

## Role in fast inactivation of conserved amino acids in the IV/S4–S5 loop of the human muscle Na<sup>+</sup> channel

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### Abstract

Since it has been shown that point mutations in the S4–S5 loop of the Shaker K<sup>+</sup> channel may disrupt fast inactivation, we investigated the role of three conserved amino acids in IV/S4–S5 of the adult human muscle Na<sup>+</sup> channel (L1471, S1478, L1482). In contrast to the K<sup>+</sup> channel mutations, the analogous substitutions in the Na<sup>+</sup> channel (S1478A/C, L1482A) did not substantially affect fast inactivation. Nevertheless, the mutations S1478A/C/Q shifted the voltage dependence of steady-state inactivation; L1471Q and S1478C slowed recovery from inactivation. In contrast, a novel non-conserved IV/S4–S5 mutation causing paramyotonia congenita (F1473S) slowed fast inactivation 2-fold and accelerated recovery from inactivation 5-fold. The results indicate involvement of the IV/S4–S5 loop of the human muscle Na<sup>+</sup> channel in fast inactivation, but different roles for conserved amino acids among Na<sup>+</sup> and K<sup>+</sup> channels.

**Keywords:** Human; Skeletal muscle; Na<sup>+</sup> channel; Inactivation; Myotonia; Patch-clamp

Voltage-gated Na<sup>+</sup> and K<sup>+</sup> channels are responsible for the initiation and propagation of action potentials in nerve and muscle cells. The Na<sup>+</sup> channel  $\alpha$ -subunit contains four domains (I–IV) of six transmembrane segments each (S1–S6), whereas K<sup>+</sup> channels are tetramers of single domains. In response to voltage changes during action potentials channels open from the resting, closed state, and then inactivate spontaneously. Different models for the gating of ion channels have been described and in the recent past, a combination of molecular biology and electrophysiology has allowed an approach towards identifying the molecular determinants of the function of these proteins.

Armstrong and Bezanilla [1] proposed a ‘ball and chain’ model for channel inactivation, in which a cytoplasmic, tethered ball occludes the internal mouth of the pore. Indeed, a cluster of 20 amino acids at the N-terminus of the Shaker K<sup>+</sup> channel, and three amino acids (IFM) in the

III–IV interlinker of the voltage-gated Na<sup>+</sup> channel were identified to form a possible inactivation particle [3,10]. There is suggestive evidence that the receptor catching the ball is located in the intracellular S4–S5 loop of the K<sup>+</sup> channel [4] whereas the location of a putative receptor for the Na<sup>+</sup> channel gate is still unknown. It has been shown that the III–IV interlinker of the Na<sup>+</sup> channel can confer fast inactivation to a slowly inactivating isoform of the K<sup>+</sup> channel [9] and for both, K<sup>+</sup> and Na<sup>+</sup> channels, hydrophobic interactions between the inactivation particle and its receptor have been proposed [3,10]. Thus, due to the structural and functional similarities between K<sup>+</sup> and Na<sup>+</sup> channels, the S4–S5 loop might play a similar important role in fast inactivation gating of the Na<sup>+</sup> channel.

Three amino acids in IV/S4–S5 of the Na<sup>+</sup> channel, L1471, S1478 and L1482, are conserved as compared to S4–S5 of the Shaker K<sup>+</sup> channel (and in most cases also in domain I–III of the Na<sup>+</sup> channel as well as in domains I–IV of the Ca<sup>++</sup> channel). To look for the function of these conserved amino acids in the adult human skeletal muscle Na<sup>+</sup> channel, we made several point mutations and com-

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pared their functional consequences to those of the naturally occurring IV/S4–S5 mutation, F1473S. F1473S causes the autosomal dominant muscle disease paramyotonia congenita (PC; Heine, Herzog, Deymeer and Lehmann-Horn, submitted) which belongs to the muscle Na<sup>+</sup> channel diseases [2].

Site-directed mutagenesis was performed using the Altered Sites system employing the plasmid vector pSELECT (Promega Corporation, [7]). Mutagenic oligonucleotides (antisense strand) were: L1471Q, 5'-CGAACAGCTGCGTACGGAT; S1478A, 5'-AGAGGGCAGGCCATCATAAGGGCGAACA; S1478Q, 5'-AGGCAGCTGCATCATAAGGGCGAACAG; L1482A, 5'-GAAAGCGGCAGGCAGCGACATCATAAGGG; F1473S, 5'-TCATGAGGGCGGACAGCAGCGTC. Two inserts were introduced at position R1469, where a unique restriction site was found for BseAI, using the following primers; insert AAPA, 5'-CCGAGCAGCACCAGC, 5'-CCGGGCTGGTGTCTGC; insert RTLLFA, 5'-CCGAGCGTCTGTTCGC, 5'-CCGGGCGAACAGCAGCGT. All mutations were verified by dideoxynucleotide sequencing. Full length wild type (WT) and mutant constructs were assembled in the mammalian expression vector pRC/CMV and transfected transiently or permanently into HEK293 cells as described [7].

Standard whole-cell recording was performed using an EPC-7 amplifier (List) and pClamp (Axon Instruments) data acquisition (prepulse protocol-P/4; 3 kHz filtering; 20 kHz sampling) as described [7]. Data were analyzed by a combination of pClamp, Excel (Microsoft) and SigmaPlot software (Jandel Scientific). Currents for WT and all mutants ranged from 1 to 5 nA. Residual series resistance was 0.5 to 1.5 M $\Omega$  (60–85% compensation) and the maximal voltage error was always <5 mV. The pipette solution contained (in mM): 130 CsCl, 5 NaCl, 2 MgCl<sub>2</sub>, 5 EGTA and 10 HEPES (pH 7.4); bathing solution: 140 NaCl, 4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 dextrose and 5 HEPES (pH 7.4). For statistic evaluation Student's *t*-test was applied. All data are shown as means  $\pm$  SEM.

Fig. 1 shows the proposed structure of the S4/S5 segments of the fourth domain of the adult human skeletal muscle Na<sup>+</sup> channel  $\alpha$  subunit (hSkM1) and the S4–S5 interlinker including all inserts and point mutations made. In order to distort the structure of IV/S4–S5 we first introduced a four amino acid insert (AAPA, Fig. 1) at the N-terminal part of IV/S4–S5 and a six amino acid insert in the middle (RTLLFA, Fig. 1). Unfortunately, we could not detect any Na<sup>+</sup> current in cells transfected with either of these two constructs.

We next focused on amino acids conserved among Na<sup>+</sup> and K<sup>+</sup> channels (Fig. 1). We introduced the same mutations known to disrupt N-type inactivation at the analogous position of the Shaker K<sup>+</sup> channel ([4], S1478A/C and L1482A) and two more 'hydrophilic' mutations (L1471Q, S1478Q). Although there might be certain similarities between K<sup>+</sup> and Na<sup>+</sup> channel fast inactivation gat-

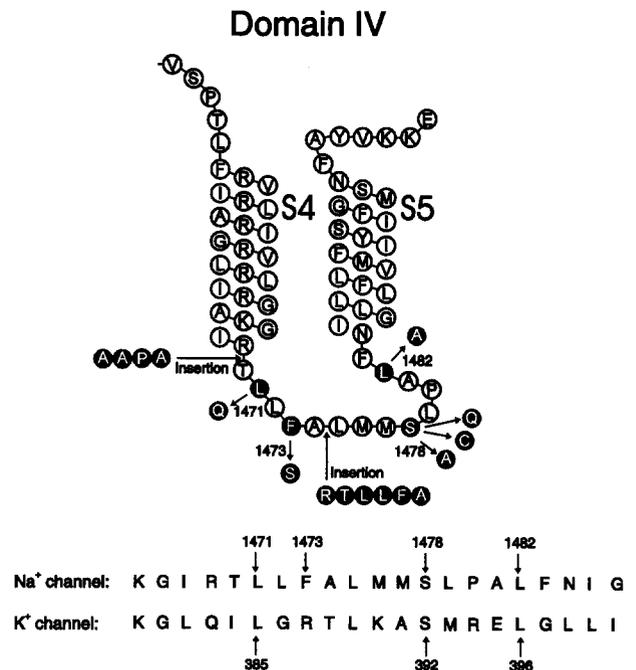


Fig. 1. Model of segments S4, S5 and the S4–S5 cytoplasmic loop in the fourth domain of the  $\alpha$ -subunit of the adult human muscle Na<sup>+</sup> channel (hSkM1). The naturally occurring mutation F1473S causing paramyotonia congenita and all other artificial mutations introduced are indicated. The amino acid sequences of the IV/S4–S5 loop of hSkM1 and S4–S5 of the Shaker K<sup>+</sup> channel are compared.

ing [9], none of the Na<sup>+</sup> channel mutations affected fast inactivation to a great extent (Fig. 2A,C; Table 1). The largest difference to WT occurred for S1478Q which increased  $\tau_{h1}$  1.5-fold and induced a small persistent Na<sup>+</sup> current of 2–3% of peak current in comparison to less than 1% for WT and all other mutants. Significant differences in the voltage dependence of steady-state inactivation (and to a less degree for steady-state activation) were detected for the S1478A/C/Q mutations (Fig. 2E; Table 1), e.g. a 17 mV shift between S1478Q and S1478C. L1471Q and S1478C slowed recovery from inactivation 1.5- to 2-fold (Fig. 2G,H; Table 1). These results indicate a destabilization of the inactivated state for S1478Q, but a stabilization for mutant S1478C (see below, discussion on F1473S).

If these amino acids of the Na<sup>+</sup> channel formed a receptor for the inactivation gate one should expect disruption of Na<sup>+</sup> channel inactivation resulting in a markedly increased steady-state current and/or markedly slowed inactivation as found for the corresponding K<sup>+</sup> channel mutations [4] or for mutations in the proposed inactivation gate of the Na<sup>+</sup> channel [10]. The most important difference in this regard between Na<sup>+</sup> and K<sup>+</sup> channels is certainly that heterologously expressed mutant K<sup>+</sup> channels are tetramers of four identical domains (i.e. introduction of a single mutation leads in fact to four mutations). Hence, it might be necessary to introduce mutations in all four domains of the Na<sup>+</sup> channel to produce similar effects as observed in the K<sup>+</sup> channel.

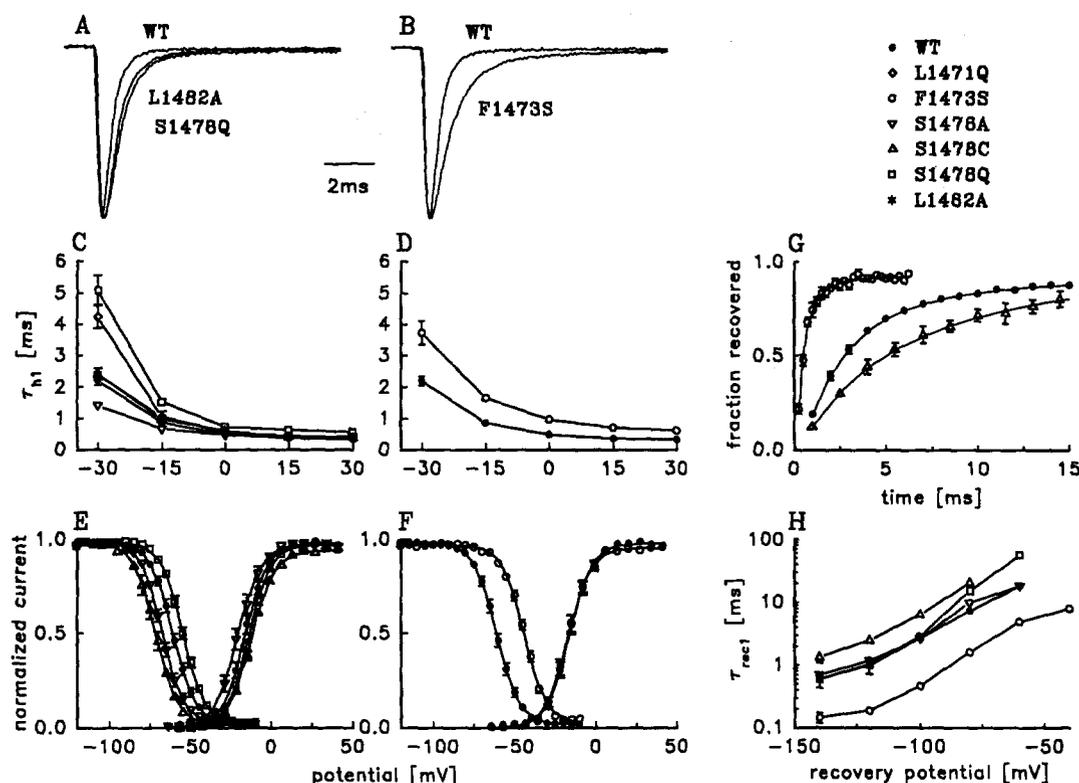


Fig. 2. Effects of IV/S4–S5 mutations on Na<sup>+</sup> channel inactivation and recovery from inactivation. (A,B) Normalized current recordings from HEK293 cells transfected with either WT or mutant human muscle Na<sup>+</sup> channels (currents were 3.0, 3.5, 1.8 and 2.6 nA for WT, L1482A, S1478Q and F1473S, respectively). Currents were elicited by 100 ms voltage steps to 0 mV after a 300 ms prepulse to  $-120$  mV (holding potential  $-85$  mV). The current decay was fitted to a second order exponential function yielding a fast ( $\tau_{h1}$ ) and a slow ( $\tau_{h2}$ ) time constant of inactivation. Both,  $\tau_{h2}$  and its relative amplitude (generally  $< 10\%$ ) were not significantly different between WT and mutant channels. (C,D) Fast time constants of inactivation ( $\tau_{h1}$ ) for WT and mutant channels versus voltage. (E,F) Steady-state inactivation was obtained using 300 ms prepulses from  $-120$  to 0 mV in 5 mV steps followed by a test pulse to 0 mV. Steady-state activation was obtained as described in (A) using test potentials from  $-64$  to 48 mV in 7 mV steps. The lines are fits to standard Boltzmann functions with parameters given in Table 1. (G) To determine recovery from inactivation, cells were held at  $-100$  mV, depolarized for 100 ms to 0 mV and then brought to different 'recovery potentials' for increasing durations before the test pulse to 0 mV. Shown are recovery time courses for WT, F1473S and S1478C at  $-100$  mV. The time course of recovery from inactivation was best fitted with a first ( $-40$  to  $-80$  mV) or a second ( $-100$  to  $-140$  mV) order exponential function. The slower time constant was not significantly different among WT and mutant channels with relative amplitudes of less than 25%. (H) Voltage dependence of the fast time constant of recovery from inactivation. Numbers for all experiments are given in Table 1.

In contrast to mutations of conserved amino acids, the paramyotonia-causing mutation F1473S produced larger alterations of inactivation. Superimposed WT and F1473S current traces are shown in Fig. 2B. The fast

time constant,  $\tau_{h1}$ , was increased 2-fold for F1473S versus WT (Fig. 2D; Table 1) over the whole potential range investigated. The steady-state Na<sup>+</sup> current at the end of a 100 ms test pulse to 0 mV was slightly, but significantly

Table 1

Activation and inactivation parameters of IV/S4–S5 mutants of the adult human muscle Na<sup>+</sup> channel

Mutation	Steady-state activation		Steady-state inactivation		Inactivation time constant at 0 mV		Time constant of recovery from inactivation at $-100$ mV	
	<i>n</i>	$V_{1/2}$ (mV)	<i>n</i>	$V_{1/2}$ (mV)	<i>n</i>	$\tau_{h1}$ (ms)	<i>n</i>	$\tau_{rec1}$ (ms)
WT	10	$-16 \pm 1$	10	$-62 \pm 1$	10	$0.48 \pm 0.01$	9	$2.7 \pm 0.2$
L1471Q	5	$-12 \pm 1^a$	5	$-66 \pm 1^a$	3	$0.58 \pm 0.05^a$	4	$5.0 \pm 0.3^c$
F1473S	14	$-17 \pm 1$	11	$-44 \pm 1^c$	10	$0.97 \pm 0.07^c$	6	$0.46 \pm 0.01^c$
S1478A	7	$-20 \pm 1^a$	8	$-67 \pm 1^a$	9	$0.46 \pm 0.01$	8	$2.6 \pm 0.2$
S1478C	5	$-12 \pm 1^a$	4	$-72 \pm 1^c$	5	$0.60 \pm 0.07^a$	4	$6.3 \pm 0.7^c$
S1478Q	8	$-13 \pm 1$	9	$-55 \pm 1^b$	9	$0.73 \pm 0.05^c$	6	$2.8 \pm 0.2$
L1482A	6	$-16 \pm 2$	7	$-64 \pm 1$	5	$0.57 \pm 0.04^a$	6	$3.0 \pm 0.4$

Boltzmann parameters of steady-state activation and inactivation, fast time constants of inactivation ( $\tau_{h1}$ ) and recovery from inactivation ( $\tau_{rec1}$ ) for WT and all studied mutations (means  $\pm$  SEM, see also Fig. 2). Compared to WT statistically significant differences are <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ , <sup>c</sup> $P < 0.001$ .

increased ( $I_{ss}/I_{peak}$  (%))  $1.9 \pm 0.4$  versus  $0.9 \pm 0.2$ ,  $P < 0.05$ ,  $n = 5-10$ ). The steady-state inactivation curve was shifted by +18 mV (Fig. 2F; Table 1). The most striking result for this mutation, however, was a 5-fold acceleration of recovery from inactivation (Fig. 2G; Table 1). The voltage dependence of the fast time constant of recovery from inactivation,  $\tau_{rec1}$ , was shifted by 20–30 mV in the depolarizing direction (Fig. 2H).

There are three lines of evidence for a destabilization of the inactivated state for F1473S, (1) the slowing of the  $Na^+$  current decay; (2) the depolarizing shift of the inactivation curve indicating a change in the relative free energy of the closed and inactivated state favoring the closed state; and (3) the marked acceleration of recovery from inactivation. For a simple closed-inactivated (C-I) two state model, the time constant of recovery from inactivation is almost exclusively determined by the reciprocal of the rate constant,  $\alpha$ , from I to C in the voltage range where no significant steady-state inactivation occurs ( $\alpha = h_{\infty}/\tau_{rec}$  and  $h_{\infty} \approx 1$ ). Hence, the rate constant,  $\alpha$ , for leaving the inactivated state was increased by about 5-fold. The increased  $\alpha$  also explains the shift of the  $h_{\infty}$  curve ( $h_{\infty} = \alpha/(\alpha + \beta)$ ), since the rate constant from C to I,  $\beta$ , was similar for WT and F1473S ( $\beta = (1-h_{\infty})/\tau_{rec}$ ; at -60 mV 31 versus 24  $s^{-1}$  for WT and F1473S, respectively), whereas  $\alpha$  was 7-fold increased ( $\alpha = h_{\infty}/\tau_{rec}$ ; at -60 mV 25 versus 180  $s^{-1}$ ).

The F1473S mutation causes paramyotonia congenita (PC), an autosomal dominant muscle disease characterized by muscle stiffness increasing with prolonged exercise. The muscle fibre membrane hyperexcitability in PC may be explained by (1) the slowed current decay; (2) the increased persistent  $Na^+$  current; and (3) the faster recovery from inactivation as observed for F1473S (and for other  $Na^+$  channel mutations causing either PC [5,11] or other  $Na^+$  channel disorders [7,8]). (1) and (2) lead to muscle depolarization due to an abnormal  $Na^+$  inward current and (3) shortens the refractory period after an action potential.

Similar  $Na^+$  channel mutants in IV/S4–S5 of the rat brain type IIA  $Na^+$  channel have been investigated by expression in *Xenopus* oocytes [6]. In this study, the mutation L1660A disrupted channel inactivation to a large extent (steady-state current of 20% of peak current) in contrast to the corresponding mutation L1482A in our study. Furthermore, F1651A showed a much larger slowing of the time course of inactivation than F1473S at the corresponding position. The different results may arise from the use of different  $Na^+$  channel types and/or expres-

sion systems, or in case of, F1651A, from the substitution of alanine instead of serine for phenylalanine.

In summary, our study indicates that the IV/S4–S5 loop of the human muscle  $Na^+$  channel is involved in fast inactivation. However, conserved amino acids proposed to be part of a receptor for the inactivation particle in the Shaker  $K^+$  channel, as well as F1473, do not seem to play this specific role in the human muscle  $Na^+$  channel.

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