Mouse heart Na\textsuperscript{+} channels: primary structure and function of two isoforms and alternatively spliced variants

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Zimmer, Thomas, Christian Bollensdorff, Volker Haufe, Eckhard Birch-Hirschfeld, and Klaus Benndorf. Mouse heart Na\textsuperscript{+} channels: primary structure and function of two isoforms and alternatively spliced variants. Am J Physiol Heart Circ Physiol 282: H1007–H1017, 2002. — We isolated two full-length cDNA clones from the adult murine heart that encode two different voltage-gated Na\textsuperscript{+} channels: mH1 and mH2. Sequence comparisons indicated that mH1 is highly homologous to rat SCN5A, whereas mH2 is highly homologous to SCN4A, expressed in rat skeletal muscle. Electrophysiological properties of mH1 channels strongly resembled the tetrodotoxin (TTX)-resistant Na\textsuperscript{+} current of mouse ventricular cells, whereas mH2 channels activated at more positive potentials and were highly sensitive to TTX [50\% inhibitory constant (IC\textsubscript{50}) = 11 nM]. We found that mH2 is not expressed in cardiac cells of neonatal mice, but appears to be upregulated during the development. Besides these Na\textsuperscript{+} channel isoforms, we also detected two alternatively spliced mH1 variants that were characterized by deletions within the sequence coding for the intracellular loop between domains II and III. One of the shortened channels, mH1–2, developed Na\textsuperscript{+} currents indistinguishable from those of mH1. The other splice variant (mH1–3) did not form functional channels. Quantitative reverse transcriptase-polymerase chain reaction indicated that RNA preparations of the adult mouse heart contain 54\% mH1, 25\% mH1–2, 16\% mH2, and 5\% mH1–3. Conclusively, mH1 generates the main portion of the mouse cardiac TTX-resistant Na\textsuperscript{+} current and mH2 is a candidate for TTX-sensitive currents previously described in adult cardiomyocytes. Furthermore, the presence of mH1–2 and mH1–3 transcripts indicates that alternative splicing plays a role in the regulation of functional Na\textsuperscript{+} channels in cardiomyocytes.

VOLTAGE-DEPENDENT Na\textsuperscript{+} channels are plasma membrane proteins that mediate the rapid increase in Na\textsuperscript{+} permeability during the initial phase of action potentials in various electrically excitable cells (11, 12). During the past decade, various Na\textsuperscript{+} channels have been cloned from different mammalian tissues, and their electrophysiological and pharmacological properties were characterized upon heterologous expression (14).

Na\textsuperscript{+} channels in the mammalian heart have been classified into two pharmacologically different groups according to their binding affinity for the specific inhibitor tetrodotoxin (TTX): investigating the TTX binding capacity of rat heart membranes, the coexistence of Na\textsuperscript{+} channels with low- (~75\%) and high-affinity (~25\%) binding sites for this drug has been shown (34, 35). Similar results were observed in human atrial cells (37). Electrophysiological measurements revealed a 50\% inhibitory constant (IC\textsubscript{50}) value of ~1 μM TTX for ventricular cells (5, 9, 10), demonstrating that the majority of Na\textsuperscript{+} channels in cardiomyocytes are relatively insensitive to the drug. Na\textsuperscript{+} channels with a significantly higher TTX sensitivity (IC\textsubscript{50} of 10–100 nM) have been suggested to contribute to the plateau phase of the action potential (15), and persistent Na\textsuperscript{+} currents with a high TTX sensitivity have been described in rat ventricular myocytes and in cultured human coronary myocytes (33, 36).

The gene coding for the TTX-resistant Na\textsuperscript{+} current (\(I_{\text{Na}}\)\textsubscript{r}) of cardiac cells has been identified by cloning and heterologous expression of SCN5A of the rat (rH1; 35) and human (hH1; 18). This isoform was shown as highly expressed in atrial and ventricular myocytes, and currents of heterologously expressed channels showed gating and blocking parameters of the native TTX-resistant \(I_{\text{Na}}\)\textsubscript{r} (13, 31, 40).

The molecular identity of cardiac TTX-sensitive Na\textsuperscript{+} channels, however, is less well understood. The neuronal isoforms SCN1A and SCN3A are expressed in the heart, and they have been discussed as candidate genes (35, 42, 44). However, respective transcripts were found in RNA preparations from both the neonatal and adult rat heart (35), whereas high-affinity TTX receptors are present only in adult, but not in newborn cardiac cells (34, 35). This result indicates that in myocytes of the newborn rat the neuronal isoforms do not produce TTX-sensitive receptors. This might be due to a too-low level of respective transcripts or due to a low efficiency of translation or intracellular channel

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trafficking. Furthermore, the possibility that these neuronal Na⁺ channel transcripts were derived from parasympathetic neurons was not excluded (42).

Beside these Na⁺ channel isoforms, two members of another Na⁺ channel subfamily (Na∞,2,1 and Na∞,2,3 of SCN6A) were isolated from the mammalian heart (17, 19). However, both isoforms have not yet been functionally expressed. Hence, their physiological relevance for the cardiac I Na remains to be elucidated.

The aim of this study was to provide more insight into the molecular basis for the cardiac I Na in the mouse heart. This organism has been developed to a powerful tool for molecular genetics, and mouse cardiomyocytes were used extensively by our group to study gating properties of single Na⁺ channels (2, 4, 8). However, sequences that code for TTX-resistant and TTX-sensitive channels have not yet been isolated. In the present study, we cloned and characterized the TTX-resistant Na⁺ channel from the mouse heart (SCN5A, termed mH1) and provide the nucleotide sequence and electrophysiological properties of a TTX-sensitive cardiac Na⁺ channel (termed mH2) that is unregulated during the development. Moreover, we screened for alternatively spliced variants of both genes by our polymerase chain reaction (PCR) approach and detected two shortened mH1 cDNAs. Implications for a possible role of mH2 channels and of alternative splicing of mH1 for cardiac excitability are discussed.

MATERIALS AND METHODS

Cloning of mH1 and mH2. Reverse transcription (RT) was performed using adult male mouse heart (strain BALB/c) total RNA purchased from Clontech, an equimolar mix of the anchored oligonucleotides dTAN, dTCN, and dTGN, and Superscript II according to the instructions of the supplier (GIBCO-BRL), followed by treatment of the final cDNA mixture with Escherichia coli RNAase H (Stratagene) for 20 min at 37°C. Aliquots of this cDNA (0.5 µl) were then used for the amplification reactions. Respectively PCR samples (volume 50 µl) contained 2 units of cloned Phusion DNA polymerase (Stratagene), the reaction buffer, and dNTP according to the instruction manual of the supplier. Cycle conditions were as follows: denaturation at 94°C for 1 min, annealing at 58°C for 1 min, and an extension time of 2 min/kb at 72°C. Product formation was observed after 30 cycles. For the isolation of mH1 and mH2, we used the primer pairs A1 to A8 (Table 1) and B1 to B4 (Table 2) to amplify eight and four fragments of the full-length mH1 and mH2 transcripts, respectively. The eight mH1 fragments (FA1 to FA8) and the four mH2 fragments (FB1 to FB4) were partially overlapping (see location of primer sequences in Tables 1 and 2) to allow for subsequent assembly either by a recombinant PCR approach or the use of common restriction sites (see below). Each of the eight mH1 and each of the four mH2 PCR fragments was subcloned into the HindIII site of plasmid pUC119. This cloning procedure resulted in the eight mH1 subclones pUC119-FA1 to pUC119-FA8 and in the four mH2 subclones pUC119-FB1 to pUC119-FB4. Because of the fidelity of thermostable DNA polymerases, PCR is known to produce partially DNA fragments with misincorporated nucleotides. The sequences of mH1 and mH2 were determined as follows. First, we sequenced two clones of each of the eight mH1 and of each of the four mH2 pUC119 derivatives. Second, we sequenced all amplicons directly using both PCR primers (see Tables 1 and 2) for the sequencing reactions. In this case, misincorporated nucleotides that are randomly distributed over the whole sequence in a certain molecule should not appear on the sequencing gel as the major band.

### Table 1. Sequences of primers for isolation and detection of mH1

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence (5’ to 3’) of the Forward/Reverse Primers</th>
<th>Location/mH1 Fragment (Length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>CCGTGGCGCCCAGAAGCGGATG/TCCTGGTAGGCATGGCCACAC</td>
<td>–28 to 1,253/FA1 (1,281 bp)</td>
</tr>
<tr>
<td>A2</td>
<td>AGCTGGGAAAGCTGTTGTTCG/GGAGGTGGAGAAGAGCAGATT</td>
<td>2,247 to 2,829/FA3 (582 bp)</td>
</tr>
<tr>
<td>A3</td>
<td>ATGTGATAGGGGAGGATGAACTTTG/AAAGAAGAGCAGGATT</td>
<td>2,772 to 3,645/FA4 (873 bp)</td>
</tr>
<tr>
<td>A4</td>
<td>CAGGGGAAAGGCTGGTCGCA/GGAGGATGAACTTTG/AAAGAAGAGCAGGATT</td>
<td>3,565 to 4,379/FA5 (814 bp)</td>
</tr>
<tr>
<td>A5</td>
<td>AAGGATGATGAACTTTG/AAAGAAGAGCAGGATT</td>
<td>4,261 to 4,940/FA6 (679 bp)</td>
</tr>
<tr>
<td>A6</td>
<td>GCAGGCAAGCTGTTGTTCG/AAAGAAGAGCAGGATT</td>
<td>4,896 to 5,689/FA7 (793 bp)</td>
</tr>
<tr>
<td>A7</td>
<td>ATGTGATAGGGGAGGATGAACTTTG/AAAGAAGAGCAGGATT</td>
<td>5,666 to 6,408/FA8 (742 bp)</td>
</tr>
<tr>
<td>A8</td>
<td>GCAGGCAAGCTGTTGTTCG/AAAGAAGAGCAGGATT</td>
<td>2,247 to 3,645/1,385bp</td>
</tr>
</tbody>
</table>

### Table 2. Sequence of primers for isolation and detection of mH2 and mouse SCN3A

<table>
<thead>
<tr>
<th>Primer Pair</th>
<th>Sequence (5’ to 3’) of the Forward/Reverse Primers</th>
<th>Location/mH2 Fragment (Length)</th>
</tr>
</thead>
<tbody>
<tr>
<td>B1</td>
<td>CTGTGGCCGCCAGAAGCGGATG/CTGAAGGAACTCAAAGAGAAGAGCCA</td>
<td>–27 to 2,400/FB1 (2,427 bp)</td>
</tr>
<tr>
<td>B2</td>
<td>ATGTGATAGGGGAGGATGAACTTTG/AAAGAAGAGCAGGATT</td>
<td>2,339 to 3,831/FB2 (1,492 bp)</td>
</tr>
<tr>
<td>B3</td>
<td>CAGGCAAGCTGTTGTTCG/AAAGAAGAGCAGGATT</td>
<td>3,761 to 5,043/FB3 (1,282 bp)</td>
</tr>
<tr>
<td>B4</td>
<td>GACITGTGATAGGGGAGGATGAACTTTG/AAAGAAGAGCAGGATT</td>
<td>4,906 to 5,913/FB4 (1,007 bp)</td>
</tr>
<tr>
<td>B5</td>
<td>GCAGGCAAGCTGTTGTTCG/AAAGAAGAGCAGGATT</td>
<td>3,127 to 3,837/(710 bp)</td>
</tr>
<tr>
<td>B6</td>
<td>GACITGTGATAGGGGAGGATGAACTTTG/AAAGAAGAGCAGGATT</td>
<td>2,077 to 2,744/667 bp</td>
</tr>
<tr>
<td>B7</td>
<td>CATCAAAATCCCTGCAGCGG/ATAAAGATGAGGAAAGAAGG</td>
<td>3,355 to 3,815/465 bp</td>
</tr>
</tbody>
</table>

*Relative to nucleotide A of start codon ATG in mH1. **Amplification with primer pair A4 produced additional fragments with a length of 714 (mH1–2) and 240 (mH1–3) bp and amplification with A9 gave two additional bands on the agarose gel at 1,239 (mH1–2) and 795 (mH1–3) bp.
The full-length mH1 clone (6.4 kb) was obtained as follows. We assembled the mH1 fragments FA1 to FA4 (encoding the mH1 region from −28 bp to 3,645 bp) with recombinant PCR (16). As the flanking primers for the recombinant PCR reaction, we used the oligonucleotides 5′-AAAGGATCCGGCTGGAGAAGATGGCGA-3′ (forward primer) and 5′-GAAGATGATGAAATCTGACCA-3′ (reverse primer). The forward primer contained—in addition to the mH1-specific region in FA1 (note underline)—a BamHI site (note italics) to allow for subsequent cloning into the expression vector pTSV40Gnew (see below). Restriction sites FsuI (overlapping region of FA4 and FA5 at 3,585 bp) and BsaBI (overlapping region of FA5 and FA6 at 4,268 bp) were used to link fragments FA5 and FA6, respectively. Fragment FA7 was fused to FA8, encoding the COOH-terminal amino acids of mH1 and a part of the 3′-untranslated region, by a recombinant PCR reaction using the following oligonucleotides: 5′-AAAGGATCCGCGATTCAGCCTAGGTGAT-3′ (forward primer) and 5′-AAAGGATCCGCGATTCAGGCTTACCT-3′ (reverse primer). The forward primer contained—in addition to the mH1-specific region in FA7 (note underline)—an XmaIII site (note italics) for the subsequent ligation to the same site in the 3′-region of FA6. The reverse primer contained—in addition to the mH1-specific region in FA8 (underline)—a SalI and NoI restriction site (italics) to allow for subsequent cloning into plasmid pUC119 (SalI) and into expression vector pTSV40Gnew (NoI; see below). The full-length mH1 fragment was finally ligated into the BamHI/SalI site of pUC119, resulting in pUC119-mH1.

To obtain the full-length mH2 clone (5.9 kb), the mH2 fragments FB1 to FB3, which were amplified with primer pairs B1 to B3 (see Table 2), respectively, were assembled with recombinant PCR using the oligonucleotides 5′-TCTGGCCCTGTGAGGAGGCTGAGATGAAATCCGATCC-3′ (pair A9, Table 1) and 5′-ACCTCTTTGGTCAGGGCAAAAGATCTCTCTCGG-3′ (reverse primer). The forward primer contained—in addition to the mH1-specific region in pair B1 (Table 2) and—a BglII and NotI restriction site (italics) to allow for subsequent cloning into plasmid pUC119 (BglII and NotI sites) and into expression vector pTSV40Gnew. Sequences of the assembled mH1 and mH2 constructs were confirmed by DNA sequencing analysis. Preparation, digestion, ligation, and sequencing of DNA were carried out according to established procedures (38).

RNA isolation and Northern blotting. For the analysis of the developmental pattern of mH1/mH2 expression, total RNA was isolated from the mouse heart (strain BALB/c) using RNeasy pure extraction solution (Qiagen, Hilden, Germany). Preparation of cDNAs and PCR were performed using the Oligotex Direct mRNA Mini Kit from Qiagen (Hilden, Germany). Finally, this cell pool was subject to mRNA isolation using the Oligotex Direct mRNA Mini Kit from Qiagen (Hilden, Germany). Preparation of cDNAs and PCR were performed as described above.

Northern blotting was carried out according to the method of Sambrook et al. (38) using 25 μg of total RNA loaded onto a formaldehyde-containing agarose gel. A 0.67-kb fragment of the mH1 cDNA encoding a part of the loop between domain I and II (1,353 to 2,026 bp) was amplified by PCR and used as the probe. Labeling reaction, hybridization, and signal detection were done using the AlkPhos Direct labeling system (Amersham Pharmacia Biotech) according to the instruction of the supplier.

Competitive PCR reactions and product quantification. The original cDNA mixtures were first prediluted 10- to 100-fold, and aliquots of these dilutions (0.5 μl) were used for the amplification reactions. To analyze the age dependency of mH1/mH2 expression (see Fig. 3), equal amounts of cDNAs were applied as PCR templates. These cDNAs were first normalized with respect to amplification of β-actin using the primers 5′-CCTGTATGCTCTGGTTCG-3′ and 5′-GTGTGTCGATAGGAGTCCT-3′. Conditions for the PCR reactions were essentially the same as described above. Signal intensity of bands of interest was determined with the use of a charge-coupled device camera and Easy Win32 software (Herolab, Wiesloch, Germany).

For the simultaneous detection of mH1 and mH2, primer pair B5 (Table 2) was used. PCR reactions produced a single band on the agarose gel (0.71 kb). To distinguish between mH1 and mH2, the PCR product was subject to the following endonuclease treatment: digestion with PsI yielded two DNA bands (at 0.57 and 0.14 kb) and the remaining mH2 signal (at 0.71 kb). PsI II produced two mH2 fragments (0.64 and 0.07 kb), whereas mH1 remained as a 0.71-kb fragment. To test for expression of SCN3A (accession NM_018732), we coamplified SCN3A and mH2 in a competitive RT-PCR reaction using primer pair B6. Digestion of the PCR products (0.67 kb for mH2 and 0.66 kb for SCN3A) with restriction endonuclease PsI II was expected to produce the following fragments (in kb): 0.60 and 0.07 for mH2 and 0.44, 0.16, and 0.06 for SCN3A.

The competitive PCR reactions for the amplification of mH1, mH1–2, and mH1–3 were carried out using primer pair A9 (Table 1). This allowed us to amplify fragments of all three isofoms at the same time in a single tube (1,398 bp for mH1, 1,239 bp for mH1–2, and 795 bp for mH1–3) and to separate and visualize the individual fragments on a 1% agarose gel.

Controls included PCR reactions using total RNA preparations as templates to prove for a possible amplification of genomic DNA. The relative mRNA ratios determined by competitive reactions were reproduced by amplification of the individual Na+ channel fragments from mixtures of respective plasmids coding for mH1, mH1–2, mH1–3, and mH2. We thereby applied different dilutions and various molecular ratios of the different plasmids. Quantitative PCR analysis confirmed the relative signal intensity of the bands on the agarose gel, indicating that our PCR system yielded reliable results.

Heterologous expression experiments. Full-length sequences of mH1, mH1–2, mH1–3, and mH2 were placed under the control of the SV40 promoter in pTSV40Gnew and expressed in HEK-293 cells. Vector pTSV40Gnew was obtained by two modifications of pTracerSV40 (Invitrogen). First, we exchanged the original coding region of the green fluorescent protein in pTracerSV40 by the coding region of the enhanced green fluorescent protein (Clontech) with PCR. Second, we placed the β-globin 5′-untranslated region of an in vitro transcription vector pGEMHEnew (26) in front of the SV40 promoter of pTracerSV40 using the Asp718I and BamHI sites. The insertion of the β-globin sequence was expected to enhance translation of the channel protein in the heterologous system. The mH1 sequence was released from pUC119-mH1 and ligated into the BamHI and NotI sites of pTSV40Gnew. The resulting vector was used to introduce the respective mH1–2 and mH1–3 deletions by an exchange of the mH1 sequence for respective mH1–2 and mH1–3 fragments using the common restriction sites BsoI and FspI.
HEK-293 cells were transfected by the calcium phosphate precipitation method and the currents were investigated 24–48 h after transfection.

Electrophysiology. All recordings were performed with the patch-clamp technique on the stage of an inverted microscope (Axiovert 100, Zeiss) using an Axopatch 200B amplifier (Axon Instruments; Foster City, CA). The measurements were carried out at room temperature. Whole cell currents were measured with standard techniques (22). For measurements of mH1 and mH1–2 currents, the bath solution contained (in mM) 20.0 NaCl, 120.0 CsCl, 0.1 CaCl2, 1.0 MgCl2, 10.0 glucose, and 10.0 HEPES, pH 7.4 (CsOH). The extracellular Na+ concentration was set to the low value of 20 mM to reduce the amplitude of IXNa and thus to improve the control of voltage. In the case of mH2-transfected cells, IXNa were significantly smaller. Therefore, we increased the current amplitude by using the following bath solution (in mM): 140.0 NaCl, 0.1 CaCl2, 1.0 MgCl2, 10.0 glucose, and 10.0 HEPES, pH 7.4 (CsOH). This bath solution was also applied when we measured mH1–3-transfected cells. Glass pipettes were pulled from borosilicate glass, and their tips were heat polished with the use of a microforge (model MF830, Narishige). The pipette resistance was between 2 and 3 MΩ when filled with the pipette solution containing (in mM) 10.0 NaCl, 130.0 CsCl, 10.0 EGTA, and 10.0 HEPES, pH 7.3 (CsOH). TTX was purchased from Biotrend (KölN, Germany) as the citrate salt and dissolved in water (1 mM stock solution). Currents were on-line filtered with a cutoff frequency of 10 kHz (4-pole Bessel). Recording and analysis of the data were performed on a personal computer with the use of ISO2 software (MFK; Niedernhausen, Germany). The sampling rate was 50 kHz.

Steady-state activation (m∞) was evaluated by fitting normalized conductance-voltage values to the Boltzmann equation 

\[ m_\infty = \frac{1 + \exp(-V - V_1/s)}{1 + \exp(V - V_0/s)} \]

Steady-state inactivation (h∞) was determined with a double-pulse protocol consisting of 500-ms prepulses to voltages between −120 and −30 mV, followed by a constant test pulse of 10-ms duration to either −30 mV (mH1) or to −10 mV (mH2) at a pulsing frequency 0.5 Hz. The amplitude of peak IXNa during the test pulse was normalized to the maximum peak current and plotted as a function of the prepulse potential. Data were fitted to the Boltzmann equation 

\[ h_\infty = \frac{1 + \exp((V - V_0)/s)}{1 + \exp(V - V_1/s)} \]

for the test potential, Vth the midactivation or inactivation potential, and s the slope factor in millivolts.

Student’s t-test was used to test for statistical significance. Statistical significance was assumed for P < 0.05.

RESULTS

Isolation of mH1 and mH2. To isolate an SCN5A channel gene that is expressed in the heart. Therefore, the mouse SCN4A cDNA encoding the α-subunit of the mouse heart Na+ channel (mH1), we separately amplified eight different subregions of the full-length transcript with RT-PCR and linked these individual fragments in frame with PCR or using common restriction sites in overlapping regions, as described in MATERIALS AND METHODS.

To ensure that the PCR fragments were indeed derived from the same transcript and to obtain finally the respective full-length sequence, the 3’-end of each fragment was overlapping with the 5’-end of the adjacent downstream fragment (for the length of respective overlapping regions compare location of primer sequences in Table 1). The primer pairs A1 to A8 (Table 1), used to generate the eight mH1 fragments FA1 to FA8, respectively (see MATERIALS AND METHODS), were designed according to the published SCN5A sequence of the rat (35) and they allowed the amplification of fragments with a size between 582 and 1,281 bp (Table 1). This strategy included the possibility to detect alternatively spliced variants that migrate differently on the agarose gel compared with the expected PCR product.

Fig. 1, top line, shows the deduced primary structure of the mH1 channel. The open reading frame predicted a 227,622-Da protein with 2,019 amino acids that consists of four large homologous domains. The nucleotide sequence of the coding region of mH1 was found to be 89.4% and 94.9% identical to SCN5A sequences of human and rat, respectively. The predicted amino acid sequence showed an identity to hH1 and rH1 of 94.0% and 98.3%, respectively. Potential sites for N-linked glycosylation and cyclic nucleotide-dependent phosphorylation as well as the proposed voltage sensors (S4 segments) and regions involved in TTX binding were identical compared with mH1 and hH1.

Besides the SCN5A isoform, we detected a second Na+ channel transcript in the adult mouse heart. PCR reactions with primer pair B2 (Table 2), originally designed to amplify a part of SCN5A, reproducibly resulted in the amplification of a second fragment that was homologous to the rat skeletal muscle Na+ channel (SCN4A) (47). The corresponding full-length clone was isolated by RT-PCR using primers based on the published sequence of rat SCN4A (Table 2, primer pairs B1 to B4). As in case of mH1 isolation, the selected primers allowed the amplification of partially overlapping fragments to ensure that the individual PCR products were derived from the same transcript (for location of primer sequences, see B1 to B4 in Table 2). Sequence analysis of the full-length cDNA revealed an open reading frame of 1,841 amino acids (Fig. 1, bottom line) and a molecular weight of the predicted protein of 208,799 Da. This second cardiac Na+ channel, termed mH2, was 63% identical to mH1 and showed high homology to SCN4A from the rat (97.7% identity) (47) and human (91.2% identity) (20).

Because the mouse SCN4A cDNA has not yet been reported, it remained unclear whether mH2 is indeed the product of SCN4A or of a closely related Na+ channel gene that is expressed in the heart. Therefore,
we analyzed a partial nucleotide sequence of the Na\(^+\) channel transcript of a skeletal muscle cDNA library (710 bp) and the corresponding chromosomal region (~3 kb) on PCR amplification of respective fragments using the primer pair B5 (see Table 2). Because the sequences of both primers corresponded to conserved regions of mH1 and mH2, we assumed that closely related Na\(^+\) channel transcripts might be detectable by PCR. As a result, the sequence of the amplified cDNA fragment and of the corresponding exon regions was identical to the corresponding mH2 region. Other Na\(^+\) channel transcripts including mH1 were not found in the skeletal muscle cDNA library.

These data indicate that mouse cardiac cells express two types of voltage-gated Na\(^+\) channels and that mH2 is the product of the mouse SCN4A gene.

Expression level of mH1 and mH2. To determine the relative ratio of the mRNA amounts of mH1 and mH2 in the heart, we performed a quantitative RT-PCR analysis. Using primer pair B5 (Table 2), we amplified a 710-bp fragment of both cDNAs in a competitive reaction and followed product formation at different cycle numbers. To distinguish between mH1 and mH2 fragments, PCR products were cleaved by specific endonucleases and analyzed on agarose gels (see MATERIALS AND METHODS). As shown in Fig. 2, the mH1 mRNA was the predominant Na\(^+\) channel transcript. For mH1/mH2, we found a molecular mRNA ratio of 0.84:0.16.

Several authors (35, 42, 44) reported the presence of a small fraction of neuronal Na\(^+\) channel transcripts in RNA preparations from the rat heart, suggesting that these Na\(^+\) channels contribute to the TTX-sensitive fraction of Na\(^+\) channels found in rat heart membranes (34). To compare the expression level of mH2 with that of the neuronal Na\(^+\) channel isoform SCN3A in mouse cardiac cells, we performed a competitive PCR. Because of the low degree of homology between SCN3A and mH2, we could not find sequences for primers that were identical in both cDNAs. The selected primers were specific for SCN3A, whereas amplification of mH2 included mismatches at two positions (see underlined nucleotides in primer pair B6, Table 2). As shown in Fig. 2A (lanes 4 and 5), the mH2 fragment generated the strongest signal on the agarose gel under these conditions (lane 5, larger fragment at 0.6 kb), whereas a minor fraction of the primary PCR product (shown in lane 4) was derived from SCN3A (lane 5, smaller fragment at 0.44 kb). This result confirms that the neuronal Na\(^+\) channel SCN3A is expressed in the heart. Compared with mH2, however, its expression level is clearly lower.

To get first insight into the transcriptional control of mH2 expression in the heart, we investigated the age-dependent level of mH2 mRNA by RT-PCR (Fig. 3). After birth and at day 9, mH2 transcripts were not detectable (P1 and P9 in Fig. 3). However, at day 38 (P38) and in adult animals, mH2 expression occurred, reaching values of up to 20% of mH1. To show that mH2 is expressed in cardiomyocytes, we collected 200 isolated myocytes from the adult mouse ventricle and used this cell pool for mRNA preparation and RT-PCR. As a result, the mH2 cDNA could be amplified using mH2-specific primers (pair B7), as confirmed by specific restriction analysis of the 0.46-kb PCR product (data not shown).

These data show that mH2 is not expressed before postnatal day 9 and that mH2 transcripts are present in adult ventricular cardiomyocytes of the mouse.

Identification of two alternatively spliced mH1 isoforms. Northern blotting analysis using a mH1-specific probe (see MATERIALS AND METHODS) yielded a distinct band at ~8.0 kb (Fig. 4A), suggesting that the primary
mH1 transcript is processed either to a single mRNA product or to alternatively spliced isoforms that do not differ significantly from the expected mH1 product. To test for the presence of such splice variants, we screened our mouse heart cDNA library by PCR using primer pairs A1 to A9 (Table 1) and isolated and sequenced all fragments that appeared in addition to the expected mH1 bands.

With the use of primer pairs A4 and A9, two additional bands appeared on the agarose gel (Fig. 4B). These fragments were smaller than the expected mH1 bands. Subcloning and sequencing analysis revealed two alternatively spliced isoforms of mH1. Respective splicing sites were located within the sequence coding for the intracellular loop connecting domains II and III of the mH1 channel (see Fig. 1). Splicing of mH1 occurred in frame and shortened the predicted full-length channel from 2,019 to either 1,966 (deletion of 201 amino acids; mH1–2; bold and boxed in Fig. 1) or 1,818 amino acids (deletion of 201 amino acids; mH1–3; bold in Fig. 1). The 3' splicing site was identical in both cases and was characterized by a motif that is conserved strictly at intron/exon boundaries (29, 30). The 5' end of the intron was different for mH1–2 and mH1–3. The respective exon/intron boundary of mH1–3 again showed a nucleotide pattern that regularly occurs at splicing positions (N/GT). The 5' splicing site of mH1–2 was marked by N/GA. Presupposing that the exon/intron architecture of the mouse SCN5A gene corresponds to the genomic organization of the human SCN5A gene (48), alternative splicing of the mH1 transcript resulted in the deletion of either exon 17 (mH1–2) or exon 17 and 18 (mH1–3; Fig. 4C).

To assess whether or not both alternatively spliced mH1 isoforms occur in myocardial cells in relevant amounts, a quantitative RT-PCR analysis was done using primer pair A9. This allowed the detection of mH1, mH1–2, and mH1–3 in a competitive reaction. Simultaneous amplification of the mH2 fragment was not observed using these primers. As shown in Fig. 5, mH1 mRNA was most abundant. At the same time, a considerable amount of mH1–2 mRNA was detectable, whereas mH1–3 occurred at a lower level. The molecular ratio of mH1/mH1–2/mH1–3 was found to be 0.65:0.29:0.06 and 0.68:0.24:0.08 after 35 and 40 PCR cycles, respectively. Because sequences for primer pair B5 used to determine the relative mRNA ratio of mH1/mH2 were located 3' to the mH1 splice sites, also mH1–2 and mH1–3 transcripts contributed to the intensity of the mH1 fragment on the agarose gel (see Fig. 2). Summarizing the relative ratio of all Na+ channel isoforms detected in this study, we conclude that RNA preparations of mouse heart contain about 54% mH1, 25% mH1–2, 16% mH2, and 5% mH1–3.

As a control, we proved that mH1, mH1–2, and mH1–3 indeed occur as full-length transcripts that do not have further nucleotide modifications within other parts of their sequence. For this purpose, we simultaneously amplified the respective full-length cDNAs by

![Fig. 3](image-url)  
Fig. 3. Developmental regulation of mH1 and mH2 transcription. A: simultaneous amplification of mH1 and mH2 in a competitive PCR reaction. RNA was prepared from the mouse heart after birth at the following developmental stages: day 1 (P1), day 9 (P9), day 38 (P38), and day 135 (adult). To distinguish between mH1 and mH2 fragments, the PvuII-digested RT-PCR products were loaded onto a 1% agarose gel. W, control PCR without cDNA template. B: fluorescence intensities of the corresponding mH1 and mH2 fragments were quantified as described in MATERIALS AND METHODS. Arbitrary units indicate signal intensities relative to the amplification of β-actin. Bars are means ± SE.

![Fig. 4](image-url)  
Fig. 4. Alternative splicing of mH1. A: Northern blot analysis with an mH1-specific probe demonstrating the presence of a ~8-kb mRNA transcript in the mouse heart. B: amplification of alternatively spliced mH1 variants using primer pair A9 (right lane). Additional fragments were excised from the agarose gel and the sequence of the shortened mH1 fragments was determined (see Fig. 1). The band between mH1 and mH1–2 was composed of an mH1/mH1–2 mixture and represented a heteroduplex between both isoforms, similarly as found for other splice variants (49). Left lane, fragments from the λ/EcoRI, HindIII marker (GIBCO-BRL). C: proposed patterns of alternative mH1 splicing. Constitutive exons (solid boxes), alternative sequences (shaded boxes), and introns (solid lines) are spliced due to 3 different pathways (dotted lines). Exon numbers are indicated according to the genomic organization of the human SCN5A gene (48).
PCR using primers that anneal in both flanking regions of the mH1 open reading frame, followed by restriction and sequencing analysis. Screening of the mouse heart cDNA library for shortened mH2 transcripts using primer pair B1 to B4 (Table 2) did not reveal alternatively spliced mH2 variants.

**Electrophysiological properties of mH1, mH1–2, mH1–3, and mH2.** The cDNAs of mH1, mH1–2, mH1–3, and mH2 were subcloned into a mammalian expression vector, and the channels were heterologously expressed in HEK-293 cells. Whole cell I\(_{\text{Na}}\) were measured with the patch-clamp technique.

Table 3 summarizes the kinetic parameters of respective I\(_{\text{Na}}\). We found that mH1 and mH1–2 generated indistinguishable I\(_{\text{Na}}\) with properties similar to those described for native cardiac Na\(^+\) channels (5, 6, 25) and other heterologously expressed SCN5A isoforms (13, 31, 40). In contrast, mH1–3 did not express functional channels (Fig. 6A). Expression of mH2 resulted in currents that could be clearly distinguished from those of mH1 and mH1–2. We found significant differences regarding the peak current amplitudes, and the activation and inactivation properties (Table 3, Fig. 6). In mH2, steady-state activation and inactivation was shifted by +15.0 mV and +10.1 mV, respectively (Fig. 6C), and the time constant of inactivation (\(\tau_{\text{i}}\)) was significantly smaller at all voltages, compared with mH1 (Fig. 6D). The kinetics of the recovery from inactivation was fitted with double-exponential functions, with a fast exponential (time constant \(\tau_f\) and amplitude \(A_f\)) and a slow exponential (time constant \(\tau_s\), amplitude \(A_s\)) function. As shown in Table 3, \(\tau_f\) was larger in mH1 than in mH2, whereas \(\tau_s\) in mH1 was smaller compared with the value in mH2.

**TTX sensitivity of mH1 and mH2 channels.** Figure 7 illustrates that mH2 channels were blocked by significantly lower toxin concentrations than mH1 channels. The IC\(_{50}\) values were ~11 nM and ~362 nM for mH2 and mH1 channels, respectively. In case of mH1–2 channels, we found an IC\(_{50}\) of ~373 nM, similar to the value observed for mH1 channels. These data indicate that mH2 channels of adult heart cells show a 33-fold higher TTX sensitivity compared with mH1.

**DISCUSSION**

The aim of the present study was to identify the types of channels underlying the voltage-dependent I\(_{\text{Na}}\) in the mouse myocardium. We cloned and characterized the TTX-insensitive cardiac Na\(^+\) channel mH1, the mouse-specific isoform of SCN5A, and we identified two alternatively spliced variants, mH1–2 and mH1–3. Furthermore, we show that cardiac cells express the TTX-sensitive Na\(^+\) channel mH2 that is highly homologous to rat SCN4A (47) and that exhibits gating properties distinct from mH1.

The fact that SCN4A is expressed in the myocardium is a new and surprising result, because this channel is the characteristic isoform of the skeletal muscle (47). We assume that mH2 channels also formed a large fraction of TTX-sensitive receptors previously found in cardiac membranes of the adult rat (34, 35). This conclusion is based on several findings. First, the developmental regulation of mH2 expression correlates with the appearance of TTX-sensitive binding sites during...
ontogeny. We did not detect mH2 transcripts in newborn mice or at day 9 but at day 38 and later (Fig. 3). Correspondingly, high-affinity TTX binding sites were not detected after birth, but their number gradually increased during development (34, 35). Second, for the ratio of the mH1 to mH2 transcripts, we found the values 84:16% in the adult heart. This ratio is in good agreement with previous TTX binding studies demonstrating a ratio between low- and high-affinity binding sites of 75:25% (34). Third, the TTX sensitivity of mH1 and mH2 channels differs by the factor of 33 (see Fig. 7 and Table 3). A similar factor was calculated from previous biochemical binding studies using a rat heart membrane fraction (34). Fourth, our competitive PCR for the simultaneous detection of mH2 and SCN3A in the adult heart indicated a clearly higher expression level of mH2 (Fig. 2, lane 5). This result indicates that the main fraction of TTX-sensitive Na\(^{+}\) channels is provided by mH2 channels and not by the neuronal

**Fig. 6.** Electrophysiological properties of heterologously expressed mH1, mH1–2, and mH2. All individual parameters are summarized in Table 3. Curves for mH1–2 were statistically not different from those of mH1. A and B: normalized peak current-to-voltage ratio (I-V) relationships. Currents were elicited for mH1 (n = 8), mH1–2 (n = 8), and mH2 (n = 9) from the holding potential of -120 mV to the indicated test potentials. The pulsing frequency was 5 Hz. HEK-293 cells transfected with mH1–3 did not express functional channels (n = 7). C: steady-state activation (m) and steady-state inactivation (h) as function of voltage. The fits were obtained as described in MATERIALS AND METHODS. D: time constant of inactivation as function of voltage for mH1 (n = 8) and mH2 (n = 7). Individual values were obtained from monoexponential fits. E: recovery from inactivation. Normalized data were fitted with double exponential functions yielding the fast and slow time constants \( \tau_f \) and \( \tau_s \), respectively (see Table 3). Bars are means ± SE.

**Fig. 7.** TTX sensitivity of mH1 (○) and mH2 (△) channels. Values from 4 independent measurements were fitted. The Hill coefficient was 1.0 for both curves. Statistically, the values for mH1–2 channels (n = 3) were indistinguishable from those for mH1 channels [for 50% inhibitory constant (IC\(_{50}\)) values, see Table 3]. \( I_{Na} \), Na\(^{+}\) current. Bars are means ± SE.
isoform SCN3A. Because of the lack of sequence information in the database, we did not investigate the cardiac mRNA levels of the other neuronal Na\(^+\) channels SCN1A and SCN2A. However, because several authors provided clear evidence for the cardiac expression of SCN1A (23, 28, 35, 42), it would be challenging to determine the respective cDNA sequence to correlate the mRNA level of SCN1A to that of mH2 by competitive RT-PCR.

The fact that mH2 channels are expressed in the heart might also answer the question why the accessory Na\(^+\) channel β1-subunit is expressed in this organ at all. Heterologously expressed SCN5A channels in Xenopus oocytes produce \(I_{Na}\) currents with normal activation and inactivation characteristics also in the absence of the β1-subunit (27, 31). This finding suggested that the β1-subunit is not required for a normal function of cardiac Na\(^+\) channels. The SCN4A channel isoform, however, unequivocally requires the accessory β1-subunit for fast activation, inactivation, and recovery from inactivation (31). We also expressed mH2 channels in frog oocytes and observed similar effects of the β1-subunit (data not shown). Therefore, we suggest that the β1-subunit is an important modulator of the gating of mH2 and of the neuronal Na\(^+\) channels in the heart.

Alternative splicing has been reported to occur in the biosynthesis of innumerable proteins (1, 24, 45), including voltage-gated Na\(^+\) channels of the rat brain (21, 32, 39, 42) and Drosophila (46). In case of the cardiac SCN5A isoform, alternatively spliced variants have not been reported so far. With respect to the physiological role of the alternatively spliced Na\(^+\) channels for the cardiac \(I_{Na}\), it is presently only possible to speculate. One possibility is that cardiac cells respond to certain stress factors via alternative splicing, rather than via a transcriptional regulation, to adjust the amplitude of \(I_{Na}\). This idea is attractive on the basis of the observation that mH1–3 channels did not functionally express and that the formation of inactive splice variants is well known for a variety of proteins as a mechanism controlling their activity in the cells (7, 43, 45). As shown for SCN3A (42) and SCN8A (32) channels, alternative splicing can also lead to a disruption of the open reading frame, resulting in the introduction of a stop codon and the translation of a significantly shorter and probably nonfunctional protein.

The functional consequence of the splicing event leading to mH1–2 channels is presently not clear. Our electrophysiological data did not reveal a significant difference between mH1 and mH1–2 channels. Furthermore, screening for a functionally relevant protein motif within the intracellular loop connecting domains II and III did not reveal a specific pattern that might be involved in the regulation of mH1 channels.

Further electrophysiological measurements and investigations on the tissue distribution might help to elucidate the role of mH1–2 and mH2 channels for cardiac excitability, possibly also for the so far inactive mH1–3 channels. These investigations may finally contribute to a better understanding of the molecular basis of heart diseases and support the development of clinically relevant antiarrhythmic drugs.

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