Introduction

The mouse has a high heart rate among experimental animal species and its myocardium has unique electrophysiological and contractile characteristics (1, 2). The action potential of the mouse ventricular myocardium has an extremely short duration that causes early termination of the Ca2+ current, resulting in less dependence of contraction on trans-sarcolemmal Ca2+ influx (3). On the other hand, the mouse myocardium was reported to have a high sarcoplasmic reticulum (SR) content and Ca2+-ATPase activity per unit myocardial mass (4), and its contraction is more highly dependent on Ca2+ released from SR than other experimental animal species (2). Such characteristics of the mouse myocardium support its rapid contraction and relaxation under high heart rate.

The immature myocardium is generally known to have electrophysiological and contractile properties different from those of the adult myocardium (2, 5, 6). In the case of the mouse heart, the beating rate increases during pre- and postnatal development; the heart rate of the fetal mouse reaches 200 on the 16th fetal day (7), is about 320 on the day of birth, and reaches the adult level of about 700 at 2 weeks after birth (1, 8). Various changes in the excitation-contraction mechanisms that enable rapid contraction and relaxation should be taking place in the developing mouse cardiomyocyte to adjust to the large increase in heart rate. The immature mouse myocardium was reported to have an action potential with a longer duration and a less developed SR compared with the adult (3, 9). However, the time course of the changes underlying the developmental shift towards a highly SR-dependent myocardium has not been systematically described.
The present study was undertaken to clarify the developmental time course of the changes in action potential properties and ultrastructural organization of the ventricular cardiomyocyte to obtain a basis for a comprehensive understanding of the excitation–contraction mechanisms of the developing myocardium. We performed pharmacological experiments, action potential measurements, and confocal microscopic analyses on isolated myocardial preparations from five different stages of pre- and postnatal development. The results obtained showed that the mouse ventricular myocardium becomes increasingly dependent on SR function with age, but the underlying mechanisms differ among developmental stages.

**Materials and Methods**

**General**

All experiments were approved by the Ethics Committee of Toho University Faculty of Pharmaceutical Sciences and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society.

**Measurement of contractile force**

Right ventricular free wall strips were rapidly isolated from 1-, 2-, and 4-week-old ddY strain mice. The approximate length and width of those preparations were 3 and 2 mm, respectively. The whole ventricle was used for the fetal (16 to 18 day) and neonatal (0 to 2 day) mice. The approximate length and width of fetal and neonatal preparations were 2 and 1 mm, respectively.

Preparations were placed horizontally in a 20-ml organ bath containing modified Ringer solution of the following composition: 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 24.9 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11.1 mM glucose (pH 7.4 at 36°C). The solution was gassed with 95% O₂–5% CO₂ and maintained at 36°C ± 0.5°C. The preparations were driven by rectangular current pulses (3 Hz, 3 ms, 1.5 × threshold voltage) through a pair of platinum plate electrodes (field stimulation) generated from an electronic stimulator (SEN-3301, Nihon Kohden). The output of a microelectrode amplifier with high input impedance and capacity neutralization (MEZ8201, Nihon Kohden) was digitalized by an A/D converting interface (Power Lab/4SP) and analyzed by a Chart 7 software. The parameters measured were resting membrane potential (RP), overshoot (OS), amplitude (AMP), maximum rate of rise (V_max), and action potential duration at 20%, 50%, and 90% repolarization (APD₂₀, APD₅₀, and APD₉₀, respectively).

**Isolation of mouse ventricular cardiomyocytes**

Ventricular myocytes from 1-, 2-, and 4-week-old mice were prepared by Langendorff perfusion and collagenase digestion as in our previous study (13). In the case of fetus and neonate, the whole ventricular tissue was immersed in collagenase solution (10 mg/ml; Wako, Tokyo) for 50 – 60 min at 36°C ± 0.5°C. The digested tissues were transported to Kraftbrühe (KB) solution, and pipetting was gently performed to make a cell suspension.

**Confocal imaging analysis**

Analyses of the cell structure and Ca²⁺ dynamics were performed with a confocal microscope (LSM 510; Carl Zeiss, Oberkochen, Germany). The objective used was C Apocromat 40×/1.2 NA water immersion (Carl Zeiss). The cells were superfused with HEPES-Tyrode solution of the following composition: 143 mM NaCl, 5.4 mM KCl, 1.8 mM MgCl₂, 2 mM CaCl₂, 0.33 mM NaH₂PO₄, 5.5 mM glucose, and 20 mM HEPES. The solution was gassed with 100% O₂. Two-dimensional imaging of the cell membrane and SR were performed with the X-Y scan mode. To stain the SR, the cells were treated with ER-TrackerTM Blue-White DPX at a concentration of 5 μM for 30 min at room temperature. They were excited at 780 nm by a 2-photon laser (Mai Tai; Spectra Physics, Santa Clara, CA, USA), and fluorescence with wavelength below 685 nm was detected. To stain the cell membrane and t-tubules, the cells were treated with PKH67-GL at a concentration of 5 μM for 5 min at room temperature. They were excited at 488 nm and fluorescence with wavelength above 505 nm was detected. The imaging of the Ca²⁺ transient was performed in the line-scan mode. Cells were placed in a chamber on the
stage of the inverted microscope, loaded with fluo 4-AM (5 μM) for 30 min at room temperature, and superfused with the Tyrode’s solution mentioned above at 36°C ± 0.5°C. Field stimulation was performed at 3 Hz through platinum wire pairs with rectangular current pulses of 3-ms duration generated by an electric stimulator (SEN-3303, Nihon Kohden). The cells were excited at 488 nm and fluorescence with wavelength above 505 nm was detected. Each line was scanned in 512 pixels at a speed of line/768 μs.

Statistics
All data are expressed as means ± S.E.M. Data were analyzed by one-way or two-way analysis of variance followed by Tukey’s test for multiple comparisons. A P-value less than 0.05 was considered statistically significant.

Chemicals
The following drugs and chemicals were used: fluo 4-AM (Dojindo Laboratories, Kumamoto), ryanodine and xestospongin C (Wako, Tokyo), PKH67-GL and nifedipine (Sigma, St. Louis, MO, USA), and ER-Tracker Blue-White DPX (Invitrogen Japan KK, Tokyo) were dissolved in DMSO. The final concentration of DMSO in the extracellular solution did not affect the parameters observed. All other drugs and chemicals were of the highest available quality.

Results

Inotropic effect of nifedipine, ryanodine and xestospongin C
Nifedipine, a blocker of the L-type Ca²⁺ channel, produced concentration-dependent negative inotropy at all ages examined; the effect became smaller as age advanced (Fig. 1: A, Ca). The developmental decrease in inotropy was prominent during the late fetal period and during the second postnatal week; nifedipine (1 μM) decreased the contractile force to 7.3% ± 1.1% in the fetus, 29.6% ± 1.8% in the neonate, 35.2% ± 3.2% in 1-week-old mice, 48.3% ± 3.0% in 2-week-old mice, and 52.5% ± 2.0% in 4-week-old mice.

Ryanodine, which inhibits Ca²⁺ release from the ryanodine receptor, produced concentration-dependent negative inotropy at all ages examined; the effect became larger as age advanced (Fig. 1: B, Cb). The developmental increase in inotropy was prominent during the late fetal period and during the second postnatal week; ryanodine (10 nM) decreased the contractile force to 76.2% ± 2.1% in the fetus, 51.3% ± 3.6% in the neonate, 45.6% ± 5.6% in 1-week-old mice, 19.6% ± 3.8% in 2-week-old mice, and 11.6% ± 1.1% in 4-week-old mice.

Xestospongin C, which inhibits Ca²⁺ release from the IP₃ receptor, produced only slight negative inotropy at all ages examined; xestospongin C (3 μM) decreased the contractile force to 95.1% ± 3.0% in the fetus, 98.5% ± 2.2% in the neonate, 96.6% ± 2.8% in 1-week-old mice, 96.3% ± 0.7% in 2-week-old mice, and 94.2% ± 1.1% in 4-week-old mice (n = 4 for each).

Action potential configuration
The action potential duration at 20% and 50% repolarization (APD₂₀ and APD₅₀) progressively decreased during development (Fig. 2, Table 1); the decrease was
prominent during the late fetal period. In contrast, the action potential duration at 90% repolarization (APD_{90}) first decreased until 1 week after birth and then turned to an increase with age. The action potential at 4 weeks had an extremely rapid early repolarization followed by a late plateau phase. The maximum rate of rise (V_{max}) increased progressively during development (Fig. 2, Table 1).

**SR and t-tubule imaging**

The size of the ventricular myocytes progressively increased with age (Fig. 3A, Table 2). The SR content of the myocytes gradually increased with age throughout the pre- and postnatal periods (Fig. 3: A, B). In 16-day-old fetal myocytes, the SR was observed slightly and only around the nucleus. In neonatal and 1-week-old myocytes, the SR was extended to the subsarcolemmal region, but was still predominant at the perinuclear region. In 2-week-old myocytes, intense SR staining was observed both at the subsarcolemmal region and at the cell center. The fraction of the fluorescence positive area for the cytoplasmic SR staining was the same as that in 4-week-old myocytes.

The t-tubules were shown to appear and increase during early postnatal development (Fig. 3: B, C). In fetal and neonatal myocytes, staining was apparent only on the surface membrane, indicating the lack of t-tubules. T-tubules were first observed at 1 week after birth in the subsarcolemmal region as short fragments of staining, but not in the cell interior. In 2-week-old myocytes, t-tubules were extended to the cell interior and the fraction of the fluorescence positive area for the t-tubule staining was the same as that in 4-week-old myocytes.

### Table 1. Developmental changes in action potential parameters

<table>
<thead>
<tr>
<th></th>
<th>fetus (16 day)</th>
<th>fetus (18 day)</th>
<th>neonate</th>
<th>1-week-old</th>
<th>2-week-old</th>
<th>4-week-old</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP (mV)</td>
<td>−75.1 ± 0.7</td>
<td>−75.1 ± 0.6</td>
<td>−76.8 ± 0.5</td>
<td>−77.4 ± 0.4</td>
<td>−77.2 ± 0.5</td>
<td>−76.6 ± 0.5</td>
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<tr>
<td>OS (mV)</td>
<td>24.3 ± 1.1</td>
<td>24.8 ± 0.8</td>
<td>23.9 ± 0.8</td>
<td>22.2 ± 1.3</td>
<td>22.3 ± 0.8</td>
<td>24.3 ± 1.5</td>
</tr>
<tr>
<td>AMP (mV)</td>
<td>99.4 ± 0.9</td>
<td>99.6 ± 1.3</td>
<td>99.1 ± 0.9</td>
<td>99.7 ± 1.3</td>
<td>99.1 ± 0.4</td>
<td>99.6 ± 1.6</td>
</tr>
<tr>
<td>APD_{20} (ms)</td>
<td>44.9 ± 2.5*</td>
<td>23.5 ± 3.5*</td>
<td>7.5 ± 0.7*</td>
<td>3.0 ± 0.2</td>
<td>2.8 ± 0.2</td>
<td>2.5 ± 0.2</td>
</tr>
<tr>
<td>APD_{50} (ms)</td>
<td>76.1 ± 3.8*</td>
<td>48.1 ± 4.1*</td>
<td>16.0 ± 1.2*</td>
<td>6.0 ± 0.4</td>
<td>5.5 ± 0.3</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>APD_{90} (ms)</td>
<td>110.5 ± 3.3*</td>
<td>86.9 ± 4.1*</td>
<td>44.5 ± 2.6</td>
<td>17.7 ± 0.6*</td>
<td>23.9 ± 1.3*</td>
<td>35.9 ± 2.2</td>
</tr>
<tr>
<td>V_{max} (V/s)</td>
<td>173.7 ± 12.9*</td>
<td>197.9 ± 15.3*</td>
<td>217.7 ± 7.7*</td>
<td>273.7 ± 11.7</td>
<td>274.2 ± 16.0</td>
<td>298.4 ± 20.3</td>
</tr>
</tbody>
</table>

Action potential parameters of ventricular myocardia from 16- and 18-day-old fetus, neonate, and 1-, 2-, and 4-week-old mice. RP, OS, AMP, and V_{max} indicate resting potential, overshoot, amplitude, and maximum rate of phase 0 depolarization, respectively. APD_{20}, APD_{50}, and APD_{90} indicate action potential duration at 20%, 50%, and 90% repolarization, respectively. Values are the mean ± S.E.M. from 8 to 15 ventricular preparations. Asterisks indicate significant difference from corresponding values in 4-week-old mice as evaluated by one-way analysis of variance followed by the Tukey’s test for multiple comparisons.
Colocalization of the SR and t-tubule occurred only faintly in the subsarcolemmal region in 1-week-old myocytes, but occurred throughout the cytoplasm in 2- and 4-week-old myocytes (Fig. 3A).

**Ca²⁺ transient measurement**

The amplitude and rate of rise of Ca²⁺ transient increased progressively with age (Fig. 4, Table 3). Fetal Ca²⁺ transients were characterized by slower rise in Ca²⁺ than those in 4-week-old myocytes both in the subsarcolemmal region and cell center. In neonatal and 1-week-old myocytes, the increase in Ca²⁺ at the center showed a slower rise than that in the subsarcolemmal region. In 2- and 4-week-old myocytes, Ca²⁺ increased rapidly and simultaneously across the entire width of the cell. Nifedipine (1 μM) decreased the amplitude of Ca²⁺ transients at all ages examined both at the subsarcolemmal region and cell center (Fig. 5). The effect of nifedipine, in other words, the nifedipine-sensitive component of the increase in Ca²⁺, became smaller as age advanced (Fig. 5, Table 4). Ryanodine (1 μM) decreased the amplitude of Ca²⁺ transients at all ages examined both at the subsarcolemmal region and cell center (Fig. 6). The effect of ryanodine, in other words, the ryanodine-sensitive component of the increase in Ca²⁺, became larger as age advanced (Fig. 6, Table 4).

**Discussion**

The negative inotropic response of the mouse ventricular myocardium to nifedipine gradually decreased during pre- and postnatal development (Fig. 1: A, Ca). This was accompanied by a decrease in the nifedipine-sensitive component of the Ca²⁺ transient (Fig. 5, Table 4), indicating that the role of trans-sarcolemmal Ca²⁺ influx in contraction decreases during development. On the other hand, the negative inotropic response to ryanodine was increased during development (Fig. 1: B, Cb), and the total amplitude of the Ca²⁺ transient progressively decreased during development.
increased during development mostly due to the increase in its ryanodine-sensitive component (Fig. 6, Table 4). These results indicate that the functional role of Ca$^{2+}$ release from the SR increases during development. Similar developmental increases in inotropic sensitivity to ryanodine have been observed in the postnatal rat (14, 15), rabbit (16), late fetal guinea pig (17), and middle age chick embryo (18), and also in the postnatal mouse (3). In the mouse ventricle, the present results revealed that, large changes in the inotropic responses to nifedipine and ryanodine occurs during the late fetal period and during the second postnatal week (Fig. 1), which indicates that important changes in the electrophysiological properties and ultrastructural organization of the cardiomyocytes take place at these stages.

The density of Ca$^{2+}$ channels on the mouse myocardial cell membrane as well as the Ca$^{2+}$ current density of mouse myocardium was reported to be doubled during postnatal development (9, 19). Thus, the developmental decrease in myocardial dependence of contraction on Ca$^{2+}$ influx could not be ascribed to a decrease in the number of functional Ca$^{2+}$ channels. Rather, there should be factors other than the Ca$^{2+}$ channel itself that underlie the developmental decrease in the relative role of transsarcolemmal Ca$^{2+}$ influx. The first is a developmental shortening of the action potential duration at depolarized

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**Table 3.** Developmental changes in Ca$^{2+}$ transient parameters

<table>
<thead>
<tr>
<th></th>
<th>Subsarcolemma</th>
<th>Center</th>
<th>Subsarcolemma</th>
<th>Center</th>
<th>Subsarcolemma</th>
<th>Center</th>
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</thead>
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<tr>
<td>Fetus</td>
<td>0.47 ± 0.05*</td>
<td>0.33 ± 0.04*</td>
<td>14.6 ± 3.3*</td>
<td>5.0 ± 1.5*</td>
<td>97.6 ± 8.3*</td>
<td>101.5 ± 5.7*</td>
</tr>
<tr>
<td>Neonate</td>
<td>0.94 ± 0.09*</td>
<td>0.53 ± 0.05*</td>
<td>45.4 ± 5.7*</td>
<td>17.9 ± 3.4*</td>
<td>79.1 ± 8.3*</td>
<td>98.2 ± 6.2*</td>
</tr>
<tr>
<td>1-week-old</td>
<td>1.00 ± 0.09*</td>
<td>0.74 ± 0.06*</td>
<td>62.6 ± 10.4*</td>
<td>38.6 ± 5.8*</td>
<td>68.9 ± 6.3*</td>
<td>86.8 ± 8.8*</td>
</tr>
<tr>
<td>2-week-old</td>
<td>1.38 ± 0.11</td>
<td>1.37 ± 0.09</td>
<td>97.5 ± 7.4</td>
<td>96.2 ± 4.8</td>
<td>47.6 ± 2.1</td>
<td>47.3 ± 2.0</td>
</tr>
<tr>
<td>4-week-old</td>
<td>1.38 ± 0.07</td>
<td>1.36 ± 0.05</td>
<td>97.7 ± 6.6</td>
<td>96.4 ± 6.7</td>
<td>47.9 ± 2.5</td>
<td>48.1 ± 3.4</td>
</tr>
</tbody>
</table>

The Ca$^{2+}$ transient parameters of isolated ventricular cardiomyocytes from the fetus, neonate, and 1-, 2-, and 4-week-old mice. Values are the mean ± S.E.M. from 14 cardiomyocytes. Asterisks indicate a significant difference from corresponding values in 4-week-old mice, and daggers indicate a significant difference from corresponding values in the subsarcolemmal region. Statistical significance was evaluated by two-way analysis of variance followed by the Tukey’s test for multiple comparisons.

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**Fig. 4.** Line-scan analyses of the Ca$^{2+}$ transient in fluo 4–loaded myocytes. A: Typical line-scan images. The arrows on the left indicate field stimulation. Horizontal and vertical bars indicate 5 μm and 100 ms, respectively. The scanning line was placed in right angle to the longitudinal axis of the cell. B: Time courses of the rise and fall in Ca$^{2+}$ fluorescence at specific sites at the subsarcolemmal (black) and center (gray). The sites quantified at the subsarcolemmal (black) and center (gray) were 1 μm in width, as shown at the top of panels in A. Fluorescence was expressed as normalized values (F/F$_0$) against the fluorescence at time zero (F$_0$) in B, at which the cells were stimulated.
membrane potentials (Fig. 2, Table 1). The repolarizing potassium current of the fetal mouse ventricular cardiomyocyte was reported to be the delayed rectifier type that requires hundreds of milliseconds for activation (20, 21). This is developmentally converted to currents that activate in a few milliseconds such as the transient outward current (13, 21–23). The developmental changes in repolarizing currents cause acceleration of repolarization and shortening of the action potential duration at depolarized membrane potentials (Fig. 2). The time window for trans-sarcolemmal Ca$^{2+}$ influx through L-type Ca$^{2+}$ channels decreases during development, especially during the late fetal period.

Increase in SR function is another factor that underlies the developmental decrease in the role of trans-sarcolemmal Ca$^{2+}$ influx. The cytoplasmic content of SR was shown to increase constantly during pre- and postnatal development of the mouse ventricular cardiomyocyte (Fig. 3: A, B). It was reported that both mRNA and protein level of ryanodine receptor, SR Ca$^{2+}$-ATPase, phospholamban increased with age in mouse myocardium (9, 24). These ultrastructural and molecular changes underlie the developmental increase in the ryanodine-sensitive component of the Ca$^{2+}$ transient (Fig. 6, Table 4). Although the expression of the IP$_3$ receptor was reported to increase during development (25), our present result that xestospongin C had only slight negative inotropic effect suggest that the role of the IP$_3$ receptor in basal myocardial contraction is small in the developing mouse ventricular myocardium.
In addition to the amount of SR itself, a factor of crucial importance is the development of the t-tubular system (Fig. 3: A, C), which greatly influenced the spatio-temporal pattern of the rise in Ca$^{2+}$ concentration (Fig. 4). In fetal cardiomyocytes, the amplitude of the Ca$^{2+}$ transient was small, which could be explained by both the less developed SR and the low expression level of sarcolemmal Ca$^{2+}$ channels. The Ca$^{2+}$ that entered the cell through sarcolemmal Ca$^{2+}$ channels must diffuse to the cell center in order to trigger Ca$^{2+}$ release from the SR through the Ca$^{2+}$-induced Ca$^{2+}$ release mechanism. Otherwise, the Ca$^{2+}$-induced Ca$^{2+}$ release may be propagated from the subsarcolemmal region to the cell center in a wave-like fashion, as was shown to be the case in atrial cardiomyocytes lacking the t-tubules (26, 27). Thus, the peak amplitude of Ca$^{2+}$ transients was much smaller and the time required to reach the peak was longer than in the adult. In the neonatal and 1-week-old cardiomyocytes, the amount of SR was increased and t-tubular invaginations were present at the subsarcolemmal region but not at the cell center. This caused a large and rapid rise in Ca$^{2+}$ concentration at the subsarcolemmal region and a smaller and slower rise at the cell center. T-tubular invaginations developed markedly during the second postnatal week and reached the cell center at 2 weeks after birth. This enabled large and rapid rise in Ca$^{2+}$ throughout the cytoplasm. The large increase in t-tubule–SR coupling during the second postnatal week underlies the increase in SR dependence of contraction, as revealed by pharmacological analysis (Fig. 1). A similar developmental change in cellular ultrastructure and Ca$^{2+}$ handling was reported in the developing rat ventricular cardiomyocyte (5, 28).

In conclusion, the present results suggest that in the mouse ventricular myocardium, the shortening of the action potential during the late fetal period and the development of t-tubule–SR coupling during the second postnatal week largely contribute to the developmental increase in the dependence of contraction on SR function. The mouse myocardium shows various changes in its responsiveness to neurotransmitters and hormones during the fetal and early postnatal period (2). Whether such changes are functionally related to the presently observed developmental changes in the basic excitation–contraction mechanisms is an interesting subject for further studies.

References


5. Tohse N, Seki S, Kobayashi T, Tsutsuura M, Nagashima M,


