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Expression and Roles of $\text{Ca}_v1.3$ (α_{1D}) L-Type Ca^{2+} Channel in Atrioventricular Node Automaticity

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Abstract

Atrioventricular node (AV node) is the hub where electrical input from the atria is propagated and conveyed to the ventricles. Despite its strategic position and role in governing impulse conduction between atria and ventricles, there is paucity of data regarding the contribution of specific ion channels to the function of the AV node. Here, we examined the roles of $\text{Ca}_v1.3$ L-type Ca^{2+} channel in AV node by taking advantage of a mouse model with null mutation of $\text{Ca}_v1.3$ ($\text{Ca}_v1.3^{-/-}$). $\text{Ca}_v1.3$ null mutant mice show evidence of AV node dysfunction with AV block, suggesting the tissue-specific function of the $\text{Ca}_v1.3$ channel. In keeping with this assertion, we demonstrate that $\text{Ca}_v1.3$ isoform is highly expressed in the isolated AV node cells. Furthermore, AV node isolated from $\text{Ca}_v1.3$ null mutant mice show a significant decrease in the firing frequency of spontaneous action potentials suggesting that $\text{Ca}_v1.3$ L-type Ca^{2+} channel plays significant roles in the automaticity of the AV node. Because of the distinct voltage-dependence of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ Ca^{2+} channels, $\text{Ca}_v1.2$ alone does not suffice to maintain normal AV node function. $\text{Ca}_v1.3$ currents activate at more hyperpolarizing voltage compared to $\text{Ca}_v1.2$ currents. Consequently, $\text{Ca}_v1.2$ Ca^{2+} channel cannot functionally substitute for $\text{Ca}_v1.3$ isoform in the AV node of $\text{Ca}_v1.3$ null mutant mice. Thus, our study demonstrates that the distinct biophysical properties of $\text{Ca}_v1.3$ Ca^{2+} channel play critical roles in the firing frequency of AV node tissues.

Keywords

$\text{Ca}_v1.3$ channel; atrioventricular nodes

INTRODUCTION

Voltage-gated Ca^{2+} channels represent one of the most common drug targets in cardiovascular diseases. Ca^{2+} channels are multi-protein complexes composing of a pore-forming transmembrane spanning α_1 subunit, a disulfide-linked complex of α_2 and γ

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DISCLOSURES

There are no conflicts to disclose.

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subunits, and an intracellular β subunit and a γ subunit [1–2]. The α_1 subunits are the largest subunit and are encoded by at least ten distinct genes [2]. The α_1 subunits incorporate the conduction pore, the voltage sensor and gating apparatus, as well as the known sites of channel regulation by second messengers, drugs, and toxins. Classically, voltage-gated Ca^{2+} channels have been classified, according to their electrophysiological and pharmacological properties, into five essential groups, termed T, L, N, P/Q and R [2–4].

Atrioventricular node (AV node) is a highly specialized pacemaking tissue located at the junction of the right atrium and ventricle. AV node represents the only electrical connection between atria and ventricles and provides the critical delay between atrial and ventricular contraction to allow for proper atrial emptying prior to the start of the ventricular contraction. Previous studies have identified an ensemble of ion channel genes and the roles of several distinct ion channels in the AV node function [5–11]. Pharmacological slowing of impulses across AV node is widely used clinically in atrial flutter and fibrillation to ensure physiological ventricular responses in these conditions. Therefore, new insights into the contribution of specific ion channels in the function of the AV node are of interest not only from physiologic point of view but are important therapeutically. Identification of AV node-specific ion channels may provide a new therapeutic target in the control of AV node conduction in atrial arrhythmias.

We have previously demonstrated that $\text{Ca}_v1.3$ (α_{1D}) L-type Ca^{2+} channels are highly expressed in the atria [12]. These findings were unexpected, yet insightful, since the main L-type Ca^{2+} channel in the heart is $\text{Ca}_v1.2$ (α_{1C}) and $\text{Ca}_v1.3$ was thought to be found mostly in neurons and neuroendocrine cells. Specifically, we have demonstrated robust expression of $\text{Ca}_v1.3$ channel both at the protein and transcript levels in mouse atria compared to ventricular tissues [12]. Indeed, several laboratories as well as ours have documented the expression of $\text{Ca}_v1.3$ Ca^{2+} channel, in addition to $\text{Ca}_v1.2$ isoform in pacemaking tissues and atria [8, 13–14]. Both $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ isoforms have similar pharmacologic properties, it is difficult to isolate one current from the other using conventional electrophysiology. Therefore, to disentangle the distinct roles played by the two Ca^{2+} channel isoforms in the heart, we have taken advantage of a mouse model with null mutation of $\text{Ca}_v1.3$ Ca^{2+} channel ($\text{Ca}_v1.3^{-/-}$) [14–15]. The homozygous null mutant mice show evidence of profound sinoatrial (SA) and AV nodes dysfunction as well as the development of atrial arrhythmias. We reason that the $\text{Ca}_v1.3$ deficient mouse model provides us a unique opportunity to directly determine the contribution of $\text{Ca}_v1.2$ vs. $\text{Ca}_v1.3$ and their roles in pacemaking tissues of the heart. Specifically, in the present investigation, using $\text{Ca}_v1.3$ null mutant mice, we focus our study on the roles of $\text{Ca}_v1.3$ on the automaticity of AV node cells. In addition, immunohistochemistry and immunofluorescence studies were further performed to document the expression of $\text{Ca}_v1.3$ Ca^{2+} channels in AV node cells.

MATERIALS AND METHODS

All animal care and procedures were approved by the University of California, Davis Institutional Animal Care and Use Committee. Animal use was in accordance with National Institutes of Health and institutional guidelines.

$\text{Ca}_v1.3$ Null Mutant Mice ($\text{Ca}_v1.3^{-/-}$)

Generation of $\text{Ca}_v1.3$ null mutant ($\text{Ca}_v1.3^{-/-}$) mice has previously been described [15]. The null mutant mice were backcrossed onto the C57Bl/6J background for greater than 7 generations. All mice were housed and kept on a 12 h light/dark cycle with food and water available *ad libitum*. Mice were weaned at 3 weeks and genotyped as previously described [14–15].

Atrioventricular Node Recordings

AV node preparation was used for microelectrode recordings [5, 10–11]. In brief, the animals were anesthetized with pentobarbital (60 mg/kg, i.p.). The heart was excised and placed in Tyrode's solution at 33–34 °C. The right atrium was separated from the ventricles and then the left atrium. The right atrium was opened under a dissecting microscope to expose the coronary sinus, the triangle of Koch and the interatrial septum. The sinoatrial (SA) node region was removed. The final preparation which included the entire AV node region and surrounding atrial muscle was pinned down (endocardial surface up) in the recording chamber. The tissue was continuously superfused with Tyrode's solution containing (mmol/L) NaCl 138, KCl 4, MgCl₂ 1, CaCl₂ 2, NaH₂ PO₄ 0.33, glucose 10, and HEPES 10, pH 7.4 with NaOH. All chemicals were purchased from Sigma Chemical (St. Louis, MO) unless stated otherwise. Spontaneous action potentials (APs) were recorded from isolated AV nodal preparations using microelectrode techniques with 3 M KCl microelectrodes at 33–34 °C [10, 14].

Whole-cell Patch-Clamp Recordings

Single AV nodal cells were isolated from WT and mutant mice as previously described with some modification [5, 10, 14, 16–17]. Whole-cell L-type Ca²⁺ current (I_{Ca}) was recorded at room temperature using patch-clamp techniques [14, 18]. The external solution contained (mmol/L): *N*-methyl-D-glucamine (NMG) 140, CsCl 5, MgCl₂ 0.5, CaCl₂ 2, 4-aminopyridine 2, glucose 10, and HEPES 10, pH 7.4 with HCl, and the internal solution contained (mmol/L): NMG 135, tetraethylammonium chloride 20, Mg-ATP 4, EGTA 10, and HEPES 10, pH 7.3 with HCl. The cell capacitance was calculated by integrating the area under an uncompensated capacitive transient elicited by a 20-mV hyperpolarizing pulse from a holding potential of –40 mV. Whole-cell current records were filtered at 2 kHz and sampled at 10 kHz.

Intracardiac electrogram recordings

Intracardiac electrograms were recorded from right atria, the HIS bundles and right ventricular apex [12]. Simultaneous surface electrocardiograms were recorded.

Immunofluorescence Confocal Microscopy

Immunofluorescent labeling was performed and imaged using a Pascal Zeiss confocal laser scanning microscopy [10, 19]. Single isolated AV node cells were fixed using 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature, washed three times with PBS, treated with 0.4% Triton X-100 in PBS for 15 min, then cells were washed and blocked with 1% bovine serum albumin in PBS, incubated overnight at 4 °C with primary antibodies. The following primary antibodies were used: (1) Anti-Ca_v1.3 antibody (Sigma, 1:100 dilution), a polyclonal antibody raised in rabbit, (2) Anti-neurofilament (NF-160) antibody (Chemicon, 1:100 dilution), a monoclonal antibody, and (3) Anti- α -actinin2 antibodies (Sigma, 1:800 dilution) was used to label atrial and ventricular myocytes as well as AV node cells. Immunofluorescence labeling for confocal microscopy was performed by treatment with FITC-conjugated goat anti-rabbit antibody (Sigma, 1:250 dilution) or Texas red-conjugated donkey anti-mouse antibody (Calbiochem, 1:250 dilution). Control experiments were performed by using secondary antibody only under the same experimental conditions. Identical settings were used for all specimens.

Immunohistochemistry of the AV Nodes

Paraffin sections of mouse hearts (4 μ m in thickness) were treated with xylene, hydrated using an alcohol gradient, then antigen retrieval was induced by heat treatment in Na-citrate buffer (10 mM, pH 6.5). The sections were treated overnight in 5% normal goat serum in

PBS at 4°C to block endogenous peroxidase activity and non-specific binding sites, after which the primary antibody (anti- $\text{Ca}_v1.3$ antibody, 1:100) in PBS containing 5% normal goat serum was added for 30 min. The sections were then treated with biotinylated secondary antibody. After washing for 5 minutes in PBS, they were incubated in VECTASTAIN ABC reagent (Vector Laboratories, Inc., Burlingame, CA) for 30 minutes. The sections were then incubated in peroxidase solution. The sections were rinsed, counterstained and mounted. AV nodal region was further evaluated in cardiac sections stained with Masson's trichrome. Immunofluorescence confocal microscopy experiments from consecutive AV node sections were performed using anti- $\text{Ca}_v1.3$ antibody (1:100 dilution) and anti-connexin 43 antibody (Cell Signaling, 1:50 dilution). Immunofluorescence labeling for confocal microscopy was performed by treatment with FITC-conjugated goat anti-rabbit antibody (Invitrogen, 1:200 dilution). Identical settings were used for all specimens.

Mathematical Modeling of the Spontaneous AP in Mouse AV Nodes

To further understand the contribution of $\text{Ca}_v1.2$ vs. $\text{Ca}_v1.3$ L-type Ca^{2+} current on the spontaneous AP of the AV node cells, we generated computer modeling to directly assess the effect of $\text{Ca}_v1.3$ Ca^{2+} current on the properties and characteristics of spontaneous AP of mouse AV node cells. As a starting point, we used the previously described model by Dokos *et al.*, which was originally established for rabbit SA node cells [20]. All programming was performed on an IBM PC desktop computer using MatLab version 6.5. Differential equations were solved using Euler method [21]. Fixed constant step of integration of 0.01 ms was used.

Data Analysis

Curve fits and data analysis was performed using Origin software (MicroCal Inc., Northampton, MA). Where appropriate, pooled data are presented as mean $\pm$ s.e.m. Statistical comparison was performed using the Student's *t*-test with $p < 0.05$ considered significant. Rate of diastolic depolarization (DDR in mV/s) was determined from recordings of the spontaneous AP by determining the first derivative of the diastolic depolarization.

RESULTS

$\text{Ca}_v1.3^{-/-}$ Mice Show Evidence of AV Node Dysfunction

To examine the effects of $\text{Ca}_v1.3$ channel ablation on AV node in the whole animal, we recorded intracardiac electrograms from the null mutant mice compared to WT littermates [12]. In addition to the prolongation in the PR interval and Wenckebach cycle-lengths in homozygous $\text{Ca}_v1.3^{-/-}$ null mice [12], null mutant mice exhibit evidence of AV node abnormalities with documented type I second degree AV block as shown in Figure 1A.

Next, we directly recorded spontaneous APs from isolated intact AV node preparation using microelectrode techniques at 33–34°C. The important landmarks were used in the identification of the AV node region [5, 10]. Representative spontaneous APs recorded from the regions within the AV node are shown in Figure 1B comparing WT, heterozygous and homozygous null mutant mice. Specifically, APs recorded from within the AV node can be identified by the presence of the spontaneous diastolic depolarization and a very slow upstroke of phase 0. Homozygous $\text{Ca}_v1.3^{-/-}$ mice show a significant decrease in the spontaneous AP activities compared to age-matched WT controls (Figure 1B). The data obtained using isolated AV node preparations suggest that the abnormalities observed are intrinsic to the AV node.

Summary data on cycle-length (CL) in ms, the maximum diastolic potential (MDP), AP amplitude (APA), maximum upstroke velocity ($V_{\max}$), rate of diastolic depolarization (DDR) and action potential duration at 50 and 80% repolarization (APD₅₀ and APD₈₀) are presented in Figure 1C. Detailed analysis of the spontaneous APs reveals a significant prolongation of the CL in homozygous $Ca_v1.3^{-/-}$ null mutant mice compared to WT littermates. The prolongation of the CL is associated with a significant decrease in DDR and MDP compared to the age-matched WT control. Heterozygous null mutant mice also show a significant decrease in DDR but to a lesser degree than homozygous animals. Specifically, the data support our proposed notion that $Ca_v1.3$ Ca^{2+} channel subtype is present in the AV node and plays important roles in determining the firing frequency of the AP in the AV node. The effects of $Ca_v1.3$ on MDP and APD are further discussed in the Discussion Section.

Whole-cell I_{Ca} Recorded From Isolated AV Node Cells

To directly test the contribution of the two Ca^{2+} channel subtypes in the observed abnormality in the AV node function in the null mutant mice, we recorded whole-cell L-type Ca^{2+} current ($I_{Ca,L}$) from single isolated AV node cells using a holding potential of -55 mV to inactivate T-type Ca^{2+} current ($I_{Ca,T}$). Figure 2A shows examples of $I_{Ca,L}$ traces elicited using different test potentials at 0, +10 and +20 mV. Currents recorded from $Ca_v1.3^{-/-}$ mice activate at more depolarized potentials compared to those from $Ca_v1.3^{+/+}$ or $Ca_v1.3^{+/-}$ animals, which express both $Ca_v1.2$ and $Ca_v1.3$ Ca^{2+} channels. Summary data for the current density-voltage relations are shown in Panel B. Even though there are no significant differences in the peak current density of $I_{Ca,L}$ from the three different genotypes, $I_{Ca,L}$ recorded from $Ca_v1.3^{-/-}$ mice shows a depolarizing shift compared to those of WT and heterozygous mutant mice. To further quantify the degree of the depolarizing shift, voltage-dependent activations were generated from WT and mutant animals (Panel C). There is a significant depolarizing shift of in the midpoint of activation of $I_{Ca,L}$ recorded from $Ca_v1.3^{-/-}$ mice, compared with $Ca_v1.3^{+/+}$ controls. To directly examine the voltage and Ca^{2+} -dependent inactivation, a two-pulse protocol was used. Summary data are shown in panel D showing similar voltage- and Ca^{2+} -dependent inactivation in WT, heterozygous and homozygous null mutant mice with no significant differences in the half-inactivation voltages. In addition, the curves exhibit the typical U-shape configuration for Ca^{2+} -dependent inactivation. In contrast, the rate of inactivation of $I_{Ca,L}$ recorded from $Ca_v1.3^{+/+}$ compared with $Ca_v1.3^{-/-}$ animals are significantly different as illustrated in Panel E. Inactivation profile of $I_{Ca,L}$ could be best fit by using two exponential time constants. Both the fast and slow time constants (τ_f and τ_s) at a test potential of 0 mV are significantly shortened in $I_{Ca,L}$ recorded from the $Ca_v1.3^{-/-}$ mutant mice compared to those of WT littermates ($P < 0.05$). Data obtained from AV nodal cells were similar to findings we have previously reported for SA nodal cells [14], supporting the important functional contribution of $Ca_v1.3$ Ca^{2+} channels in both types of pacemaking tissues.

Expression of $Ca_v1.3$ Ca^{2+} Channels in Single Isolated AV Node Cells

To further document the expression of the $Ca_v1.3$ channel in AV node, we performed immunofluorescence confocal laser scanning microscopy using isolated single AV node cells. Figure 3A shows confocal microscopic images of single isolated AV node cells (a&b) compared to atrial myocyte (c) illustrating positive staining using anti- $Ca_v1.3$ and anti- α -actinin2 antibodies. Panel b represents negative control using secondary antibodies only. In Figure 3B, neurofilament (NF)-160 was used as a specific AV node cell marker. It has previously been demonstrated that NF-160 is a marker of the pacemaker and conduction system [22–24] and was used in this study to distinguish AV node cells from atrial myocytes. In our study, NF-160 is only expressed in AV node cells (Figure 3B, panel a) and not atrial myocytes (panel c) further documenting the specific cell types used in our study.

Panel **b** shows lack of staining with secondary antibodies only. Finally, the presence of $\text{Ca}_v1.3$ isoform was further demonstrated using single staining with anti- $\text{Ca}_v1.3$ antibody in isolated AV node cells and atrial myocytes from WT animals. AV node cells and atrial myocytes from $\text{Ca}_v1.3^{-/-}$ animals were used as negative control (Figure 3C).

Histologic Sections Through Mouse AV Nodes Show Positive Staining for $\text{Ca}_v1.3 \text{ Ca}^{2+}$ Channels

Expression of $\text{Ca}_v1.3$ channel protein in the mouse AV node was further evaluated using immunostaining of cardiac section through AV nodes [10]. Figure 4A shows photomicrographs of histologic sections of AV node and working myocardium from WT mice at low (left panels) and high (right panels) magnification. Masson's trichrome was used to stain fibrous tissues to view the AV node demonstrating an oval shape of compact node cells next to the central fibrous body and the ventricular septum. Expression of $\text{Ca}_v1.3$ protein in AV node of WT animals was documented as peroxidase-positive staining (Panels **B**). Absence of $\text{Ca}_v1.3$ protein expression in the AV node and working myocardium in $\text{Ca}_v1.3^{-/-}$ mouse heart is shown in Panels **C** as a negative control. In addition, immunofluorescence confocal microscopy was performed using consecutive sections of AV nodes to label $\text{Ca}_v1.3$ channel and connexin 43 (Figure 4D). $\text{Ca}_v1.3$ channel is highly expressed in AV node. In contrast, connexin 43 protein is expressed in the working myocardium as documented previously [25].

Mathematical Modeling of the Spontaneous AP in Mouse AV node

To directly verify that the documented hyperpolarizing shift in the steady-state voltage-dependent activation of $\text{Ca}_v1.3$ compared to $\text{Ca}_v1.2$ L-type Ca^{2+} channel contributes importantly to the observed decrease in the AV node firing frequency in the null mutant mice, we generated computer modeling to directly assess the effect of $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ currents on the properties and characteristics of spontaneous APs of mouse AV node cells. We used the previously described model by Dokos *et al.*, which was originally established for rabbit SA node cells [20]. Modifications were made by the addition of transient outward K^+ current (I_{to}), the slow outward rectifier K^+ current ($I_{K,slow}$) and sustained component of outward K^+ currents as previously documented for mouse SA and AV node cells [11, 26] as well as the cytosolic Ca^{2+} buffering components [27]. Spontaneous APs of AV node were generated as shown in Figure 5A for WT animals (solid line). $I_{Ca,L}$ was then shifted in the depolarizing direction by 6.6 mV as observed experimentally for $\text{Ca}_v1.3^{-/-}$ mice. This single modification was sufficient in producing a significant reduction in the firing frequency as well as DDR (Figure 5B) of the spontaneous APs similar to what were observed experimentally.

DISCUSSION

$\text{Ca}_v1.3$ null mutant mice show evidence of AV node dysfunction with AV block, suggesting the tissue-specific function of the $\text{Ca}_v1.3$ channel. Using immunofluorescence confocal microscopy, we demonstrate that $\text{Ca}_v1.3$ isoform is highly expressed in the isolated AV node cells. Furthermore, $\text{Ca}_v1.3$ L-type Ca^{2+} channel plays significant roles in the automaticity of AV node. Specifically, AV node isolated from $\text{Ca}_v1.3$ null mutant mice show a significant decrease in the firing frequency of spontaneous action potentials. Whole-cell patch-clamp recordings of single isolated AV node cells further reveal a significant depolarizing shift in the voltage-dependent activation of $I_{Ca,L}$ in $\text{Ca}_v1.3$ null mutant mice compared to wild-type littermates. Thus, our study demonstrates that the distinct biophysical properties of $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channel contribute importantly to the automaticity of AV node tissues.

Diversity and Complexity of Voltage-Gated Ca^{2+} Channels

Voltage-gated Ca^{2+} channels are pivotal in normal cardiac function. The abundant message of Ca^{2+} channel genes in the heart and the abnormality of cardiac function resulted from gene-targeted ablation all testify to the importance of these membrane proteins in cardiovascular functions. Classically, Ca^{2+} channels have been classified, according to their electrophysiological and pharmacological properties, into five essential groups, termed T, L, N, P/Q and R [3–4, 28–29]. T-type channels are low-voltage activated and has been shown to be important for cardiac pacemaker activity and the oscillatory activity of thalamic neurons. The kinetic hallmark of T-type channels is slow activation and fast voltage-dependent inactivation. The high-voltage activated (HVA) channels, N-type and P/Q-type Ca^{2+} channels, co-localize at the synapses where they control exocytosis [30–32]. L-type channels are ubiquitous, particularly in skeletal and cardiac muscle, where they play an essential role in excitation-contraction coupling. The primary structures of these subunits have been determined. In most neurons, L-type channels contain $\text{Ca}_v1.2$ (α_{1C}) or $\text{Ca}_v1.3$ (α_{1D}) subunits, N-type channels contain $\text{Ca}_v2.2$ (α_{1B}) subunits, P- and Q-types contain alternatively spliced forms of $\text{Ca}_v2.1$ (α_{1A}) subunits, R-type channels contain $\text{Ca}_v2.3$ (α_{1E}) subunits, and T-type channels contain $\text{Ca}_v3.1$ (α_{1G}), $\text{Ca}_v3.2$ (α_{1H}) or $\text{Ca}_v3.3$ (α_{1I}) subunits. In addition, association with different β subunits also influences Ca^{2+} channel gating substantially, yielding a remarkable diversity of functionally distinct molecular species of Ca^{2+} channels.

AV Node Electrophysiology

AV node is a highly complex and heterogenous structure with intricate organization of multiple nodal and nodal-like myocytes. AV node is defined as the region within the Koch's triangle. This includes the central fibrous body with the compact node (CN) as well as the posterior nodal extension (PNE) projecting in the isthmus below the coronary sinus. Indeed, AV node is the only electrical connection between atria and ventricles and as such, AV node plays a critical role in governing the conduction and provides a physiologic delay between the two chambers and represents an intrinsic safety feature for the heart to prevent rapid ventricular response during atrial arrhythmias.

A large number of recent studies have provided insights into distinct ion channel proteins which are expressed in AV nodes as well as distribution of the specific connexin within the AV node structure [5–9, 11, 33–35]. Pacemaker current or I_f has been shown to play important roles in pacemaking activities by initiating the early phase of the spontaneous diastolic depolarization. However, because of the slow activation kinetics of I_f in addition to the voltage threshold of activation which is relatively hyperpolarized compared to the maximum diastolic potential, it is likely that I_f is not the sole initiator of pacemaking activities. More recent studies have demonstrated that the critical events in the spontaneous diastolic depolarization can be linked to rhythmic intracellular Ca^{2+} signals initiated by sarcoplasmic reticulum Ca^{2+} release and inwards current *via* Na^+ - Ca^{2+} exchanger [36–37].

L-type Ca^{2+} channels in the AV nodes

Ca^{2+} channels are important in the generation of the APs in the pacemaking tissues. $\text{Ca}_v3.1$ and $\text{Ca}_v3.2$ isoforms have been shown to be expressed in SA and AV nodes, however, expression of the $\text{Ca}_v3.3$ isoform has not been found in the myocardium, SA or AV nodes [9, 38]. Using *Ca_v3.1* null mutant mouse model, it was demonstrated that $\text{Ca}_v3.1$ Ca^{2+} channels contribute functionally to SA pacemaker activity and AV conduction [9].

For L-type Ca^{2+} channel, previous data suggest that L-type Ca^{2+} channels containing $\text{Ca}_v1.3$ subunit are expressed mainly in neurons and neuroendocrine cells, while those containing $\text{Ca}_v1.2$ subunit are found in the brain, vascular smooth muscle and cardiac tissue. Recently,

we as well as others, have documented the expression of $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channel, in addition to $\text{Ca}_v1.2$ isoform in pacemaking tissues and atria [8, 13–14]. Disruption of the gene encoding for $\text{Ca}_v1.3$ channels resulting in SA and AV nodes dysfunction as well as atrial arrhythmias. Indeed, there is differential expression of $\text{Ca}_v1.3$ subunit between atrial and ventricular tissues. We have previously documented using $\text{Ca}_v1.3$ null mutant mouse model as well as heterologous expression system that there are significant differences in the voltage-dependent activation between $\text{Ca}_v1.2$ and $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channel subtypes [14]. Specifically, $\text{Ca}_v1.2 \text{ Ca}^{2+}$ channel shows a significant depolarizing shift in the voltage-dependent activation compared to that of the $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channel [14, 39–40]. The data from the present study further suggest that $\text{Ca}_v1.2 \text{ Ca}^{2+}$ channel cannot functionally substitute for $\text{Ca}_v1.3$ isoform in the pacemaking tissues leading to SA and AV nodes abnormalities in $\text{Ca}_v1.3$ null mutant mice.

In addition, we and others have recently documented the expression of small conductance Ca^{2+} -activated K^+ channels (SK channels) in AV nodes [10,11]. Using gene-targeted mouse models, we demonstrated that overexpression of SK2 channels results in the shortening of the spontaneous action potentials of the AV node cells and an increase in the firing frequency. On the other hand, ablation of the SK2 channel results in the opposite effects on the spontaneous action potentials of the AV nodes [10]. We further demonstrate that SK channels associate with $\text{Ca}_v1.3$ and $\text{Ca}_v1.2$ channels through a cytoskeletal protein, α -actinin2 in cardiac myocytes [41]. The functions of SK2 channels in atrial myocytes are critically dependent on the normal expression of $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channels. Null deletion of $\text{Ca}_v1.3$ channel results in abnormal function of SK2 channel and prolongation of repolarization and atrial arrhythmias [41]. Consistent with our previous studies, null deletion of $\text{Ca}_v1.3 \text{ Ca}^{2+}$ channel also results in prolongation of the APD and a decrease in the MDP in the AV node cells likely from abnormal function of SK2 channel in the AV nodes in the null mutant mice (Figure 1C).

Even though $I_{\text{Ca,L}}$ recorded from $\text{Ca}_v1.3^{-/-}$ mice shows a depolarizing shift compared to those of WT and heterozygous mutant mice, there are no significant differences in the peak current density of $I_{\text{Ca,L}}$ from the three different genotypes. The lack of a significant decrease in the peak current density of $I_{\text{Ca,L}}$ in the null mutant mice may represent a compensatory changes which had been described in different models of gene-targeted mouse models.

Limitations of the present study

There is well documented existence of distinct cell types within the AV node tissue. Since the study was performed using isolated AV node cells, the specific roles of different AV node cell types cannot be directly correlated with their in situ function. Furthermore, data on primary pacemaker site with the AV node and the resulting AV nodal activation pattern were not obtained in the present study. Hence, the proposed relationships between cell and tissue behavior remain largely speculative at this time. Future studies are required to further address this critical issue.

Physiological significance of the present study

Even though $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ channels have similar pharmacologic properties, the channels show distinct voltage- and time-dependent kinetics; specifically, $\text{Ca}_v1.3$ channel exhibits a low threshold of activation with slower time-dependent inactivation compared to $\text{Ca}_v1.2$ isoform. Such differences in the gating kinetics confer specific function in the pacemaking tissues. Consistent with the findings we have previously reported for SA node [14], $\text{Ca}_v1.2$ channel is important during the slow upstroke of the spontaneous AP of the pacemaking cells while $\text{Ca}_v1.3$ channel plays a critical role during phase 4 depolarization.

Ablation of Ca_v1.3 channel results in a decrease in DDR in AV node cells. Importantly, Ca_v1.2 cannot functionally substitute for Ca_v1.3 isoform in these pacemaking tissues.

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Figure 1.

Functional roles of $Ca_v1.3$ Ca^{2+} channel in intact animals. **A**, *in vivo* electrophysiologic studies in $Ca_v1.3$ null mutant mice showing evidence of type I second degree AV block during sinus rhythm. Upper tracings are surface ECG (Lead I, II and aVF). Lower tracings are intracardiac electrograms showing atrial (A) and ventricular (V) electrograms and His bundle potential (H). **B**, Representative examples of spontaneous APs recorded from intact AV nodes from $Ca_v1.3^{+/+}$, $Ca_v1.3^{+/-}$ and $Ca_v1.3^{-/-}$ mice showing a significant decrease in the AV node firing frequency in $Ca_v1.3^{-/-}$ mice. **C**, Summary data from the three groups of animals for DDR (mV/s), CL (ms), MDP (mV), APA (mV), V_{max} (V/s), APD₅₀ (ms), and APD₈₀ (ms). * $P < 0.05$, $n = 9-12$ animals.

Figure 2. $I_{Ca,L}$ recorded from AV node cells from $Ca_v1.3^{-/-}$ shows depolarizing shift in the voltage-dependent activation. **A**, Examples of normalized whole-cell $I_{Ca,L}$ recorded from single isolated AV node cells from $Ca_v1.3^{+/+}$, $Ca_v1.3^{+/-}$, and $Ca_v1.3^{-/-}$ mice from a holding potential of -55 mV. The test potentials used are shown to the left of the current traces. **B**, Summary data for $I_{Ca,L}$ density-voltage relations from AV nodes isolated from the three groups of animals. **C**, Voltage-dependent activation curves showing normalized conductances (G/G_{max}) from $Ca_v1.3^{+/+}$ vs. $Ca_v1.3^{-/-}$ mice ($n=7-8$ for each group). The solid lines represent fits to the Boltzmann function yielding $V_{1/2}$ values of -11.8 vs. -5.2 mV and slope factors of 4.3 vs. 5.1 mV for $Ca_v1.3^{+/+}$ vs. $Ca_v1.3^{-/-}$, respectively (same symbol representation as in **B**). **D**, Voltage-dependent inactivation obtained by using two-pulse protocols (600-ms prepulses to various voltages followed by a test pulse to $+10$ mV) showing a typical U-shaped inactivation curve for Ca^{2+} -dependent inactivation of $I_{Ca,L}$ recorded from the three groups of animals. No significant difference in the Ca^{2+} -dependent inactivation was documented. **E**, Summary data of the fast and slow time constants of inactivation (τ_f and τ_s , respectively) from the three groups of animals. $*P < 0.05$.

Figure 3.

Subcellular distribution of $Ca_v1.3$ channel in mouse AV node cells. **A**, Photomicrographs of confocal laser scanning microscopy of immunostaining of AV node cells and atrial myocytes with anti- $Ca_v1.3$ and anti- α -actinin2 antibodies: (a) double staining with anti- $Ca_v1.3$ (green) and anti- α -actinin2 antibodies (red) in an AV node cell, (b) treatment with secondary antibodies only (anti-rabbit IgG-FITC conjugated and anti-mouse IgG-Texas Red conjugated antibodies) as a negative control, (c) double staining with anti- $Ca_v1.3$ (green) and anti- α -actinin2 antibodies (red) in a single atrial myocyte, respectively. Merged images are shown in the right panels. **B**, Photomicrographs of confocal laser scanning microscopy as in **A** except that anti-neurofilament 160 (NF 160) antibody (red) was used instead of anti- α -actinin2 antibody in AV node cells and atrial myocytes. NF 160 was used as an AV node marker. **C**, Photomicrographs of confocal laser scanning microscopy from single staining using anti- $Ca_v1.3$ antibody in AV node cells and atrial myocytes isolated from WT and $Ca_v1.3^{-/-}$ animals. Scale bars = 10 μ m.

Figure 4.

Expression of $\text{Ca}_v1.3$ channel protein in AV nodes. Photomicrographs of histologic sections of AV nodes and working myocardium from WT and $\text{Ca}_v1.3^{-/-}$ mice at low (left panels) and high magnification (right panels). **A**, Sections stained with Masson's trichrome. The broken yellow line outlines the AV node area. **B**, Expression of $\text{Ca}_v1.3$ channel protein (peroxidase positive) in the AV node and working myocardium in WT animals. **C**, Absence of $\text{Ca}_v1.3$ channel protein expression in the AV node and working myocardium in $\text{Ca}_v1.3^{-/-}$ mice. **D**, Photomicrographs of confocal laser scanning microscopy of immunofluorescence staining of consecutive AV node sections using anti- $\text{Ca}_v1.3$ ($\text{Ca}_v1.3$, upper panel) and anti-connexin 43 (Con43, lower panel) antibodies. DAPI was used for nuclear stain and are shown in the 2nd panels from the left. Differential interference contrast images are shown in the 3rd panels from the left (labeled as DIC). Merged images are shown in the 4th panels from the left (labeled as Merged). The 5th panels from the left represent the AV node region at higher magnification. Scale Bars=50 μm , AVN, AV node; VS, ventricular septum.

Figure 5. Mathematical Modeling of Spontaneous APs in Mouse AV node. **A**, Computer simulations of spontaneous APs from AV nodes in WT animals (solid line). A significant decrease in the firing frequency was observed in $Ca_v1.3^{-/-}$ animals (dashed line) by shifting the voltage-dependent activation of $I_{Ca,L}$ by 6.6 mV in the depolarizing direction as observed experimentally for $Ca_v1.3^{-/-}$ mice. **B**, First derivative (dV/dt) of the spontaneous APs from **A**. Inset shows the first derivative in larger scale illustrating a decrease in DDR in the AV node from $Ca_v1.3^{-/-}$ animals (dashed line) compared to WT animals (solid line).