Modulation of Na\textsubscript{\text{\textalpha}} 1.5 Channel Function by an Alternatively Spliced Sequence in the DII/DIII Linker Region*

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In the present study, we identified a novel splice variant of the human cardiac Na\textsuperscript{\textplus} channel Na\textsubscript{\textalpha} 1.5 (Na\textsubscript{\textalpha} 1.5d), in which a 40-amino acid sequence of the DII/DIII intracellular linker is missing due to a partial deletion of exon 17. Expression of Na\textsubscript{\textalpha} 1.5d occurred in embryonic and adult hearts of either sex, indicating that the respective alternative splicing is neither age-dependent nor gender-specific. In contrast, Na\textsubscript{\textalpha} 1.5d was not detected in the mouse heart, indicating that alternative splicing of Na\textsubscript{\textalpha} 1.5 is species-dependent. In HEK293 cells, splice variant Na\textsubscript{\textalpha} 1.5d generated voltage-dependent Na\textsuperscript{\textplus} currents that were markedly reduced compared with wild-type Na\textsubscript{\textalpha} 1.5. Experiments with mexiletine and 8-bromo-cyclic AMP suggested that the trafficking of Na\textsubscript{\textalpha} 1.5d channels was not impaired. However, single-channel recordings showed that the whole-cell current reduction was largely due to a significantly reduced open probability. Additionally, steady-state activation and inactivation were shifted to depolarized potentials by 15.9 and 5.1 mV, respectively. Systematic mutagenesis analysis of the spliced region provided evidence that a short amphiphilic region in the DII/DIII linker resembling an S4 voltage sensor of voltage-gated ion channels is an important determinant of Na\textsubscript{\textalpha} 1.5 channel gating. Moreover, the present study identified novel short sequence motifs within this amphiphilic region that specifically affect the voltage dependence of steady-state activation and inactivation and current amplitude of human Na\textsubscript{\textalpha} 1.5.

Voltage-gated Na\textsuperscript{\textplus} channels of mammals constitute a family of 10 different members (1) that can be extensively modified by alternative RNA splicing (2). The respective transcript variability includes (a) the alternative usage of 5¢- and 3¢-untranslated regions (3), (b) exon skipping events within the coding region (4–9), (c) mutually exclusive exon splicing (4, 9–11), (d) intron-to-exon conversion (12), and (f) extension or partial deletion of an exon due to the usage of alternative donor/acceptor splice sites (9, 13–17). Modifications of mammalian Na\textsuperscript{\textplus} channel transcripts by alternative splicing result either in the formation of functional proteins that show unchanged or only marginally altered electrophysiological properties compared with the wild-type channel (7, 14), or, as in most cases, in truncated proteins that are not expected to form functional Na\textsuperscript{\textplus} channels (4, 7, 8, 12, 13, 17). Suppression of Na\textsuperscript{\textplus} channel activity could be physiologically important for embryonic excitable cells or for adult nonexcitable tissues that express an undesired Na\textsuperscript{\textplus} channel isoform constitutively (4, 18).

In the mammalian heart, the Na\textsuperscript{\textplus} channel isoform Na\textsubscript{\textalpha} 1.5 is thought to determine the major electrophysiological and pharmacological properties of the I\textsubscript{\textalpha}EK in atrial and ventricular cardiomyocytes (1, 19). The mouse heart expresses two alternatively spliced variants of Na\textsubscript{\textalpha} 1.5, Na\textsubscript{\textalpha} 1.5a (mH1–2) and Na\textsubscript{\textalpha} 1.5b (mH1–3), both with deletions in the DII/DIII intracellular loop (7). Splice variant Na\textsubscript{\textalpha} 1.5a, which has been also discovered previously in a rat hippocampal progenitor stem cell line (HiB5) (6), generates currents indistinguishable from wild-type Na\textsubscript{\textalpha} 1.5, whereas Na\textsubscript{\textalpha} 1.5b does not form functional channels. The first human Na\textsubscript{\textalpha} 1.5 splice variant detected was Na\textsubscript{\textalpha} 1.5c (hH1c) (15), which lacks a single amino acid at position 1077 and represents the preferred alternatively spliced variant in the heart. Electrophysiological measurements in HEK293 cells revealed a faster recovery from inactivation and a shift of steady-state inactivation by 7 mV toward depolarized potentials in Na\textsubscript{\textalpha} 1.5c (hH1c) versus Na\textsubscript{\textalpha} 1.5 (hH1) channels. In addition to these naturally occurring splice variants, two different mutations in intronic sequences of the human cardiac Na\textsuperscript{\textplus} channel were shown to be responsible for completely skipping exon 22 or for partially deleting exon 27, causing Lenègre disease (8) or Brugada syndrome (17), respectively.

In the present study, we describe a novel splice variant of Na\textsubscript{\textalpha} 1.5 (named Na\textsubscript{\textalpha} 1.5d) that could be detected in all human heart RNA samples investigated. This splice variant was characterized structurally by a 40-amino acid deletion within the intracellular DII/DIII loop, and functionally by altered Na\textsuperscript{\textplus} current amplitude and gating compared with wild-type Na\textsubscript{\textalpha} 1.5. Moreover, structure-function analysis within the DII/DIII linker provides evidence for the importance of short sequence motifs for steady-state activation and inactivation of the human heart Na\textsubscript{\textalpha} 1.5 channel.

EXPERIMENTAL PROCEDURES

Source of Human RNA and cDNA Synthesis—Total RNA samples from whole human hearts were purchased from Invitrogen and from Stratagene. The RNA preparation from Invitrogen was obtained from a pool of eight male and female hearts (25–73 years; Fig. 1, lane 2). The samples from Stratagene were obtained from five male embryonic hearts (18 (two hearts), 19, 20, and 21 weeks) (Fig. 1, lane 3), from three female embryonic hearts (16, 20, and 22 weeks) (Fig. 1, lane 4), from a single male patient (72 years) (Fig. 1, lane 5), and from three adult female hearts (23, 27, and 48 years) (Fig. 1, lane 6). Reverse transcription was performed using an equimolar mix of the poly(A)-anchored oligonucleotides dTAN, dTCN, and dTGN and Superscript II according to the suggestions of the supplier (Invitrogen), followed by treatment of the cDNA mixture for 20 min at 37°C with Escherichia coli RNase H (MBI Fermentas).

Competitive PCRs and Product Quantification—The original cDNA mixtures were first prediluted 10-fold, and aliquots of these dilutions
(0.5 μl) were used for the amplification reactions. Competitive PCRs were performed with Pfu DNA polymerase (Promega, Madison, WI) according to the recommendation of the supplier. Cycle conditions were as follows: denaturation at 94 °C for 1 min, annealing at 58 °C for 1.5 min, extension at 72 °C for 3 min (30 cycles). Simultaneous amplification of the human Na1.5 variants was carried out using forward primer 5′-AAAGAATTCTTTCAGTGACAACTCCTCAC-3′ and reverse primer 5′-GAAGATGATGAAGTGTCAGACAA-3′, resulting in signals at 825 bp (Na1.5) and 705 bp (Na1.5d). Mouse Na1.5 variants were amplified using forward primer 5′-GGCGTGTTGCTGAGTCAAGACAC-3′ and reverse primer 5′-TTGACACCCACCCGGCATGGCCTCTAAA-3′, resulting in signals at 823 bp (Na1.5) and 664 bp (Na1.5a). Na1.5b was amplified only from the mouse heart cDNA and appeared as a faint band only after >35 cycles (not shown in this study; for Na1.5b data, see Ref. 7). Product quantification was done by determining the fluorescence intensities of bands of interest from an agarose gel (1%), using a CCD camera and the E.A.S.Y. Win32 software from Herolab (Wiesloch, Germany). In the present study, we always corrected the respective intensity values according to the product sizes in order to obtain the molar ratios. Controls included PCRs using total RNA preparations as templates to prove for a possible amplification of genomic DNA. The reliability of the assay conditions was tested by amplification of the Na1.5 and Na1.5d fragments using mixtures of respective plasmids, similarly as previously described (7).

**Construction of Expression Plasmids**—Wild-type human Na1.5 (hH1; kindly provided by Dr. George and Dr. Kallen) (20) was placed under the control of the SV40 promoter in expression plasmid pTSV40G-Na1.5 by inserting its coding region as a HindIII/XbaI fragment into the Asp718/SpeI site of pTSV40G after Klenow treatment of the HindIII and Asp718 sites. Vector pTSV40G was obtained by exchanging the original coding region of the green fluorescent protein (GFP)7 in pTracerSV40 (Invitrogen) by the coding region of the enhanced GFP from Clontech by means of PCR. Expression plasmids for splice variant Na1.5d and all genetically modified variants were obtained by recombinant PCR as follows. We used specific primers to amplify hH1 nucleotide regions located directly upstream (1.1 kb) and downstream (1.8 kb) to the sequences that had to be deleted. Respective fragments were then assembled using a unique restriction site that was incorporated into the PCR primers. Finally, the resulting fragment was exchanged for the wild-type region in pTSV40G-Na1.5 using the EcoR1/Asp718 sites in the human Na1.5 sequence. For in vitro transfection, the coding region of all constructs were recloned into a pSP64Poly(A) vector (Promega) using the unique sites HindIII/XbaI. Plasmids for expression of Na1.5-GFP and Na1.5d-GFP in HEK293 cells were constructed as previously described (21). Pfu Turbo DNA polymerase (Stratagene) was used for all PCRs to minimize PCR-mediated nucleotide exchanges. Two independent PCR clones of each mutated Na1.5 construct were tested in the heterologous expression system. The correctness of the DNA constructs was checked by the dyeoxy-DNA sequencing method. Preparation, digestion, and ligation of DNA were carried out according to standard procedures (22).

**Heterologous Expression in HEK293 and Xenopus Oocytes**—HEK293 cells were cultured in minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B. Cells were transfected by a standard calcium phosphate precipitation method using 1–2 μg of plasmid DNA per transfection (60-mm cell culture dishes). After an incubation time of 24 h, the transfection mixture was removed, and the cells were seeded onto poly-L-lysine-coated glass coverslips and cultured in fresh growth medium. Currents were investigated 24–48 h after transfection. Mexiletine treatment was done as recently described (23). Briefly, 24 h after transfection, cells were incubated in mexiletine-containing medium (500 μM) for 24 h before measuring whole-cell currents.

Preparation of oocytes from Xenopus laevis, in vitro transcription, and cRNA injection was done as previously described (24). Aliquots of the different cRNA samples were loaded onto an agarose gel, and relative fluorescence intensities of the bands were measured with the gel documentation system from Herolab (Wiesloch, Germany). The different cRNAs were diluted accordingly. Whole-cell currents in oocytes were between 0.4 and 5 μA.

**Laser-scanning Microscopy**—Confocal imaging of HEK293 cells was performed with a Zeiss LSM 510 (Carl Zeiss GmbH, Jena, Germany), as previously described (21).

**Electrophysiology**—Recordings using HEK293 cells were performed with the patch clamp technique on the stage of an inverted microscope (Axiovert 100, Carl Zeiss Jena GmbH, Germany), 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 (25). The bath solution contained 140.0 mM NaCl, 0.1 mM CaCl2, 1.0 mM MgCl2, 10.0 mM glucose, 10.0 mM HEPES, pH 7.35 (CsOH). The pipette solution contained 10.0 mM NaCl, 130.0 mM CsCl, 10.0 mM EGTA, 10.0 mM HEPES, pH 7.35 (CsOH). Currents were elicited by test potentials from −120 to 40 mV in 10-mV increments at a pulsing frequency of 0.2 Hz. We used only cells that produced a peak current amplitude <5 nA. Steady-state activation (m∞) was evaluated by fitting the Boltzmann equation m∞ = (1 + exp((V − Vm)/s))−1 to the normalized conductance as a function of voltage. 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 −10 mV at a pulsing frequency of 0.5 Hz. The amplitude of peak h∞ 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∞ = (1 + exp((V − Vm)/s))−1. V is the test potential, Vm and Vs are the midactivation and midinactivation potentials, respectively, and s is the slope factor in mV. Glass pipettes were pulled from boro-silicate glass, and their tips were heat-polished by the microforge MF 830 (Narishige, Japan). The pipette resistance was between 2 and 3 megahms. Series resistance compensation was adjusted so that any oscillations were avoided, leaving at most 25% of the series resistance uncompensated. Currents were on-line filtered with a cut-off frequency of 10 kHz (4-pole Bessel). Recording and analysis of the data were performed on a personal computer with the ISO2 software (MFK, Niernhausen, Germany). The sampling rate was 50 kHz.

For mexiletine-treated HEK293 cells, anti-CD8 beads (Dynal) were used to identify transfected cells (23). In parallel experiments, we performed initial studies on Na+ channel subcellular localization, and therefore we used at the same time the yellow fluorescent protein-tagged channel variants for mexiletine treatment. As shown previously (21), GFP/yellow fluorescent protein tagging does not alter Na+ channel properties. Patch clamp recordings were carried out using an internal solution containing 60.0 mM CsCl, 70.0 mM CsAsp, 11.0 mM EGTA, 1.0 mM MgCl2, 1.0 mM CaCl2, 10.0 mM HEPES, and 5.0 mM Na2ATP, pH 7.2 (CsOH). The external solution contained 130.0 mM NaCl, 2.0 mM CaCl2, 1.2 mM MgCl2, 5.0 mM CsCl, 10.0 mM HEPES, and 5.0 mM glucose, pH 7.4 (CsOH). The holding potential was −100 mV. For these

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2 The abbreviations used are: GFP, green fluorescent protein; PKA, protein kinase A; 8-Br-cAMP, 8-bromo-cyclic AMP; CaM, calmodulin.
whole-cell measurements, we used a VE-2 amplifier (Alembic Instruments). The resistance of the pipettes was between 1.5 and 3.0 megaohms.

Single-channel recording was performed similarly as described previously (21). Briefly, the pipette resistance was between 10 and 50 megaohms when filled with the following solution: 140.0 mM NaCl, 2.5 mM CaCl$_2$, 4.0 mM KCl, 1.0 mM MgCl$_2$, 5.0 mM HEPES, pH 7.4 (NaOH). The bath solution contained 140.0 mM KCl, 1.0 mM MgCl$_2$, 5.0 mM EGTA, 10.0 mM HEPES, pH 7.4 (KOH). Recordings were on-line filtered with a cut-off frequency of 5 kHz (4-pole Bessel).

Whole-cell Na$^+$ currents in oocytes were recorded with the two-microelectrode voltage clamp technique. The glass microelectrodes were filled with 3 M KCl. The microelectrode resistance was between 0.2 and 0.5 megaohms. The bath solution contained 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl$_2$, 1 mM MgCl$_2$, 10 mM HEPES, pH 7.2 (KOH). The currents were elicited by test potentials from −80 to 40 mV in 5-mV increments from a holding potential of −120 mV. The pulsing frequency was 0.2 Hz. For stimulation of protein kinase A (PKA), 8-bromoadenosine-3′-5′-cyclic monophosphate (8-Br-cAMP; JenaBioscience) was added to the bath solution at a final concentration of 100 μM, similarly as described previously (26).

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

**RESULTS**

**Identification of a Novel Human Na$_{1.5}$ Splice Variant—**Detection of the splice variant Na$_{1.5}$a in both rat and mouse (6, 7) suggested that alternative splicing of exon 18 of the cardiac Na$^+$ channel is conserved among mammals. To test for the presence of Na$_{1.5}$a in the human heart, we purchased total RNA isolated from a pool of eight male and female hearts (25–73 years) and performed a respective reverse transcription-PCR analysis. Forward and reverse primers were specific for a region within the flanking exons 17 and 20, respectively. As a control, we first used a mouse heart cDNA library to reproduce the previously obtained Na$_{1.5}$a pattern (see Ref. 7). As shown in Fig. 1 (lane 1), the fragment corresponding to mouse Na$_{1.5}$a was most abundant (−65%), followed by lower levels of Na$_{1.5}$a (−35%). In the human heart, Na$_{1.5}$a was not detected (Fig. 1, lane 2). Instead, we found, in addition to the Na$_{1.5}$ signal, a band that was larger than splice variant Na$_{1.5}$a. Isolation of this fragment and sequencing analysis revealed a novel human Na$_{1.5}$ splice variant. We termed this splice variant Na$_{1.5}$d. It was characterized by an in-frame deletion of a 120-bp fragment of exon 17 (Fig. 2A). As further shown in Fig. 1, the level of Na$_{1.5}$d in the whole heart was relatively low (−2–3% of Na$_{1.5}$). When investigating Na$_{1.5}$d levels in male and female hearts at two different developmental stages (embryonic, adult), we did not find significant differences in the Na$_{1.5}$/Na$_{1.5}$d ratio (Fig. 1, lanes 3–6). The fact that Na$_{1.5}$d appeared over a long range during ontogeny in both male and female hearts indicates that this splice variant is constantly produced in the human heart under normal physiological conditions. Interestingly, alternative splicing of Na$_{1.5}$ is species-dependent; we neither detected Na$_{1.5}$a in humans nor Na$_{1.5}$d in mice.

**Effect of Alternative Splicing on Whole-cell Currents—**In order to investigate the functional consequences of the deletion in Na$_{1.5}$d, we...
expressed Na\(_{1.5d}\) heterologously in HEK293 cells and analyzed the whole-cell currents. Na\(_{1.5d}\) generated voltage-dependent Na\(^+\) currents that were markedly reduced compared with wild-type Na\(_{1.5}\) (Fig. 3A). Because of the significant cell-to-cell variability of whole-cell currents often observed upon transient transfection, we verified functional surface expression in the Xenopus oocyte system. When injecting equal amounts of wild-type Na\(_{1.5}\) and Na\(_{1.5d}\) cRNA, the whole-cell currents of Na\(_{1.5d}\) were reduced to a similar degree (Fig. 3B). This suggests that current reduction is caused neither by an insufficient transfection nor reduced transcription efficiency. Consequently, the reduction in current density can only be due to intrinsic properties of Na\(_{1.5d}\) in the plasma membrane or due to abnormal trafficking and reduced surface expression.

Abnormal cardiac ion channel trafficking has previously been rescued pharmacologically or by temperature reduction (23, 27, 28). To investigate whether or not Na\(_{1.5d}\) is a trafficking-deficient splice variant, we tested the effect of mexiletine, a Na\(^+\) channel blocker that is capable to restore a deficient trafficking phenotype (27). As shown in Fig. 4A, a 24-h mexiletine treatment of Na\(_{1.5d}\) channels in HEK293 cells did not produce whole-cell currents that were comparable with those generated by wild-type Na\(_{1.5}\). The drug enhanced cell surface expression in both Na\(_{1.5}\) (1.9-fold) and Na\(_{1.5d}\) (2.6-fold). Similar data were obtained when expressing the channels at a lower temperature (28°C) in HEK293 cells; Na\(_{1.5d}\) currents remained significantly smaller compared with Na\(_{1.5}\) (data not shown). Oocyte expression was also performed at a low temperature (18°C), and as shown in Fig. 3B, currents remained significantly lower in Na\(_{1.5d}\) compared with Na\(_{1.5}\).

Finally, we tested whether or not PKA differentially modulates surface expression of both channels. It has been shown previously that PKA enhanced trafficking of the human cardiac Na\(^+\) channel in Xenopus oocytes (26). We speculated that the loss of the potential PKA site in Na\(_{1.5d}\) (Fig. 2B) may underlie this current decrease and that a PKA-dependent activation of channel trafficking occurs only in Na\(_{1.5}\) and not in Na\(_{1.5d}\) channels. We added 8-Br-cAMP, a membrane-permeable PKA activator, to the bath solution of injected oocytes and determined peak Na\(^+\) currents by the two-microelectrode voltage clamp technique. Current mediated by Na\(_{1.5d}\) channels could be increased similarly to the wild-type current (Fig. 4B). This indicates that the potential PKA site in the DII/DIII linker of Na\(_{1.5}\) is not responsible for the PKA-mediated rise in Na\(^+\) current and suggests that reduced whole-cell currents in Na\(_{1.5d}\) are not due to the lack of the potential PKA site in this splice variant.

**Subcellular Localization of Na\(_{1.5}\) and Na\(_{1.5d}\) in HEK293 Cells**—Current enhancement by mexiletine and 8-Br-cAMP was similar in Na\(_{1.5}\) and Na\(_{1.5d}\), and Na\(_{1.5d}\) whole-cell currents were only marginally affected at a reduced temperature. In order to investigate the trafficking and subcellular localization if this Na\(_{1.5d}\) variant, we studied Na\(_{1.5}\) and Na\(_{1.5d}\) channels labeled with the green fluorescence protein (GFP) in HEK293 cells (Fig. 5). We did not observe an obvious difference between cell populations expressing either Na\(_{1.5}\)-GFP or Na\(_{1.5d}\)-GFP. In both cases, we found similar fluorescent structures as observed previously for Na\(_{1.5}\) (21, 29); when using higher concentrations of plasmid DNA for transfection (1–2 μg per 5-mm dish), most of the cells showed a strong intracellular fluorescence and a clustering at the plasma membrane (Fig. 5, Na\(_{1.5}\)-GFP, left). Lower DNA amounts (<0.05 μg) resulted in reduction of the overall cellular fluorescence, and signals at the outer membrane became more prominent (Fig. 5, Na\(_{1.5}\)-GFP, right). In the case of Na\(_{1.5d}\), we also found, in addition to the staining of intracellular membranes, a clear fluorescence in the outer membrane of the cells. Occasionally, cells even showed a marked fluorescence in the outer membrane only (Fig. 5, Na\(_{1.5d}\)-GFP, bottom), similarly as observed for Na\(_{1.5}\) (29). This substantiates that subcellular localization of the splice variant is not impaired.

**Properties of Single Na\(_{1.5d}\) Channels**—Because the trafficking and subcellular localization is not altered in Na\(_{1.5d}\) channels, we tested whether single-channel properties of Na\(_{1.5d}\) are different from those in Na\(_{1.5}\). The amplitude of single cardiac Na\(^+\) channels is nearly 2 pA at −40 mV (20, 30). In the present study, the channel number in the patch was determined as a lower estimate from the maximal overlap per 1000 consecutive sweeps. In the case of Na\(_{1.5}\), open probability is rel-
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In addition to the intracellular fluorescence previously reported for Na<sub>1.5</sub> channels (21), plasma membrane staining was observed in both Na<sub>1.5-GFP</sub> and Na<sub>1.5d-GFP</sub> (arrows; see also outer membrane staining in Na<sub>1.5-GFP</sub> on the right). Occasionally, a uniform labeling of the cell periphery was seen in both Na<sub>1.5d-GFP</sub> (bottom) and Na<sub>1.5-GFP</sub> (not shown). The bars represent 10 μm.

Identification of Sequence Motifs within the DII/DIII Linker Modulating Na<sub>1.5</sub> Gating—To identify the molecular region within the DII/DIII linker that is responsible for the observed effect on Na<sub>1.5d</sub> gating, we first constructed and tested two deletion variants of Na<sub>1.5</sub> (Fig. 8; Na<sub>1.5Δ1</sub> and Na<sub>1.5Δ2</sub>). Electrophysiological properties of Na<sub>1.5Δ1</sub> channels were similar to those of splice variant Na<sub>1.5d</sub>, whereas Na<sub>1.5Δ2</sub> could not be distinguished from wild-type Na<sub>1.5</sub>. Inspection of the amino acid sequence deleted in Na<sub>1.5Δ1</sub> showed two interesting protein motifs: (a) a putative amphiphilic α helix carrying regularly arranged positive charges and thus resembling an S4 segment of voltage-gated ion channels (called the S4-like region in Figs. 2B and 8) and (b) a potential phosphorylation site for PKA attached to this putative amphiphilic structure (Figs. 2B and 8). The results on Na<sub>1.5Δ1</sub> argued that at least one of those motifs is responsible for the markedly different properties of Na<sub>1.5d</sub> compared with Na<sub>1.5</sub> channels.

Next, we deleted the S4-like region in Na<sub>1.5</sub> (Na<sub>1.5Δ5</sub>; Fig. 8). Steady-state activation and inactivation was shifted toward depolarized...
potentials, and the current amplitude was reduced, similarly as observed for Nav1.5d (p > 0.1 for Nav1.5d versus Nav1.5Δ5, and p < 0.001 for Nav1.5Δ5 versus Nav1.5). A clear effect was also observed in a similar deletion variant (Nav1.5Δ6; Fig. 8) in which the S4-like region (except for lysine 974) but not the potential PKA site was removed (Nav1.5R3A; Fig. 8). The result was that the current amplitude was reduced (Table 2), whereas $V_m$ and $V_i$ values were indistinguishable from those of the wild-type channels (Fig. 8). In both HEK293 and Xenopus oocytes, reduction of the current amplitude in Nav1.5Δ3A was significantly less pronounced compared with Nav1.5d or Nav1.5Δ5 (p < 0.05), suggesting that further amino acids contribute to Nav1.5 current amplitude in the plasma membrane. Interestingly, removal of the S4-like region, except for the N-terminal motif LALA, produced channels with unchanged steady-state activation but with significantly altered steady-state inactivation (Nav1.5Δ7; Fig. 8). This suggests that steady-state activation and inactivation properties are modulated by different regions; the motif LALA modulates steady-state activation, and the amphiphilic region modulates steady-state inactivation.

To prove this hypothesis, we constructed another deletion variant in which only the motif LALA was deleted (Nav1.5Δ8). Indeed, these channels showed markedly altered steady-state activation ($p < 0.001$ for Nav1.5Δ8 versus Nav1.5) but unchanged steady-state inactivation values ($p > 0.1$ for Nav1.5Δ8 versus Nav1.5). The observed reduction of the current amplitude indicates that, in addition to the adjacent arginine residues, also the motif LALA affects current amplitude of Nav1.5 (see also Nav1.5R3A data). The observed shift of steady-state activation toward depolarized potentials was significant, but not as much pronounced as in case of splice variant Nav1.5d or Nav1.5Δ5 ($p < 0.001$ for Nav1.5Δ8 versus Nav1.5d or Nav1.5Δ5). This indicates that the motif LALA is an important determinant of steady-state activation in Nav1.5. In addition to this motif, however, also the adjacent S4-like region must be removed in order to obtain channels with Nav1.5d properties.
Similar to Na$_{1.5}$R3A, an exchange of the positively charged amino acids by a neutral alanine in Na$_{1.5}$/H$_9004$ had no further effect (Na$_{1.5}$/H$_9004$; Fig. 8); $V_m$ was similarly affected in Na$_{1.5}$/H$_9004$ as in Na$_{1.5}$/H$_9004$, whereas $V_h$ remained unaffected in both mutant channels. Interestingly, a further deletion of the motif RIQ in Na$_{1.5}$/H$_9004$ significantly shifted also steady-state inactivation (Na$_{1.5}$/H$_9004$; Fig. 8), suggesting that these amino acids are important determinants for steady-state inactivation of Na$_{1.5}$.

DISCUSSION

In the present study, we show that alternative splicing of the heart-specific Na$^+$ channel creates a Na$_{1.5}$ variant with novel electrophysiological properties. We conclude that Na$_{1.5}$d represents a naturally occurring splice variant that is produced in all human hearts under physiological conditions; respective transcripts were detected in heart RNA samples of both sexes already before birth, and they occurred in various adult hearts of different ages at nearly constant Na$_{1.5}$/Na$_{1.5}$d ratios. The transcript levels of Na$_{1.5}$d were observed to be relatively low compared with those of Na$_{1.5}$, suggesting at most a minor contribution of Na$_{1.5}$d currents to excitation of ventricular and atrial cardiomyocytes. However, one may speculate that this splice variant is predominantly expressed in specialized cardiac cells like those of the sinus node or of Purkinje fibers. It is well known that alternative splicing of Na$^+$ channels occurs in a tissue-dependent manner (4, 9, 14, 18). Specific expression of Na$_{1.5}$d in the conduction system would not only explain its low RNA levels in the whole human heart but would also point to an important contribution of Na$_{1.5}$d to cardiac excitability by facilitating the action potential initiation also at more depolarized potentials. Unfortunately, it is difficult to test for this hypothesis,
because Na$\text{\textsubscript{1.5d}}$ seems to occur only in the human heart. We could not detect even traces of Na$\text{\textsubscript{1.5d}}$ in mouse or rat hearts. Moreover, we recently started to analyze Na$\text{\textsubscript{1.5}}$ splicing in different dog heart regions, but so far we have failed to detect Na$\text{\textsubscript{1.5d}}$ also in this species. Thus, the physiological role of Na$\text{\textsubscript{1.5d}}$ for the human heart is still unknown.

However, the splice variant Na$\text{\textsubscript{1.5d}}$ led us to an intracellular linker region surprisingly involved in the voltage-dependent gating. Whereas subcellular localization and trafficking was not affected, single-channel properties of the splice variant were clearly different compared with the wild-type channels. We found a dramatically reduced single-channel open probability at an unchanged single-channel amplitude. Moreover, the splice variant showed altered steady-state activation and inactivation parameters. We tested various Na$\text{\textsubscript{1.5}}$ mutant channels in HEK293 cells and detected short sequence motifs in the DII/DIII linker affecting the gating of Na$\text{\textsubscript{1.5}}$. As the main result, we found that the motif LALARIQGRVFVR plays a crucial role for Na$\text{\textsubscript{1.5}}$ steady-state activation, steady-state inactivation, and current amplitude. Interestingly, respective changes of these three parameters were not linked to the same amino acids within this short region. Instead, deletion of the motif LAL was essential to shift steady-state activation of Na$\text{\textsubscript{1.5}}$ toward depolarized potentials, whereas steady-state inactivation was unaltered (Na$\text{\textsubscript{1.5ΔL}}$ in Fig. 8). In contrast, deletion of the adjacent amphiphilic region, resembling an S4 of voltage-gated ion channels, was sufficient to shift steady-state inactivation (Na$\text{\textsubscript{1.5ΔS4}}$ in Fig. 8) but did not alter steady-state activation of Na$\text{\textsubscript{1.5}}$. An exchange of the respective positively charged amino acids by alanine attenuated whole-cell currents but had no obvious effect on channel gating (Na$\text{\textsubscript{1.5R3A}}$ in Fig. 8).

An amino acid alignment of all Na$\text{\textsubscript{v}}$ sequences revealed that the motif LALARIQGRVFVR is unique to the cardiac isoform Na$\text{\textsubscript{1.5}}$. This isoform activates and inactivates at significantly more hyperpolarized potentials than neuronal or skeletal muscle Na$\text{\textsuperscript{+}}$ channels (7, 30–35). Based on the finding that deletion of this motif shifted the gating parameters into the depolarized direction, we hypothesized that a transfer of this region into Na$\text{\textsubscript{1.4}}$ at the corresponding position respectively shifts the gating of Na$\text{\textsubscript{1.4}}$ toward hyperpolarized potentials. However, we did not observe this shift (data not shown). Therefore, the motif LALARIQGRVFVR is only effective in a Na$\text{\textsubscript{1.5}}$ background, possibly by interacting with the DI/DII linker or with other amino acids of the Na$\text{\textsubscript{1.5}}$ DII/DIII loop. This idea agrees with the results of several previous studies on cardiac and skeletal muscle Na$\text{\textsuperscript{+}}$ channels. Bennett (36, 37) pointed out the importance of the DI/DII and DII/DIII cytoplasmic loops for Na$\text{\textsuperscript{+}}$ channel activation. The author conclusively showed that the isoform-specific channel activation is coupled to the presence of both intracellular loops. The DII/DIV linker and the C terminus do obviously not contribute significantly to the differences of steady-state activation and inactivation between Na$\text{\textsubscript{1.4}}$ and Na$\text{\textsubscript{1.5}}$ channels (32, 36, 38).

Beside the proposed interaction of the motif LALARIQGRVFVR with other intracellular Na$\text{\textsubscript{1.5}}$ loop regions, one should consider the possibility whether this $\text{\textsuperscript{S4-like}}$ region contributes to the gating by dipping into the transmembrane field, thereby facilitating the opening of the pore already at more hyperpolarized potentials. However, this mechanism is unlikely, because an exchange of the positively charged residues did not alter steady-state activation (Na$\text{\textsubscript{1.5Δ3A}}$ in Fig. 8). A further possibility is that this short motif interacts with membrane lipids or with other proteins. For example, it is well known that calmodulin (CaM) is capable of recognizing positively charged, amphiphilic α-helical peptides independent of their precise amino acid sequences (39).

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2 V. Haufe, Y. S. Wu, R. Dumaine, and T. Zimmer, unpublished data.
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