OVERCOMING HISTORICAL CHALLENGES OF NAV1.9 VOLTAGE-GATED SODIUM CHANNEL AS A DRUG DISCOVERY TARGET FOR TREATMENT OF PAIN

Brett Antonio, Karen Padilla, Zhixin Lin, Sonia Santos, Theresa Mersch, Doug McIlvaine, Neil Castle
Icagen, 4222 Emperor Boulevard, Suite 350, Durham, NC 27703 USA

INTRODUCTION

Voltage-gated ion channels that conduct sodium ions (Nav channels) play important roles in many physiological processes including the generation and propagation of electrical signals required for peripheral and central nervous system function. As such, Nav channels are of considerable interest as targets for drug development for treatment of pain and other neurological disorders (1). To reduce the potential for side effects, targeting specific sodium channel subtypes expressed in tissue/organ of interest has been a goal of the pharmaceutical industry. For example, of the nine known Nav channel subtypes (Nav1.1-Nav1.9), Nav1.9 is expressed exclusively in sensory neurons and has recently been demonstrated to be involved in human genetic disorders associated with both loss and gain of sensitivity to pain (2,3). Despite its importance as a potential pain treatment target, Nav1.9 has proved to be challenging for drug discovery, primarily due to the historical inability to express functional recombinant channels, and consequently the absence of robust compound screening assays.

Here, we report the first successful stable functional expression of recombinant human Nav1.9 in HEK-293 cells, and demonstrate that their biological and pharmacological properties are comparable to Nav1.9 natively expressed in sensory neurons. We also present the development and implementation of a fluorescence based sodium flux high throughput screening assay. We also show the implementation of automated electrophysiology platform for follow up evaluation of HTS active compounds for further defining potency and Nav channel subtype and species selectivity. The successful development of recombinant Nav1.9 cell lines carrying human Nav1.9 mutations, as well as pharmacological binding site ablation mutations have also allowed detailed analysis of mode and site of action. In summary, our data show for the first time that Nav1.9 can be the subject of a robust drug discovery program, which opens up the potential for future novel pain treatment.

MATERIALS AND METHODS

cDNA constructs and Cell line generation: All Nav channel cDNAs were cloned into either pcDNA3.1 or pLCK2 mammalian expression vectors using standard molecular biology methods. A stable recombinant mammalian cell line carrying full length human Nav1.9, encoded by SCN1A (accession NM_00139.2), was generated in Hek293 cells. SCN1A cDNA (PCR cloned into pcDNA3.1 (Invitrogen, Carlsbad, CA), sequence-verified, and then transfected into HEK293 using Fugene (Roche Applied Science). Human TRX (TRK) and human Nav1.9 channel subunit B2 (SCN2B) were similarly co-transfected with hSCN1A. Human Nav1.9 containing mutation of the consensus homologous DIV 56 local anesthetic binding site residues F152A and V159A, Nav1.9 K799N, Nav1.7 and Nav1.7 HMK4 channel cDNAs were still expressed in a HEK-293-B12 cell line in which the human B1 and B2 subunits were present. Mouse and rat Nav1.9 cell lines were generated by stably expressing Nav1.9 in HEK-293-mouse B1/B2 and HEK-293-rat B1/B2 lines.

Electrophysiology: Whole-cell voltage clamp recordings were performed using PatchMaster eletrophysiological platform at 22-24°C. Extracellular solution contained (in mM): NaCl 135, CaCl2 2, KCl 5, MgCl2 1, Glucose 5, Heps (pH 7.4 with NaOH) and 300 mM tetrodotoxin (TTX) and an internal solution (in mM): CaCl2 15, MgCl2 5, NaCl 120, Heps, 10 mM (pH 7.4 with NaOH) plus 50 mM GTP-5-s. To determine voltage dependence of activation and inactivation, clamps were at membrane potential of -140 mV and current amplitude was measured during a 40 ms depolarizing test pulse to potentials from -140 mV to -40 mV in 10 mV increments (activation) or at a membrane potential of -140 mV followed by a 500 or 500 ms conditioning prepulse to potential from -140 mV to -160 mV in 10 mV increments and a 40 ms test pulse to -40 mV (inactivation). For pharmacology studies, compounds were dissolved in DMSO to make 10 mM stock solutions, which were diluted into extracellular solution to obtain the final concentrations desired. Test compound effects were evaluated using a protocol in which each cell was clamped at -120 mV and sodium currents elicited by 40 ms pulses to -40 mV and repeated 3-5 min at a frequency of 0.5 Hz.

FLUPR: HEK-293 cells stably expressing NNAV1.9 along with TRB2 and B2 were plated (15,000 cells/250 well) in 384 well black-walled clear bottom plates coated with Poly-D-lysine, and cultured overnight in DMEM containing 10% FBS, 2mM Sodium Pyruvate, 10mM HEPS, 0.001% L G4, 100mM Gcins, and 5mM Glutathion at 37°C in the presence of 10% CO2. The following day, cells were removed from the plate and replaced with 5sul of Ang II dye solution. Plates are incubated for 60 to 90 minutes at room temperature, protected from light. After incubation, dye was removed from the plate and replaced with 10sul of assay buffer. Assay plates were loaded into FLUPR3 fluorescence imaging system (Molecular Devices). The FLUPR system monitored an initial baseline of 10 seconds (excitation 488nm/emission 520nm) before the internal 384 well pipette head added 10sul of either stimulation buffer (500mM TCS 1752, DMSO vehicle, or test compound made up in assay buffer containing 100mM Oubain (Sigma). Compounds were incubated on the cells for 5 minutes while the FLUPR monitored the plate. After incubation, the FLUPR pipette added 20sul of stimulation buffer (assay buffer containing 100mM Oubain, 3mM TCS 32Sigma), and 50mM Deltamethrin (Calbiochem) final assay concentrations. Following addition of the stimulation buffer, the FLUPR monitored fluorescence response for 10 minutes. Data export metrics set to report responses over a 10 - minute period to a 200 files and from just before the stimulation addition through the end of the read time. Data was normalized to mask the baseline and baseline responses and percent inhibition.

Data Analysis: Data was acquired and analyzed using DataPlex 2.0 (Molecular Devices) and GraphPad Prism (GraphPad Software, Inc.). Sodium current amplitudes following activation were converted to sodium conductance and plotted as a function of test potential using the Boltzmann equation. Similarly, currents from steady-state inactivation were also plotted as a function of prepulse potential and fitted to the Boltzmann equation. Concentration response curves were generated using logistic equation to calculate an IC50 for each compound. All data are presented as mean ± SEM or mean ± (95% Confidence Limits).


Stable Expression of hNav1.9 In HEK-293 Cells

Characterization of human Nav1.9 stably expressed in HEK-293 cells. (A) Representative families of current traces and current-voltage relationship from HEK-293 cells stably expressing human Nav1.9. (B) Structure of Nav channel inhibitor TC-N 1752 and representative traces of Nav1.9 currents in presence and absence of 3 µM TC-N 1752. (C) Concentration response curves for inhibition of hNav1.9 by known sodium channel inhibitors. (D) IC50 comparison for four sodium channel inhibitors against Nav1.9, Nav1.7 and Nav1.8.

Activity Across Nav1.9 Species Orthologs

Characterization of mouse and rat Nav1.9 stably expressed in HEK-293 cells. Representative families of current traces from HEK-293 cells stably expressing mouse (A) or rat (B) Nav1.9. Comparison of concentration dependence of inhibition of human (open circle), mouse (square) and rat (triangle) Nav1.9 by tetracaine (C) or TC-N 1752 (D).

Localizing Nav1.9 Ligand Interactions

(A) Sequence alignment showing that the Nav1.9 unique lysine residue K799 on D16 (asparagine in all other Nav channel subtypes) aligns spatially with DIV 56 F152O (putative local anesthetic binding site). (B) Homology model of the pore region of human Nav1.9 based on Nav1.1 crystal structure (PDB: 3G21) indicating the position of lysine (K) in relation to residues important for local anesthetic binding. (C) Effect of changing putative local anesthetic binding site residues on potency of TC-N 1752. (D) Effect of mutating unique D16 lysine to consensus asparagine on potency of TC-N 1752.

Evaluating Disease Associated Mutations

Characterization of a human Nav1.9 mutation causing complete inactivity to pain (CIS). (A) Representative families of current traces from HEK-293 cells stably expressing hNav1.9 (black) or hNav1.9 with C5 associated L1320F mutation (blue). (B) Comparison of current voltage relationship for Nav1.9 (black) and the L1320F mutant (blue). Note that L1320F mutant activated much more hyperpolarised membrane potential than Nav1.9 (IC50 Concentration response curve for inhibition of Nav1.9 (black) or L1320F mutation (blue) by tetracaine.

CONCLUSION

Current study demonstrates successful stable functional expression of human, mouse and rat Nav1.9 in HEK-293 cells.

Known local anesthetic compounds are all significantly less potent against Nav1.9 compared to other TTx (Nav1.7) or TTx (Nav1.8) channels.

Successful development and execution of a 384 well high-throughput assav for Nav1.9 measuring Na+ influx using ANG-2

The reported Nav1.7 inhibitor, TC-N 1752 found to be the most potent inhibitor of Nav1.9 (IC50 ~1µM) Potency of TC-N 1752 decreased by ~200 fold following mutation of amino acid residues important for local anesthetic interaction.