β-Adrenergic modulation of L-type Ca$^{2+}$-channel currents in early-stage embryonic mouse heart

WEIRAN LIU, KENJI YASUI, AKIKO ARAI, KAICHIRO KAMIYA, JIANHUA CHENG, ITSUO KODAMA, AND JUNJI TOYAMA
Department of Circulation, Division of Regulation of Organ Function, Research Institute of Environmental Medicine, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan

β-Adrenergic modulation of L-type Ca$^{2+}$-channel currents in early-stage embryonic mouse heart. Am. J. Physiol. 276 (Heart Circ. Physiol. 45): H608–H613, 1999.—Little information is available concerning the regulation of cardiac function by β-adrenergic agonists in early-stage embryonic mammalian heart. We have examined the effects of isoproterenol (Iso) on the spontaneous beating rate and action potential (AP) configuration in embryonic mouse hearts at 9.5 days postcoitum (dpc), just 1 day after they started to beat. Iso (3 µM) increased the spontaneous beating rate in whole hearts, dissected ventricles, and isolated ventricular myocytes. In ventricular myocytes, Iso also increased the slope of the pacemaker potential and the action potential duration but decreased the maximum upstroke velocity. In whole cell voltage-clamp experiments, the Ca$^{2+}$-channel currents were measured as Ba$^{2+}$ currents (I$\text{Ba}$). In 9.5-dpc myocytes, I$\text{Ba}$ was enhanced significantly from $-6.7 \pm 1.2$ pA/pF (by 52.4 ± 14.8%, n = 10) after the application of Iso. Propranolol (3 µM) reversed the effect of Iso. Forskolin (For, 10 µM) produced an increase in I$\text{Ba}$ by 95.5 ± 18.8% (n = 8). In ventricular myocytes at a late embryonic stage (18 dpc), 3 µM Iso caused an appreciably greater increase in I$\text{Ba}$ from $-6.2 \pm 0.5$ to $-14.5 \pm 2.2$ pA/pF (by 137.8 ± 33.0%, n = 8), whereas the increase in I$\text{Ba}$ by 10 µM For (by 120.0 ± 23.0%, n = 7) was comparable to that observed in the early stage (9.5 dpc). These results indicate that the L-type Ca$^{2+}$-channel currents are modulated by β-adrenergic receptors in the embryonic mouse heart as early as 9.5 dpc, probably via a cAMP-dependent pathway.

calcium ion channels; β-adrenergic receptor; isoproterenol

IT IS WELL KNOWN that the β-adrenergic system plays an important role in the regulation of cardiac excitation and contraction in mature mammals. The modulation of L-type Ca$^{2+}$-channel currents in cardiomyocytes by β-adrenergic receptors is the main process in the regulation of heart rate and excitation-contraction coupling. Although it is well established that β-adrenergic agonists, by activating the G$\beta\gamma$ protein, stimulate cardiac Ca$^{2+}$-channel currents via dual pathways, cAMP dependent and cAMP independent (24, 25), the β-adrenergic modulation of Ca$^{2+}$ channels in the embryonic heart is still ambiguous. There is a considerable discrepancy in the literature as to the role of β-adrenergic receptors in embryonic hearts at an early stage (within a couple of days after the initiation of beating). An et al. (1) reported that in the embryonic mouse heart, L-type Ca$^{2+}$ channels are responsive to isoproterenol (Iso) in late-stage (17–20 days postcoitum (dpc)) cardiomyocytes but not in early-stage (11–13 dpc) cardiomyocytes. In rat cardiomyocytes, Iso has little effect on Ca$^{2+}$-channel currents at fetal days 15 and 18 but has a marked stimulatory effect from fetal day 20 (14). However, biochemical studies proved the expression of β-adrenergic receptors in 13-day-old fetal mouse heart or 12-day-old fetal rat heart (4, 5, 22). On the other hand, Robkin et al. (18, 19) reported that the chronotropic β-adrenergic response of the rat heart appears at fetal day 10.5 or 11 in the explanted whole embryo, concomitant with the onset of heart beating (a rat embryo at day 11 of gestation corresponds to a mouse embryo at day 10 in terms of the stage of development). Similarly, Hall (8) found chronotropic β-adrenergic response of isolated embryonic rat hearts at 10.5–13.5 dpc.

To clarify the functional role of β-adrenergic receptors in the early-stage embryonic mouse heart, we examined the effect of Iso on the heart beating rate and electrophysiological properties of ventricular myocytes (action potential configuration and L-type Ca$^{2+}$-channel currents) at 9.5 dpc. For comparison, we also examined the effect of Iso on L-type Ca$^{2+}$-channel currents in 18-dpc ventricular myocytes. Our results indicate that activation of β-adrenergic receptors does modulate heart beating and L-type Ca$^{2+}$ channels in the 9.5-dpc embryonic mouse heart, although the modulatory potency is appreciably less than in the 18-dpc embryonic heart.

MATERIALS AND METHODS

Dissection of 9.5-dpc mouse embryos and ventricles. Pregnant (9.5 dpc) mice were killed by cervical dislocation, the uteri were isolated, and the whole embryos were exposed. Ventricles were cut from the exposed embryos. During the dissection procedures, the tissues were kept in Hanks’ balanced salt solution (GIBCO). Isolation of 9.5-dpc cardiomyocytes. The dissected ventricles were incubated in Ca$^{2+}$-free saline with 0.3 mg/ml collagenase type II (Worthington Biochemical) containing (in mM) 116 NaCl, 20 HEPES, 1.0 NaH$_2$PO$_4$, 5.5 glucose, 5.0 KCl, and 0.8 MgSO$_4$ (pH 7.35) for 20 min at 37°C and then rinsed with the same buffer solution without enzyme. The ventricles were mechanically dissociated by trituration. The cell suspensions were centrifuged at 1,000 rpm for 5 min, and the isolated cells were plated on collagen-coated glass coverslips and incubated in Dulbecco’s modified Eagle medium (GIBCO) with 10% fetal bovine serum (GIBCO) and 10 µg/ml gentamicin at 37°C in a humidified CO$_2$ incubator.

H608 0363-6135/99 $5.00 Copyright © 1999 the American Physiological Society
Isolation of 18-dpc cardiomyocytes. Ventricles were dissected from 18-dpc embryonic hearts and cut into pieces. The ventricular pieces were incubated in Dulbecco’s phosphate-buffered saline containing 0.16 mg/ml collagenase (Yakult) with stirring at 37°C for 12 min, and cell suspension was collected; this was repeated four times. The collected suspension was centrifuged at 1,000 rpm for 5 min, and the isolated cells were cultured in the same way as 9.5-dpc cardiomyocytes.

Observation of heart beating. Embryos or ventricles (9.5 dpc) were transferred to a chamber perfused with normal Tyrode solution containing (in mM) 143 NaCl, 5.4 KCl, 1.8 CaCl2, 0.5 MgCl2, 5.5 glucose, and 5 HEPES (pH 7.4) maintained at 37°C. The heart rate was measured by the naked eye under an inverted microscope (Nikon).

Action potential recording. Action potentials (AP) were recorded from spontaneously beating myocytes, cultured for ~20 h, in a normal Tyrode solution. Pipettes (15–20 MΩ) filled with an internal solution containing (in mM) 60 KOh, 80 KCl, 40 aspartate, 5 HEPES, 10 EGTA, 5 MgATP, 5 Na2-phosphocreatine, and 0.65 CaCl2 (pH 7.2) were sealed to the myocytes, and whole cell recording was initiated by suction with negative pressure. Electrical signals were fed into an Axopatch-1D (Axon Instruments), filtered at 2 kHz, and digitized at a sampling rate of 5 kHz.

Measurement of Ca2+-channel currents. Whole cell voltage-clamp recordings were performed from cardiac myocytes 20–28 h after plating. To isolate Ca2+-channel currents, the cells were perfused with a Na+- and K+-free solution containing (in mM) 2 BaCl2, 50 tetraethylammonium chloride, 100 Tris·Cl, 0.5 MgCl2, 3 4-aminopyridine, 5 HEPES, and 5.5 glucose (pH 7.4). Pipettes were pulled to resistances of 3.5–5.0 MΩ when filled with an internal solution containing (in mM) 80 CsCl, 60 CsOH, 40 aspartate, 5 HEPES, 10 EGTA, 5 MgATP, 5 Na2-phosphocreatine, and 0.65 CaCl2 (pH 7.2).

Data were recorded with an Axopatch-1D (Axon Instruments), filtered at 2 kHz, digitized at 5 kHz, and stored on a microcomputer disk for subsequent off-line analysis. Experiments were performed at 33°C.

Chemicals. l-Isoproterenol, forskolin, and dl-propranolol were purchased from Sigma (St. Louis, MO).

Data analysis. Data were analyzed using the pCLAMP program (Axon Instruments). Current density was calculated using the measured membrane capacitance. The cell capacitance was determined by applying a ramp voltage pulse of ±0.5 V/s at a potential ranging between −50 mV and +70 mV. Data are expressed as means ± SE. The mean percentage of change was the average value of individual percentage. Statistical analysis was performed using Student’s t-test, and values of P < 0.05 were considered to indicate a significant difference.

RESULTS

Effect of Iso on beating rates of 9.5-dpc whole hearts, ventricles, and ventricular myocytes. Table 1 compares the effects of Iso on beating rates of the whole hearts in vivo and those of the dissected ventricles and firing rates of AP recorded from isolated ventricular myocytes. There was no significant difference in the control beating rates between whole hearts and dissected ventricles. The spontaneous firing rate of isolated ventricular myocytes was slightly higher (by 13–15%) than the beating rates of whole hearts and of dissected ventricles. The increase in beating rate after Iso (3 µM) application in the whole hearts (by 17.0 ± 4.9%, n = 8) was similar to that in the dissected ventricles (by 16.5 ± 6.5%, n = 10). The corresponding value in ventricular myocytes (29.4 ± 3.8%, n = 8) was slightly larger than those in the whole hearts and dissected ventricles.

Effects of Iso on AP in ventricular myocytes. Figure 1 shows examples of AP before, during, and after application of Iso (3 µM), AP fired spontaneously (101/min), with a slow diastolic depolarization. The maximal upstroke velocity (Vmax) under the control condition amounted to 70.4 V/s (Fig. 1A). During the application of Iso (3 µM), the AP firing rate increased to 138/min (Fig. 1B). This positive chronotropic effect was associated with an increase in the slope of slow diastolic depolarization and a prolongation of AP duration. The AP duration measured at −50 mV (APD−50) was prolonged by 30 ms. However, Vmax was decreased to 56.2 V/s. These AP changes partially recovered after the withdrawal of Iso (Fig. 1C).

Table 1. Effect of isoproterenol on whole hearts, dissected ventricles, and isolated ventricular myocytes

<table>
<thead>
<tr>
<th>Heart Beating Rate, beats/min (n = 8)</th>
<th>Ventricle Beating Rate, beats/min (n = 10)</th>
<th>Myocyte Firing Rate, min−1 (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>108 ± 5.4</td>
<td>110 ± 3.2</td>
</tr>
<tr>
<td>Iso</td>
<td>127 ± 4.9†</td>
<td>128 ± 6.9†</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = no. of cells or preparations. Iso, isoproterenol (3 µM). *P < 0.05; †P < 0.01 vs. control.
Table 2. Action potential parameters in absence and presence of Iso

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>MDP, mV</th>
<th>APA, mV</th>
<th>$V_{\text{max}}$, V/s</th>
<th>$V_{\text{in}}$, V/s</th>
<th>APD$_{50}$, ms</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>-71.5 ± 0.5</td>
<td>116.3 ± 5.9</td>
<td>90.4 ± 17.2</td>
<td>0.034 ± 0.005</td>
<td>85.9 ± 8.3</td>
</tr>
<tr>
<td>Iso (3 µM)</td>
<td>8</td>
<td>-71.0 ± 0.6</td>
<td>114.8 ± 4.9</td>
<td>78.6 ± 15.3$^*$</td>
<td>0.054 ± 0.008$^t$</td>
<td>100.8 ± 9.8$^t$</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. MDP, maximum diastolic potential; APA, action potential amplitude; $V_{\text{max}}$, maximum upstroke velocity; $V_{\text{in}}$, slope of pacemaker potential; APD$_{50}$, duration of action potential at -50 mV. *P < 0.05; †P < 0.01 vs. control.

Table 2 summarizes the effects of Iso on AP parameters. Neither maximum diastolic potential (MDP) nor AP amplitude (APA) was affected by Iso. However, $V_{\text{max}}$ was significantly increased by 28.6% after Iso (3 µM) application and recovered to control level after the addition of its antagonists, propranolol (3 µM). Pro (3 µM). Pro (3 µM).

Effects of Iso and forskolin on L-type Ca$^{2+}$-channel currents in ventricular myocytes. Whole cell voltage-clamp recordings were performed on dissociated ventricular myocytes at 9.5 dpc to examine the effect of Iso on L-type Ca$^{2+}$-channel currents. To minimize rundown of Ca$^{2+}$-channel currents and to obtain higher conductance, Ba$^{2+}$ was used as a charge carrier of Ca$^{2+}$-channel currents instead of Ca$^{2+}$. The inward currents, evoked by 200-ms depolarizing pulses from a holding potential of -50 mV to 0 mV, were identified as an L-type Ca$^{2+}$-channel current ($I_{\text{Ba}}$) because of their complete blockade by 1 µM nisoldipine (data not shown). Figure 2, A and B, shows an example: $I_{\text{Ba}}$ was augmented by 28.6% after Iso (3 µM) application and recovered to control level after the addition of its antagonist, propranolol (3 µM).

Figure 3 shows the average current-voltage (I-V) relationship of the peak $I_{\text{Ba}}$ density in the absence and presence of Iso (3 µM). $I_{\text{Ba}}$ was elicited by depolarizing pulses from a holding potential of -50 mV in 10-mV increments. Iso increased the maximum value of peak $I_{\text{Ba}}$ obtained at 0-mV test pulse) from $-4.7 ± 0.9$ to $-6.7 ± 1.2$ pA/pF ($P < 0.01$, n = 10) without a discernible shift of the voltage dependence of $I_{\text{Ba}}$.

To investigate possible mechanisms underlying the response to Iso, we studied the effect of forskolin (For), a direct adenylate cyclase (AC) activator. For (10 µM) increased $I_{\text{Ba}}$ by 95.5 ± 18.8% (n = 8). Representative current traces are shown in Fig. 2C.

Furthermore, to compare the developmental changes in responses to Iso and For on $I_{\text{Ba}}$ in late-stage (18 dpc) ventricular myocytes. Summarized whole cell voltage-clamp data are shown in Table 3. In 18-dpc myocytes, 3 µM Iso caused an appreciably greater increase in $I_{\text{Ba}}$ (by 37.8 ± 33.0% n = 8) than in 9.5-dpc myocytes (by 52.4 ± 14.8%, n = 10, P < 0.05). In contrast, the increase in $I_{\text{Ba}}$ by 10 µM For at 18 dpc (by 120.0 ± 23.0%, n = 7) was comparable to that observed at 9.5 dpc (P > 0.05).

**DISCUSSION**

Initiation of heartbeat and Iso. During mouse embryogenesis, the primitive heart normally begins to contract...
irregularly at 8.5 dpc, whereas at 9 dpc of development the primitive atria and ventricles beat regularly and powerfully (10). In this study, an Iso-induced increase in the heart beating rate was observed at 9.5 dpc.

Studies on initiation of heart beating of embryos have been carried out extensively in chick embryos, but there are few reports regarding mammals. Hall (8) first reported that a stable heartbeat (140 beats/min) appeared in 10.5-dpc rat embryos and that the beating rate increased by 21% after exposure to epinephrine (27.3 µM). Similarly, Robkin et al. (19) observed an increase (by 13%) in the heart rate of 10.5-dpc rat embryos after Iso (1.6 µM) application. To our knowledge, we are the first to demonstrate that the heartbeat can be regulated by the β-adrenergic signaling system in mouse embryos at such an early stage as 9.5 dpc.

AP and Iso. Couch et al. (6) reported developmental changes in AP of ventricular muscles of prenatal rats (10.5–20.5 dpc). However, the AP recorded from these rat hearts had limited magnitudes of both resting potentials (positive to −50 mV in average) and $V_{\text{max}}$ (∼25 V/s), suggesting that the tip of a Woodbury-Brady type of floating electrode may not be able to be impaled completely into the intracellular space of such immature and downsized ventricular muscle cells. AP of single ventricular myocytes recorded through a suction pipette electrode in the present study were comparable to those of myocytes in prenatal or neonatal rat hearts in terms of MDP, APA, and $V_{\text{max}}$ (7, 11). In addition, Iso significantly reduced the $V_{\text{max}}$ of AP (see Table 2). This is consistent with an inhibition of Na$^{+}$-channel currents by Iso in adult mammalian ventricular myocytes, which has been linked to the Gs protein pathway (17, 20, 21).

The slow diastolic depolarization was enhanced by Iso suggests the possible modulatory effect of Iso on pacemaker currents. Because the I-V curve shows that L-type Ca$^{2+}$-channel currents at such negative potentials are almost inactive, other pacemaker currents may be involved in the response to Iso.

Stimulation of L-type Ca$^{2+}$-channel currents by Iso. Our main finding in this report is that Iso, a β-adrenergic agonist, enhanced $I_{\text{Ba}}$ in 9.5-dpc mouse cardiomyocytes. The effect of Iso on $I_{\text{Ba}}$ was mediated by β-adrenergic receptors because of its reversible blockade by propranolol.

It has been well documented that β-adrenergic agonists enhance L-type Ca$^{2+}$-channel currents via Gs/AC signaling pathway in mature cardiomyocytes. In accordance, For also enhanced $I_{\text{Ba}}$ in 9.5-dpc mouse cardiomyocytes, suggesting that a similar transducing mechanism may underlie the modulation of $I_{\text{Ba}}$ by β-adrenergic receptors at such an early stage.

The stimulatory effect of Iso on $I_{\text{Ba}}$ was increased from 9.5-dpc to 18-dpc cardiomyocytes, reflecting some developmental changes in β-adrenergic receptors or their transducing mechanism. Because the effect of For on $I_{\text{Ba}}$ did not show significant increase from 9.5-dpc to 18-dpc cardiomyocytes, the signaling cascade downstream from the AC may develop before β-adrenergic receptors.

Recently, several studies focused on age-related changes in β-adrenergic modulation of Ca$^{2+}$ channels. In the embryonic mouse heart, An et al. (1) reported that L-type Ca$^{2+}$-channel currents of cardiomyocytes were enhanced by Iso (3 µM) at a late stage (17–20 dpc) but unaffected at an earlier stage (11–13 dpc). Masuda et al. (14) also observed in embryonic rat hearts that Iso had little effect at 15–18 dpc but caused a substantial increase in L-type Ca$^{2+}$-channel currents at stages later than 20 dpc. The present results indicate a significant Iso-induced increase in L-type Ca$^{2+}$-channel currents at an earlier stage of development (9.5 dpc) in the embryonic mouse heart.

Different experimental protocols might have contributed to such a discrepancy. One possibility is the difference in the temperatures used in experiments. Our experiments were conducted at 33°C, but Masuda's experiments were conducted at 37°C.

### Table 3. Effects of Iso and For on $I_{\text{Ba}}$ in mouse embryonic cardiomyocytes

<table>
<thead>
<tr>
<th>Embryonic Stage</th>
<th>Control, $I_{\text{Ba}}$ (pA/pF)</th>
<th>Iso (3 µM), $I_{\text{Ba}}$ (pA/pF)</th>
<th>Increase, %</th>
<th>n</th>
<th>Control, For (10 µM), $I_{\text{Ba}}$ (pA/pF)</th>
<th>For, Increase, %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>9.5 dpc</td>
<td>$-4.7 \pm 0.9$</td>
<td>$-6.7 \pm 1.2$†</td>
<td>52.4 ± 14.8</td>
<td>10</td>
<td>$-4.7 \pm 0.7$</td>
<td>$-9.2 \pm 1.5$†</td>
<td>95.5 ± 18.8‡</td>
</tr>
<tr>
<td>18 dpc</td>
<td>$-6.2 \pm 0.5$</td>
<td>$-14.5 \pm 2.2$‡</td>
<td>137.8 ± 33.0*</td>
<td>8</td>
<td>$-6.8 \pm 1.2$</td>
<td>$-14.6 \pm 2.5$‡</td>
<td>120.0 ± 23.0</td>
</tr>
</tbody>
</table>

Values are means ± SE; n, no. of cells. For, forskolin; dpc, days postcoitum. Ca$^{2+}$-channel current measured with Ba$^{2+}$ as charge carrier ($I_{\text{Ba}}$) was elicited by a 200 ms-depolarizing pulse from −50 mV to 0 mV. *P < 0.05 vs. 9.5 dpc; †P < 0.01 vs. control; ‡P < 0.05 vs. Iso at 9.5 dpc.
et al. (14) used a lower temperature (22–25°C) and An et al. (1) did not mention the temperature used. The other possibility is the variance in the cell isolation procedures, especially those for enzymatic treatment. In any case, our findings are in agreement with the functionally effective β-adrenergic receptors being expressed in 10.5- to 11.5-dpc rat embryos (8, 19).

In adult cardiomyocytes, the response of L-type $\text{Ca}^{2+}$-channel currents to Iso is accompanied by a negative shift in the I-V relationship (15). In our data, however, Iso did not cause such a negative shift in the I-V relationship of $I_{\text{Ca}}$ at 9.5 dpc. This is consistent with previous reports by An et al. (1) in 17- and 19-dpc embryonic mouse hearts and by Masuda et al. (14) in 20-dpc embryonic rat hearts. The key regulatory subunits of L-type $\text{Ca}^{2+}$ channels expressed in embryonic hearts might be different from those expressed in adult hearts.

Our data may suggest that even in the 9.5-dpc embryonic mouse heart, both L-type $\text{Ca}^{2+}$ and β-adrenergic receptors are developed and that the activation of β-adrenergic receptors enhances L-type $\text{Ca}^{2+}$-channel currents, possibly via a cAMP-dependent pathway.

Physiological significance. In mature mammalian cardiomyocytes, excitation-contraction coupling is mediated mainly by $\text{Ca}^{2+}$ release from the sarcoplasmic reticulum (SR), which is triggered by $\text{Ca}^{2+}$ entry through sarcolemmal L-type $\text{Ca}^{2+}$ channels (2, 9, 16). However, during early embryonic stages of development, cardiac contraction is more dependent on transsarcolemmal $\text{Ca}^{2+}$ influx through Ca2 channels because of immature $\text{Ca}^{2+}$ regulatory properties of SR (3, 12). The expression of functional L-type $\text{Ca}^{2+}$ channels in 9.5-dpc mouse embryos is in agreement with the fact that the primitive mouse heart begins to beat rhythmically at 9.0 dpc (10).

Pharmacological and biochemical experiments have indicated that the embryonic heart possesses β-adrenergic receptors before the heart is innervated by the sympathetic division. Rat sympathetic innervation of the heart cannot be detected until gestation 19–20 (12). Thus cardiac β-adrenergic responses occur before sympathetic innervation, as indicated by our results and another previous paper (8). Norepinephrine was detected in 10.5-dpc fetal mouse (23), and catecholamines were reported to be essential for early fetal mouse development (11.5 dpc) (23, 26). We propose that the embryonic heart should be under the control of hormonal catecholamines even in the 9.5-dpc mouse.

The authors thank Drs. Philip T. Palace (University of Texas Medical Branch at Galveston) and Noritsugu Tohse (Sapporo Medical University) for comments on the manuscript.

This study was supported by the grants from the Japanese Ministry of Science, Education, Sports and Culture (nos. 09770480 and 09877129).


Received 22 April 1998; accepted in final form 9 October 1998.

REFERENCES


