C) and EGTA concentration within the pipette was kept relatively low (20 μM) to allow Ca-dependent inactivation of Ca current. The fast Na current and T-type Ca current were inactivated by depolarizing holding potential (−45 mV). Rat myocytes have strong K currents, which were eliminated by replacing K with equimolar Cs in both external and internal solutions, and adding 15 mM TEA in the pipette solution. The inward current recorded under these conditions was completely blocked by Cd (0.5 mM) (Fig. 2).

To assess the voltage dependence of Ca current, 200 ms duration depolarizing pulses were applied at a frequency of

![Fig. 3. Voltage-dependence of L-type Ca current in neonatal and adult rat cardiac myocytes. Cd-sensitive Ca currents from 6-day-old (A) and 30-day-old (B) rat ventricular cells. Currents were elicited by 200 ms depolarizing voltage steps from a holding potential of −45 mV to −40, −25, 0 and +20 mV as indicated for each current tracing, with 1.8 mM Ca as the charge carrier. Membrane capacitances were 30 and 77 pF, giving maximal current densities of 21.9 and 15.8 pA/pF at 0 mV for 6-day-old and 30-day-old cells, respectively. (C) and (D) show current–voltage relations of peak \(I_{Ca}\) at the six different postnatal stages of the rat. (C) shows absolute amplitudes of \(I_{Ca}\) as means ± s.e. from 7 to 20 cells, (D) the same curves after normalization to the peak current. Ca current was determined as the difference between peak inward current and current at the end of 200 ms voltage pulse (visual estimate) from the holding potential of −45 mV.](image-url)
of Ca current increases very prominently during the postnatal development (Fig. 3C), but comparison of the normalized currents indicates that voltage dependence of $I_{Ca}$ is very similar at all developmental stages (Fig. 3D).

Amplitude of L-type Ca current was $277 \pm 254$ pA (mean $\pm$ s.e., $n = 16$) in 1-day-old rat, reached a value of $489 \pm 35$ pA at the age of 10 days ($n = 18$) and increased with advancing age to the adult value of $1961 \pm 98$ pA ($n = 16$). Thus, over 7-fold increase in peak Ca current occurs in rat ventricular myocytes from birth to adulthood. The membrane capacitance of the same cell population increased from $17.7 \pm 1.6$ pF at day 1, to $21.3 \pm 2.0$ pF at day 10, and to $125.0 \pm 5.5$ pF in the adult rat. The increase in Ca current amplitude was quite closely matched with the increase in capacitive cell surface area; accordingly Ca current density remained largely unchanged during the postnatal maturation (Fig. 4). In 10-day-old rats the current density ($22.8 \pm 1.4$ pA/pF) was slightly higher than in newborn and adult rats ($P < 0.05$) (Table 1).

The decay of L-type Ca current is both voltage- and Ca$^{2+}$-dependent. Inactivation kinetics of Ca current were determined by fitting the current decays with a biexponential equation. Fast and slow time constants and their magnitudes were calculated for a voltage step from $-45$ to 0 mV. It is evident from Fig. 5 that the inactivation rate of Ca current is faster in adult than 1-day-old rat. Time constant of the fast component was much shorter in adult (3.4 ms) than in 1-day-old rat (7.2 ms). Also the slow component inactivated faster in adults (27.7 ms) than in the newborn (29.1 ms). Mean results show that in 1- and 6-day-old rats the inactivation time constant of the fast component ($5.90 \pm 0.62$ and $5.71 \pm 0.41$ ms) is significantly longer than in the adult animals ($4.50 \pm 0.39$ ms) (Table 1). A similar trend is evident for the slow component although the differences were not statistically significant ($27.70 \pm 1.97$ ms vs. $21.73 \pm 2.98$ ms for 1-day-old and adult rats, respectively; $P = 0.26$). The fast component of Ca current represents 65–81% of the ampli-

### Table 1

Properties of $I_{Ca}$ (visual estimate) in enzymatically isolated ventricular myocytes of postnatally developing rat heart

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>n</th>
<th>$I_{Ca}$ (pA)</th>
<th>pF</th>
<th>pA/pF</th>
<th>$\tau_{f}$ (ms)</th>
<th>$\tau_{s}$ (ms)</th>
<th>$A_{slow}$ (%)</th>
<th>$A_{fast}$ (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16</td>
<td>277 ± 25</td>
<td>17.7 ± 1.6</td>
<td>16.9 ± 1.8</td>
<td>5.92 ± 0.62</td>
<td>27.74 ± 1.97</td>
<td>66 ± 6</td>
<td>34 ± 6</td>
</tr>
<tr>
<td>6</td>
<td>13</td>
<td>388 ± 74</td>
<td>21.3 ± 2.0</td>
<td>17.0 ± 1.8</td>
<td>5.71 ± 0.41</td>
<td>28.27 ± 1.69</td>
<td>65 ± 4</td>
<td>35 ± 4</td>
</tr>
<tr>
<td>10</td>
<td>18</td>
<td>489 ± 35</td>
<td>21.3 ± 1.6</td>
<td>22.8 ± 1.4</td>
<td>4.84 ± 0.68</td>
<td>23.78 ± 1.97</td>
<td>79 ± 4</td>
<td>21 ± 4</td>
</tr>
<tr>
<td>15</td>
<td>20</td>
<td>692 ± 58</td>
<td>32.4 ± 1.7</td>
<td>21.2 ± 1.5</td>
<td>3.77 ± 0.19</td>
<td>25.97 ± 1.42</td>
<td>80 ± 1</td>
<td>20 ± 1</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>1502 ± 211</td>
<td>91.8 ± 4.5</td>
<td>16.2 ± 2.0</td>
<td>4.15 ± 0.96</td>
<td>26.13 ± 3.25</td>
<td>81 ± 1</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Adult</td>
<td>16</td>
<td>1961 ± 98</td>
<td>125.0 ± 5.5</td>
<td>15.9 ± 0.7</td>
<td>4.50 ± 0.39</td>
<td>21.73 ± 2.98</td>
<td>76 ± 2</td>
<td>24 ± 2</td>
</tr>
</tbody>
</table>

The results are means $\pm$ s.e. of 7 to 20 myocytes.

$I_{Ca}$, peak amplitude of L-type Ca current; pF, cell capacitance; pA/pF, the peak density of Ca current; $\tau_{f}$, inactivation time constant of the fast component; $\tau_{s}$, inactivation time constant of the slow component; $A_{slow}$ and $A_{fast}$, relative amplitudes of slow and fast components of Ca current.

$P$ values indicate the level of statistical significance by one-way analysis of variance; NS = not significant ($P > 0.05$).

Percent values were tested after arcsin transformation of the primary results.
Fig. 5. Rate of Ca current inactivation in 1-day-old newborn (A) and adult (B) rat ventricular myocytes. Currents were elicited by 200 ms voltage pulse from the holding potential of −45 mV to 0 mV. Current inactivation was fitted by biexponential equation (dashed line) and the time constants for fast (τf) and slow (τs) components are shown in the lower right corner of the figures. (C) shows comparison of normalized currents.

plitude of the total current and was slightly smaller in newborn than adult rats (Table 1).

Since inactivation rate of Ca current increases with age the peak current density underestimates the true transsarcolemmal Ca influx in the younger age-groups. To avoid this charge transfer through L-type Ca channels was determined by calculating the time integral of the inactivating part of Ca-sensitive Ca current for a voltage step from −45 mV to 0 mV. Fig. 6 shows representative tracings of Cd-sensitive Ca currents and their time integrals for 1, 6, and 30-day-old and adult rats. It is obvious that strong increase in both peak amplitude of ICa and its time integral occur during the postnatal maturation of the rat heart. The time integral of Cd-sensitive Ca current increased 3.45-fold from 7.18 ± 1.00 pC at day one to 24.80 ± 1.80 pC in the adult rat (P < 0.001). This increase is less than the 6.4-fold increase in the amplitude of Ca current (Table 2). When the integral of Ca current is expressed per unit cell surface area it is evident that Ca influx through sarcolemmal Ca channels decreases strongly from 0.429 ± 0.074 pC/pF at day one to 0.188 ± 0.016 pC/pF of the adult rat (Table 2). In addition to the inactivating part of Ca inward current a maintained or noninactivating component of Ca current seems to be also present (see Fig. 3B and Fig. 6 for 30-day-old and adult rat myocytes). Calculation of the window current from the product of the steady-state inactivation and activation parameters (fi, di) indicated that the noninactivating component of Ca current is maximal around −20 mV in all age-groups and contributes slightly more than 30% to the total inward current at this potential (data not shown). Integration of Ca current was accomplished at 0 mV which is quite at the edge of the current window; at 0 mV the amplitude of the noninactivating Ca current was on average 3% (range from 1.6 to 5.2%) of the peak inward current. Three percent of the peak current makes a clearly discernable noninactivating current of 60 pA in adult myocytes but only a tiny 10 pA current in the

Table 2
Amplitude, density and time integral of Cd-sensitive Ca current and its contribution to total cellular Ca in postnatally developing rat heart ventricles.

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>n</th>
<th>pA</th>
<th>pF</th>
<th>pA/pF</th>
<th>pC</th>
<th>pC/pF</th>
<th>V (×10^3 μm^3)</th>
<th>ΔCa (μmol l^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7</td>
<td>363±51</td>
<td>19.1±3.3</td>
<td>20.8±2.7</td>
<td>7.18±1.00</td>
<td>0.429±0.074</td>
<td>1.138±0.202</td>
<td>24±9</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>578±116</td>
<td>25.5±3.9</td>
<td>22.7±1.8</td>
<td>10.37±1.96</td>
<td>0.408±0.034</td>
<td>1.820±0.284</td>
<td>42±3</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>715±49</td>
<td>28.3±2.8</td>
<td>23.2±2.5</td>
<td>8.42±0.78</td>
<td>0.314±0.042</td>
<td>2.082±0.207</td>
<td>32±4</td>
</tr>
<tr>
<td>15</td>
<td>12</td>
<td>799±59</td>
<td>31.6±1.1</td>
<td>25.4±1.9</td>
<td>9.43±0.72</td>
<td>0.301±0.023</td>
<td>2.780±0.093</td>
<td>25±7</td>
</tr>
<tr>
<td>30</td>
<td>7</td>
<td>1564±228</td>
<td>91.8±4.5</td>
<td>16.8±2.0</td>
<td>17.08±1.03</td>
<td>0.188±0.014</td>
<td>11.040±0.539</td>
<td>12±1</td>
</tr>
<tr>
<td>Adult</td>
<td>11</td>
<td>2322±120</td>
<td>133.7±6.0</td>
<td>17.9±1.1</td>
<td>24.80±1.80</td>
<td>0.188±0.016</td>
<td>23.216±1.045</td>
<td>8±1</td>
</tr>
<tr>
<td>P value &lt;</td>
<td></td>
<td>0.001</td>
<td>0.001</td>
<td>0.05</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

The results are means ± s.e. of 6 to 12 myocytes. pA, peak amplitude of Cd-sensitive Ca current; pA/pF, density of Cd-sensitive Ca current; pC, charge transfer by Cd-sensitive Ca current; pC/pF, charge transfer by Ca current per unit capacitive cell surface area; V, volume of the myocytes as determined from the cell capacitance; ΔCa, increase in total cellular Ca by Ca influx through Cd-sensitive Ca current.
newborn myocytes, which is difficult to separate from the noise. Integration of the whole Cd-sensitive difference current gave only marginally higher charge values in comparison to the integral of the inactivating current in newborn rats (7.24 vs. 7.18 pC) but clearly bigger values in the adults (28.7 vs. 24.80).

3.2. Postnatal changes in cell size

To be able to calculate the contribution of Ca current to intracellular Ca concentration the myocyte volume must be also known. Therefore, average cell size was determined from the same cell isolations, which were used for electrophysiological experiments. Maximum length and average width of the myocyte were used to calculate sarcolemmal surface area and volume of single ventricular cells (Table 3). The mean length of the myocytes increased from 45.3 ± 1.2 μm at the age of 1 day to 126.6 ± 2.9 μm in the adult rat. The corresponding increase in the width was from 9.1 ± 0.2 to 27.6 ± 1.1 μm (Table 3). The lean surface area of sarcolemma increases only 8.7 times from 1.001 ± 0.025 × 10^3 to 8.675 ± 0.426 × 10^3 μm², while the increase in cell volume is as high as 28-fold from 0.991 ± 0.042 × 10^3 to 27.741 ± 2.489 μm³. Accordingly, surface-to-volume ratio decreased from 1.05 ± 0.02 in the newborn rat to 0.36 ± 0.01 in the adult animal (P < 0.001).

The capacitive surface area was measured for those cells which were used for characterization and quantification of Ca current. Cell capacitance increased 5.18-fold from birth to day 30 (Table 1). This corresponds quite well with the morphological determination of external cell surface area which increased during the same developmental period 4.18-fold (Table 3). The slightly higher increase in capacitive cell surface area may be explained by increase in cell surface area due to T-tubules. The specific membrane capacitance, calculated by dividing the mean cell capacitance by the mean sarcolemmal surface area (different cell populations), gave values between 1.26 and 2.19 μF/cm² without any clear age-dependent trend. In a smaller sample of cells, where both membrane capacitance and sarcolemmal surface area were determined for the same cell, specific membrane capacitance varied between 1.2 and 1.7 μF/cm² with a mean value of 1.6 μF/cm².

The relative constancy of specific membrane capacitance suggests that the contribution of membrane infoldings to total surface area does not change decisively during the whole postnatal period. Supposing that the specific capacitance of biological membranes is 1 μF/cm², sarcolemmal infoldings would increase cell surface area by approximately 60%.

3.3. Contribution of L-type Ca current to total cellular Ca

Integral of Ca current and cell volume were determined for the same cell to get an estimate of intracellular Ca increase. Cell volume was calculated from the cell capacitance, which was transformed to sarcolemmal surface area using the specific membrane capacitance of 1.6 μF/cm². Sarcolemmal surface area was further transformed to cell volume by utilizing the morphometrically determined surface-to-volume ratios. The increment in total cellular Ca was also calculated be dividing the time integral of \(I_{Ca}\) by the mean volume of the myocytes for the respective age-group (Fig. 7). The latter way of calculation is independent of the measurements of cell capacitance and surface-to-volume ratios of the cells. Both methods give similar results. The results are means ± s.e. of 6 to 12 myocytes.

![Fig. 7. Increment in total cellular Ca due to sarcolemmal Ca influx through Cd-sensitive Ca channels at six developmental stages of the rat heart.](image-url)
results but the difference between newborn and adult animals is slightly more pronounced when morphometrically determined cell volume is used. In the newborn rat the increment of total cellular Ca was as high as 54 ± 9 μmol 1⁻¹. It decreased very prominently during the whole postnatal period reaching the adult value of 8 ± 1 μmol 1⁻¹ (P < 0.001) (Fig. 7). Thus our analysis shows that the contribution of L-type Ca current to total cellular Ca is over 6 times higher in 1-day-old than adult rat heart.

4. Discussion

During early postnatal development of the rat heart contractility goes through a series of rapid changes, which are based on age-dependent alterations in the isoforms of contractile proteins and changes in Ca regulation of contraction. Direct measurements of Ca fluxes through putative Ca transport mechanisms at various developmental stages are necessary to elucidate maturational changes in cardiac excitation–contraction coupling. The present experiments were designed to delineate the contribution of Ca influx through L-type Ca channels to total cellular Ca in postnatally developing rat heart. The evaluation requires measurement of time integral of Ca current and determination of myocyte volume for each developmental stage. This kind of analysis has not been previously done for the developing heart of rat or any other mammalian species.

Cultured cardiac cells of neonatal rat heart are a useful preparation for many research problems but inadequate in studies where physiological developmental pattern is the main interest. Culture conditions change growth rate of myocytes and density of K and Ca currents in a nonphysiological way [17,18]. To avoid these problems freshly isolated cells were used in the present study. The same isolation procedure and enzymes were used for both neonatal and adult rat hearts, so that the comparison of the results between different age groups should be valid. With the current method, isolation of neonatal cardiac cells takes maximally 10 min and dissociation of adult heart approximately 15 min from the cannulation of the aorta. Myocytes are subjected to enzymes only for 3–5 min in neonates and 10 min in the adults.

4.1. Density of Ca current

The present results show that although the amplitude of Ica increases strongly during postnatal maturation, Ica density remains largely unaltered in the rat heart. A slight increase in Ica density occurs during the first 10 days of postnatal development followed by an equal decline during later development. Previous reports on the Ca current density of fetal and postnatally developing rat heart are somewhat variable. Cohen and Lederer [19] mention that the density of Ica in cultured cardiac cells from 2–7-day-old rats is larger than the current in freshly isolated adult rat cells, although quantitative data for current densities were not given. Quite in contrast to the results of Cohen and Lederer, Gomez et al. [18] showed that in freshly isolated cells from 1–2-day-old rats Ica density was only about 1/4 of that in adult rat heart cells. Masuda et al. [20], also using freshly isolated rat ventricular cells, found that density of Ca current increases during the fetal period but did not change any more during the early postnatal development from birth to the age of 10 days. Our results agree with those of Masuda et al. showing little change during early maturation (from day 1 to day 6) and are concordant with the results of Cohen et al. in that a small decline of Ica density may be present during later postnatal development (from 10-day-old neonates to the adults), but disagree with those of Gomez et al. [18]. The reason for the variability of the results is not clear but could include differences in experimental conditions and cell isolation.

We have tried to minimize the effect of isolation procedure by using coronary perfusion for all age groups. By this means we could avoid long exposures to dissociating enzymes, which is necessary when myocytes are isolated by incubation of tissue chunks in enzymic solutions.

Maturation changes in dihydropyridine receptor density of rat heart have been reported. [H]-nitrendipine binding to cardiac homogenate proteins suggests a very prominent (over 3-fold) increase in Ca channel density during the first 7–9 days of postnatal development in rat [21]. Results of Wibo et al. [11] with 3H(+)–PN200–110 binding to cardiac microsomes show only slight (1.35-fold) increase in Ca channel density between birth and 30 days of age. In isolated adult rat ventricular cells, which are practically free from nonmyocyte membranes, the number of dihydropyridine binding sites is calculated to be 380 000/cell and in cultured neonatal rat cells 54 000/cell [22,23]. When the channel numbers are divided by sarcollemmal surface areas (1001 and 8675 μm²) these values transform to 54 and 44 sites/μm² for neonatal and adult rat heart, respectively, suggesting a slight decrease in Ca channel density from birth to adulthood. Thus, binding experiments with relatively pure myocyte preparations show only minor changes in Ca channel density during the postnatal maturation of rat heart and accord well with the present results on Ca current density. Further, charge movement associated with Ca channel gating is very similar in newborn and adult rat ventricular myocytes (4 vs. 5 nC/μF; [24,25]), suggesting that the number of functional Ca channels does not change markedly between birth and adulthood.

4.2. Size and surface to volume ratio of myocytes

For the calculation of sarcomemma surface area and volume of the cells, myocytes were considered to be right elliptical cylinders with the ratio of longer and shorter radius of 3:1. That the relatively flat myocyte shape approximates well the true cellular morphology is shown by
the close match of the present results and Coulter counter determinations of Gerdes et al. [26,27]; cell volume by Coulter counter analysis for adult rats (28.690 μm³) fits very well with our determination (27.741 μm³) for the same age-group. Also the results for 31-day-old rats of Gerdes et al. are close to the values measured by us for 30-day-old rats (length 94.46 vs. 92.8 μm; width 18.2 vs 18.3 μm; volume 10633 vs. 8543 μm³). The cell volume of 12-day-old rat heart by Coulter counter analysis (3135 μm³) [27] settles between the volumes of 10-day-old and 15-day-old rats of the present study (2185 and 3694 μm³). Our experiments extend the analysis of cell size to younger rats and show that relatively modest increases in cell dimensions occur during the first 10 postnatal days. This developmental pattern is evident from both morphological determination of the cell size and measurement of cell capacitance.

External sarcolemmal surface-to-cell-volume ratio is physiologically important since Ca influx through sarcolemma may directly control contraction in the fetal and early postnatal period. In newborn mammals T-tubules are rudimentary or absent and plane cell surface area should be representative for the whole cell surface area. In accordance with this our values for newborn rat (1.05) agree well with former electron microscopic findings in newborn rabbits (1.03) [7] and cats (1.01) [28]. Stewart and Page [29] showed that surface-to-volume ratio of rat ventricular myocytes declines from 0.61 for 40.5-g rat (corresponding approximately the age of 20 days) to 0.47 for the adult animal. They did not study smaller animals but our results show that surface-to-volume ratio increases further towards smaller body sizes. The calculated surface-to-volume ratio for adult rat myocytes (0.36) of the present study is similar to morphometrically determined values in several mammalian species. For adult rats, rabbits, and cats values of 0.46, 0.47 and 0.47 have been reported [7,28–30]. The good match of the present values with the electron microscopic determinations is slightly surprising since our values are for the plane cell surface area but the electron microscopic measurements include T-tubular system. It seems that the contribution of the T-system to cell surface area is not sufficient to counterbalance the decrease in surface-volume ratio which is due to the increase in cell dimensions. In adult rat T-tubules increase surface-to-volume ratio from 0.30 to 0.46 [30] and in adult rabbit from 0.30 to 0.47 [7]. That sarcolemmal infoldings, in the form of T-tubules and caveolae, do not decisively change the surface-to-volume ratio of postnatally developing heart is supported by specific cell capacitance which stays relatively constant during the whole postnatal maturation. Cell capacitance of newborn rat myocytes is typically around 18 pF ([17,31], present study) and in 3-month-old adult rats approximately 165 pF [37], which means a 9.2-fold increase in membrane capacitance during the postnatal development of the rat heart. This is not far from the morphologically determined increase in cell surface area (8.7-fold) of the present study. It seems that surface-to-volume ratio of ventricular cells is 2- to 3-fold higher in newborn than adult mammals and accordingly Ca fluxes through sarcolemma have higher importance in newborn than adults, even if fluxes per unit sarcolemma remain constant.

4.3. Contribution of Ca current to total cellular Ca

The present analysis shows that contribution of Ca current to total cellular Ca is over 6 times larger in newborn than adult rats. Our value for the adult rat (8 μmol l⁻¹) agrees well with the determination of Negretti et al. [33] (10.3 μmol l⁻¹) in the same species. In our calculations Ca-accessible (or nonmitochondrial) cell compartment was supposed to be 65% of the total myocyte volume. This approximates well the true mitochondrial volume density of the adult rat heart myocytes (36%) but slightly underestimates Ca accessible volume of the newborn rat heart cells in which mitochondria occupy only 20% of the myocyte volume [5]. Using 80% value instead of 65% for the newborns will reduce the total increment in cellular Ca from 54 to 47 μmol l⁻¹, which is still almost 6 times higher than the adult value. Furthermore, action potential duration of the adult rat heart is much less than 200 ms and thus the time integral of Ca current may be slightly overestimated in the adult group. With these corrections in mind our calculations should approximate the physiological situation.

The increment in total cellular Ca is dependent upon three factors: amplitude and inactivation time course of Ca current and surface-to-volume ratio of the cells. Our results show that the density of Ca current remains fairly constant during the postnatal maturation but inactivation rate of I_{Ca} and surface-to-volume ratio of myocytes change markedly during postnatal development. Although the peak Ca current density does not change very much, the integrated Ca influx per unit surface area through L-type Ca channels is bigger in neonates than adults due to slower inactivation rate of I_{Ca} in the neonates. I_{Ca} is inactivated by voltage- and Ca-dependent mechanisms [34]. Since the SR is less well-developed in neonates than adults, a weaker Ca-release from intracellular stores may exert less potent Ca-dependent inactivation on Ca current in young than more mature rats. The opening behaviour of L-type Ca current is reported change from brief openings (mode-1) to long-lasting openings (mode-2) during fetal and early postnatal development [20]. Therefore, it is also possible that developmental decrease in mode 2 behaviour increases the inactivation rate of I_{Ca}. The growth of myocytes is associated with decrease in surface-to-volume ratio of the cells and as a consequence the smaller Ca influx of adult rat myocytes is diluted to three times larger myoplasmic volume when compared to the myocytes of newborn rat heart.

The postnatal decline of sarcolemmal Ca influx is complementary to the finding that the recirculating fraction of
Ca increases during postnatal maturation of the rat heart [8]. Others [11] have shown that in 3–4-day-old rat ventricle ryanodine (1 μM) causes approximately 27% inhibition of contraction but nifedipine (1 μM) practically abolishes contraction. This suggests that contraction of neonatal rat ventricle is totally dependent on nifedipine-sensitive Ca influx (Ca current) and that a major portion of this influx is directly accessible to myofilaments and only a minor part is secondarily due to Ca release from the SR. The total nifedipine sensitiveness of contraction does not exclude reverse Na–Ca exchange as an alternative Ca influx route, because blockade of Ca channels reduces action potential duration and thereby also Na–Ca exchange [35].

Peak Ca current density in neonatal rabbit heart is only 25–50% of the current of the adult animals. When the larger surface-to-volume ratio of neonatal cells is taken into consideration Ca current is calculated to provide approximately the same amount of Ca in neonates and adults [36–39]. Since Ca influx through Ca channels seems insufficient to account for tension generation, the Na–Ca exchanger is supposed to be a significant influx route for activator Ca in neonatal rabbit heart [35,40]. This is supported by biochemical analyses: mRNA of Na–Ca exchanger is high in fetal and newborn rabbit heart and declines during the postnatal period, while mRNA level of sarcosomal Ca channels is low at birth and rises during maturation [41,42]. As in the rabbit mRNA level of the Na–Ca exchanger [41] and Na-dependent Ca uptake of the rat heart [43] peak near birth and are less prominent in the adult. The relatively great Ca influx through the channels suggests, however, that reverse Na–Ca exchange may not be as important in the rat heart as it is in the rabbit. Due to the rudimentary nature of SR in neonatal rats the high Na–Ca exchange activity is, anyway, necessary for the relaxation.

In conclusion, analysis of L-type Ca current and size of freshly isolated ventricular myocytes show that contribution of sarcosomal influx through Ca channels to total cellular Ca decreases prominently during postnatal maturation of the rat heart. The larger Ca influx in neonates is partly due to the slower inactivation of Ca current in young animals, partly contributed by decreasing sarcosomal surface-to-volume ratio of growing myocytes.

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References


