Profiling endogenous sodium channels in the ND7-23 neuroblastoma cell line: implications for use as a heterologous ion channel expression system and native tissue model suitable for automated patch clamp screening

Marc Rogers[1], Nace Zidar[2], Danijel Kikelj[2] and Robert Kirby[1]

[1]Metrion Biosciences, Babraham Research Campus, Cambridge CB22 3AT, U.K; [2]Faculty of Pharmacy, University of Ljubljana, Slovenia

Introduction

Voltage-dependent sodium channels (Nav) are implicated in a wide range of diseases, with their role in triggering and modulating membrane excitability making them key drug discovery targets for cardiac and neurological indications. Neuronal Nav's are divided into TTX-sensitive (Nav1.1, Nav1.2, Nav1.3, Nav1.6 and Nav1.7) and TTX-resistant channels (Nav1.8 and Nav1.9), with those found in the CNS underlying various types of epilepsy and those expressed in the periphery implicated in many types of pain behaviour such as inflammatory, neuropathic, chemotherapy and cancer-induced pain, as well as visceral pain conditions such as irritable bowel syndrome (IBS).[1] Key to the role of Navs in pain is their specific distribution and function in peripheral sensory nociceptors of the dorsal root (DRG) and other sensory ganglia, where Nav1.x channel function changes after injury through the effects of inflammatory mediators and signalling pathways, and channels are redistributed from the soma to axons.[2] In this way Nav activity increases neuronal excitability and induces spontaneous, persistent, repetitive and ectopic action potential firing. The role of peripheral Navs in pain are supported by the association of SNPs and genetic mutations in Nav1.7 with several pain several phenotypes (CIP, PEPD and IEM)[3] and gain-of-function mutations in Nav1.7 and Nav1.8 in human patients suffering from small fibre neuropathy (SFN).[4]

Translating *in vitro* Nav1.x pain drug discovery compounds successfully to preclinical animal models and human clinical trails requires access to and use of predictive native tissue assays, but there is a growing trend to reduce animal use (NC3Rs). We have therefore characterised the endogenous rodent Nav1.x channels expressed in the ND7-23 neuroblastoma cell line, which is a hybrid of mouse N18Tg2 neuroblastoma and rat DRG neurons that is amenable to automated patch clamp (APC) high throughput screening platforms such as the QPatch. Molecular profiling indicates a range of possible Nav1.x channels expressed in these cells[5,6] but the functional repertoire remains to be determined. Our data suggests that ND7-23 cells are a useful translational screening system for pain drug discovery, moving DRG-based species selectivity and native tissue assays higher up the screening cascade for the discovery of potent, selective and state-dependent Nav ligands.

Methods & Materials

Cell culture

CHO and HEK-293 cell lines stably expressing human Nav1.3, Nav1.6 and Nav1.7 α subunits were cultured according to Millipore protocols (typically DMEM ± F12 media supplemented with non-essential amino acids and 10% FBS). Undifferentiated ND7-23 cells (ECACC) were grown in DMEM media supplemented with 10% FBS. T-175 flasks were routinely passaged every 2-3 days to keep cell confluency <80%, while flasks for QPatch experiments were grown for 2 - 5 days to achieve optimal cell density and Nav1.x channel expression Cells were dissociated with enzyme-free reagents (e.g. Accutase) to obtain single cell suspensions with optimal cell viability and "patchability" for APC.

Automated patch-clamp (APC) electrophysiology

All $hNa_v1.x$ cell lines were previously validated biophysically and pharmacologically on the QPatch platforms (Sophion, Denmark). We used standard single hole chips (~2.5 MΩ resistance) except for hNav1.3 assay (10 hole X-plates which sum current amplitude and divide seal resistance) .

Solutions: Internal solution contained (in mM): 120 CsF, 10 NaCl, 10 HEPES, 5 Na_2-ATP, 10 EGTA, 1 $MgCl_2$; pH 7.2, ~290mOsm. External solution contained (in mM): 140 NaCl, 5 KCl, 10 Glucose, 10 HEPES, 2 $MgCl_2$ and 1 $CaCl_2$; pH 7.4, ~310mOsm.

Voltage protocols: All recordings were in conventional whole-cell configuration, achieved through a combination of suction and voltage zaps. Nav currents were elicited from a negative holding potential (typically Vh -100 mV) with brief (20 - 50 msec) activating test pulses from -20 to 0 mV depending on the Nav1.x channel, and -10 mV in ND7-23 cells. Compound screening utilised a 2 pulse protocol delivered every 15s to assess resting (Peak 1) and inactivated state inhibition (Peak 2), following a 5 sec conditioning prepulse before Peak 2 designed to achieve ~50% inactivation for each Nav1.x channel cell line on each assay day. Series resistance (4-15MΩ) was compensated by 65-85%.

Data analysis: Peak current (minimum amplitude) during the activating test step was measured with cursors (1-20% duration) in QPatch Assay Software. Subtraction of responses in the presence of a saturating dose of 300 nM TTX added at the end of each experiment allowed for leak subtraction.

Compound screening

Vehicle (0.1% DMSO or 0.05% BSA for toxins) was applied to achieve a stable control recording (4 min) before compound potency was determined from single or multiple cumulative applications of test compound (5-10μL), and time-matched vehicle control data was obtained for all Nav1.x assays to ensure run-up or rundown was neglible (<2%/min) after the initial stabilisation period. Concentration response curves (four parameter logistic curve) were fitted to %inhibition data using Prism (GraphPad) from which IC_{50} (50% inhibitory concentration) and Hill coefficients were determined (Hill slope constrained to $0.5>n_h<2.0$). All %inhibition and IC_{50} data is N ≥ 3.

Compounds: 4,9 anhydro TTX was supplied by Mr Ari Alexandrou (Pfizer-Neusentis) and Protoxin-II was purchased from PeptaNova GmbH. Icagen patent compound 68 was synthesised according to Gonzalez *et al.*, (2006).[7]

Results

1. Confirming selectivity of Nav1.x ligands

We first determined the potency and selectivity of the reference ligands against human Nav1.3, Nav1.6 and Nav1.7 cell lines on the QPatch, using the same 2 pulse voltage protocol that would be employed to profile the repertoire of Nav1.x channels in ND7-23 cells on the same platform.

The selective Nav1.x antagonists are:

- Icagen Nav1.3 patent compound 68,[7] which we have shown previously to have no effect against Nav1.4, Nav1.5, Nav1.6 and Nav1.7 channels.[8]
- 4,9 anhydro TTX, a selective nM blocker of rodent Nav1.6 channels in *Xenopus* oocytes[9]
- Protoxin-II, a moderately selective inhibitor of Nav1.7 channels via the voltage sensor domain

Icagen compound inhibition of Nav1.3

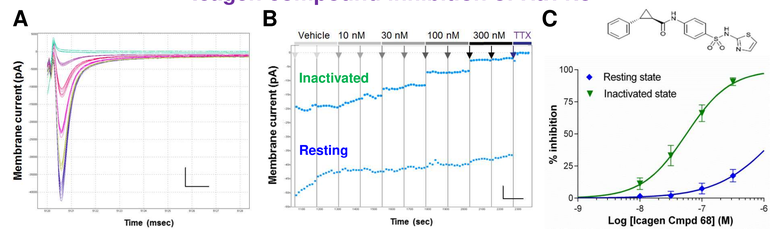

Figure 1: **Potent and state-dependent inhibition of Nav1.3 currents by Icagen patent compound**
A: Raw current traces from multi-hole Xplate showing rapidly inactivating Nav1.3 currents inhibited by increasing concentrations of Icagen patent compound (vehicle=blue, 10 nM=green, 30 nM=pink, 100 nM=red and 300 nM=purple; Cyan = complete block by 300 nM TTX). Scale bar = 1 msec and 5 nA in **A**, and 100 sec and 5 nA in **B**.
B: Current-time plot showing dose- and state-dependent block of resting (■) *vs* inactivated state (◆) Nav1.3 currents.
C: Fitting % inhibition at each dose reveals IC_{50} of 56 nM and 18 μM for inactivated and resting and state, respectively.

Nav1.6 *vs* Nav1.7 selectivity of 4,9 anhydro TTX

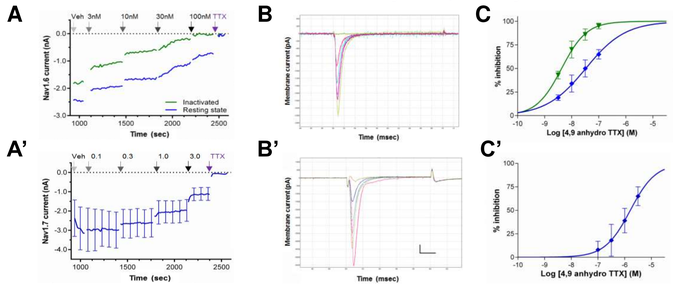

Figure 2: **4,9 anhydro TTX exhibits 50 fold selectivity for Nav1.6 *vs* Nav1.7 currents**
A, A': Current-time plots showing dose- dependent block of resting and inactivated state Nav1.6 and Nav1.7 currents.
B, B':. Raw current traces of rapidly inactivating Nav1.6 and Nav1.7 currents inhibited by increasing concentrations of 4,9 anhydro TTX (3-100 nM for Nav1.6, 0.1-3 μM for Nav1.7); all currents are completely blocked by 300 nM TTX.
C, C': IC_{50} for inhibition of Nav1.6 resting and inactivated state are 32.9 and 4.3 nM; Nav1.7 resting state IC_{50} is 1.62 μM.

Nav1.7 potency and state-dependency of Protoxin-II

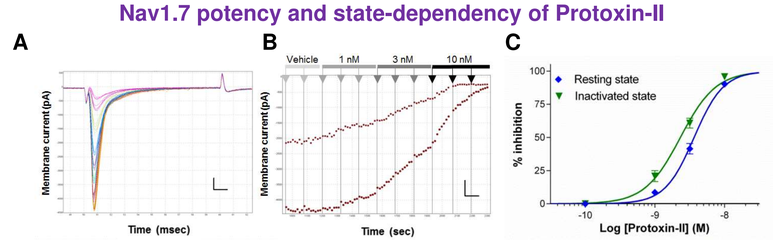

Figure 3: **Potent inhibition of Nav1.7 currents by Protoxin-II on QPatch**
A: Raw data traces of Nav1.7 currents in presence of increasing concentrations of Protoxin-II (vehicle=orange, followed by triple additions of 1, 3 and 10 nM Protoxin in 0.05% BSA). Scale bar; 500pA, 1 msec and 100 sec in **A** and **B**.
B: Current-time plot showing dose but not state-dependent block of resting (●) *vs* inactivated state (■) Nav1.7 currents.
C: Fitting % inhibition at each dose reveals an IC_{50} of 3.65 and 2.32 nM for resting and inactivated state, respectively.

2. Profiling endogenous TTX-s Nav1.x channels in ND7-23 cells

Molecular techniques detect Nav1.7 as the predominant species of TTX-sensitive Nav1.x subunit, with lower levels of Nav1.2, Nav1.3 and Nav1.6 subunits.[5,6] We determined the repertoire of *functional* Nav1.x TTX-s channels by applying toxins and small molecules shown to be selective for Nav1.3 (Icagen),[7] Nav1.6 (4,9 anhydro TTX)[9] and Nav1.7 channels (Protoxin-II) on the same QPatch platform.

Minor contribution from Nav1.3 and Nav1.6 channels

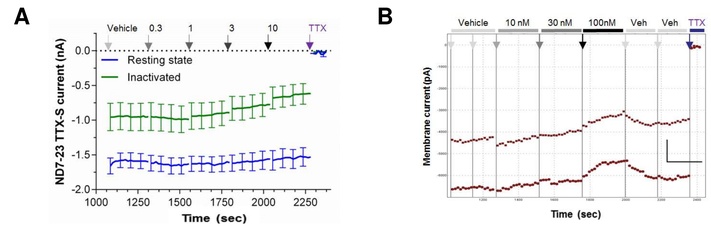

Figure 4: Little effect of selective Nav1.3 and Nav1.6 antagonists on ND7-23 TTX-s current
A: No change in TTX-sensitive I_{Na} in ND7-23 cells by Nav1.3-selective doses (0.3-1 μM) of Icagen patent compound.
B: Small effect of Nav1.6-selective concentrations (10-100 nM) of 4,9 anhydro TTX; complete block by 300 nM TTX.

Major contribution from Nav1.7 channels

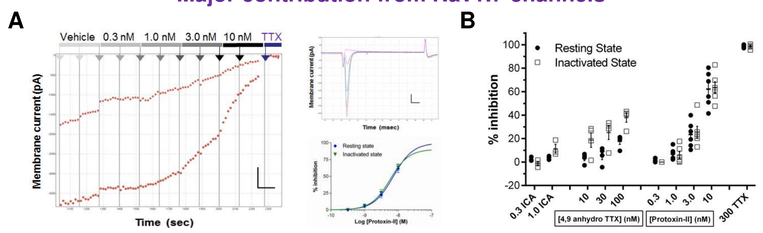

Figure 5: **Predominant expression of Nav1.7 >> Nav1.6 TTX-S channels in ND7-23 cells**
A: Inhibition of whole-cell Nav currents by Nav1.7-selective doses of Protoxin-II (0.3-10 nM), followed by complete inhibition in TTX (300 nM). Protoxin IC_{50} of 7.2 and 5.7 nM for resting and inactivated state, respectively.
B: Contributions from Nav1.3, Nav1.6 and Nav1.7 channels to state-dependent TTX-S currents in single cells.

Conclusions

- ND7-23 cells are an excellent *in vitro* model of DRG neurons amenable to automated patch clamp screening of endogenous ion channels and receptors, making them suitable for high throughput species selectivity and native tissue translational studies in pain drug discovery screening cascades.
- Undifferentiated ND7-23 cells express rodent Nav1.7 > Nav1.6 >> Nav1.3 channels, similar to the repertoire of TTX-S currents in small diameter DRG neurons and confirming published PCR data.
- We confirm that 4,9 anhydro TTX is a potent and selective antagonist of rodent Nav1.6 channels, and is a useful toolbox compound as it exhibits a similar profile against human Nav1.6 channels.

References

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