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Slow-inactivation induced conformational change in domain 2-segment 6 of cardiac Na^+ channel

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Abstract

To examine conformational changes during slow inactivation involving domain 2-segment 6 (D2-S6) of human cardiac Na^+ channel (hNav1.5), we applied the substituted-cysteine accessibility method (SCAM) using methanethiosulfonate ethylammonium (MTSEA). We substituted cysteine (C) for native valine (V) at position 930 of D2-S6 in the MTSEA-resistant hNav1.5 mutant C373Y to produce the double mutant C373Y-V930C. Whole-cell Na^+ currents were recorded using patch-clamp techniques in transiently transfected HEK cells. In C373Y-V930C, we find that MTSEA (1.5 mM) applied in the closed state (−160 mV) has no significant effect on whole-cell Na^+ current, while MTSEA applied in the slow-inactivated state (prolonged depolarization at 0 mV) decreases current. We propose that D2-S6 in hNav1.5 undergoes molecular rearrangement during slow inactivation exposing the side chain of residue 930 such that it becomes accessible to modification by MTSEA.

Keywords: Na^+ channels; Site-directed mutagenesis; D2-S6; hNav1.5; Methanethiosulfonate (MTS); Slow inactivation

Voltage-gated Na^+ channels (Navs) are transmembrane proteins, the main component of which is an α-subunit consisting of four homologous domains (D1–D4), each with six transmembrane segments (S1–S6). Although several Nav isoforms are associated with modulatory accessory β-subunits, the α-subunit is capable of channel function, i.e., voltage-sensitivity, activation, ion selectivity, and inactivation, when expressed alone in heterologous expression systems such as *Xenopus oocytes* and mammalian HEK cells [1–3].

Navs respond rapidly to depolarization of the membrane, allowing Na^+ ions, driven by their electrochemical gradient, to cross the membrane via an intraprotein permeation pore. This process, termed activation, is responsible for the initiation and propagation of action potentials in most excitable tissues such as those comprising the nervous system, cardiac muscle, and skeletal muscle. In response to the same stimulus (i.e., depolarization) Navs enter a non-conducting state called inactivation from which they rarely activate. This process is observed to occur over two different time scales. One type of inactivation occurs within milliseconds of depolarization and is appropriately termed fast inactivation. This inactivation is important for the termination of action potentials [4]. The other type of inactivation, which occurs with prolonged depolarizations (seconds to minutes), is called slow inactivation and likely plays a role in membrane excitability and action potential firing patterns [5,6]. In addition to differences between the kinetics of fast and slow inactivation, these processes involve different molecular mechanisms. For example, fast inactivation can be eliminated by internal perfusion of protease or amino acid substitutions in the D3–D4 linker (the fast inactivation "gate"), manipulations that do not eliminate slow inactivation [7,8].

Because electrophysiological function of Navs is critically dependent on molecular structure, it is not surprising that amino acid alterations in Navs can disrupt normal function of excitable tissues. Nearly twenty human disease states have been attributed to heritable mutations in Navs

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or "channelopathies" [9]. For example, more than 150 mutations in neuronal Navs have been identified in patients with epilepsy [10], more than 25 mutations in skeletal muscle Navs have been found that produce myotonias [11], and at least five mutations in human heart Navs are found in patients with long QT syndrome and Brugada's syndrome [12]. Many of these mutant Navs have been studied in heterologous expression systems such as *Xenopus oocytes* and HEK cells, and diverse effects caused by the mutations on activation, fast inactivation, and/or slow inactivation have been described [13].

Understanding the relationship between molecular structure and physiological function has been greatly aided by the cloning and expression of Navs. For example, activation is dependent on the voltage-sensing S4 segments [14], and the cytoplasmic linker between D3 and D4 appears to be the fast-inactivation gate or hinge [15]. However, the molecular events occurring during slow inactivation are not well defined. One hypothesis poses that during slow inactivation of Navs, the outer mouth of the pore collapses, a mechanism similar to C-type inactivation of K^+ channels [16]. A second hypothesis involves a rearrangement of amino acid residues within the putative pore-lining S6 segments [17] that would constrict the inner pore region [18]. In support of the latter hypothesis are studies showing that mutations in S6 have effects on slow-inactivation gating [18–21].

In the present study, we substituted cysteine (C) for native valine (V) at the inner pore residue 930 (V930C) in D2-S6 of the human cardiac muscle Nav hNav1.5. We chose this residue in hNav1.5 because we previously found state-dependent molecular rearrangement at the homologous site (V787 in D2-S6) in rat skeletal muscle rNav1.4 [18]. We were interested in determining if Nav1.5 and Nav1.4 isoforms share similar molecular mechanisms of slow inactivation despite the fact that Nav1.5 is relatively resistant to slow inactivation compared with Nav1.4 [22,23].

In an effort to detect conformational changes in D2-S6 during slow inactivation in hNav1.5, we used methanethiosulfonate ethylammonium (MTSEA) and the substituted-cysteine accessibility method (SCAM) [24]. We found that application of MTSEA to wild-type hNav1.5 rapidly reduces current, an effect that is also found in the rat cardiac Na^+ channel rNav1.5 [25]. Therefore, we studied the V930C mutant in the MTSEA-resistant hNav1.5 mutant C373Y background (C373Y-V930C). Our data extend previous studies and support the hypothesis that D2-S6 in hNav1.5 undergoes conformational change during slow inactivation enhancing MTSEA-accessibility at residue 930.

Materials and methods

Site-directed mutagenesis. Nav mutagenesis was carried out using an adapted 2-stage modification of the Quick Change XL Site Directed Mutagenesis Kit Protocol (Stratagene, La Jolla, CA, USA, and personal communication, Sho-Ya Wang, SUNY Albany, NY, USA) [26,27]. pcDNA1 (Invitrogen, Carlsbad, CA, USA) containing hNav1.5 cDNA was used as the template for mutagenesis. For each mutation, a mutagenesis mix (Stratagene, La Jolla, CA, USA) was prepared containing 74 ng of template plasmid, 2.1% dNTP mix, 6.3% QuickSolution, 10.5% of 10× Reaction Buffer, and 1.5 U *PfuTurbo* DNA polymerase in a final volume of 28 μl. 13.3 μl mix was added to each of two 200 μl thin-walled PCR tubes. Seventy nanograms of top strand mutagenic oligonucleotide was added to one tube containing mix (Stage 1, 14 μl total volume) and 70 ng lower strand mutagenic oligonucleotide was added to the other tube containing mix (Stage 2, 14 μl total volume). For Stage 1 reaction, PCR was carried out as follows: Step 1, 94 °C, 30 s; Step 2, 94 °C, 30 s; Step 3, 55 °C, 1 min; Step 4, 68 °C, 23 min; followed by Step 5, 3 cycles to Step 2. Stage 1 reaction product was then combined with Stage 2 tube (28 μl total combined volume), an additional 0.5 U *PfuTurbo* DNA polymerase was added, and mixture subjected to Stage 2 PCR as follows: Step 1, 94 °C, 30 s; Step 2, 94 °C, 30 s; Step 3, 55 °C, 1 min; Step 4, 68 °C, 23 min; Step 5, 17 cycles to Step 2; Step 6, 68 °C, 7 min. Ten microliters of product was digested for 1 h at 37 °C with 2 U *Dpn*I, digestion was repeated, and product transformed into XL10-Gold as recommended by the supplier (Stratagene, La Jolla, CA, USA). Colonies growing on LB-ampicillin plates were picked, grown in LB containing 50 μg/ml ampicillin, and plasmid DNA isolated using a Wizard Plus SV miniprep kit (Promega, Madison, WI, USA). Plasmid DNA was digested with *Eco*RI to check overall integrity, sequenced on both strands in regions encompassing putative mutations (Pennington Biomedical Genomics Core Facility, Baton Rouge, LA, USA), and intact clones containing mutations selected for study. Plasmid DNA for transfection was purified using maxi prep columns (Qiagen, Valencia, CA, USA). Oligonucleotides for mutagenesis included; hH1-C373Y-top CGCCTGATGACGCAGGACTACTGGG AGCGCCTCTATCAG, hH1-C373Y-bot CTGATAGAGGCGCTCCC AGTAGTCCTGCGTCATCAGGCG, hH1-V930C-top GTCATTGGC AACCTTGTGTGCCTGAATCTCTTCCTGGCC, hH1-V930C-bot GG CCAGGAAGAGATTCAGGCACACAAGGTTGCCAATGAC. DNA sequencing primers included; hH1C373Y-seqfor CTGTGTGGGAACAG CTCTGAC, hH1C373Y-seqrev GTAGAAGGACCCCAGGAAGATG, (D2-S6)mut-seqfor CCTAATCATCTTCCGCATCC, and (D2-S6)mut-seqrev GGAGACCACAGCAGAAATCC. All oligonucleotides were obtained commercially (Operon Biotechnologies, Huntsville, AL, USA).

Transient transfection. Plasmid expression constructs containing wild-type and mutant Nav cDNA clones were transiently transfected into HEK cells using calcium phosphate precipitation as previously described [22,28]. The transfection included 1–5 μg of a plasmid encoding cell surface antigen CD8 (OriGene, Rockville, MD, USA) and 5–10 μg of Nav cDNA expressed from pcDNA1 (Invitrogen, Carlsbad, CA, USA).

Electrophysiology. Using standard patch-clamp techniques [29], we recorded whole-cell peak Na^+ current (I_{Na}) from transiently transfected HEK cells. Recordings were performed at room temperature (21–22 °C), without correction for liquid junction potential. Activation (conductance–voltage) and steady-state fast inactivation (h_∞) curves were obtained approximately 5 min after rupture of the membrane. Recording micropipettes (Drummond Scientific, Broomall, PA, USA) were pulled on a Model P-97 Flaming-Brown puller (Sutter Instruments, Novato, CA, USA). Pipette resistance ranged from 0.5 to 1.5 MΩ. The extracellular recording solution was (in mM): 65 NaCl, 85 choline-Cl, 2 $CaCl_2$, and 10 Hepes, titrated to pH 7.4 with TMA-OH (tetramethylammonium hydroxide). After establishing whole-cell conditions, cells were continuously bathed in the extracellular solution with a gravity-fed superfusion system at a rate of approximately 0.1 ml/min. The intracellular (pipette) solution was (in mM): 100 NaF, 30 NaCl, 10 EGTA, and 10 Hepes, titrated to pH 7.2 with CsOH. These solutions create an outward Na^+ gradient and an outward Na^+ current at the test pulse of +50 mV, thus reducing potential problems associated with space clamp or series resistance errors [30]. Series resistance was compensated at 80%. Linear leak subtraction based on five hyperpolarizing pulses was used for all recordings. Any endogenous K^+ currents were blocked with Cs^+ in the pipette, and HEK cells express no native Ca^{2+} current [31]. Cells were selected for recording on the basis of positive immunoreaction with anti-CD8 Dynabeads (Dynal Biotech, Inc., Lake Success, NY, USA). Recordings were performed 1–3 days post-transfection.