Subtype Switching of T-Type Ca\(^{2+}\) Channels From Cav3.2 to Cav3.1 During Differentiation of Embryonic Stem Cells to Cardiac Cell Lineage

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Background  The developmental changes of Ni\(^{2+}\)-sensitivity to automaticity of Nkx2.5-positive cells derived from mouse embryonic stem cell have been identified, suggesting developmental regulation of expressing Ni\(^{2+}\)-sensitive T-type Ca\(^{2+}\) channel, although the mechanism of the change has not been fully studied.

Methods and Results  Transcripts of Cav3.2, Cav3.1 and Cav1.2 genes of beating Nkx2.5-positive cells, which encode the Ni\(^{2+}\)-sensitive T-type Ca\(^{2+}\) channel, Ni\(^{2+}\)-insensitive T-type Ca\(^{2+}\) channel, and L-type Ca\(^{2+}\) channel, respectively, were investigated by real-time reverse-transcriptase-polymerase chain reaction, and the current density of each channel was measured by patch-clamp techniques at the early and late stages of differentiation. The expression of the Cav3.2 transcript predominated in the early stage whereas those of Cav3.1 and Cav1.2 transcripts were upregulated in the late stage, which was consistent with the change in each current density, suggesting the expression of channel proteins is largely determined at the transcriptional level.

Conclusion  The results indicate that the mechanism of change of Ni\(^{2+}\)-sensitivity is partly, if not completely, the subtype switch of T-type Ca\(^{2+}\) channel from Cav3.2 to Cav3.1 at the transcriptional level, and that the expression of the L-type Ca\(^{2+}\) channel might have an attenuating effect on Ni\(^{2+}\)-sensitivity to automaticity in the late stage of differentiation. (Circ J 2005; 69: 1284–1289)

Key Words: Cardiac cell lineage; Differentiation; Nkx2.5; Subtype switch; T-type Ca\(^{2+}\) channels

The pacemaker cells of the sinoatrial node\(^{1,2}\) and conduction-system cells of Purkinje fibers\(^3\) are enriched in cardiac T-type Ca\(^{2+}\) channels, and it is this localization and their activation in a voltage range consistent with a pacemaker activity\(^4\) that suggest their role in cardiac pacemaker activity. Expression of these Ca\(^{2+}\) channels is developmentally regulated, as indicated by their higher density in embryonic and neonatal myocytes than in mature myocytes\(^5\).

T-type Ca\(^{2+}\) channels are classified into 2 subtypes, Ni\(^{2+}\)-insensitive (\(\Omega\)IG) and -sensitive (\(\Omega\)IH) channels, encoded by the Cav3.1 and Cav3.2 genes, respectively.\(^6,7\) It has been reported that in the mid-gestational fetal human myocardium only Cav3.1 is responsible for the functional T-type Ca\(^{2+}\) channels\(^8\) whereas another report indicated that in rat hearts from the middle-to-late embryonic and perinatal periods both Cav3.1 and Cav3.2 participated in T-type Ca\(^{2+}\) channel development.\(^9\) Both Niwa et al\(^9\) and Xu et al\(^10\) have shown that at the transcriptional level, there is a subtype switching from Cav3.2 to 3.1 in the perinatal period of the mouse, which is in agreement with reports by Larsen et al\(^11\) and Ferron et al\(^8\) both of whom demonstrated a rapid decline in Cav3.2 transcripts in the activity after the birth of mice.

Murine pluripotent embryonic stem cells (ES cells) retain their developmental capacity to differentiate into cardiomyocytes. By tracking myosin light chain (MLC) 2v-expressing cells, Zhang et al suggested that Cav3.1 mainly underlies the development of T-type Ca\(^{2+}\) channels in ES cell-derived cardiomyocytes.\(^12\) However, MLC2v(+) cells represent a subset of cardiac lineage cells with the potential to differentiate into ventricles, because MLC2v is specifically expressed in ventricles at the early stage of embryonic diversification. Cardiac precursor cells with the potential to differentiate into atrium, ventricle and pacemaker cells would be necessary for an investigation of the developmental changes of cardiac T-type Ca\(^{2+}\) channels during the earliest stage of cardiogenesis.

Nkx2.5 is expressed throughout the development of the heart primordium, beginning in the cardiogenic mesoderm before \(-\)-cardiac actin and -myosin heavy chain genes are turned on.\(^13\) Nkx2.5/GFP(+) cells are cardiac precursor cells derived from ES cells and their expression of GFP is driven by a Nkx2.5 promoter. They differentiate into sinus node- or conduction-system-type cells, as well as myocytes.\(^14\) We previously reported that the Nkx2.5/GFP(+) cells showed Ni\(^{2+}\)-sensitive automaticity in the early and intermediate
stages of their differentiation, but not in the late stage; a result that suggested the subtypes of cardiac T-type Ca2+ channels changed during their differentiation. The purpose of the current study was to study the subtype switch of the T-type Ca2+ channels in beating Nkx2.5/GFP(+) cells.

Methods

Culture of Nkx2.5/GFP(+) ES Cells

H7 is an ES cell line derived from 129/Ola and carries a hygromycin resistance gene in one of the Oct-3/4 loci, which allows selection of Oct-3/4-positive undifferentiated stem cells. Nkx2.5/GFP(+) cells are derived from H7 and carry a GFP reporter gene in one of the Nkx2.5 loci. The cells were grown and maintained on gelatin-coated dishes without feeder cells in Glasgow-modified Eagle’s medium (GMEM, Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated fetal calf serum (Equitech-Bio, Kerrville, TX, USA), 1× non-essential amino acids (Invitrogen), 0.1 mmol/L 2-mercaptoethanol, 1 mmol/L sodium pyruvate, 0.1 g/ml penicillin, 0.1 mg/ml streptomycin, 100 μg/ml hygromycin (Gibco-BRL), and 1,000 units/ml leukemia inhibitory factor (ESGRO™, Chemicon International Inc, Temecula, CA, USA). Differentiation of Nkx2.5/GFP(+) cells was induced by formation of embryo bodies (EBs). Briefly, EBs were generated by plating 20 000 cells in suspension (2.5 × 104 cells/ml) on the lid of bacterial dishes. The message in the early stage was collected from day 7 EBs and further cultured on gelatin-coated dishes. The cells were dissociated from day 8 EBs. The cells were obtained from day 7 EBs and further cultured on gelatin-coated dishes. The message in the early stage was collected from EBs on days 5–10 and that in the late stage on day 15.

Fluorescence Activated Cell Sorter Analysis

Cells were dissociated from EBs by trypsinization and gentle pipetting. They were resuspended in Hank’s Balanced Salt Solution containing 1% bovine serum albumin, and 2.5 μg/ml proidium iodide (PI) and subjected to flow cytometry (EPICS XL, Beckman Coulter, USA) with WinMDI software. The sort gate for GFP(+) cells was established on the basis of the forward-scattered light, side-scattered light, and PI and GFP fluorescence intensities of control ES cells. Cells were sorted into culture medium and cultured as floating EBs for N days.

Electrophysiological Recordings

Whole-cell Ca2+ currents were recorded from the beating Nkx2.5/GFP(+) cells using patch-clamp techniques. All experiments were carried out at 37°C. We measured the Ca2+ currents of cells in the early stage (days 8–10) and the late stage (days 15–17) of differentiation.

T-Type Ca2+ Channel Current

The cells were perfused with Na+- and K+-free external solution and internal pipette solution. The external solution contained (in mmol/L) 140 N-methyl-D-glucamine, 0.5 MgCl2, 10 HEPES, 10 D-glucose, 2 CaCl2, 5 CsCl, and 0.005 tetrodotoxin (pH 7.4 with HCl); the internal pipette solution contained 20 TEA-C12, 125 CsCl, 10 HEPES, 10 EGTA, and 4 Mg-ATP (pH 7.3 with CsOH). The recording pipettes had tip resistances ranging from 2 to 5 MΩ. Current recordings were made with an Axopatch-200B amplifier (Axon Instruments). The average cell capacitance was 34.5±3.5 pF in the early stage, and 41.0±4.8 pF in the late stage. Command voltage pulse generation, data acquisition, and data analysis were performed with pCLAMP9 software (Axon Instruments). T-type Ca2+ channel currents (I_{Ca,T}) were elicited every 3 s by 500 ms step pulses from a holding potential of −90 mV to test potentials of −90 to 50 mV at 10 mV intervals (5 μmol/L nifedipine was included to eliminate L-type Ca2+ channels). The current amplitude was divided by cell capacitance to obtain current density.

L-Type Ca2+ Channel Current

The external solution contained (in mmol/L) 140 NaCl, 5.4 KCl, 1.8 CaCl2, 1 MgCl2, 10 HEPES, and 10 glucose (pH 7.4 with NaOH). The internal solution was the same as that for the T-type Ca2+ channel current. The recording pipettes had tip resistances ranging from 5 to 13 MΩ. The average cell capacitance was 31.8±3.9 pF in the early stage, and 57.7±8.6 pF in the late stage. L-type Ca2+ channel currents (I_{Ca,L}) were elicited every 2 s by 300 ms step pulses from a holding potential of −40 mV to test potentials of −40 to +60 mV at 10 mV intervals, after a short −90 mV depolarization pulse applied to exclude the Na+ channel current.

Real-Time Reverse-Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from EBs using an RNaseasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s instructions. RNA samples were treated with DNaseI (Promega) to eliminate genomic DNA contamination, and cDNA was synthesized using SuperScript™ II reverse transcriptase (Gibco-BRL). Real-time PCR was performed with a Light Cycler SYBR Green I kit according to the manufacturer’s instructions (Roche Diagnostics, Tokyo, Japan) and products were analyzed with an ABI PRISM R 7700 Sequence Detection System (Applied Biosystems). The gene-specific primers used are shown in Table 1.

Table 1. Sequence of Oligonucleotides Used in Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Target</th>
<th>Accession number</th>
<th>Left primer</th>
<th>Right primer</th>
<th>Sequence (5’→3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cav3.1</td>
<td>NM_009783</td>
<td>ACCCTCCCCAAGAAGAT</td>
<td>GCTGTTGAGGGACTTAGAAT</td>
<td>Left primer ACCCTCCCCAAGAAGAT Right primer GCTGTTGAGGGACTTAGAAT</td>
</tr>
<tr>
<td>Cav3.2</td>
<td>NM_021415</td>
<td>GCTGTTGAGGGACTTAGAAT</td>
<td>CGAAGGTGACGAAGTAGC</td>
<td>Left primer GCTGTTGAGGGACTTAGAAT Right primer CGAAGGTGACGAAGTAGC</td>
</tr>
<tr>
<td>Cav1.2</td>
<td>NM_009781</td>
<td>TCTGCTCTCTAGTGGCAA</td>
<td>GGGAATGTTGGTGAGGAAATG</td>
<td>Left primer TCTGCTCTCTAGTGGCAA Right primer GGGAATGTTGGTGAGGAAATG</td>
</tr>
<tr>
<td>β-actin</td>
<td>X03672</td>
<td>CACCGGTGAAAAGATGAC</td>
<td>CAGGATCTCATCGTGATAGT</td>
<td>Left primer CACCGGTGAAAAGATGAC Right primer CAGGATCTCATCGTGATAGT</td>
</tr>
</tbody>
</table>

RT-PCR, reverse-transcriptase-polymerase chain reaction.
Effects of Ni\textsuperscript{2+} on ICa,T of Nkx2.5/GFP(+) Cells in the Early and Late Stages of Differentiation

Ni\textsuperscript{2+} at a low concentration (ie, <50\,\mu\text{mol/L}) has been used to selectively block ICa,T in several cell types\textsuperscript{1,20} Fig 1A,B shows the effects of 40\,\mu\text{mol/L Ni}\textsuperscript{2+} on ICa,T in the early and late stages of differentiation. In the early stage, Ni\textsuperscript{2+} strongly decreased ICa,T (from 1.62 to 0.65 nA), whereas Ni\textsuperscript{2+} at the same concentration barely had an effect (from 1.94 to 1.60 nA) in the late stage. Panels C and D in Fig 1 show current-voltage curves of ICa,T elicited in a voltage range from –60 mV to +40 mV. In the early stage of differentiation, Ni\textsuperscript{2+} caused a significant reduction of ICa,T within the voltage range of –30 and 0 mV [peak current: 3.8±0.7 pA/pF (in control) and 1.3±0.5 pA/pF (40\,\mu\text{mol/L Ni}\textsuperscript{2+})], whereas in the late stage, Ni\textsuperscript{2+} caused no significant decrease in ICa,T [peak current: 3.7±0.6 pA/pF (in control), and 2.5±0.6 pA/pF (40\,\mu\text{mol/L Ni}\textsuperscript{2+})]. The Ni\textsuperscript{2+}-sensitive fractions of ICa,T constituted 66.5±5.9% and 42.8±7.4% of total ICa,T, in the early and late stages of differentiation, respectively (Fig 2).

The presence of the GFP reporter gene in one of the Nkx2.5 loci might have influenced the development of cardiac precursor cells, as suggested by the association of Nkx2.5 dysfunction with congenital heart diseases\textsuperscript{18} Therefore, we repeated the same experiments using cardiac precursor cells from a different murine ES cell line with intact Nkx2.5 function (data not shown). We obtained similar results from these cells, excluding the effects of Nkx2.5 gene dosage or GFP gene expression on the developmental changes in Ni\textsuperscript{2+} sensitivity of ICa,T.

Expression of mRNA Encoding T-Type Ca\textsuperscript{2+} Channels in EBs During Differentiation

Fig 3 shows developmental changes in the mRNA expression of Cav3.1 and Cav3.2, quantified by real-time RT-PCR. Levels of Cav3.1 mRNA (Fig 3A) were relatively low in the early stage and showed a steady increase during differentiation, reaching the maximum level in the late stage (day 15). In contrast, levels of Cav3.2 mRNA (Fig 3B) were highest on day 6 (in the early stage) and gradually decreased thereafter.
It is interesting to study the changes in the transcription of L-type Ca\textsuperscript{2+} channels in EBs and their activity of Nkx2.5/GFP(+) cells during differentiation and to compare them with those of T-type Ca\textsuperscript{2+} channels. Fig.4A,B shows representative L-type Ca\textsuperscript{2+} currents and the results indicated that their amplitude was remarkably larger in the late stage than in the early stage. Current-voltage curves of ICa,L.
obtained from 4–5 individual cells (Fig 4C) showed a significantly larger amplitude in the late stage than the early stage within the test potentials ranging from −20 to 30 mV without changes in either threshold, peak or equilibrium potential. The level of Cav1.2 mRNA expression was relatively low in the early stage, but gradually increased to reach its maximum level on day 15, which was consistent with their channel activity (Fig 4D).

**Discussion**

We previously showed that the Ni²⁺-sensitive T-type Ca²⁺ current plays an important role in the automaticity of Nkx2.5/GFP(+) cells during the early stage of cell differentiation and that the Ni²⁺-sensitivity to automaticity was attenuated during differentiation, indicating the possibility that Cav3.2 is developmentally regulated to be predominantly expressed during the early stage of differentiation. However, neither the messenger level nor the functional level of Cav3.2 has ever been examined. Furthermore, our previous results suggested subtype switching of the T-type Ca²⁺ channel from Cav3.2 to Cav3.1 as another mechanism for the change in Ni²⁺-sensitivity to automaticity during cellular differentiation.15 Both the contribution of Cav3.1, but not Cav3.2, to chamber-specified Mlc2v-positive cells derived from ES cells12 and the subtype switching of T-type Ca²⁺ channel during differentiation have been reported5,10 supporting the subtype switching identified in the present study. On the other hand, the expression of the L-type Ca²⁺ channel, another depolarizing current responsible for automaticity, may explain the change in Ni²⁺-sensitivity to automaticity during differentiation. Therefore, we studied the message levels and current densities of Ni²⁺-sensitive and Ni²⁺-insensitive T-type and L-type Ca²⁺ channels during cell differentiation.

The present results indicate that the transcription of Cav3.2 was initiated at onset of beating and then waned during the very early stage of differentiation before chamber specific genes were upregulated. On the other hand, transcription of Cav3.1 and Cav1.2 increased after the transient expression of the Cav3.2 transcript. In addition, the transcriptional levels of each channel during differentiation were consistent with the current densities encoded by the Cav3.2, Cav3.1 and Cav1.2 genes. These results suggest that the expression of each channel is mainly determined at the transcriptional level.

The subtype switching of T-type Ca²⁺ channel is also considered to be regulated at the transcriptional level. Growth factors and transcription factors are important regulators in early cardiogenesis19 and they have been reported to play a role in the expression of cardiac ion channels. Insulin-like growth factor, for example, regulates the expression of the T-type Ca²⁺ channel20 and one of the transcriptional systems activated by the canonical Wnt signaling, NRSE-NRSF, induces Ni²⁺-sensitive Cav3.2 during differentiation.21 Despite these facts, the precise mechanisms of the control at the transcription level of the subtype switch for the T-type Ca²⁺ channel remain to be elucidated.

The developmental role of the subtype switching of the T-type Ca²⁺ channel is obscure. Cardiogenesis is considered to be determined by the regulation of the balance between proliferation and differentiation. Highly differentiated mammalian cells exit the cell cycle and are thought to be incapable of proliferation. Recent studies indicated that T-type Ca²⁺ channels were required for cell-cycle progression and proliferation of human pulmonary artery smooth muscle cells.22–23 and in addition, automaticity of cardiac precursor cells was reported to be required for cardiac differentiation. Although these reports suggest that T-type Ca²⁺ channels play a crucial role in both proliferation and differentiation during cardiogenesis, the precise role of the subtype switching remains to be clarified.

Our result indicate the possibility that the L-type Ca²⁺ channel is an important depolarizing current in the development of automaticity during the late stage, but not in the early stage of differentiation. This channel can modulate the rate of automaticity by changing the maximum upslope velocity or by changing the action potential duration. Furthermore, the expression of this channel enables cells to respond to sympathetic tone by increasing calcium influx via the channel. However, further investigation is required to define the mechanisms of controlling the expression during differentiation and the functional roles of this channel in automaticity.

In conclusion, our results indicate that the mechanism of the change in Ni²⁺-sensitivity is partly, if not completely, related to switching of the T-type Ca²⁺ channel from Ni²⁺-sensitive Cav3.2 to Ni²⁺-insensitive Cav3.1 at the transcriptional level. In addition, the expression of the L-type Ca²⁺ channel during the late stage of differentiation might modulate the automaticity of Nkx2.5-positive cells to be insensitive to Ni²⁺.

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Subtype Switch of \( I_{Ca} \) During Differentiation

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