Molecular and Electrophysiological Differences in the L-Type Ca^{2+} Channel of the Atrium and Ventricle of Rat Hearts

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Background Many pathological conditions induce electrical remodeling, possibly through intracellular Ca^{2+} overload, but the currently available L-type Ca^{2+} channel blockers may be detrimental because of their global negative inotropic effects.

Methods and Results To determine whether the L-type Ca^{2+} channel is identical throughout the heart, the distribution of the mRNAs and proteins comprising the L-type Ca^{2+} channel and its electrophysiological properties were analyzed in rat atria and ventricles. The mRNA of $\alpha_2\text{-}2$ (*Cacna2d2*) was more abundantly expressed in the atrium (~5-fold) than in the ventricle. In contrast, $\alpha_1\text{c}$ (*Cacna1c*) (*Cav1.2*) mRNA was significantly less abundant in the atrium. The level of the $\alpha_1\text{c}$ (*Cacna1c*) (*Cav1.2*) protein was decreased (~0.5-fold) and that of $\alpha_2\text{-}1$ (*Cacna2d1*) was increased (~2-fold) in the atrium compared with the ventricle. Although the peak $\text{I}_{\text{Ca,L}}$ density showed no significant differences, voltage dependence of inactivation and activation of the current showed a more depolarized shift in the atrium than in the ventricle.

Conclusion These results indicate that in the rat heart the L-type Ca^{2+} channel differs between the atrium and ventricle with regard to gene expression and electrophysiological properties. (Circ J 2006; 70: 610–614)

Key Words: Atrial fibrillation; Atrium and ventricle; L-type Ca^{2+} channel; Remodeling; Subunit

The L-type Ca^{2+} channel expressed in cardiac muscle plays an important role in determining the intracellular Ca^{2+} and thus the action potential duration of cardiomyocytes.¹ Although Ca^{2+} overload is a major cause of myocardial cell injury,² blocking this channel could be either beneficial or detrimental. In fact, the available L-type Ca^{2+} channel blockers should be useful for treating several types of cardiac arrhythmias, but might cause negative inotropic effects that lead to pump failure. A T-type Ca^{2+} channel blocker, mibefradil, has shown potential for preventing tachycardia-induced atrial fibrillation remodeling,³ although its underlying mechanism of prevention remains unclear. Because the T-type Ca^{2+} channel blockers are one of the “atrium specific Ca^{2+} channel blockers”, regional differences in the L-type Ca^{2+} channel blocker, if any, would open a new paradigm for controlling regional intracellular Ca^{2+} .

The function of the L-type Ca^{2+} channel is characterized by its main subunit, $\alpha_1\text{c}$ (*Cacna1c*) (*Cav1.2*), and also the auxiliary subunits α_2 (*Cacna2d*) and αnb (*Cacnb*). However, there are few reports investigating regional differences in Ca^{2+} channel composition. Because previous studies have focused on regional or developmental differences of a single subunit,^{4,5} it would be inappropriate to discuss regional

differences of the L-type Ca^{2+} channel with special reference to the relative expression of the composite subunits. In the present study, to determine whether the composition of the L-type Ca^{2+} channel is identical throughout the heart, we analyzed both the distribution of the mRNAs and proteins that comprise the L-type Ca^{2+} channel and its electrophysiological properties in rat atria and ventricles.

Methods

mRNA Analysis and Ribonuclease Protection Assay

Hearts were harvested from 10-week-old female Sprague-Dawley (SD) rats. For mRNA analysis, the excised hearts were divided into the sinus-node, the right atrium, the left atrium, the intraventricular septum, the epicardial side of the left ventricular free wall, the endocardial side, the left

Table 1 PCR Primers Used for Amplification of L-Type Ca^{2+} Channel Subunit Genes

$\alpha_1\text{c}$ (<i>Cav1.2</i>)	Sense	5'-GATGCAAGACGCTATGGGCTATGAG
	Antisense	5'-GCATGCTCATGTTTCGGGGTTTGTC
$\alpha_2\text{-}1$ (<i>Cacna2d1</i>)	Sense	5'-TGCAATTGATCCTAATGGTTATGTG
	Antisense	5'-GTATTCCCTTGGTGCTATGAAAGTG
$\alpha_2\text{-}2$ (<i>Cacna2d2</i>)	Sense	5'-CTGTGGCTGCTGCTGCCGCTTCTAC
	Antisense	5'-CCTCCTTGATGTTGTCTGCCAGCG
α_2 (<i>Cacnb2</i>)	Sense	5'-AATTCACAGGGTTCTCAAGGTGATC
	Antisense	5'-GCTTCGTTGTTGCTGCACACTCAITG
α_3 (<i>Cacnb3</i>)	Sense	5'-CTGCTGGGGGAGCGAGGTGAGGAGC
	Antisense	5'-CTGTCCTTAGGCCAAGGCCCGGTTAC

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ventricular apex and the right ventricle, and then immediately snapfrozen in liquid nitrogen. RNA was extracted by the AGPC (acid guanidinium-phenol chloroform) method.

Digoxigenin-labeled RNA probes were made using a RT-PCR kit (access RT-PCR system, Promega, Madison, WI, USA) with subunit-specific primers (Table 1) and myocardial RNA as a template. The RT-PCR products were subcloned into a PCRII vector (Invitrogen, San Diego, CA, USA), and the digoxigenin (Boehringer Mannheim)-labeled RNA probes were made with the cDNA as a template. All of the prepared RNA probes were partial lengths confirmed not to cross-react with each other. Ribonuclease protection assays were performed using a RPAIII kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions.

Protein Analysis and Western-Blot Analysis

For protein analysis, hearts harvested from 10-week-old female SD rats were divided into the atrium and the ventricle. The samples were immediately homogenized while gently suspended in Tris/EDTA (TE) buffer and 1/10 volume of a protease inhibitor cocktail (Halt Protease Inhibitor Cocktail, EDTA free Pierce, Rockford, IL, USA). Tissue lysates were centrifuged for 20 min at 15,000 rpm. Soluble proteins were removed and the precipitate was dissolved in TE buffer containing 10% trichloroacetic acid. After centrifuging for 30 min, the pellet was dissolved in TE buffer containing 25% urea, 4% β -mercaptoethanol, 2% Triton-X and 2.5% LDS, and finally prepared as membranous fraction of the proteins. All subsequent manipulations were performed on ice.

Proteins were separated using SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis), electrophoresed on PVDF (polyvinylidene difluoride) membrane, blocked overnight by 5% skim milk TBST (Tris-buffered saline Tween-20), and thereafter incubated overnight with the specific primary antibodies (Alamone Labs, Jerusalem, Israel). The prepared antibody concentrations were 1:833 for anti- α_1 and anti- α_3 , and 1:1,000 for anti- α_2 -1. Anti-rabbit HRP (horseradish peroxidase)-linked IgG (Cell Signaling, Beverly, MA, USA) was used as the secondary antibody. Protein levels were detected with chemiluminescence reagents (Western Lightning Chemiluminescence Reagent Plus, Perkin Elmer Life Science, Boston, MA, USA) and quantitatively analysed using microcomputer imaging software (ATTO Bioscience & Biotechnology, Tokyo, Japan).

Patch-Clamp Analysis

Hearts from 10-week-old female SD rats were removed and immediately retrogradely perfused on a Langendorff apparatus with oxygenated 37°C normal Tyrode solution. Thereafter, oxygenated Ca^{2+} -free Tyrode solution was superfused for 10 min followed by 15-min perfusion with 1% collagenase-containing solution. The heart was divided into the atrium and the ventricle, and cells were separated on ice from the tissue soaked in modified KB solution (in mmol/L: KOH 70, L-glutamic acid 50, KCl 40, taurine 20, KH_2PO_4 20, MgCl_2 3, glucose 10, EGTA 1, HEPES 10 (pH 7.4)).

A small aliquot of isolated cells was set on the stage of an inverted microscope, superfused with normal Tyrode solution at room temperature. Whole-cell patch clamp recording was performed with 2–5 M Ω glass pipettes (Clark Electromedical Instruments, Pangbourne Reading, UK) filled with pipette solution (in mmol/L: CsCl 13, MgCl_2

$6\text{H}_2\text{O}$ 0.1, EGTA 1, GTP 0.01, ATP-Mg 0.5, HEPES 1 (pH 7.4)), connected to a patch-clamp amplifier (L/M-EPC 7, List Electronic, Darmstadt-Eberstadt, Germany). Command pulses were generated by a digital-to-analog converter (VB-10B digital data recorder CRC), which was controlled by Pulse+ pulsefit software (HEKA Elektronik, Lambrecht, Germany). Recordings were stored on the hard disk of the computer. The cells were superfused with the extracellular solution (in mmol/L: TEA-Cl 126, CsCl 5.4, MgCl_2 1, CaCl_2 2, dextrose 10, HEPES 10, ryanodine 2×10^{-3} (pH 7.4)) while recording $I_{\text{Ca,L}}$. Only clearly striated rod-shaped cells were studied.

Data Analysis and Statistical Analysis

The mRNA expression level of each subunit was normalized with that of cyclophilin. Statistical analysis was performed with ANOVA and multiple comparison was done by Bonferroni's modified t-test. The protein levels and the current densities were analyzed using unpaired t-test. Activation and inactivation of the current were fitted to the Boltzmann formula ($f = 1 / \{1 + \exp[(V + V_{1/2})/b]\}$), where f is the steady-state parameter, V is the membrane voltage potential, and $V_{1/2}$ is the membrane voltage potential at half steady-state function. Recovery from inactivation was fitted to a biexponential function.

Values of $p < 0.05$ were considered to be significantly different.

Results

Ribonuclease Protection Assay

The main subunit α_1 (Cacna1c) (Cav1.2) and the auxiliary subunits α_2 (-1 with splice variants c, d and e and -2 with splice variants I and II) (Cacna2d1 and Cacna2d2) and α_3 (Cacnb2 and Cacnb3) showed homogeneous mRNA expression throughout the atrial regions investigated (ie, the sinus-node and the right and left atria). The mRNA expressions of these molecules were also homogeneously distributed through the ventricle. However, when we compared the mRNA level of the α_1 subunit (Cacna1c) (Cav1.2) in the atrium with that in the ventricle, it was significantly less abundant in the atrium, approximately 50% of that in the ventricle. In contrast, the mRNA level of the auxiliary subunit α_2 -2 (Cacna2d2) (splice variants I and II) was approximately 5-fold more abundant in the atrium than in the ventricle. The mRNA levels of the other auxiliary subunits α_2 -1 (Cacna2d1), α_2 (Cacnb2), and α_3 (Cacnb3) showed no significant regional differences between the atrium and the ventricle (Fig 1A).

Western-Blot Analysis

The α_1 (Cacna1c) (Cav1.2) immunoreactive protein was significantly decreased in the atrium to approximately 50% of the level in the ventricle, which was consistent with the results of its mRNA expression. On the other hand, the α_2 1 (Cacna2d1) immunoreactive protein was approximately 2-fold more abundant in the atrium than in the ventricle. The α_3 (Cacnb3) auxiliary subunit showed no significant differences between the atrium and the ventricle (Fig 1B). The protein levels of α_2 -2 (Cacna2d2) and α_2 (Cacnb2) could not be determined in the present study because their antibodies were not available.

Patch-Clamp Analysis

The $I_{\text{Ca,L}}$ was elicited by 240 ms depolarizing pulses to

Fig 1. (A) The mRNA levels of the main subunit α_{1C} (Cav1.2) (Cacna1c) and the auxiliary subunit α_{2-2} (Cacna2d2) (splice variants I and II), α_{2-1} (Cacna2d1) (splice variants c, d and e), β_2 (Cacnb2) and β_3 (Cacnb3) in the atria and ventricles. The mRNA expression of the α_{1C} (Cav1.2) (Cacna1c) subunit was homogeneous in the sinus-node, right atrium and left atrium. It did not show any regional differences in the ventricular regions investigated, but was clearly less abundant in the atrium than in the ventricle. The mRNA expression of the α_{2-2} (Cacna2d2) was homogeneously distributed in both the atria and ventricles. However, the subunit mRNA was ~5-fold more abundant in the atrium than in the ventricle in both of the 2 splice variants. The mRNA levels in each region are shown in the lower panel (n=5, p<0.05 when the mean values of the atrial regions were compared with those of the ventricular regions). The α_{2-1} (Cacna2d1), β_2 (Cacnb2) and β_3 (Cacnb3) auxiliary subunits showed no regional differences in mRNA expression. (B) The protein levels of the main subunit α_{1C} (Cav1.2) (Cacna1c) and auxiliary subunits α_{2-1} (Cacna2d1) and β_3 (Cacnb3) in the atrium and ventricle. The α_{1C} (Cav1.2) (Cacna1c) immunoreactive protein was significantly less abundant in the atrium than in the ventricle. In contrast, the α_{2-1} (Cacna2d1) immunoreactive protein level showed an approximately 2-fold increase in the atrium compared with the ventricle. The β_3 (Cacnb3) immunoreactive protein level showed no significant differences between the atrium and ventricle. The mean values are shown in the lower panel (n=5, *p<0.05 when the atrium compared with the ventricle). N, negative control; SN, sinus node; RA, right atrium; LA, left atrium; IVS, intraventricular septum; epi, left epicardial ventricle; end, left endocardial ventricle; apex, left ventricular apex; RV, right ventricle.

–40 mV to ~+60 mV with +10 mV increments from a holding potential of –40 mV, and the $I_{Ca,L}$ density was obtained by normalizing the $I_{Ca,L}$ with the membrane capacitance. Representative recordings at –20, 0, +10, +20 mV test pulses are shown in Fig 2A, and the mean current density-voltage relations for the atrial and ventricular cells are shown in Fig 2B. Though the peak $I_{Ca,L}$ density was slightly smaller in the atrial cells than in the ventricular cells, the difference was not statistically significant. However, in the atrial cells, the potential of the peak $I_{Ca,L}$ shifted to a more significantly depolarized level than in the ventricular cells. Figs 2C,D shows the voltage dependence of inactivation and activation of $I_{Ca,L}$. The $V_{1/2}$ of the voltage dependence of inactivation was -24.4 ± 0.6 mV in the atrium and -29.0 ± 0.1 mV in the ventricle. The $V_{1/2}$ of the voltage dependence of activation was -7.8 ± 22.0 mV in the atrium, and -15.7 ± 4.9 mV in the ventricle. Accordingly, in the atrial cells, both the voltage dependence of inactivation and activation curves showed a significantly more depolar-

ized shift than in the ventricular cells, although the current density was similar throughout the heart.

Discussion

The major findings of the present study are as follows. (1) Rat hearts have inhomogeneous mRNA expression of the subunits encoding the L-type Ca^{2+} channel. The auxiliary subunit α_{2-2} (Cacna2d2) mRNA is abundantly expressed in the atrium, whereas the main subunit α_{1C} (Cav1.2) (Cacna1c) mRNA is predominantly expressed in the ventricle. (2) There are differences in the expression of the immunoreactive protein in the atrium and the ventricle. The atrium is characterized by decreased α_{1C} (Cacna1c) (Cav1.2) protein accompanied by increased α_{2-1} (Cacna2d1) protein. (3) Irrespective of the different protein levels, peak $I_{Ca,L}$ density showed no significant difference between the atrium and the ventricle. However, the voltage-dependence of the $I_{Ca,L}$ inactivation and activation showed a more

Fig 2. (A, B) Representative recordings of the L-type Ca^{2+} current ($I_{\text{Ca,L}}$) at -20, 0, +10, +20 mV test pulses, and the mean current density-voltage relation for atrial and ventricular cells. Although the peak $I_{\text{Ca,L}}$ density of the atrial cells was slightly less than that in the ventricular cells, the difference was not statistically significant. However, the potential of the peak $I_{\text{Ca,L}}$ density shifted to a significantly depolarized level in the atrial cells ($n=7$). Voltage dependence of inactivation (C) and activation (D) of $I_{\text{Ca,L}}$. The $V_{1/2}$ of the voltage dependence of inactivation was -24.4 ± 0.6 mV in the atrium, and -29.0 ± 0.1 mV in the ventricle. The $V_{1/2}$ of the voltage dependence of activation was -7.8 ± 2.0 mV in the atrium, -15.7 ± 4.9 mV in the ventricle. In the atrial cells, both the voltage dependence of inactivation and activation curves showed a significantly more depolarized shift, which could reflect the differences in the voltage-dependence of $I_{\text{Ca,L}}$ ($n=7$).

depolarized shift in atrial cells than in ventricular cells.

In cardiac myocytes, the L-type Ca^{2+} channel is composed of multiple subunits, including the main subunit $\alpha_1\text{C}$ (Cacna1c) (Cav1.2) and the auxiliary subunits α_2 (Cacna2d) and $\alpha_2\text{-1}$ (Cacna2d1). Variations have been reported in the auxiliary subunits, including $\alpha_2\text{-1-4}$ (Cacna2d1-4) and $\alpha_2\text{-1-4}$ (Cacnb1-4). The subunits synergistically control the L-type Ca^{2+} channel, thereby regulating the excitation-contraction coupling process of the myocytes. Many pathological conditions in several experimental models are reported to have induced reduction in the $I_{\text{Ca,L}}$ density,^{6,7} which is ascribed partially to the transcriptional down-regulation of the main subunit $\alpha_1\text{C}$ (Cacna1c) (Cav1.2). A typical example is atrial fibrillation, in which the $I_{\text{Ca,L}}$ density decreases progressively during the maintenance of the arrhythmia.⁸ In accordance with the reduced $I_{\text{Ca,L}}$, the mRNA level of the main subunit has been reported to be decreased by atrial fibrillation itself.⁹ However, little is known about the gene expression of the channel auxiliary subunits.

A recent study reported inhomogeneous distribution of the L-type Ca^{2+} channel subunits between the atrium and the ventricle of rat hearts: the mRNA of the main subunit $\alpha_1\text{C}$ (Cacna1c) (Cav1.2) was more abundantly expressed in the ventricle than in the atrium.¹⁰ Moreover, the mRNA levels of $\alpha_2\text{-2}$ (Cacna2d2) and $\alpha_2\text{-3}$ (Cacna2d3) were higher in the atrium than in the ventricle.¹¹ As for the

protein expressions, α_2 (Cacnb2) is reported to be more abundant in the ventricle than in the atrium.¹² These previous reports clearly demonstrate that the molecular basis of the L-type Ca^{2+} channel differs between the atrium and the ventricle. Our present study also supports this concept, adding the new finding that the rat atrium is characterized by a lower level of the immunoreactive protein of the main subunit $\alpha_1\text{C}$ (Cacna1c) (Cav1.2) and a higher level of the auxiliary subunit $\alpha_2\text{-1}$ (Cacna2d1). However, surprisingly, our electrophysiological data demonstrated that the peak density of the L-type Ca^{2+} channel was similar in the atrium and the ventricle, irrespective of the inhomogeneous distribution of the subunits.

The function of the L-type Ca^{2+} channel should be determined by the composition of the co-expressed subunits. Although the main subunit $\alpha_1\text{C}$ (Cacna1c) (Cav1.2) accounts for the Ca^{2+} ion channel pore, the voltage sensor, the selectivity filter and the drug binding sites and is alone able to function as the L-type Ca^{2+} channel, co-expression of the auxiliary subunits modifies the electrophysiological function of the main subunit. The α_2 (Cacna2d) auxiliary subunit is known to increase the peak $I_{\text{Ca,L}}$ density and to shift the voltage dependence of inactivation and activation to a more hyperpolarized level.^{13,14} Also, the peak $I_{\text{Ca,L}}$ density is known to be increased by co-expression of the α_2 (Cacnb) auxiliary subunit,¹⁴⁻¹⁶ although there have been contro-

versal results reported for α_3 (Cacnb3).¹⁷ On the basis of these reports, the present electrophysiological observations would be explained simply by the decreased amount of the main subunit α_1C (Cacna1c) (Cav1.2) in the atrium being compensated for by the increased auxiliary subunit α_2 (Cacna2d), leading to similar $I_{Ca,L}$ density in the atrium and the ventricle. In this consideration, the voltage dependence of inactivation and activation of $I_{Ca,L}$ should show a hyperpolarized shift in the atrium through the increased α_2 (Cacna2d), contradictory to our observation of a depolarized shift. However, that could be attributed to glycosylation of the α_2 (Cacna2d) subunit, which is known to have many glycosylation sites that cause surface charge effects.¹⁸

The subunit composition and electrophysiological function of the L-type Ca^{2+} channel might differ among animal species. Human hearts are known to express α_2-1-3 (Cacna2d1–3) mRNA, and the mouse heart lacks α_2-3 (Cacna2d3) mRNA.¹⁹ Electrophysiological study using mouse hearts has demonstrated that the peak $I_{Ca,L}$ density is significantly smaller in the atrium than the ventricle,²⁰ but its molecular basis remains unknown. Therefore, further information of the subunit composition of the L-type Ca^{2+} channel in various animals may be required to conclude the regional differences throughout the heart. However, the present study clearly demonstrated that in rat hearts the L-type Ca^{2+} channel differs between the atrium and the ventricle at the molecular level and also in electrophysiological function. This finding could lead to new therapeutic tools, such as auxiliary subunit-targeted drugs, which may exert atrial-specific effects.

Study Limitations

First, because the antibodies were unavailable, the levels of all the proteins comprising the L-type Ca^{2+} channel could not be determined in the present study. Second, we could not explain the inconsistent result between the mRNA and protein levels of α_2-1 (Cacna2d1). This raises the possibility of differing regional turnover of α_2-1 (Cacna2d1) mRNA or protein. Third, subtype-switching^{21,22} which is known as the shift of the Ca^{2+} -channel constituent subunits during development, may have played a role in our present results. Regional differences in the L-type Ca^{2+} channel should be determined at all developmental stages, which would be a future study. Lastly, as the subunit composition of the L-type Ca^{2+} channel might differ among animal species, the results of this study cannot be directly applied to humans. Though limited for these reasons, the present results conclusively demonstrated regional differences of the L-type Ca^{2+} channel in the atrium and the ventricle.

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