Characterization of Sodium Channel α- and β-Subunits in Rat and Mouse Cardiac Myocytes

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Background—Sodium channels isolated from mammalian brain are composed of α-, β1-, and β2-subunits. The composition of sodium channels in cardiac muscle, however, has not been defined, and disagreement exists over which β-subunits are expressed in the myocytes. Some investigators have demonstrated β1 expression in heart. Others have not detected any auxiliary subunits. On the basis of Northern blot analysis of total RNA, β2 expression has been thought to be exclusive to neurons and absent from cardiac muscle.

Methods and Results—The goal of this study was to define the subunit composition of cardiac sodium channels in myocytes. We show that cardiac sodium channels are composed of α-, β1-, and β2-subunits. Nav1.5 and Nav1.1 are expressed in myocytes and are associated with β1- and β2-subunits. Immunocytochemical localization of Nav1.1, β1, and β2 in adult heart sections showed that these subunits are expressed at the Z lines, as shown previously for Nav1.5. Coexpression of Nav1.5 with β2 in transfected cells resulted in no detectable changes in sodium current.

Conclusions—Cardiac sodium channels are composed of α-(Nav1.1 or Nav1.5), β1-, and β2-subunits. Although β1-subunits modulate cardiac sodium channel current, β2-subunit function in heart may be limited to cell adhesion.

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Key Words: cells ▬ signal transduction ▬ genes ▬ ion channels

Voltage-gated sodium channels initiate action potentials in excitable cells. Brain sodium channels are composed of a single pore-forming α-subunit and 2 auxiliary β-subunits, with a stoichiometry of 1:1:1. β-Subunits do not form the ion-conducting pore but rather modulate channel gating and cell surface expression levels and interact with extracellular matrix and cell adhesion molecules. Recently, 2 additional members of the β-subunit gene family have been identified: β1A and β1B.

The subunit structure of cardiac sodium channels has not been well defined. At least 2 α-subunit mRNAs are expressed in heart: Nav1.5 and Nav1.1. β1-Subunit mRNA is expressed in rat and human heart but was not detected in mouse heart. β1-Subunit polypeptides have been demonstrated in rat heart; however, purified preparations of cardiac sodium channels from chicken and rat did not show detectable associated β-subunits after immunoprecipitation with α-subunit antibodies. Nav1.5 + β1 coexpression has been studied in heterologous expression systems with variable and conflicting results. β1A polypeptides and β1 mRNA have also been detected in heart. β2 transcripts are not detectable in heart by Northern blotting techniques. Thus, it was postulated that β1, β1A, and β2 may be expressed in heart, whereas β2 was most likely absent. Recent studies, however, have compelled us to reexamine this hypothesis. Nav1.5 sodium channels become permeable to Ca2+ after activation of protein kinase A. This mode of the channel, called slip-mode conductance, requires β1 and β2 coexpression with α. Nav1.5 α- and β2-subunits covalently associate in HEK293 cells; it was not determined, however, whether β2-subunits are expressed in cardiac muscle.

The purpose of this study was to define the sodium channel α- and β-subunits expressed in cardiac myocytes. Using specific antibodies, we identified Nav1.1, Nav1.5, β1, and β2. The developmental time course of β1 expression in heart shows that it is detectable by postnatal day 15. Nav1.5, β1, and β2 associate in cardiac myocytes, as do Nav1.1, β1, and β2. Immunocytochemistry revealed Nav1.1, β1, and β2 expression in adult cardiac muscle along the Z lines. Coexpression of Nav1.5 and β2-subunits in tsA201 cells did not result in any detectable changes in sodium current over α alone. We conclude that cardiac sodium channels contain β1- and β2-subunits and that either Nav1.1 or Nav1.5 α-subunits can form the ion-conducting pore.

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Antibodies

Antibodies to β1 or β2 used for Western blots were described previously. An extracellular epitope β1 antibody was obtained from Dr W.A. Catterall, University of Washington, and used for immunolocalization. Anti-β1-specific antibody or anti-rH1 antibody, each at 1:500 dilution, followed by secondary antibody conjugates were from Vector Laboratories.

Human Fetal Heart β2 cDNA Clone

A cDNA clone for a human fetal heart sodium channel β2-subunit was obtained as previously described (accession number AF107028). An extracellular epitope β2 antibody was obtained from Alomone Laboratories. The anti-rH1 antibody was raised in rabbit against the peptide D_{10}RLPKSDSEDPRALNQLS_{19}(C) with an additional C-terminal cysteine and affinity-purified. Anti–cardiac α-sarcomeric actin and anti–α-actinin were from Sigma. Fluorescent secondary antibody conjugates were from Vector Laboratories.

RT-PCR Analysis of Rat and Mouse mRNA

Poly-A mRNA was obtained from Clonetech. Reverse transcription–polymerase chain reaction (RT-PCR) was performed by use of the Titan One Tube PCR system (Roche Molecular Biochemicals). Primers CTFNS (5′-CTGTAACCCTCAACTCTCTTACCC-3′) and E3′ (5′-ATGACTGCCACCGTGAGTCCCGCTCTG-3′) were used. Each reaction contained 50 ng of mRNA. First-strand cDNA was synthesized at 55°C for 30 minutes, and then PCR was performed as follows: 94°C for 2 minutes; 10 cycles of 94°C for 15 seconds, 65°C for 30 seconds, and 68°C for 30 seconds; 25 cycles of 94°C for 15 seconds, 65°C for 30 seconds, and 68°C for 30 seconds, plus 5 additional seconds for each cycle. A final step for 7 minutes at 68°C was performed. This PCR amplifies a 335-bp product from mRNA and a 1061-bp product from genomic DNA that includes introns 1 and 2 of SCN2B.

Northern Blot Analysis of Total Mouse RNA

Samples of total mouse mRNA were purified with Trizol reagent (Life Technologies). Northern blot analysis was performed as previously described.
Figure 3. Cardiac β2-subunit expression. A, Rat heart membranes (50 μg per sample) were boiled in reducing (5% β-mercaptoethanol) or nonreducing SDS-PAGE sample buffer, separated on 10% acrylamide SDS-PAGE gels, and transferred to nitrocellulose. Blot was probed with anti-β2 antibody (1:500 dilution) and then with secondary antibody (1:100 000 dilution). B, Cardiac myocytes: total cell lysate (100 μg) of cardiac myocytes was separated on 10% acrylamide SDS-PAGE gel and transferred to nitrocellulose. Western blot was probed with anti-β2 antibody as in A. C, Developmental expression pattern of β2-subunits: Mouse hearts were dissected at indicated time points (except for lane A, in which whole embryos were homogenized), and membranes were prepared. Of each preparation, 50 μg was separated on 10% acrylamide SDS-PAGE gels and transferred to nitrocellulose. Western blot was probed with anti-β2 antibody as in A. Lane A, whole early embryos, ~E10; lane B, E19; lane C, P2; lane D, P10; lane E, P15; lane F, P22; lane G, P30; lane H, P40; lane I, P77; lane J, P138; lane K, P173 (E indicates embryonic; P, postnatal).

Figure 4. Coimmunoprecipitation of α- and β-subunits from heart membranes. Membranes were prepared and solubilized as described in Methods. Soluble fraction was immunoprecipitated with anti-rH1 antibody (1:500 dilution) as described. Western blots were probed with antibodies to β1 (left) or β2 (right) (1:500 dilution for each) and then with secondary antibody (1:100 000 dilution). NI indicates nonimmune serum; α, anti-rH1 antibody; β1, anti-β1 antibody; and β2, anti-β2 antibody.

Figure 5. Coimmunoprecipitation of α- and β-subunits from cardiac myocytes. Total myocyte lysates were solubilized and immunoprecipitated with anti-rH1 antibody (1:500 dilution) (A) or anti-SP11-I antibody (1:500 dilution) (B) as described in Methods. Western blots were probed as in Figure 4. In B, α indicates anti-SP11-I antibody; other abbreviations as in Figure 4.

Electrophysiology

Cell Culture

TSa201 cells (a gift from Dr Mohamed Chahine, University of Laval) were grown under standard conditions and transfected with Nav1.5 alone or Nav1.5+hβ2-, Nav1.5+hβ2+, or Nav1.5+hβ2-subunits with lipofectamine as described.23 hβ2 cDNA was a gift from Dr A. George, Vanderbilt University.

mRNA Extraction and RT-PCR

mRNA extraction and RT-PCR were performed with the mRNA Capture Kit and the Titan One Tube RT-PCR kit (Roche Molecular Biochemicals). The primers used were, for the detection of hβ2: SCN1B-F(5'-GACCAACGCTGAGACCTTC-3')/SCN1B-R(5'-CAGGAGCCATATGGTCAACAC-3'); for the detection of hβ1: hβ1n90 (5'-GGAGGTCACTACAGTCCACCCTGC-3')/SCN1A-R(5'-GACCAACGCTGAGACCTTC-3'); and for the detection of human β-actin: hβ-ACTIN556F (5'-CAGTGTTCCACTCTCGAGG-3')/hβ-ACTIN1169R(5'-CGGACTCGTCACTACTCC-TGCTT-3'). First-strand cDNA was synthesized at 60°C for 30 minutes, and then PCR was performed as follows: 94°C for 2 minutes, then 10 cycles of 94°C for 30 seconds, 55°C (for hβ2 and hβ1) or 45°C (for hβ2-actin) for 30 seconds, and 68°C for 45 seconds, followed by 25 cycles of 94°C for 30 seconds, 55°C or 45°C as described above for 30 seconds, and 68°C for 45 seconds, plus 10 additional seconds for each cycle. A final step for 7 minutes at 68°C was then performed.
Whole-Cell Voltage Clamp

Currents were measured at room temperature by whole-cell patch-clamp procedures with Axopatch 200B amplifiers (Axon Instruments) with previously described recording solutions and voltage protocols. Data were collected and analyzed with pClamp8 and Origin software (Axon Instruments and Microcal Software). The voltage-dependence of inactivation was determined by measuring current in response to pulses to \(-20\) mV that had been preceded by conditioning pulses (500 ms) to a series of voltages. Holding potentials were \(-100\) mV, and \([\text{Na}^+]_o\) was 130 mmol/L. For the voltage-dependence of activation, current was measured in response to pulses from \(-80\) to \(+60\) mV, and \([\text{Na}^+]_o\) was 10 mmol/L (with \(N\)-methyl-glucamine used as an equimolar \(\text{Na}^+\) substitute). Data are presented as mean\(\pm\)SEM. Two-tailed Student’s \(t\) test was used to compare means; a value of \(P<0.05\) was considered statistically significant. Data were filtered with a Boltzmann relationship, where \(V_{1/2}\) is the voltage where half of the channels are available (or activated) and \(k\) is the slope factor.

Results

Antibody Specificity

To determine the specificity of the rH1 antibody for Nav1.5, we performed Western blots of heart and brain membranes using anti-rH1 versus anti–SP11-I antibodies. Using anti–SP11-I, we observed that Nav1.1 is expressed both in brain and in cardiac myocytes, whereas Nav1.5 could be detected.
in myocytes but not in brain membranes with anti-rH1 (Figure 1A). Figure 1B shows that anti-rH1, but not anti–SP11-I, recognizes Nav1.5 sodium channels expressed in HEK 293 cells. These results indicated that the 2 antibodies are selective for the 2 channel subtypes and that cross-reactivity would not complicate our experiments.

Expression of $\beta_2$ mRNA in Heart
Our previous studies suggested that $\beta_2$ expression is limited to neuronal tissues.17 Thus, we decided to retest cardiac RNA for $\beta_2$ expression by more sensitive methods. RT-PCR showed that $\beta_2$ mRNA is expressed in mouse heart (Figure 2A). This experiment was designed with
Figure 8. RT-PCR analysis of tsA201 cells, performed as described in Methods. 8 μL of each RT-PCR product was separated on an agarose gel and stained with ethidium bromide. Left, SCN1B-F/SCN1B-R and hACTIN556F/hACTIN1169R reactions. Lane 1, standards (100-bp DNA ladder, GIBCO); lane 2, tsA201 nontransfected; lane 3, tsA201 transfected with Nav1.5, hβ1, and hβ2; lane 4, pcDNA3 vector containing hβ1 cDNA; lane 5, H2O. Right, hαβ2, tsA201 nontransfected; lane 3, tsA201 transfected with Nav1.5, hβ1, and hβ2; lane 4, pcDNA3 vector containing hβ2 cDNA; lane 5, H2O.

oligonucleotide primers flanking introns 1 and 2 of the β2 gene17 (Figure 2B) such that contaminating genomic DNA could be clearly separated from the lower-molecular-weight mRNA (cDNA) band (Figure 2A, arrows). Northern blot experiments confirmed our previous results that β2 transcript is not detectable in total heart RNA (data not shown). We conclude that β2 mRNA is present in cardiac muscle tissue, but at significantly lower levels than in brain.

β1 and β2 Associate With Cardiac Sodium Channels

Analysis of heart membranes in the presence and absence of β-mercaptoethanol showed the presence of an immunoreactive β2 band that shifted on reduction from >200 kDa to 33 kDa, indicating α-β2 covalent interactions (Figure 3A). Western blot analysis of primary cardiac myocytes with anti-β2 antibody revealed an immunoreactive band at ≈40 kDa (Figure 3B). Figure 3C shows the developmental time course of β2 expression in heart from early embryo to adulthood. β2–Subunits are expressed only after birth, becoming detectable at postnatal day 15.

Immunoprecipitation experiments showed that in heart membranes (Figure 4) as well as in myocytes (Figure 5A), Nav1.5 associates with β1 and β2. Figure 5B demonstrates that β1 and β2 are also associated with Nav1.1 in cardiac myocytes. Thus, sodium channels in cardiac myocytes are composed of α-, β1- and β2-subunits, and either Nav1.1 or Nav1.5 can form the ion-conducting pore.

Immunolocalization of Nav1.1 α, β1, and β2 in Heart

Previous immunolocalization of Nav1.5 revealed labeling of surface and T-tubular membrane systems of atrial and ventricular myocytes when viewed in cross section. In longitudinal sections, labeling was also observed at terminal intercalated disks in ventricular muscle in accordance with Z-line appearance.25 We used Nav1.1, β1, and β2 antibodies to investigate the localization of these subunits in longitudinal sections of cardiac muscle. As shown in Figure 6, A and D, both β1 and β2 colocalized with α-actinin (Figure 6, B and E), a marker for cardiac-muscle Z lines. As shown in Figure 7, A and B, Nav1.1 also showed a labeling pattern similar to that of α-actinin. Because anti–SP11-I antibody does not recognize Nav1.5 (Figure 1B), we could be confident that our results were not complicated by antibody cross-reactivity and that Nav1.1 is indeed expressed in the myocytes. Antibodies preadsorbed with peptides showed no specific signals (Figure 6, C and F; Figure 7C). The Nav1.1 α-, β1-, and β2-labeling results are similar to previous results for Nav1.5.25 Thus, Nav1.1, Nav1.5, and β-subunits are colocalized in heart muscle.

Electrophysiological Analysis

We used RT-PCR to investigate the presence of endogenous sodium channel auxiliary subunits in tsA201 cells. Figure 8 indicates that endogenous expression of β1 is not detected in our assays. In contrast, tsA201 cells do express β2 mRNA. Transfection with β1 strongly increased the level of β1 mRNA, however, suggesting that it might be possible to detect functional consequences of coexpression of Nav1.5 with both β-subunits in this cell line. We expressed Nav1.5 with and without hβ1 and hβ2 in tsA201 cells and studied the properties of the expressed channels (Table). We found no differences in peak current density due to hβ1 or hβ2, but did detect an effect of hβ2 on the voltage-dependence of inactivation. Coexpression of Nav1.5 with hβ2 caused a significant +5-mV shift in the half-maximal voltage-dependence of inactivation (V1/2), similar to previous reports.16 These data indicate that the endogenous levels of β1 expression in tsA201 cells are not sufficient to saturate the effect of β1 on

<table>
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<tr>
<th>Transfected Subunits</th>
<th>Current Density, pA/pF</th>
<th>V1/2 Inactivation, mV</th>
<th>k Inactivation</th>
<th>V1/2 Activation, mV</th>
<th>k Activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>SCN5A</td>
<td>−305 ± 57 (5)</td>
<td>−77.1 ± 0.5</td>
<td>4.9 ± 0.1 (5)</td>
<td>−25.6 ± 1</td>
<td>6.0 ± 0.3 (4)</td>
</tr>
<tr>
<td>SCN5A + hβ1</td>
<td>−296 ± 64 (5)</td>
<td>−72.9 ± 1.0*</td>
<td>4.7 ± 0.2 (5)</td>
<td>−24.9 ± 1</td>
<td>5.9 ± 0.1 (4)</td>
</tr>
<tr>
<td>SCN5A + hβ2</td>
<td>−308 ± 42 (7)</td>
<td>−71.1 ± 1.1</td>
<td>5.1 ± 0.1 (7)</td>
<td>−23.2 ± 1</td>
<td>5.9 ± 0.2 (5)</td>
</tr>
<tr>
<td>SCN5A + hβ1 + hβ2</td>
<td>−441 ± 63 (7)</td>
<td>−74.0 ± 1.0*</td>
<td>4.9 ± 0.2 (7)</td>
<td>−24.3 ± 2</td>
<td>5.8 ± 0.2 (5)</td>
</tr>
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Steady-state inactivation and activation curves were determined as described in Methods for a series of cells transfected with various subunit combinations. Inactivation and activation curves were fitted by Boltzmann functions. V1/2 is the voltage for which half the channels are inactivated or activated; k is the slope factor of the Boltzmann curve. Numbers of cells are in parentheses.
channels. Cardiac myocytes express was to define the subunit composition of cardiac sodium nels were most likely composed of Nav1.1 and Nav1.5, respectively, have also been identified in adult rat heart, with high-affinity receptors estimated to make up 25% to 50% of the total population of sodium channels.\(^5\) \(\beta_1\) mRNA and protein are expressed in heart tissue at high levels\(^6,9\); however, its association with \(\alpha\)-subunits has not been demonstrated. \(\beta_2\) transcripts are not detectable in total cardiac RNA by Northern blotting techniques.\(^17\) In purified preparations of chicken and rat cardiac sodium channels, \(\beta_2\)-subunits could not be detected at all.\(^10,11\) Thus, although a number of investigators presumed that cardiac sodium channels were most likely composed of \(\alpha\)- and \(\beta\)-subunits, this has remained controversial. The purpose of the present study was to define the subunit composition of cardiac sodium channels. Cardiac myocytes express \(\beta_1\) and \(\beta_2\) polypeptides, and Nav1.5 physically associates with both \(\beta\)-subunits. \(\beta_2\)-Subunit polypeptides are expressed postnatally in heart. \(\alpha\)-, \(\beta_1\)-, and \(\beta_2\)-subunits are localized to the Z lines in heart sections. We also identified Nav1.1 in cardiac myocytes and showed that it associates with \(\beta_1\) and \(\beta_2\). We conclude that sodium channels expressed in cardiac myocytes are composed of either Nav1.1 or Nav1.5 and that both associate with \(\beta_1\) and \(\beta_2\). Although \(\beta_2\) has modulatory effects on Nav1.5, \(\beta_1\) has no detectable effects in our system, suggesting that the effects of \(\beta_2\) in heart in vivo may involve cell adhesion and cytoskeletal communication as opposed to channel gating.

What is the physiological role of \(\beta\)-subunits in heart? Brain and skeletal muscle sodium channels expressed in oocytes exhibit slow inactivation kinetics. Coexpression of \(\beta_2\)-subunits produces a significant increase in the rate of inactivation of these channels.\(^27,28\) In contrast, expression of Nav1.5 in oocytes produces channels that inactivate rapidly in the absence of \(\beta\)-subunits.\(^15\) Some groups have reported that \(\beta_1\) has no observable effects on Nav1.5 functional expression.\(^7,12\) Others reported that coexpression of \(\beta_1\) and Nav1.5 results in increased current density with no detectable effects on channel kinetics or voltage-dependence.\(^13,14\) Some groups have found modulation of channel sensitivity to lidocaine block and subtle changes in channel kinetics and gating properties in response to \(\beta_2\) expression,\(^15\) whereas others have reported significant shifts in the voltage-dependence of steady-state inactivation, similar to the present results.\(^16\) A Nav1.5 mutation associated with long-QT syndrome affects the voltage-dependence of channel inactivation by altering the interaction of Nav1.5 and \(\beta_1\).\(^16\) Finally, Nav1.1 \(\alpha\)-subunits are modulated by \(\beta_1\)- and \(\beta_2\)-subunits when expressed in oocytes.\(^28\) Thus, \(\beta\)-subunits may modulate cardiac sodium channels and play a role in cardiac physiology.

\(\beta_1\) and \(\beta_2\) are cell adhesion molecules of the immunoglobulin superfamily.\(^27\) Both interact with extracellular matrix molecules and participate in homophilic cell adhesion, resulting in cellular aggregation and recruitment of ankyrin to the plasma membrane at points of cell-cell contact.\(^2\) We have proposed that a major function of \(\beta\)-subunits is cell adhesion, contributing to channel localization, clustering, and nodal formation in brain and peripheral nerve.\(^2\) Cardiac sodium channels reside at specific locations as well.\(^23\) In the present study, we observed Nav1.1, \(\beta_1\), and \(\beta_2\) labeling along Z lines in longitudinal sections. We have also observed \(\beta_1\) staining of surface membranes of cardiac myocytes when viewed in cross section.\(^2\) It has been proposed that cardiac sodium channels may be targeted and clustered to specific locations in a manner similar to that observed for sodium channels in brain.\(^25\) The presence of \(\beta\)-subunits in cardiac myocytes may facilitate sodium channel localization and clustering to discrete functional domains via cell-adhesive interactions. Treatment of inside-out patches of ventricular cells with cytochalasin-D induced sodium channels to enter a mode characterized by lower peak open probability with a greater persistent activity, consistent with a decrease in the rate of inactivation.\(^29\) Sodium channels in ankyrin\(\beta_2\)-knockout mice display reduced current density and abnormal kinetics that contribute to prolonged action potential duration and abnormal QT-rate adaptation.\(^30\) Thus, cytoskeletal interactions may be critical to sodium channel localization and gating in the heart as well as in the brain. We propose that the presence of \(\beta_1\)- and \(\beta_2\)-subunits in cardiac myocytes may facilitate channel-cytoskeletal interactions and play a key role in the regulation of the cardiac action potential.

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