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Subtype Switching of L-Type Ca²⁺ Channel From Caᵥ1.3 to Caᵥ1.2 in Embryonic Murine Ventricle

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Background  Embryonic hearts exhibit spontaneous electrical activity, which depends on Ca²⁺ influx through L-type Ca²⁺ channels. In this study the expression of the L-type Ca²⁺ channel α₁ subunit gene in the developing mouse heart was investigated.

Methods and Results  Mouse cardiac ventricles 9.5 days post coitum (dpc), 18 dpc and adult were used. At 9.5 dpc the level of Caᵥ1.3 mRNA was higher than that of Caᵥ1.2 mRNA. With development, Caᵥ1.2 mRNA increased and Caᵥ1.3 mRNA decreased. Analysis of Caᵥ1.3 splicing variants showed that Caᵥ1.3(1b) mRNA was expressed at a higher density than Caᵥ1.3(1a) mRNA. Caᵥ1.3 protein was detected only at 9.5 dpc, whereas Caᵥ1.2 protein was expressed from 9.5 dpc and its expression increased with development. L-type Ca²⁺ currents were prominent at 9.5 dpc. The Ca²⁺ current amplitude at 9.5 dpc was comparable to that at 18 dpc, and was larger in adults than at the embryonic stage. L-type Ca²⁺ current at 9.5 dpc was activated and/or inactivated at more negative membrane potentials than at 18 dpc or adult. L-type Ca²⁺ channels at 9.5 dpc were less sensitive to inhibition by nisoldipine than at adult.

Conclusions  The Caᵥ1.3 channel is functionally expressed in early embryonic mouse ventricular myocytes and potentially underlies ventricular automaticity. (Circ J 2005; 69: 1405 – 1411)

Key Words:  Ca²⁺ channel; Cardiac development; Gene expression
Cav1.3 (1a) and Cav1.3 (1b), containing exon 1a and exon 1b, were used for the present study. All animal procedures were approved by the Animal Care and Use Committee, Research Institute of Environmental Medicine, Nagoya University.

Animals

PCR (Institute of Cancer Research, Philadelphia, PA, USA) mice (9.5 dpc, 18 dpc and 10-week-old adult) were used for the present study. All animal procedures were approved by the Animal Care and Use Committee, Research Institute of Environmental Medicine, Nagoya University.

Analysis of mRNA Expression of L-Type Ca²⁺ Channels

Total RNA of the cardiac ventricle was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) from the 9.5 dpc mouse embryo and adult mouse. Single-stranded cDNA synthesis was performed with total RNA using oligo d(T) primer using SuperScript II reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA) after DNase treatment of total RNA.

To investigate which type of L-type Ca²⁺ channel genes (Ca.1.1, Ca.1.2, Ca.1.3 or Ca.1.4) are expressed in embryonic cardiac muscle, we performed classical PCR. For the quantitative analysis of the mRNA of L-type Ca²⁺ channel genes, we used real-time fluorogenic 5'-nuclease PCR assay (Perkin-Elmer ABI Prism 7700). We also analyzed the mRNA expression of 2 splicing variants of Ca v1.3: Cav1.3 (1a) and Cav1.3 (1b). For the quantitative analysis of the mRNA of L-type Ca²⁺ channel genes, we used real-time fluorogenic 5'-nuclease PCR assay (Perkin-Elmer ABI Prism 7700).

Western Blotting

Immunoblotting for the Ca.1.2 Ca²⁺ channel protein was performed by membrane fraction. Cardiac ventricles were homogenized in lysis A (0.25 mol/L sucrose, 0.25 mol/L KCl, 10 mmol/L imidazol (pH 7.4), 5 mmol/L MgCl₂, 10 mmol/L ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) and protease inhibitors). The homogenates were centrifuged at 100,000 g for 60 min to remove debris and nuclei. The pellets were resuspended for 60 min with lysis A containing 0.6 mol/L KCl to extract myosin. After centrifugation at 100,000 g for 60 min, the pellets (membrane protein) were resuspended in lysis B (50 mmol/L Tris-HCl (pH 7.4), 2 mmol/L EDTA, 2 mmol/L ethylene glycol bis(N,N,N',N'-tetraacetic acid (EGTA), 1% sodium dodecyl sulfate (SDS) and protease inhibitors). For Ca.1.3 Ca²⁺ channel protein, the crude homogenate was used for blotting. Cardiac ventricles were homogenized in lysis buffer (0.9% NaCl, 10 mmol/L Tris-maleate (pH 6.8), 1% SDS and protease inhibitors). The amount of protein was determined by a CS Saver and Analyzer (CCD camera, ATTO & Rise Corporation). We also analyzed the mRNA expression of Ca.1.3 (1a) and Ca.1.3 (1b), containing exon 1a and exon 1b, respectively. The specific primers and TaqMan probes were designed by BCA assay (Pierce Biotechnology, Rockford, IL, USA: 34075). The intensity of protein bands by chemiluminescence was quantified by chemiluminescence (SuperSignal West Dura Extended Duration Substrate (Pierce Biotechnology, #34075)). The intensity of protein bands by chemiluminescence was quantified by a CS Saver and Analyzer (CCD camera, ATTO & Rise Corporation).
L-Type Ca\textsuperscript{2+} Channel in Embryonic Heart

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#611463) was used as control. The blotting for Cav1.1 Ca\textsuperscript{2+} channel protein (anti-Ca\textsubscript{v}1.1 antibody: 1/500, Santa Cruz Biotechnology, Santa Cruz, CA, USA: #sc-16257) was also performed by membrane fraction and the crude homogenate. The signal for Cav1.1 Ca\textsuperscript{2+} channel protein was not detected in cardiac ventricles at any stage.

**Electrophysiological Experiments**

Cultured single ventricular myocytes were prepared from ventricles of 9.5 dpc and 18 dpc mouse embryonic hearts by the same method previously described. Briefly, cardiac ventricles were dissected from the exposed embryos and single myocytes were isolated by collagenase treatment. The ventricular myocytes were then cultured on collagen-coated glass coverslips in minimum essential medium including 10% fetal bovine serum and 100\mu g/ml gentamycin for 18–24 h before recording the current. Fresh single adult ventricular myocytes were used for patch clamp experiments. Adult myocytes were isolated by collagenase treatment with Langendorff perfusion.

Whole-cell voltage clamp recording was performed using Axopatch 200B (Axon Instruments, USA). To isolate the L-type Ca\textsuperscript{2+} channel currents, myocytes were superfused with a Na\textsuperscript{+}-free and K\textsuperscript{+}-free external solution containing (mmol/L) TEA-Cl 140, MgCl\textsubscript{2} 1, HEPES 5 (pH 7.4), glucose 10, CaCl\textsubscript{2} 5 and 30\mu mol/L tetrodotoxin. Internal solution contained (mmol/L) CsOH 60, CsCl 80, l-aspartate 40, HEPES 5 (pH 7.2), MgATP 5, Na\textsubscript{2}-phosphocreatinine 5, EGTA 10, CaCl\textsubscript{2} 0.65 (pCa 7.96). Cell capacitance was measured by the application of a ramp voltage pulse of 0.5 V/s at a potential ranging between –50 mV and +70 mV. For inactivation-curve assessment, conductance (g) was obtained by dividing peak Ca\textsuperscript{2+} channel current at the test potential by the difference between test and reverse potential. Inactivation and activation curves were fitted by the Boltzmann equation: $I/Imax = 1/(1+exp[(Vm –V1/2)/k])$ and $g/gmax =1/[1+exp[(V1/2 –Vm)/k]]$, where $V_m$ is the membrane voltage, $V_{1/2}$ is the voltage at half-maximal inactivation or activation, and $k$ is the slope factor. All electrophysiological experiments were carried out at 35–37°C.

**Statistics**

Data are presented as means±SE. Statistical analysis was performed using paired and non-paired Student’s t-test (patch clamp data), or ANOVA (mRNA and protein data). Differences were considered significant at $p<0.05$.

**Results**

**mRNA Expression of L-Type Ca\textsuperscript{2+} Channel Genes During Development**

Classical PCR revealed the expression of Cav1.1, Cav1.2 and Cav1.3 mRNA at 9.5 dpc, but Cav1.4 mRNA expression was not detected at any stage (9.5 dpc, 18 dpc or adult) (data not shown). Next, we analyzed quantitatively the mRNA expression of Cav1.1, Cav1.2 and Cav1.3 using real-time PCR. Fig 1A shows the level of Cav1.1, Cav1.2 and Cav1.3 mRNA expression at 9.5 dpc, 18 dpc and adult stages. Interestingly, at 9.5 dpc the level of Cav1.3 mRNA was higher than that of Cav1.2 mRNA (474±59 vs 295±113 molecules/10\textsuperscript{5} GAPDH mRNA molecules, n=4, p<0.05). The level of Cav1.1 mRNA was very low (72±31 molecules/10\textsuperscript{5} GAPDH mRNA molecules, n=4). With development, Cav1.2 mRNA increased (429±18 molecules/10\textsuperscript{5} GAPDH mRNA molecules at 18 dpc, n=4; 1104±154 molecules/10\textsuperscript{5} GAPDH mRNA molecules at adult, n=4), Cav1.3 mRNA decreased (31±6 molecules/10\textsuperscript{5} GAPDH mRNA molecules at 18 dpc,
Fig 2. Western blotting of the L-type Ca\(^{2+}\) channel protein in mouse cardiac ventricles at 9.5 days post coitum (dpc), 18 dpc and adult. (A) Cav1.2 protein expression. Upper panel shows Western blotting of Cav1.2 protein, which was detected at all stages. Cav1.2 protein expression was strongest in the adult. Immunoblot densities were normalized to positive control (rat cerebrum lysate of 10\(\mu\)g). Quantified data are summarized in the lower panel. (B) Western blotting of Cav1.3 protein, which was detected at 9.5 dpc, but not at either 18 dpc or adult. The difference was significant at *p<0.05 and **p<0.01.

Fig 3. Developmental change in the L-type Ca\(^{2+}\) channel current in mouse ventricular myocytes at 9.5 days post coitum (dpc), 18 dpc and adult. (A) L-type Ca\(^{2+}\) channel currents were elicited by depolarization steps with 10 mV increment from a holding potential of –50 mV. L-type Ca\(^{2+}\) channel current at 9.5 dpc was clearly recognized by depolarization to –30 mV. (B) Averaged current–voltage relationships of the Ca\(^{2+}\) current at 9.5 dpc (n=9), at 18 dpc (n=6) and at adult (n=8). (C) Steady-state activation curves for the L-type Ca\(^{2+}\) channel current. The threshold of activation of the Ca\(^{2+}\) current at 9.5 dpc (n=9) was more negative potential as compared with those at 18 dpc (n=6) and adult (n=8). D. Inactivation curves were obtained by depolarization pulse to +10 mV from conditioning pulses of 1 s. The L-type Ca\(^{2+}\) channel current at 9.5 dpc (n=5) was inactivated at more negative potentials than those at 18 dpc (n=5) and adult (n=6). Values are presented as means ± SE.
n=4; 11±2 molecules/10^5 GAPDH mRNA molecules at adult, n=4) and Cav1.1 mRNA became undetectable. Thus, in the early embryonic stage, Cav1.3 is the predominant type of L-type Ca^{2+} channel, but during the second half of embryonic life, Cav1.2 becomes the main type.

Because 2 splicing variants of Cav1.3 have been reported in the embryonic heart (Cav1.3(1a) corresponding to exon 1a and Cav1.3(1b) corresponding to exon 1b), we also studied the mRNA expression of these splicing variants by quantitative PCR. Fig 1B shows that Cav1.3(1b) mRNA expression is larger than Cav1.3(1a) mRNA expression in cardiac ventricles at 9.5 dpc (272±90 vs 115±44 molecules/10^5 GAPDH mRNA molecules, n=4, p<0.05). With development, the mRNA expression of both splicing variants decreased in association with the reduction in the total mRNA of Cav1.3 (see also Fig 1A).

Western Blotting of L-Type Ca^{2+} Channels

Fig 2 shows the protein level of L-type Ca^{2+} channels during development. Cav1.2 protein expression increases with development (0.63±0.13 at 9.5 dpc, 1.02±0.17 at 18 dpc, 2.82±0.70 at adult, n=6, normalized to control peptide). Cav1.3 protein could only be detected at 9.5 dpc, but not at 18 dpc or adult. Cav1.1 protein was below the level of detection at any stage (data not shown).

Characteristics of the L-Type Ca^{2+} Channel Current During Development

We applied depolarization pulses for 200 ms to various potentials from the holding potential of –50 mV to elicit L-type Ca^{2+} channel currents. The holding potential of –50 mV inactivated almost completely the T-type Ca^{2+} channel (data not shown), which has been reported to be present in embryonic mouse cardiac ventricle.

Fig 3A shows representative membrane currents in response to depolarizing pulses ranging from –40 mV to 0 mV in ventricular myocytes at 9.5 dpc, 18 dpc and adult. Depolarization to –30 mV elicits an inward Ca^{2+} current at 9.5 dpc, minimum current at 18 dpc, and no inward current at adult. In contrast, a depolarizing pulse to 0 mV induces a larger inward Ca^{2+} current at adult than at 9.5 or 18 dpc. Fig 3B summarizes the current–voltage relationships (I–V curves) of Ca^{2+} current obtained from ventricular myocytes at 9.5 dpc, 18 dpc and adult. The amplitude of the Ca^{2+} current by depolarization to 10 mV was 6.6±0.6 pA/pF (n=9) at 9.5 dpc, 5.7±5.6 pA/pF (n=6) at 18 dpc and 13.1±2.2 pA/pF (n=8) at adult. Fig 3C shows the activation curves of Ca^{2+} the current. In agreement with Fig 3A, the threshold membrane potential for activation of the Ca^{2+} current at 9.5 dpc was more negative than those at 18 dpc and at adult. The potential of half-maximal activation (V_1/2) was –14.6±2.4 mV (n=9) at 9.5 dpc, –3.7±1.1 mV (n=6) at 18 dpc, and –4.8±2.4 mV (n=8) at adult. V_1/2 at 9.5 dpc was significantly more negative than at 18 dpc and at adult. The slope factor (k) was 10.5±1.8 at 9.5 dpc, 7.6±0.5 at 18 dpc, and 5.6±0.3 at adult. We also measured the inactivation curves of the Ca^{2+} current from ventricular myocytes at 9.5 dpc, 18 dpc and adult (Fig 3D). The potential of half-maximal inactivation was also shifted to a more negative membrane potential at 9.5 dpc (V_1/2=–29.7±1.4 mV, n=5) than at 18 dpc (–29.3±1.2 mV, n=5) and at adult (–31.0±1.8 mV, n=6). Thus, Ca^{2+} channels at 9.5 dpc were inactivated at significantly more negative potentials than those at 18 dpc and

| Table 2 Kinetics of the L-Type Ca^{2+} Current During Murine Development |
|-----------------------------|-----------------------------|-----------------------------|
|                             | 9.5 dpc (n=12)              | 18 dpc (n=5)                | Adult (n=9)                     |
| Activation (ms)             | 1.08±0.17                   | 0.98±0.34                   | 1.14±0.16                      |
| Inactivation (ms)           | 79.0±20.3                   | 45.2±5.63                   | 51.8±16.8                      |

Kinetics of Ca^{2+} current at the potential of the peak current amplitude were analyzed. Activation or inactivation process of current was fitted by a single or a double exponential function, respectively. Values are expressed as mean±SE.
adult. The slope factor (k) was 9.2±0.9 at 9.5 dpc, 10.7±1.2 at 18 dpc, and 8.9±0.4 at adult.

Table 2 summarizes the time constants of activation and inactivation of the Ca2+ current at the potential of peak current amplitude. Although there were no statistical differences between these values among the 3 developmental stages, the time constant of inactivation at 9.5 dpc tended to be longer than those at 18 dpc and at adult.

We studied the nisoldipine sensitivity of the L-type Ca2+ current of ventricular myocytes at both 9.5 dpc and at adult (Fig 4). We used 2 different holding potentials (−50 mV and −100 mV) for current recording. When the holding potential was set to −50 mV, 1 μmol/L nisoldipine inhibited the L-type Ca2+ currents almost completely at 9.5 dpc and at adult. The Ca2+ current blockade at peak potential (0 mV for 9.5 dpc and +10 mV for adult) was 88.8±1.1% (n=5) and by 95.9±1.0% (n=7). The shift of the holding potential to −100 mV attenuated the blockade by 1 μmol/L nisoldipine. The blockade of Ca2+ current was 55.4±5.6% (n=5) at 9.5 dpc (200 μmol/L Ni2+ was added to the external solution to eliminate the T-type Ca2+ current) and 73.0±4.6% (n=5) at adult. L-type Ca2+ channels in ventricular myocytes at 9.5 dpc may therefore be slightly less sensitive to nisoldipine than those of adults.

Discussion

We investigated the L-type Ca2+ channel in mouse ventricular myocytes during development from 9.5 dpc to adulthood and substantial L-type Ca2+ channel currents were recorded in the early embryonic stage. The total amplitude of the Ca2+ current at 9.5 dpc was comparable to that at 18 dpc, but the Ca2+ current at adult was larger than at the embryonic stage. The L-type Ca2+ current at 9.5 dpc was activated and/or inactivated at more negative membrane potentials than at 18 dpc and at adult. The L-type Ca2+ channels at 9.5 dpc were slightly less sensitive to inhibition by nisoldipine than at adult. Quantitative PCR and Western blotting showed expression of both Ca1.2 and (larger) Ca1.3 at 9.5 dpc. A loss of Ca1.3 expression and an increase of Ca1.2 were observed with development. The analysis of 2 splicing variants of Ca1.3 revealed higher expression of Ca1.3(1b) than of Ca1.3(1a) at 9.5 dpc. Ca1.1 mRNA was expressed only at 9.5 dpc.

Molecular Basis of L-Type Ca2+ Channel in Cardiac Ventricle at an Early Embryonic Stage

In our electrophysiological study, the L-type Ca2+ channels at 9.5 dpc were activated and/or inactivated at more negative potentials than at 18 dpc and at adult, which indicates different phenotypes of the L-type Ca2+ channel in ventricular myocytes at 9.5 dpc, 18 dpc and at adult. In a study of heterologous expression of Ca1.3, it was reported that the Ca1.3 Ca2+ channel current activates at more negative potentials (by 14.2 mV) than the Ca1.2 current.12 In the present study quantitative PCR revealed that Ca1.1, Ca1.2 and Ca1.3 mRNA was expressed at 9.5 dpc, and that Ca1.3 was the predominant type. Ca1.2 and Ca1.3 proteins were also detected at 9.5 dpc by Western blotting. These findings indicate that the Ca1.3 subtype, in association with Ca1.2, does contribute to the L-type Ca2+ channel current at the early embryonic stage.

The presence of 2 splicing variants of Ca1.3 at the early embryonic stage has been reported previously13,15 and our study confirms the higher expression of Ca1.3(1b) mRNA than of Ca1.3(1a) at 9.5 dpc. Xu et al reported that Ca1.3(1b) Ca2+ channels are less sensitive to nisoldipine than Ca1.3(1a) Ca2+ channels and that Ca1.3(1b) Ca2+ channels might be responsible for the L-type Ca2+ channel current in Ca1.2 knockout cardiac myocytes.13 The slightly lower sensitivity to nisoldipine of the L-type Ca2+ currents in the 9.5 dpc myocytes in our experiments may therefore result from abundant functional expression of Ca1.3(1b) Ca2+ channels in early embryonic cardiac ventricular myocytes.

Physiological Meaning

Platzer et al reported that Ca1.3 Ca2+ channels may underlie cardiac pacing in the SA node, because SA node dysfunction occurs in Ca1.3 knockout mice. At the early-embryonic stage, the cardiac ventricles beat spontaneously, as SA node cells do. In the present study we show that Ca1.3 Ca2+ channels are functionally expressed in cardiac ventricular myocyte at 9.5 dpc in the normal, wild-type embryo. Ca1.3 Ca2+ channels activate at a more negative membrane potential than Ca1.2 Ca2+ channels and potentially contribute to the automatocity of the embryonic heart. Under pathological conditions, such as cardiac hypertrophy and failure, recapitulation of fetal gene programs may underlie ionic remodeling. In previous work16 we showed that the pacemaker channel gene (HCN4; SA nodal type) encoding the hyperpolarization-activated inward current (If) is expressed in murine embryonic ventricle at the 9.5 dpc stage and that it produces ventricular automaticity in these immature embryos. Its expression declines just before birth, which is compatible to what occurred with the expression of Ca1.3 in the present study. Increase of the If current in the failing human heart has been described previously14 and it has recently been reported that both the SA nodal type of gene (HCN4) and the ventricular type of gene (HCN2) are indeed upregulated in hypertrophied rat ventricular muscle.17 A comparable change in (SA nodal) Ca1.3 expression might play a role in the increased excitability of the diseased heart. As to the expression of Ca1.3 Ca2+ channels in diseased hearts, however, no experimental or clinical evidence has been presented to date, and its implication in the pathogenesis of arrhythmias remains to be studied.

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References


