Subtype Switching of L-Type Ca^{2+} Channel From $\text{Ca}_v1.3$ to $\text{Ca}_v1.2$ in Embryonic Murine Ventricle

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Background Embryonic hearts exhibit spontaneous electrical activity, which depends on Ca^{2+} influx through L-type Ca^{2+} channels. In this study the expression of the L-type Ca^{2+} channel α_1 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 $\text{Ca}_v1.3$ mRNA was higher than that of $\text{Ca}_v1.2$ mRNA. With development, $\text{Ca}_v1.2$ mRNA increased and $\text{Ca}_v1.3$ mRNA decreased. Analysis of $\text{Ca}_v1.3$ splicing variants showed that $\text{Ca}_v1.3(1b)$ mRNA was expressed at a higher density than $\text{Ca}_v1.3(1a)$ mRNA. $\text{Ca}_v1.3$ protein was detected only at 9.5 dpc, whereas $\text{Ca}_v1.2$ protein was expressed from 9.5 dpc and its expression increased with development. L-type Ca^{2+} currents were prominent at 9.5 dpc. The Ca^{2+} 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 Ca^{2+} current at 9.5 dpc was activated and/or inactivated at more negative membrane potentials than at 18 dpc or adult. L-type Ca^{2+} channels at 9.5 dpc were less sensitive to inhibition by nisoldipine than at adult.

Conclusions The $\text{Ca}_v1.3$ channel is functionally expressed in early embryonic mouse ventricular myocytes and potentially underlies ventricular automaticity. (Circ J 2005; 69: 1405–1411)

Key Words: Ca^{2+} channel; Cardiac development; Gene expression

L-type Ca^{2+} channels are pivotal for cardiac excitation and contraction¹ and they are coded by 4 distinct genes: $\text{Ca}_v1.1$ (α_1S , skeletal muscle), $\text{Ca}_v1.2$ (α_1C , cardiac and smooth muscle), $\text{Ca}_v1.3$ (α_1D , neuron, neurosecretory cells and heart) and $\text{Ca}_v1.4$ (α_1F , retina)^{2–4}. The $\text{Ca}_v1.2$ Ca^{2+} channel is predominant in cardiac muscle^{5,6}. Ca^{2+} entry into the cytosol through this channel triggers Ca^{2+} release from the sarcoplasmic reticulum and underlies the Ca^{2+} transient for contraction¹. The $\text{Ca}_v1.3$ Ca^{2+} channel activates at more negative membrane potentials than the $\text{Ca}_v1.2$ channel⁷ and contributes to diastolic depolarization of sinoatrial (SA) nodal cells. This role of $\text{Ca}_v1.3$ Ca^{2+} channels in SA nodal automaticity is further substantiated by the observation that $\text{Ca}_v1.3$ ablation leads to SA node dysfunction^{8–10}.

The murine embryonic heart starts to contract at 8.5 days post coitum (dpc) and the heart beat becomes regular at 9.5 dpc, when the heart shape is still tube-like¹¹. Ventricles isolated from embryonic hearts at 9.5 dpc also beat spontaneously and regularly. Cardiac excitation and contraction in this early embryonic stage depend on Ca^{2+} influx through

L-type Ca^{2+} channels¹². The $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ genes, encoding the α_1C and α_1D subunits of L-type Ca^{2+} channels, respectively, have been reported to be expressed at the embryonic stage^{13–15}. Two splicing variants of $\text{Ca}_v1.3$, $\text{Ca}_v1.3(1a)$ and $\text{Ca}_v1.3(1b)$, correspond to exon 1a and exon 1b, respectively¹³. $\text{Ca}_v1.2$ knockout mice die before 14.5 dpc and in these mice the expression of the $\text{Ca}_v1.3$ channel, especially $\text{Ca}_v1.3(1b)$, is upregulated and functions as the main L-type Ca^{2+} channel¹⁵. The expression of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ and their functional status, including their development, has thus far not been studied in detail in the normal, wild-type murine embryo.

In this study, therefore, we investigated the developmental changes of L-type Ca^{2+} channels in mouse cardiac ventricles (at 9.5 dpc, 18 dpc and adult) using whole-cell patch clamp, Western blotting and real-time polymerase chain reaction (PCR).

Our study reveals that the $\text{Ca}_v1.3$ Ca^{2+} channel is expressed functionally at 9.5 dpc. The fact that its expression is higher than that of $\text{Ca}_v1.2$ (the adult type) at 9.5 dpc during normal embryonic development constitutes a novel finding. Furthermore, $\text{Ca}_v1.3(1b)$ mRNA expression was higher than that of $\text{Ca}_v1.3(1a)$. The $\text{Ca}_v1.3$ Ca^{2+} channel downregulates during the second half of embryonic development and the $\text{Ca}_v1.2$ Ca^{2+} has already become the dominant type of L-type Ca^{2+} channels before birth. A comparable scenario with a sinus nodal type pacemaker current (If) encoded by the HCN4 in ventricular myocytes (expression at the early embryonic stage and disappearance before birth) has been described by us previously¹⁶.

(Received July 4, 2005; revised manuscript received August 3, 2005; accepted August 11, 2005)

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Table 1 Sequence of PCR Primers and Sequence Specific Probes

Target sequence	Accession no.*	Primer	Sequence (5' 3')	Position	Amplicon length (bp)
Cav1.2	L01776	Sense	caacctggaacgactggagtatct	1637–1660	157
		Probe	tacggacttctctccaccaacgcttac	1707–1736	
		Antisense	cgtggtgttagggcttttagt	1772–1793	
Cav1.3	AB086123	Sense	cccdaaaaccataccagtataa	3646–3668	100
		Probe	tacgtggtgaactctcgccttcgaat	3676–3703	
		Antisense	tcaacacgctctgcctgg	3728–3745	
Cav1.3 (1a)	AJ437291	Sense	atcaacggcagcaccaaga	35–53	117
		Probe	caccagactccctatttctgggaaggacc	87–116	
		Antisense	aacagctccaagcaaaactgtcc	130–151	
Cav1.3 (1b)	AJ437292	Sense	ggcagagttgttaagtgcgtaga	134–156	139
		Probe	caccagactccctatttctgggaaggacc	208–237	
		Antisense	aacagctccaagcaaaactgtcc	251–272	
Cav1.1	L06234	Sense	gagcttgatgaatcgaagcct	1304–1326	101
		Probe	catccagttcattcgccactggcg	1377–1400	
		Antisense	tggaagtgcacacactagta	1383–1404	
Cav1.4	NM019582	Sense	ttccatcatgaagcgctt	732–750	101
		Antisense	actcgagctattctcggac	813–832	
GAPDH	M32599	Sense	cttcaccacatggagaaggc	343–363	238
		Probe	cctggccaaggctcatgcatacaacttt	517–544	
		Antisense	ctcatgaccacagtcctgcc	560–580	

*In GenBank release (October 2003). PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Methods

Animals

ICR (Institute of Cancer Research, Philadelphia, PA, USA) mice (9.5dpc, 18dpc 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 Ca^{2+} Channels

Total RNA of the cardiac ventricle was extracted with the RNeasy Mini Kit (Qiagen, Hilden, Germany) from the 9.5dpc mouse embryo and with the AGPC (Acid Guanidinium Thiocyanate-Phenol-Chloroform) method from the 18dpc mouse embryo and adult mouse.¹⁶ 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 Ca^{2+} channel genes (Cav1.1, Cav1.2, Cav1.3 or Cav1.4) are expressed in embryonic cardiac muscle, we performed classical PCR. For the quantitative analysis of the mRNA of L-type Ca^{2+} channel genes, we used real-time fluorogenic 5'-nuclease PCR assay (Perkin-Elmer ABI Prism 7700).^{16,20} We also analyzed the mRNA expression of 2 splicing variants of Cav1.3: Cav1.3(1a) and Cav1.3(1b), containing exon 1a and exon 1b, respectively.¹³ The specific primers and TaqMan probes we designed are shown in Table 1. A cDNA sample (150 ng) was added to each PCR tube. The threshold cycle (Ct) from the baseline to reach a statistically significant increase in fluorescence signal was measured. The Ct value predicts the quantity of target cDNA in the sample. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as an endogenous control. PCR products were subcloned using TA cloning (pGEMR-T Easy, Promega, Madison, WI, USA) and were verified by sequencing. cDNA standards were obtained by digesting plasmid by EcoRI. Five different molecules of cDNA standards for target genes (1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3) were amplified to determine

the standard curves between Ct and the log starting molecule number of cDNA standards.

Western Blotting

Immunoblotting for the Cav1.2 Ca^{2+} 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 imidazole (pH 7.4), 5 mmol/L $MgCl_2$, 10 mmol/L ethylenediamine-N,N,N',N'-tetraacetic acid (EDTA) and protease inhibitors). The homogenates were centrifuged at 5,000 G for 10 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 Cav1.3 Ca^{2+} 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 BCA assay (Pierce Biotechnology, Rockford, IL, USA). Protein samples (20 μ g for Cav1.2, 50 μ g for Cav1.3) were loaded on 7% polyacrylamide-SDS gels and transferred to phosphate buffered saline membranes, which were blocked with 2.5% (for Cav1.2) or 0.3% (for Cav1.3) non-fat milk in phosphate-buffered saline and incubated overnight at 4°C with a rabbit polyclonal antibody solution (anti-Cav1.2 antibody: 1/500, BD Bioscience, #550716; anti-Cav1.3 antibody: 1/500, BD Bioscience, #550712). The immunoblots were developed with horseradish peroxidase-labeled goat anti-rabbit IgG antibody (1/15,000, Sigma, St Louis, MO, USA: A0545) for 1 h, followed by enhanced 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).¹⁷ For analyzing Cav1.2 protein expression, rat cerebrum lysate (10 μ g, BD Transduction Laboratories,

Fig 1. Quantification of mRNA expression of L-type Ca^{2+} channel in mouse cardiac ventricles at 9.5 days post coitum (dpc) (white bar), 18dpc (hatched bar) and adult (black bar). (A) Expression of mRNA for Cav1.1, Cav1.2 and Cav1.3. Expression of Cav1.2 mRNA increased with development. Cav1.3 mRNA was expressed abundantly at 9.5 dpc. Cav1.1 mRNA was detected slightly only at 9.5 dpc. (B) mRNA expression of 2 splicing variants of Cav1.3: (Cav1.3 (1a) and Cav1.3 (1b)). Large amounts of Cav1.3 (1a) and Cav1.3 (1b) mRNA were detected at 9.5 dpc. Cav1.3 (1b) mRNA expression was larger than that of Cav1.3 (1a) at 9.5 dpc ($p < 0.05$). mRNA expression was quantified by real-time polymerase chain reaction. Levels of expression of the Cav genes were normalized to 10^5 molecules of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. Values are presented as means $\pm$ SE. N.D., below the level of detection. The difference was significant at * $p < 0.05$ and ** $p < 0.01$.

#611463) was used as control. The blotting for Cav1.1 Ca^{2+} channel protein (anti-Cav1.1 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^{2+} channel protein was not detected in cardiac ventricles at any stage.

Electrophysiological Experiments

Cultured single ventricular myocytes were prepared from ventricles of 9.5dpc and 18dpc mouse embryonic hearts by the same method previously described.¹⁸ 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 10 $\mu\text{g}/\text{ml}$ 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.¹⁹

Whole-cell voltage clamp recording was performed using Axopatch 200B (Axon Instruments, USA). To isolate the L-type Ca^{2+} channel currents, myocytes were superfused with a Na^+ -free and K^+ -free external solution containing (mmol/L) TEA-Cl 140, MgCl_2 1, HEPES 5 (pH 7.4), glucose 10, CaCl_2 5 and 30 $\mu\text{mol}/\text{L}$ tetrodotoxin. Internal solution contained (mmol/L) CsOH 60, CsCl 80, l-aspartate 40, HEPES 5 (pH 7.2), MgATP 5, Na₂-phosphocreatinine 5, EGTA 10, CaCl_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, 200 $\mu\text{mol}/\text{L}$ NiCl_2 was added to external solution to inhibit the T-type Ca^{2+} channel.^{20–22} Test pulses to $+10$ mV were preceded by a conditioning pulse ranging from -100 mV to 10 mV for 1 s from the holding potential of -50 mV. For activation-curve

assessment, conductance (g) was obtained by dividing peak Ca^{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: $I/I_{\text{max}} = 1/\{1 + \exp[(V_m - V_{1/2})/k]\}$ and $g/g_{\text{max}} = 1/\{1 + \exp[(V_{1/2} - V_m)/k]\}$, 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 $\pm$ 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^{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/ 10^5 GAPDH mRNA molecules, $n=4$, $p < 0.05$). The level of Cav1.1 mRNA was very low (72 ± 31 molecules/ 10^5 GAPDH mRNA molecules, $n=4$). With development, Cav1.2 mRNA increased (429 ± 118 molecules/ 10^5 GAPDH mRNA molecules at 18 dpc, $n=4$; 1104 ± 154 molecules/ 10^5 GAPDH mRNA molecules at adult, $n=4$), Cav1.3 mRNA decreased (31 ± 6 molecules/ 10^5 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 μg). 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 a 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 $\pm$ 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),¹³ 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, 1.00 ± 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),^{23,24} which has been reported to be present in embryonic mouse cardiac ventricle.^{21,25}

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

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)			
act	1.08 ± 0.17	0.98 ± 0.34	1.14 ± 0.16
Inactivation (ms)			
slow	79.0 ± 20.3	45.2 ± 5.63	51.8 ± 16.8
fast	7.21 ± 0.73	6.53 ± 0.41	6.92 ± 1.03

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 $\pm$ SE.

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 ± 0.6 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 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 ($V_{1/2} = -40.1 \pm 2.1$ mV, $n=5$) than at 18 dpc (-29.7 ± 1.4 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

Fig 4. Nisoldipine sensitivity of the L-type Ca^{2+} channel current in mouse ventricular myocytes at 9.5 days post coitum (dpc) and adult. To test voltage-dependency of nisoldipine, 2 holding potentials of -50 mV (A) and -100 mV (B) were used. Peak L-type Ca^{2+} channel currents were recorded at 0 mV and $+10$ mV, at 9.5 dpc and adult, respectively. When the holding potential was set to -100 mV at 9.5 dpc, Ni^{2+} ($200 \mu\text{mol/L}$) was added to eliminate the T-type Ca^{2+} channel current. Open and closed circles indicate control and 5 min after application of $1 \mu\text{mol/L}$ nisoldipine, respectively. HP, holding potential.

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 Ca^{2+} 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 Ca^{2+} 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, $1 \mu\text{mol/L}$ nisoldipine inhibited the L-type Ca^{2+} currents almost completely at 9.5 dpc and at adult. The Ca^{2+} current blockade at peak potential (0 mV for 9.5 dpc and $+10$ mV for adult) was $88.8 \pm 1.1\%$ ($n=5$) and by $95.9 \pm 1.0\%$ ($n=7$). The shift of the holding potential to -100 mV attenuated the blockade by $1 \mu\text{mol/L}$ nisoldipine. The blockade of Ca^{2+} current was $55.4 \pm 5.6\%$ ($n=5$) at 9.5 dpc ($200 \mu\text{mol/L}$ Ni^{2+} was added to the external solution to eliminate the T-type Ca^{2+} current) and $73.0 \pm 4.6\%$ ($n=5$) at adult. L-type Ca^{2+} 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 Ca^{2+} channel in mouse ventricular myocytes during development from 9.5 dpc to adulthood and substantial L-type Ca^{2+} channel currents were recorded in the early embryonic stage. The total amplitude of the Ca^{2+} current at 9.5 dpc was comparable to that at 18 dpc, but the Ca^{2+} current at adult was larger than at the embryonic stage. The L-type Ca^{2+} 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 Ca^{2+} 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 $\text{Cav}1.2$ and (larger) $\text{Cav}1.3$ at 9.5 dpc. A loss of $\text{Cav}1.3$ expression and an increase of $\text{Cav}1.2$ were observed with development. The analysis of 2 splicing variants of $\text{Cav}1.3$ revealed higher expression of $\text{Cav}1.3(1b)$ than of $\text{Cav}1.3(1a)$ at 9.5 dpc. $\text{Cav}1.1$ mRNA was expressed only at 9.5 dpc.

Molecular Basis of L-Type Ca^{2+} Channel in Cardiac Ventricle at an Early Embryonic Stage

In our electrophysiological study, the L-type Ca^{2+} channels at 9.5 dpc were activated and/or inactivated at more negative potentials than those at 18 dpc and at adult, which indicates different phenotypes of the L-type Ca^{2+} channel in ventricular myocytes at 9.5 dpc, 18 dpc and at adult. In a study of heterologous expression of $\text{Cav}1.3$, it was reported that the $\text{Cav}1.3$ Ca^{2+} channel current activates at more negative potentials (by 14.2 mV) than the $\text{Cav}1.2$ current.⁷ In the present study quantitative PCR revealed that $\text{Cav}1.1$, $\text{Cav}1.2$ and $\text{Cav}1.3$ mRNA was expressed at 9.5 dpc, and that $\text{Cav}1.3$ was the predominant type. $\text{Cav}1.2$ and $\text{Cav}1.3$ proteins were also detected at 9.5 dpc by Western blotting. These findings indicate that the $\text{Cav}1.3$ subtype, in association with $\text{Cav}1.2$, does contribute to the L-type Ca^{2+} channel current at the early embryonic stage.

The presence of 2 splicing variants of $\text{Cav}1.3$ at the early embryonic stage has been reported previously^{13,15} and our study confirms the higher expression of $\text{Cav}1.3(1b)$ mRNA

than of $\text{Cav}1.3(1a)$ at 9.5 dpc.⁴ Xu et al reported that $\text{Cav}1.3(1b)$ Ca^{2+} channels are less sensitive to nisoldipine than $\text{Cav}1.3(1a)$ Ca^{2+} channels and that $\text{Cav}1.3(1b)$ Ca^{2+} channels might be responsible for the L-type Ca^{2+} channel current in $\text{Cav}1.2$ knockout cardiac myocytes.¹⁵ The slightly lower sensitivity to nisoldipine of the L-type Ca^{2+} currents in the 9.5 dpc myocytes in our experiments may therefore result from abundant functional expression of $\text{Cav}1.3(1b)$ Ca^{2+} channels in early embryonic cardiac ventricular myocytes.

Physiological Meaning

Platzer et al reported that $\text{Cav}1.3$ Ca^{2+} channels may underlie cardiac pacing in the SA node, because SA node dysfunction occurs in $\text{Cav}1.3$ knockout mice.⁹ At the early-embryonic stage, the cardiac ventricles beat spontaneously, as SA node cells do. In the present study we show that $\text{Cav}1.3$ Ca^{2+} channels are functionally expressed in cardiac ventricular myocyte at 9.5 dpc in the normal, wild-type embryo. $\text{Cav}1.3$ Ca^{2+} channels activate at a more negative membrane potential than $\text{Cav}1.2$ Ca^{2+} channels and potentially contribute to the automaticity 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 (I_f) 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 $\text{Cav}1.3$ in the present study. Increase of the I_f current in the failing human heart has been described previously²⁶ 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.²⁷ A comparable change in (SA nodal) $\text{Cav}1.3$ expression might play a role in the increased excitability of the diseased heart. As to the expression of $\text{Cav}1.3$ Ca^{2+} 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.

Acknowledgments

This study was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports, and Culture, Japan. We thank Mrs Mayumi Hojo for technical assistance.

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