RESEARCH ARTICLE

UPDATED **Electrophysiological properties of mouse and epitope-tagged human cardiac sodium channel Na$_v$1.5 expressed in HEK293 cells** [version 2; referees: 2 approved]

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Abstract

Background: The pore-forming subunit of the cardiac sodium channel, Na$_v$1.5, has been previously found to be mutated in genetically determined arrhythmias. Na$_v$1.5 associates with many proteins that regulate its function and cellular localisation. In order to identify more *in situ* Na$_v$1.5 interacting proteins, genetically-modified mice with a high-affinity epitope in the sequence of Na$_v$1.5 can be generated.

Methods: In this short study, we (1) compared the biophysical properties of the sodium current (I_{Na}) generated by the mouse Na$_v$1.5 (mNa$_v$1.5) and human Na$_v$1.5 (hNa$_v$1.5) constructs that were expressed in HEK293 cells, and (2) investigated the possible alterations of the biophysical properties of the human Na$_v$1.5 construct that was modified with specific epitopes.

Results: The biophysical properties of mNa$_v$1.5 were similar to the human homolog. Addition of epitopes either up-stream of the N-terminus of hNa$_v$1.5 or in the extracellular loop between the S5 and S6 transmembrane segments of domain 1, significantly decreased the amount of I_{Na} and slightly altered its biophysical properties. Adding green fluorescent protein (GFP) to the N-terminus did not modify any of the measured biophysical properties of hNa$_v$1.5.

Conclusions: These findings have to be taken into account when planning to generate genetically-modified mouse models that harbour specific epitopes in the gene encoding mNa$_v$1.5.

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	Invited Referees	
	1	**2**
UPDATED **version 2** published 05 Apr 2013	☑	☑ report
version 1 published 13 Feb 2013	☑	? report

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Comments (0)

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Competing interests: No competing interests were disclosed.

Introduction

The voltage-gated cardiac sodium channel $Na_v1.5$ is responsible for the initial phase of the cardiac action potential and plays a central role in cardiac impulse propagation[1]. Its role in human disorders has been underlined by the findings of several hundred mutations in its gene, *SCN5A*, that are linked to inherited cardiac electrical disorders such as congenital long QT syndrome and Brugada syndrome[2]. In recent years, it has been demonstrated that $Na_v1.5$ interacts with and is regulated by different proteins (recently reviewed by Shy *et al.*[3]). Many of these interacting proteins were also found to be mutated in patients with genetically-determined cardiac arrhythmias[4]. The generation of genetically-modified mouse models, harbouring mutations in the *Scn5a* gene, has proven to be a very informative approach to investigate the various human phenotypes that are linked to the genetic variants of this gene[5]. Since $Na_v1.5$ interacts with many proteins during its life cycle in cardiac cells, it would be of great interest to generate a mouse model that permits the biochemical purification of $Na_v1.5$ with high efficiency, hence allowing the co-purification of interacting proteins. The identity of these co-purified proteins may then be determined by using mass spectrometry-based technologies. In order to do this, one needs to first generate a knock-in mouse model, where a high-affinity epitope would be added to the mouse *Scn5a* gene that codes for $Na_v1.5$.

The goals of this short study were (1) to compare the biophysical properties of the sodium current (I_{Na}) generated by mouse $Na_v1.5$ and human $Na_v1.5$ constructs expressed in HEK293 cells, and (2) to investigate the possible alterations of the biophysical properties of human $Na_v1.5$ constructs that were modified with specific epitopes. We used the common fluorescent GFP and YFP proteins as epitopes, which provide the advantage of being detectable without the use of antibodies. However, these tags can only be added to the N- and C-termini, which are both intracellular in $Na_v1.5$, and which are thus, not easily accessible. Therefore, we additionally chose the FLAG-epitope (Sigma-Aldrich), which consists of a short sequence that can be inserted into the extracellular loops of $Na_v1.5$. The results of these studies will have to be taken into account when planning the generation of a mouse line bearing an epitope-tagged $Na_v1.5$ channel.

Methods

Transfection and culture of HEK293 cells

HEK293 cells (Robert S Kass laboratory, Columbia University, New York) were transfected by Lipofectamine LTX (Invitrogen) according to the manufacturer's instructions. The plasmids used were the 2019 amino acid isoform of the mouse voltage-gated sodium channel (pcDNA3-mNa$_v$1.5; a gift from Thomas Zimmer, University of Jena, Germany[6]), human Na$_v$1.5 (pcDNA3.1-hNa$_v$1.5), and three differently tagged hNa$_v$1.5 (pcDNA3.1-hNa$_v$1.5-GFP-N-terminal,

pEYFP-hNa$_v$1.5, and pcDNA3.1-FLAG(299/300)-hNa$_v$1.5). The FLAG-tag is an eight amino acid-long epitope (DYKDDDDK) that was inserted previously (by Robert S Kass laboratory) into the extra-cellular loop linking the transmembrane segments S5 to S6 of domain I, between the residues Leu-299 and Val-300; GFP and YFP were previously added by T. Zimmer to the N-terminal[7]. For wild-type and tagged hNa$_v$1.5, 1 μg of one of the listed plasmids, 1 μg of empty pcDNA3.1 (Invitrogen), and 0.4 μg of DNA coding for CD8 (Robert S Kass laboratory) were used for transfection. In order to measure currents of comparable size, 0.01–1 μg of mNa$_v$1.5 was co-transfected with 1 μg of empty pcDNA3.1, and 0.4 μg of DNA coding for CD8. Transfected HEK293 cells were then grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco) with 10% calf serum (Gibco), 0.2% glutamine (Sigma), and 20 mg/ml gentamycin (Gibco), and incubated at 37°C with 95%O_2/5%CO_2.

Cellular electrophysiology

All experiments were performed in the whole-cell voltage-clamp mode. The extracellular solution contained (in mM): 50 NaCl, 80 NMDG-Cl, 5 CsCl, 2 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES, 5 Glucose, adjusted to pH 7.4 with CsOH, and with an osmolality of 280–290 mOsm. The internal solution consisted of (in mM): 70 CsAsp, 60 CsCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES; 11 Cs_2EGTA, 5 Na_2ATP, adjusted to pH 7.2 with CsOH, and with an osmolality of 297 mOsm. Recordings were performed at room temperature (20–22°C) using a VE-2 amplifier (Alembic Instruments, Montreal, Canada). Data was acquired by Clampex 10.2 (Axon Instruments, Union City, Canada). Membrane resistance was ≥ 1 GΩ and access resistance ≤ 6.1 MΩ. Transfected cells were recognized by the addition of 1 μl/ml Dynabeads CD8 (Invitrogen) into the extracellular solution. Current-voltage (I/V) curves were assessed by depolarising cells from a holding potential of -100 mV to voltages of between -80 and 40 mV during 20 ms. Steady-state inactivation properties were measured by the following protocol: the cells were kept at a holding potential of -100 mV and then hyper- and depolarised during 500 ms to voltages of between -120 and 0 mV in steps of 5 mV, followed by 20 ms at the voltage that elicited the maximal response during the I/V-protocol. Voltage-dependent activation was read either from the I/V- or the steady-state inactivation-protocol. To characterise the recovery from inactivation, the cells were depolarised from a holding potential of -100 mV for 100 ms, repolarised to -100 mV at a recovery time of 0.25–3000 ms, and depolarised again for 25 ms. By varying the time of the first depolarisation step from 3 to 3000 ms followed by 25 ms of repolarisation, the onset of slow inactivation was determined (see insets of Figure 2 and Figure 4).

Data analyses and statistics

Peak values for all protocols were detected and measured by Clampfit 10.2 and I/V-relationships were fitted using KaleidaGraph 3.5 (Synergy Software, Reading, USA). Values were normalised to membrane capacitance. The following formula was used to fit I/V-curves and to calculate reversal potentials: $I_{Na} = (G_{max}(V-V_{rev,Na}))/(1+e^{V-V0.5/K})$ with I_{Na} = sodium current in pA, G_{max} = max. conductance = 60 Ω⁻¹, $V_{rev,Na}$ = reversal potential = 40 mV, K = $(-z\delta F)/FR$ = equilibrium constant = -5, $V_{0.5}$ = voltage for 50% of maximum current = -20 mV. Activation and inactivation curves were fitted with the Boltzmann equation $f_0 = 1/(1+ e^{V-V0.5/K})$ with f_0 = fraction of open

channels/total available channels. Statistical analyses were performed using two-tailed Student's t-tests. A p value <0.05 was considered statistically significant.

Results
Electrophysiological properties of human and mouse Na$_v$1.5 are comparable
To compare the biophysical properties of the cardiac sodium channel Na$_v$1.5 from the human (hNa$_v$1.5) or the mouse sequence (mNa$_v$1.5), we measured the electrophysiological properties of

hNa$_v$1.5 and mNa$_v$1.5, transiently expressed in HEK293 cells. Representative I$_{Na}$ recordings are shown in Figure 1. The responses to all applied protocols revealed similar characteristics for both channels, except for the reversal potential and the slope of steady-state inactivation (Figure 2 and Table 1). The peak currents from the I/V-protocol were at -15 mV for both channels (Figure 2A). Furthermore, activation and inactivation of 50% of the channels occurred for both channels at ~-28 mV and ~-71 mV, respectively. In addition, the slopes of the activation curve were comparable for both channels (6.00 mV/e-fold in human and 6.24 mV/e-fold

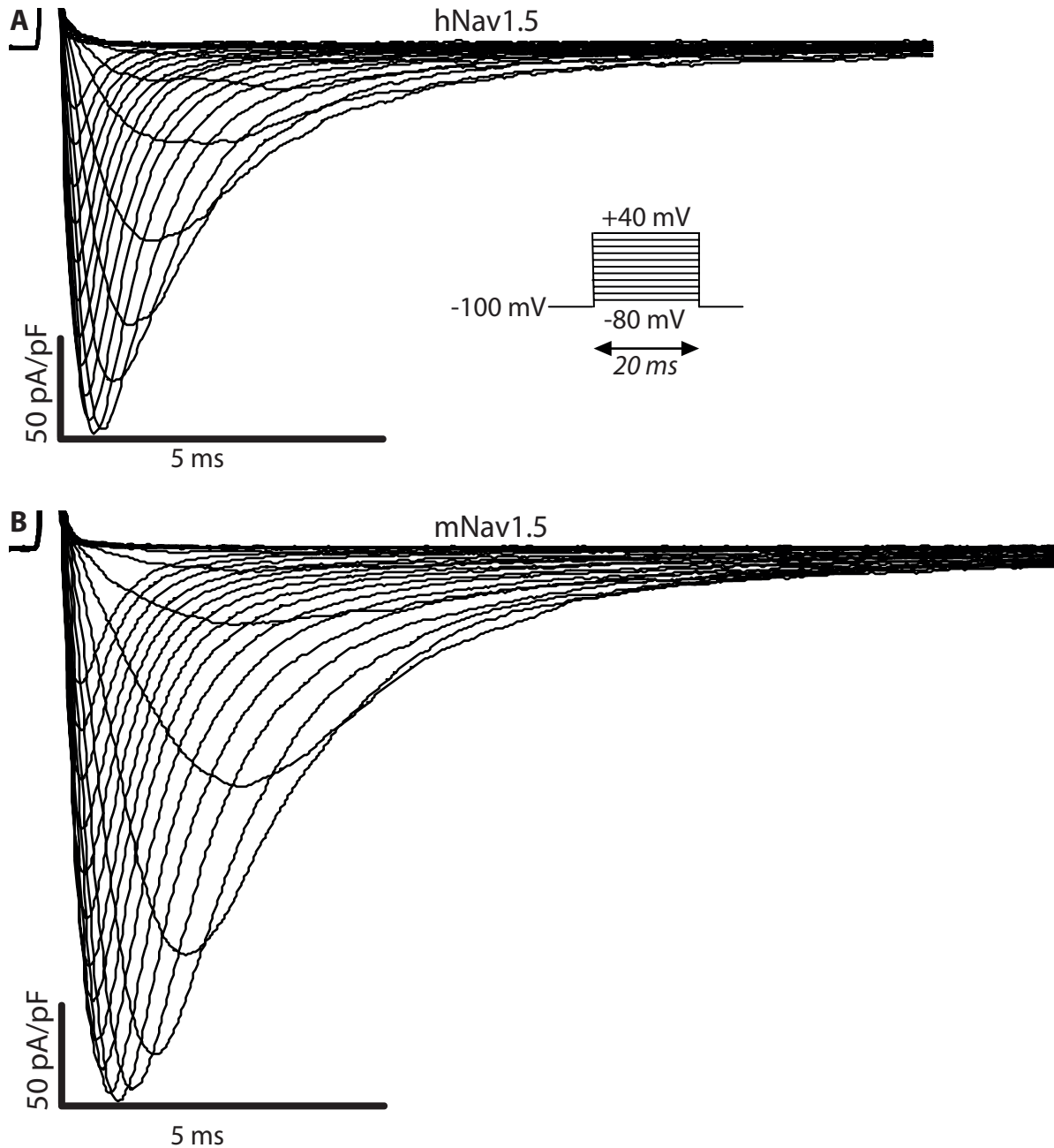**Figure 1. Representative I$_{Na}$ recordings following the current voltage (I/V)-protocol described in the Methods**. (**A**) Voltage-dependent currents measured for hNa$_v$1.5 expressed in a HEK293 cell. (**B**) Data from the same protocol for mNa$_v$1.5.
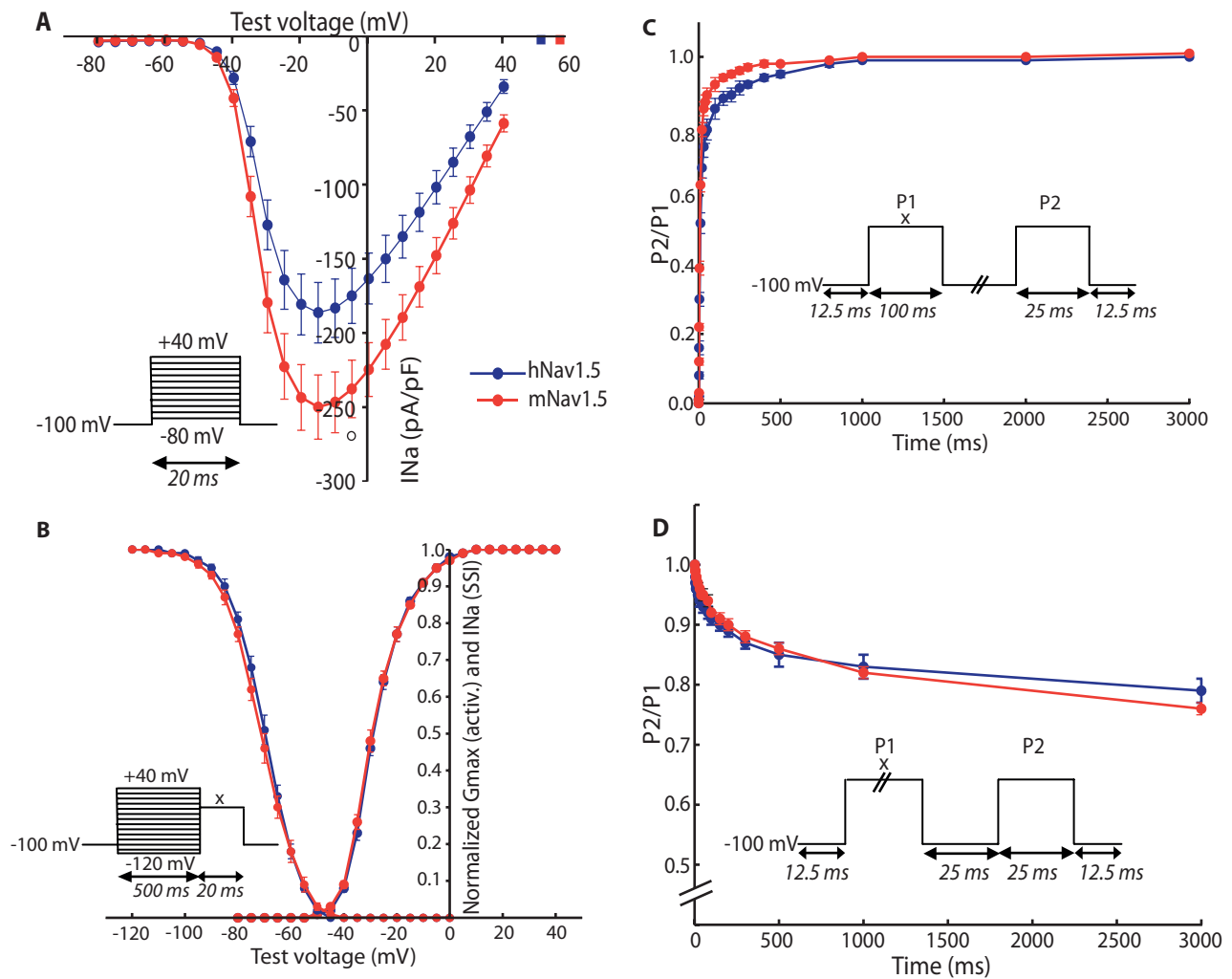
Figure 2. Electrophysiological properties of human and mouse Na$_v$1.5. The voltage-clamp protocols used are shown in the corresponding insets. For **B–D**, the voltage x was adjusted to the voltage that elicited maximum current during the current voltage (I/V)-protocol (-10 to -30 mV). (**A**) I/V-protocol for assessment of reversal potentials. Peak currents were measured for both channels at -15 mV. Calculated reversal potentials are marked with square data points. (**B**) Voltage-dependence of activation and steady-state inactivation (SSI). The data was fitted with the Boltzmann formula. Only the slope of the inactivation curve differs between mouse and human sodium channels (shallower in mNa$_v$1.5). (**C**) Recovery from inactivation. The duration between the depolarising steps was varied from 0.25 to 3000 ms. mNa$_v$1.5 had a slight tendency to recover faster than hNa$_v$1.5. (**D**) Onset of slow inactivation. The duration of the first step was varied from 0.25 to 3000 ms. The relative number of channels entering slow inactivation is similar for both types. (**A–B**) n(hNa$_v$1.5) = 22, n(mNa$_v$1.5) = 17. (**C–D**) n(hNa$_v$1.5) = 9, n(mNa$_v$1.5) = 7. **P<0.01 obtained by two-tailed Student's t-tests; error bars indicate standard errors.

in mouse). Significant differences could be detected in the reversal potential V_{rev} (51.0 mV and 56.6 mV, P<0.01) and in the slope of the inactivation curve (5.95 mV/e-fold and 6.67 mV/e-fold, P<0.01) (Figure 2B). In addition, mNa$_v$1.5 had a tendency to recover faster from inactivation (Figure 2C). The fraction of channels entering into a slow inactivation state was similar for both channel types (Figure 2D).

> **Raw data from sodium current recordings obtained from untagged human and mouse sodium channels (Na$_v$1.5) expressed in HEK293 cells**
>
> *1 Data File*
>
> **http://dx.doi.org/10.6084/m9.figshare.155795**

FLAG-tag inserted at the L299/V300 site alters voltage-dependent activation of hNa$_v$1.5

The second set of experiments addressed the effects of adding epitopes to Na$_v$1.5 on its biophysical properties. To do this, we assessed the influence of these epitopes on I$_{Na}$ by expressing differently tagged hNa$_v$1.5 in HEK293 cells and performing whole-cell voltage-clamp experiments similar to those described above. YFP- and GFP-tags were added to the N-terminus; the FLAG-tag was inserted into the extracellular loop linking S5 to S6 of domain I, between residues Leu-299 and Val-300. Representative I$_{Na}$ recordings for all transfected constructs are shown in Figure 3 and the data is summarised in Table 2. With the exception of the GFP-tagged construct, tagging of hNa$_v$1.5 led to a significant decrease in peak current I$_{max}$ (Figure 4A) compared to the control WT hNa$_v$1.5 (FLAG: 57 pA/pF with

Table 1. Summarized properties of human and mouse Na$_v$1.5. Data was obtained with current voltage (I/V)- and steady-state inactivation protocols. Mean values and standard errors are shown. **P<0.01 obtained by two-tailed Student's t-tests.

		hNa$_v$1.5	mNa$_v$1.5
		mean ± sem	mean ± sem
I/V	V$_{rev}$ (mV)	51.0 ± 0.9	**56.6 ± 0.8
Activation	V$_{1/2}$ (mV)	-27.8 ± 0.5	-28.2 ± 0.7
	Slope (mV/e-fold)	6.00 ± 0.17	6.24 ± 0.16
Inactivation	V$_{1/2}$ (mV)	-70.0 ± 1.0	-71.7 ± 1.1
	Slope (mV/e-fold)	5.95 ± 0.12	**6.67 ± 0.19
Cell capacitance	pF	16.0 ± 1.2	14.2 ± 0.7
I$_{max}$	pA/pF	-185 ± 21	-249 ± 22
n		22	17

P<0.01, YFP: 120 pA/pF with P<0.05, WT hNa$_v$1.5: 240 pA/pF). Adding GFP did not affect any of the biophysical properties of the human sodium channel, while a shallower activation slope (6.87 vs. 5.91 mV/e-fold, P<0.05, Figure 4B and Table 2) was observed for the YFP-tagged channel. The most pronounced effects were observed for the FLAG-tagged hNa$_v$1.5. The activation slope was significantly shallower (6.96 vs. 5.91 mV/e-fold, Figure 4B and Table 2), indicating that the activation of this channel is less sensitive to voltage changes. In addition, the V$_{1/2}$ of activation was shifted towards more positive voltages by about 5 mV, with -23.9 mV in FLAG-hNa$_v$1.5, compared to -28.9 mV in untagged hNa$_v$1.5. Finally, the reversal potential was decreased in the FLAG-hNa$_v$1.5 (FLAG 39.3 mV and untagged 51.8 mV, Figure 4B). Recovery from inactivation (Figure 4C) and onset of slow inactivation (Figure 4D) were comparable for all channels.

Discussion

The present study demonstrates (1) that the biophysical properties of mouse Na$_v$1.5 are essentially similar to the human homolog when expressed in HEK293 cells, and (2) that adding epitopes either upstream of the N-terminus of human Na$_v$1.5 or in one of the extracellular loops reduces the amount of I$_{Na}$ and alters some of its biophysical properties. Interestingly, GFP in the N-terminus was the only epitope that did not modify any of the measured biophysical properties of hNa$_v$1.5. The most pronounced effects could be observed by the insertion of the FLAG-tag in an extracellular loop. In this construct, not only was the amount of I$_{Na}$ drastically decreased, but also the activation properties of the sodium channel were altered. The smaller changes found in the properties of YFP-hNa$_v$1.5 might be partially linked to the different vector used for this epitope, especially since no alterations could be observed for GFP-hNa$_v$1.5.

The limitations of studying mutant Na$_v$1.5 channels in mammalian cells have been demonstrated in two recent studies. First, Mohler and colleagues[8] observed that the Brugada syndrome causing mutant p.E1053K Na$_v$1.5 channel did not display any trafficking

Table 2. Summarized properties of wild-type and tagged hNa$_v$1.5. Data was obtained with current-voltage (I/V)-, and steady-state inactivation protocols. Mean values and standard errors are shown. *P<0.05, **P<0.01 obtained by two-tailed Student's t-tests (all statistics were calculated with untagged hNa$_v$1.5 channel as a reference).

|		hNa$_v$1.5	FLAG-hNa$_v$1.5	YFP-hNa$_v$1.5	GFP-hNa$_v$1.5
		mean ± sem	mean ± sem	mean ± sem	mean ± sem
I/V	V$_{rev}$ (mV)	51.8 ± 0.9	**39.3 ± 2.2	49.1 ± 1.5	53.8 ± 1.9
Activation	V$_{1/2}$ (mV)	-28.9 ± 0.6	**-23.9 ± 0.5	-27.5 ± 1.1	-29.8 ± 1.1
	Slope (mV/e-fold)	5.91 ± 0.17	**6.96 ± 0.14	*6.87 ± 0.27	5.40 ± 0.36
Inactivation	V$_{1/2}$ (mV)	-70.6 ± 1.2	-70.0 ± 1.1	-71.2 ± 1.4	-68.9 ± 1.2
	Slope (mV/e-fold)	5.95 ± 0.24	5.33 ± 0.15	5.69 ± 0.19	6.37 ± 0.19
Cell capacitance	pF	14.4 ± 1.6	16.3 ± 1.1	14.5 ± 0.9	14.0 ± 0.8
I$_{max}$	pA/pF	240 ± 36	**57 ± 11	*120 ± 17	214 ± 33
n		7	11	11	8
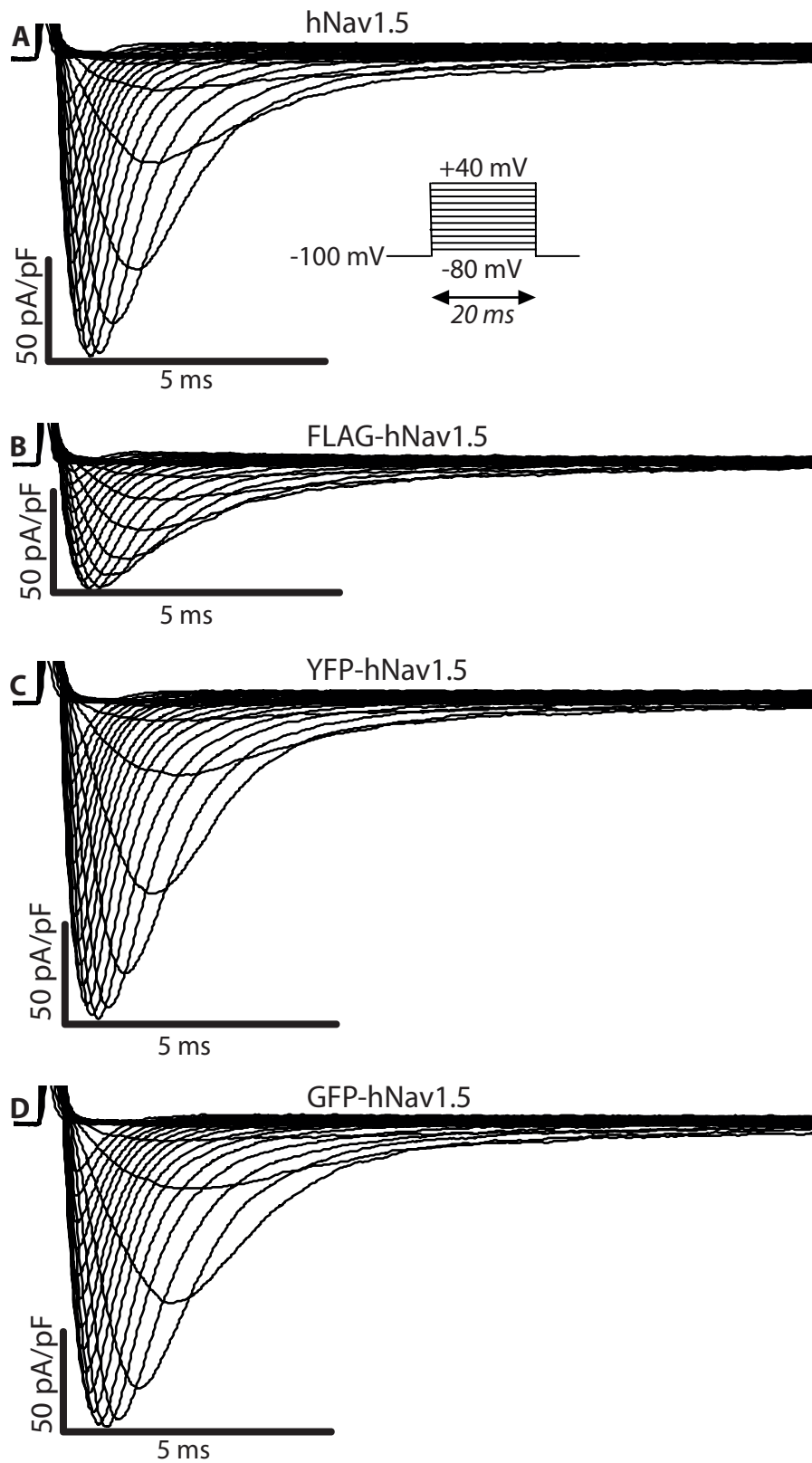
Figure 3. Representative sodium current (I$_{Na}$) recordings. (**A**) Voltage-dependent currents measured for hNa$_v$1.5 expressed in a HEK293 cell. The same data for (**B**) FLAG-hNa$_v$1.5 (**C**) YFP-hNa$_v$1.5, and (**D**) GFP-hNa$_v$1.5.
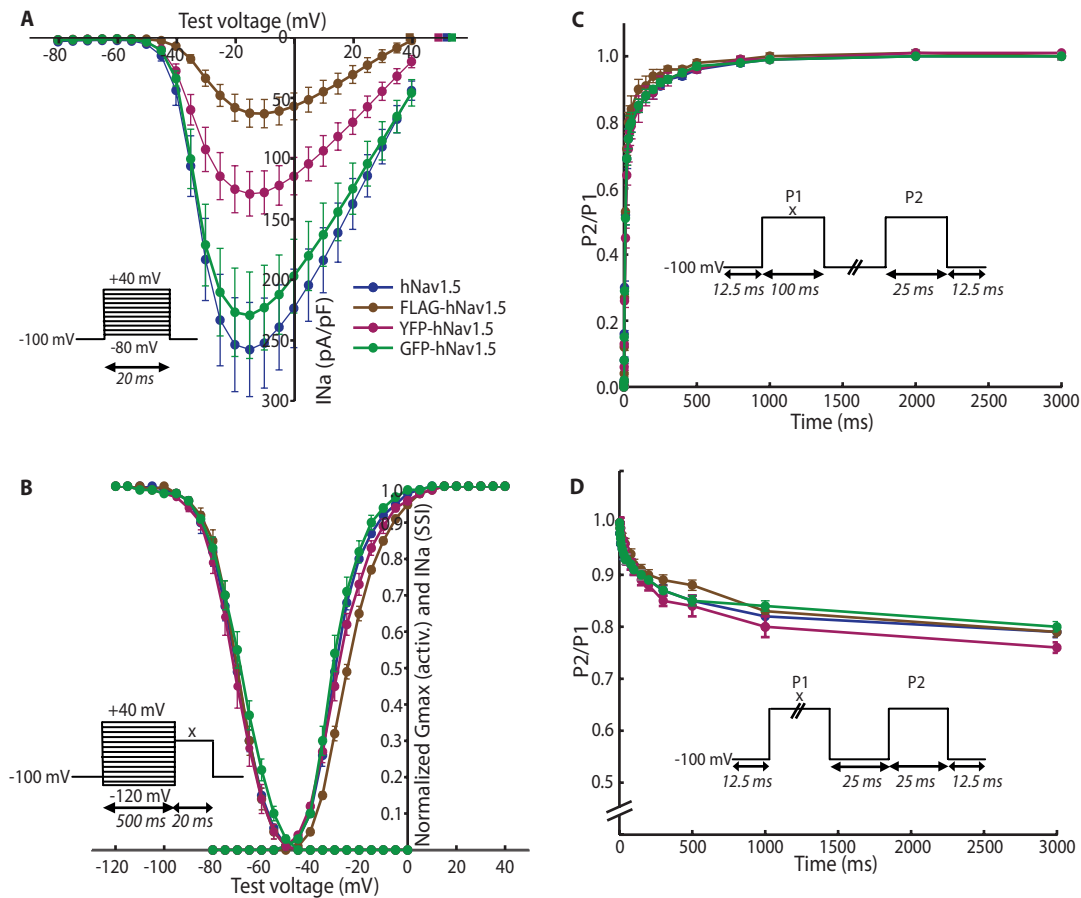
Figure 4. Electrophysiological properties of untagged and tagged hNa$_v$1.5. The voltage-clamp protocols used are shown in the corresponding insets. For **B–D**, the voltage x was adjusted to the voltage that elicited maximum current during the current voltage (I/V)-protocol. (**A**) I/V-protocol for assessment of reversal potentials. Tagging with N-terminal YFP and FLAG (L299/V300) significantly decreases peak currents. Calculated reversal potentials are marked with square data points. (**B**) Voltage-dependence of activation and steady-state inactivation. The data was fitted with the Boltzmann formula. The activation slope of FLAG- and YFP-tagged channels is shallower compared to the untagged hNa$_v$1.5. V$_{1/2}$ is shifted by 5 mV for FLAG-hNa$_v$1.5. (**C**) Recovery from inactivation. The duration between the depolarising steps was varied from 0.25 to 3000 ms. No differences between the different channels could be detected. (**D**) Onset of slow inactivation. The duration of the first step was varied from 0.25 to 3000 ms. The relative number of channels entering slow inactivation is similar for all four channel types. (**A–B**): n(untagged) = 7, n(FLAG) = 11, n(YFP) = 11, n(GFP) = 8. (**C**): n(untagged) = 12, n(FLAG) = 5, n(YFP) = 11, n(GFP) = 8. (**D**): n(untagged) = 10, n(FLAG) = 8, n(YFP) = 10, n(GFP) = 8. **P<0.01 obtained by two-tailed Student's t-tests; error bars indicate standard errors.

defect in HEK293 cells, while it failed to traffic to the intercalated discs when expressed in rat ventricular cells. Second, the Na$_v$1.5 p.D1275N variant, found in patients with dilated cardiomyopathy and various arrhythmias and conduction disease, was also found to display reduced expression in knocked-in mouse cardiac tissue and defective expression at the lateral membrane of ventricular myocytes[9]. However, when expressed in chinese ovary cells, the p.D1275N variant had properties that were undistinguishable from wt channels. These observations demonstrate that, while useful to study their intrinsic biophysical properties, the mammalian cells that are used as expression systems have clear limitations when studying the trafficking properties of ion channels. Generation of

genetically-modified animal models is one of the most powerful, albeit time-consuming, approaches.

However the findings of the present study have to be taken into account when planning to generate such mouse models that harbour specific epitopes in the mouse Na$_v$1.5 gene. Different combinations of epitopes and insertion sites might reveal better candidates for *in-vivo* approaches. Furthermore, additional studies should be performed in HEK293 cells co-expressing other subunits and regulating proteins, and in native cardiomyocytes in order to assess the effects of added epitopes on the interactions with these proteins.

Author contributions

KR, JSR, JO, and HA designed the experiments. KR performed the experiments and analysed the data. KR and HA wrote the manuscript. JSR, JO, and HA supervised the project. All authors commented on the manuscript and approved the final manuscript for publication.

Competing interests

No competing interests were disclosed.

Grant information

Acknowledgments

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   PubMed Abstract | **Publisher Full Text**

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Céline Fiset
Faculty of Pharmacy, Centre de Recherche de l'Institut de Cardiologie de Montréal, Université de Montréal, Montréal, QC, Canada

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Referee Report 20 May 2013

doi:10.5256/f1000research.1309.r958

Jamie Vandenberg
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The discussion has been appropriately modified to take into account the suggestions I made after reviewing the first version of the manuscript. The authors have not however added in any more data or analysis of the trafficking phenotypes of the tagged proteins. I still believe that data comparing the forward trafficking and stability of the different epitope tagged constructs would be worth having. This could be achieved using Western blot / immunohistochemistry and/or live cell imaging.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.

Version 1

Referee Report 25 February 2013

doi:10.5256/f1000research.1119.r798

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This manuscript from Reinhard and colleagues describes the electrophysiology of epitope tagged sodium channels expressed in mammalian cell lines. This system is commonly used for the characterizing the electrophysiological and/or trafficking phenotypes of clinically occurring mutants. Some of the constructs described in this study have been reported before (see e.g. reference 7 in the manuscript: Zimmer T, Biskup C, Dugarmaa S, *et al.*: Functional expression of GFP-linked human heart sodium channel (hH1) and subcellular localization of the a subunit in HEK293 cells and dog cardiac myocytes. *J Membr Biol*. 2002; **186** (1): 1–12) but to my knowledge the construct with the insertion of a FLAG-tag into the extracellular loop linking S5 to S6 of domain I, between residues Leu-299 and Val-300, is novel. Whilst it is unfortunate that this construct has altered electrophysiological properties, it should be useful for live cell trafficking assays as the extracellular epitope can be recognized without having to lyse the cells. Reagents such as those described in this study have been extremely useful and will continue to be so. In this context it is useful to have proper baseline characterization of their properties, which can be used as a reference point for other users.

The manuscript however could have benefited from a more thorough analysis of the trafficking properties of the tagged channels and a broader discussion of the advantages and limitations of the use of the mammalian expression system for characterizing mutant sodium channels. One particularly pertinent recent example of the limitations of the heterologous expression system is that of the D1275N cardiac sodium channel which causes minimal perturbation to gating when expressed *in vitro* but has a marked loss of function when expressed *in vivo* in gene-targeted mice (see Watanabe H *et al.*, Striking In Vivo Phenotype of a Disease-Associated Human SCN5A Mutation Producing Minimal Changes in Vitro, *Circulation*. 2011; **124**:1001-1011.)

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Competing Interests: No competing interests were disclosed.

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I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Competing Interests: No competing interests were disclosed.