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ION CHANNELS, TRANSPORTERS

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Impaired slow inactivation due to a polymorphism and substitutions of Ser-906 in the II-III loop of the human $Na_v1.4$ channel

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Abstract The loop connecting domains II and III of the sodium channel α-subunit is not known to have a major effect on channel gating. Recently mutations in the II-III loop of various sodium channel isoforms have been reported to cause channelopathies suggesting the functional importance of this region. In the II-III loop of the skeletal muscle isoform $Na_v1.4$, we found a Ser-to-Thr substitution at position 906 in 5% of patients with dyskalemic periodic paralysis but also in 4% of healthy human individuals. To investigate whether this position is important for channel gating, we characterized the following amino acids at 906 by whole-cell patch-clamp experiments: Gln, Ser, Thr, Cys, Pro, Val, ordered according to their hydrophobicity. All substitutions mainly affected slow inactivation. For example, Gln caused a +13-mV right-shift of the steady-state slow inactivation curve, and entry into slow inactivation was 6 times slower compared with Ser, leading to a destabilization of the slow inactivated state; in contrast, Val, at the other end of the hydrophobicity spectrum, shifted the steady-state slow inactivation curve by –6 mV and slowed the recovery from the slow inactivated state threefold compared with Ser, resulting in an enhancement of slow inactivation. Recovery from the slow inactivated state was also slowed by Pro, Cys and Thr. Our results suggest that (1) a benign polymorphism affects channel function, (2) the II-III loop is important for slow inactivation, and (3) the effects on slow inactivation may depend on the hydrophobicity of the residue at position 906.

Keywords Amino acid hydrophobicity · Benign polymorphism · II-III loop · Skeletal muscle sodium channel

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Introduction

The term "mutation" is commonly used to denote a DNA change which modifies the arrangement, number or molecular sequence of at least one gene [24]. These changes are generally considered to alter protein function causing plenty of hereditary diseases. For these disease-causing mutations, there are several criteria widely accepted by the scientific community: (1) the mutation segregates with the clinical status without recombinations in affected families; (2) it is absent in a large number of non-related unaffected individuals; (3) the encoded protein is expressed in the affected tissue; (4) the mutation leads to a change of the amino acid sequence of the encoded protein in a highly conserved region, or results in a splice variant or exon skipping [9]; and (5) the mutant protein shows functional alterations in a cellular expression system. These functional changes ordinarily serve as a final "proof" that an identified base exchange is a disease-causing mutation.

If at least one of the five criteria is not fulfilled, the DNA change is usually considered to be a "polymorphism", a word derived from the Greek meaning "many appearances". Clinically this term is habitually used to designate a DNA modification which can be found in a part of a population without being present exclusively in persons suffering from some disorder. These DNA alterations do not cause a disease; however, the association with the susceptibility to a so far unknown, e.g. pharmacogenetic disorder, cannot be excluded [19]. Although the polymorphic DNA variations can also result in changes of the amino acid sequence of the encoded protein, these amino acid substitutions are commonly assumed not to affect the protein function and to be of less functional importance.

Even further, many researchers working in molecular biology and particularly in the field of channelopathies believe that the finding of a functional alteration of the protein encoded by DNA different from the "wild type" can serve as the final proof for a disease-causing mutation and is not compatible with a "benign polymorphism".

Regarding sodium channels, we can find this assumption reflected in several statements: (1) a proposed sodium channel mutation was identified as a benign polymorphism, because, beside one other criterion, the expression demonstrated no functional differences from the normal channel [13]; and (2) there is apparently "no margin for error" in sodium channel gating [7]. This opinion is based on the observation that all disease-causing ion channel mutations so far functionally expressed revealed only subtle changes.

Here, we show an example of a sodium channel amino acid substitution, S906T, that we originally thought causes periodic paralysis. However, we found the same substitution in a similar percentage of normal controls and, therefore, we concluded that it is a polymorphism. This substitution is located within a loop joining the second and the third transmembrane domains (II-III loop) of the human skeletal muscle sodium channel α-subunit [$Na_v1.4$ (*SCN4A*)]. As several mutations causing epilepsy [generalized epilepsy with febrile seizures plus (GEFS+), intractable childhood epilepsy with generalized tonic-clonic seizures (ICEGTC)] or cardiac arrhythmia [long QT syndrome type 3 (LQT-3)] were discovered in the II-III loop of various sodium channel isoforms, suggesting the functional importance of this region, we electrophysiologically examined an S906T polymorphism as well as additionally designed substitutions at position 906.

Materials and methods

Genetics

Patients and healthy controls participating in the genetic study gave their informed consent. Experiments were approved by the Ethics Committee of Ulm University and were in concordance with the Declaration of Helsinki. Mutation analysis from genomic DNA using the single strand conformation analysis (SSCA) technique was performed using the human skeletal muscle sodium channel α-subunit *SCN4A* gene in exons 14–16 encoding the II-III loop and parts of the adjacent transmembrane segments II/S6 and III/S1. Aberrant bands were sequenced using an automated 373A sequencer (Applied Biosystems). An allele specific polymerase chain reaction (PCR) test was developed to screen for the *SCN4A* base exchanges identified.

Mutagenesis and patch-clamp experiments

Site-directed mutagenesis was performed using an overlapping PCR-based technique. Subsequently, the mutants were reassembled in the pRC/CMV plasmid (Invitrogen, San Diego, Calif., USA) for transfection by the calcium phosphate precipitation method in tsA201, a mammalian cell line. Cells were incubated at 37°C.

Standard whole-cell recording methods were used as previously described [14]. Individual cells were allowed to equilibrate for 10 min after achieving internal access before acquiring data. The pulse protocols are given in the figure legends. Capacity transients were eliminated by a -P/4 protocol. Series resistance errors were <4 mV. Usually 70% or more of series resistance errors were compensated. Data were filtered at 3 kHz, and obtained using pCLAMP (Axon Instruments, Foster City, Calif., USA). Pipette resistance was in the range 0.8–1.2 MΩ. Patch electrodes contained (in mM): 105 CsF, 35 NaCl, 10 EGTA, 10 Cs-HEPES, pH 7.4. The bath contained 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 Cs-HEPES, pH 7.4. For adjusting pH, CsOH and HCl were used. Corrections were made for liquid junction potentials. All experiments were performed at room temperature (20–24°C). Whole-cell data were analysed by a combination of pCLAMP and ORIGIN (MicroCal) programs. Data are presented as mean ± standard error of the mean. Student's *t* test was applied for statistical evaluation. Significance level was set at $P<0.05$.

Results

S906T substitution in patients and healthy controls

We identified a G2717C base exchange in exon 14 of the *SCN4A* gene predicting an S906T substitution in 6 of 154 healthy controls (4%), in 3 of 54 individuals with neurological diseases not related to channelopathies (6%), and in 8 of 166 unrelated patients (5%) with either hypokalemic periodic paralysis (4 of 89 patients) or hyperkalemic periodic paralysis (4 of 77 patients). This amino acid substitution is located in the II-III intracellular loop of $Na_v1.4$. The frequency of substitution between patients and controls did not significantly differ.

Effects of the S906 substitutions on the fast gating mode

Figure 1 shows typical recordings of sodium currents through wild-type (WT) channels and S906 substitutions. The corresponding current–voltage (*I–V*) and conductance–voltage (*G–V*) relationships were not different among the constructs (Fig. 2A; Table 1). For all the substitutions except for the threonine, time constants of fast inactivation (τ_h) were 1.3- to –1.6-fold increased over the range of −30 mV to +15 mV (n=6–13, $P<0.05$ compared with the WT; Fig. 2D). Recovery from fast inactivation studied at –100 mV was 1.7-fold slower for S906P and S906C mutants, compared with the WT (Fig. 2C; Table 1). For the S906C mutant, fits to a Boltzmann function revealed a significant −4-mV left-shift of the steady-state fast inactivation curve, compared with the WT (Fig. 2B; Table 1), indicating a mild stabilization of the fast inactivated state for this mutant. Thus, the effects of the S906 substitutions on the fast gating process were rather small and non-specific.

Current density

Current density for the S906Q, S906C, S906P and S906V mutants was similar to that of the WT. For the S906T mutant the current density was 1.9-fold reduced compared with the WT (Table 1).

Residue-specific effects of the S906 substitutions on slow channel gating

The most profound effects of the S906 substitutions referred to a slow gating mode. These effects were

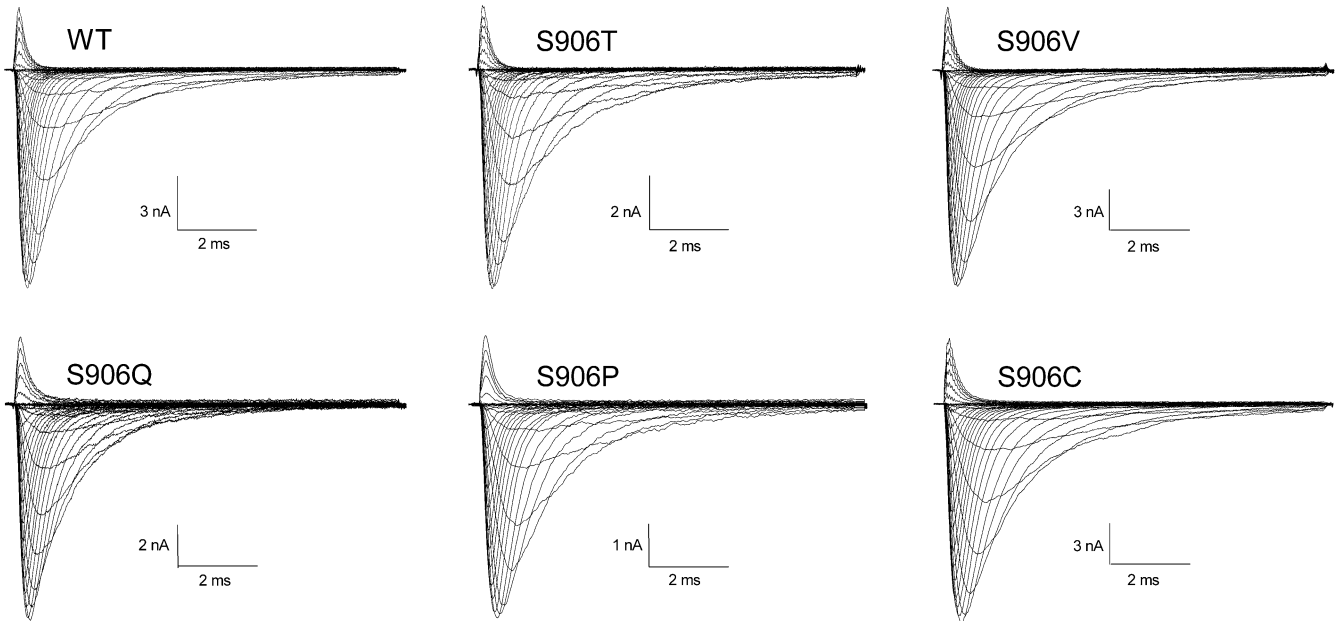

Fig. 1 Sodium currents elicited by a family of 10-ms depolarizations from a –140 mV holding potential to voltages ranging from –90 to +65 mV in 5-mV steps recorded from tsA201 cells expressing WT and S906 mutant α-subunit channels

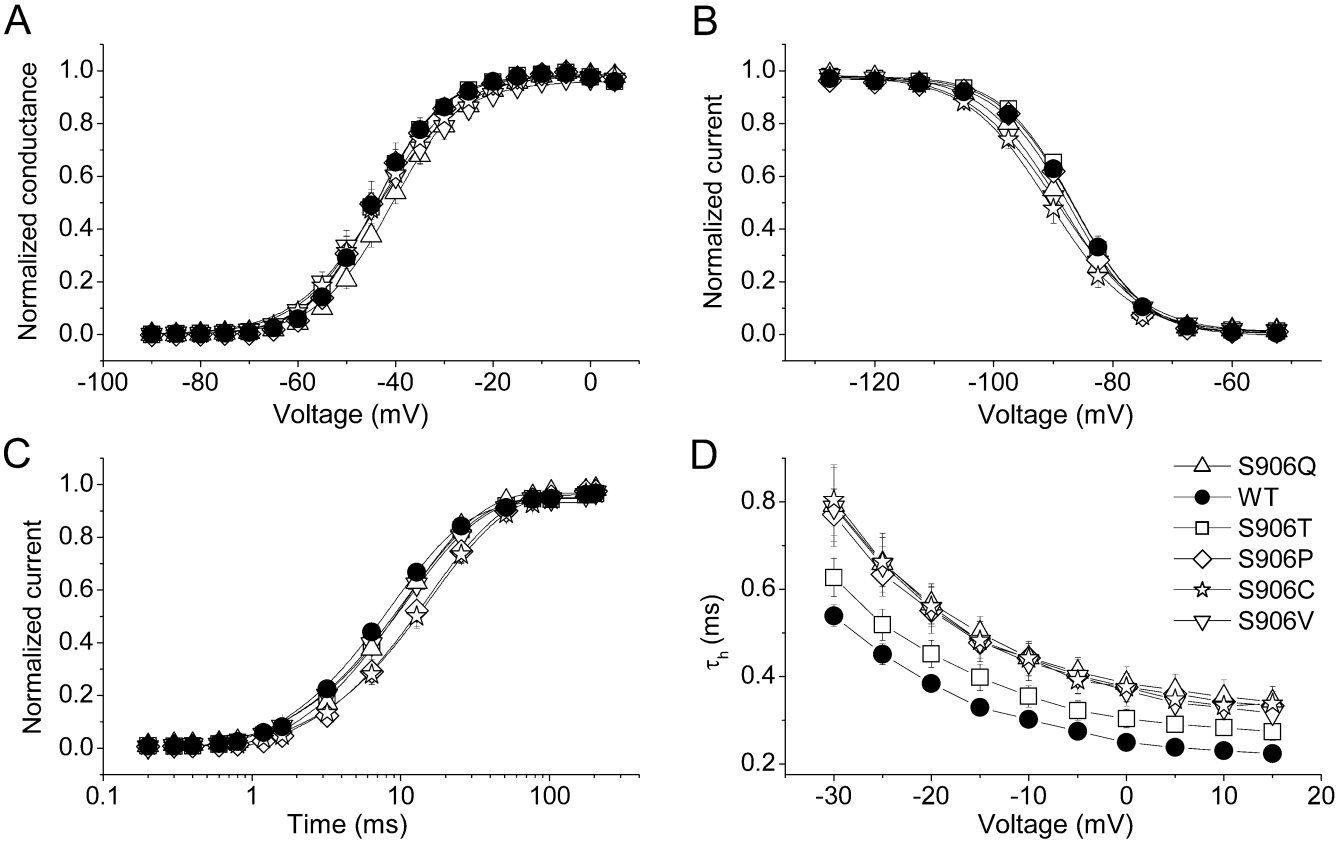

Fig. 2A–D Fast gating parameters. **A** Conductance–voltage relationships for WT and S906 mutants. $V_{1/2}$ of the curve and slope factor k were obtained from a fit of the mean data with a Boltzmann function. **B** Steady-state fast inactivation was determined from a holding potential of -150 mV using a series of 300-ms prepulses from –150 to –45 mV in 7.5-mV increments prior to the test pulse to -20 mV. The mean data were fitted with the Boltzmann function. **C** Recovery from fast inactivation for a holding potential of –100 mV was determined by a 100-ms depolarization to –20 mV followed by a variable-duration return to –100 mV. The mean data were fitted with the monoexponential function. **D** Voltage dependence of fast inactivation time constants, τ_h

Table 1 Electrophysiological parameters for WT and S906 mutants. (k Slope, n number of cells, $V_{1/2}$ half-maximal voltage of activation/inactivation, τ time constant, ω current density.) All data are presented as mean ±standard error of the mean

Channel		S906Q	WT	S906T	S906C	S906P	S906 V
Conductance–voltage relationships	$V_{1/2}$ (mV)	–41±1	–44±2	–44±1	–44±2	–44±2	–43±2
	k	–6.7±0.3	–6.1±0.3	–6.3±0.2	–7.0±0.3	–5.9±0.4	–7.0±0.3
	ω (pA/pF),	466±88	690±101	371±88*	486±108	790±290	456±78
	n	11	10	9	10	6	13
Steady-state fast inactivation	$V_{1/2}$ (mV)	–89±1	–87±1	–87±1	–91±1*	–87±1	–89±1
	k	5.6±0.2	5.3±0.2	5.1±0.1	5.5±0.3	5.0±0.1	5.6±0.2
	n	13	16	10	13	7	22
Recovery from fast inactivation	τ (ms)	11.8±0.9	10.0±0.6	11.8±1.2	17.2±1.8**	16.5±2.7**	13.1±1.8
	n	12	10	11	12	5	14
Steady-state slow inactivation	$V_{1/2}$ (mV)	–55±3***	–68±2	–69±2	–68±3	–69±2	–74±1*
	k	21.1±1.0***	11.3±0.4	12.4±0.7	14.7±1.2**	13.3±0.5**	12.6±1.4
	n	6	11	6	6	7	7
Entry into slow inactivation	τ (s)	11.3±0.8***	2.1±0.3	3.8±0.4**	5.3±1.4*	5.5±0.6***	5.0±0.5***
	n	8	7	7	5	6	6
Recovery from slow inactivation	τ_1 (ms)	70±25	99±11	162±14**	180±13***	195±16***	160±15**
	τ_2 (s)	1.3±0.4	1.2±0.1	1.8±0.1*	3.1±0.5**	5.9±0.9***	4.0±0.8**
	n	5	7	5	6	5	6

*P<0.05, **P<0.01, ***P<0.001

residue-specific and characterized the stability of the slow inactivated state, which can be theoretically quantified from results obtained in experiments measuring steady-state slow inactivation, entry into slow inactivation and recovery from slow inactivation.

The steady-state slow inactivation curve for the S906Q mutant was shifted by +13 mV to depolarized potentials (Fig. 3A; Table 1). Consistent with the right-shift of steady-state slow inactivation, entry into slow inactivation for the S906Q mutant was 5.4-fold slower compared with the WT and 2- to 3-fold slower compared with all the other mutants studied (Fig. 3B; Table 1). These results apparently indicate a destabilization of the slow inactivated state by the S906Q substitution. On the contrary, the S906 V mutant revealed a –6-mV left-shift of the steady-state slow inactivation curve; consistent with this, recovery from slow inactivation for this mutant was slowed, compared with the WT (Fig. 3A, C; Table 1), suggesting a stabilization of slow inactivation by the S906V substitution, i.e. the opposite effect compared with the S906Q mutant.

S906Q and S906V are the mutants with the most destabilized and the most stabilized slow inactivated state, respectively. S906T, S906P and S906C mutants have the slow inactivated state of intermediary stability, because the mid-points ($V_{1/2}$) of the steady-state slow inactivation curves for these mutants were not different from the mid-point for the WT (Table 1). While comparing S906T, S906C and S906P among them, we see that there are no differences in steady-state and entry parameters (Fig. 3A, B; Table 1), but the rate of recovery from slow inactivation for S906 substitutions decreased [increment of the second time constant (τ_2) of recovery] in a sequence: WT<S906T<S906C<S906P (Fig. 3C; Table 1). It may be concluded that the stability of the slow inactivated state increases in the same order, but this statement should be taken with caution, because it is based on only one parameter; and steady-state slow inactivation curves which presumably reflect the balance between entry and recovery processes and may have the highest importance for quantifying stability of the slow inactivated state were not changed. Nevertheless, the general trend is likely to be correct and we believe that the stability of the slow inactivated state for the WT and the S906 mutants increases in the order: S906Q<WT≤S906T≤S906C≤S906P<S906V.

For the amino acid side chains at the position 906 the relations transform in a sequence: Gln<Ser≤Thr≤Cys≤Pro<Val.

This order completely corresponds to the trend of hydrophobicity parameters for the amino acid side chains [5]. Thus, we suggest that position S906C in the II-III loop is involved in slow gating of the skeletal muscle sodium channel and that hydrophilic-hydrophobic balance at this place is important for unimpaired slow inactivation.

Discussion

Benign polymorphism at position S906 modifies channel function

One of most important findings of this study is an effect of the benign polymorphism on channel function. S906T represents the first naturally occurring sodium channel variant that shows affected gating but does not cause a disease, thus contradicting the commonly accepted assumption that a benign polymorphism does not alter channel function.

The reason for this benign character of S906T may be a unique modification of slow gating transitions. Usually, for disease-causing sodium channel mutations which

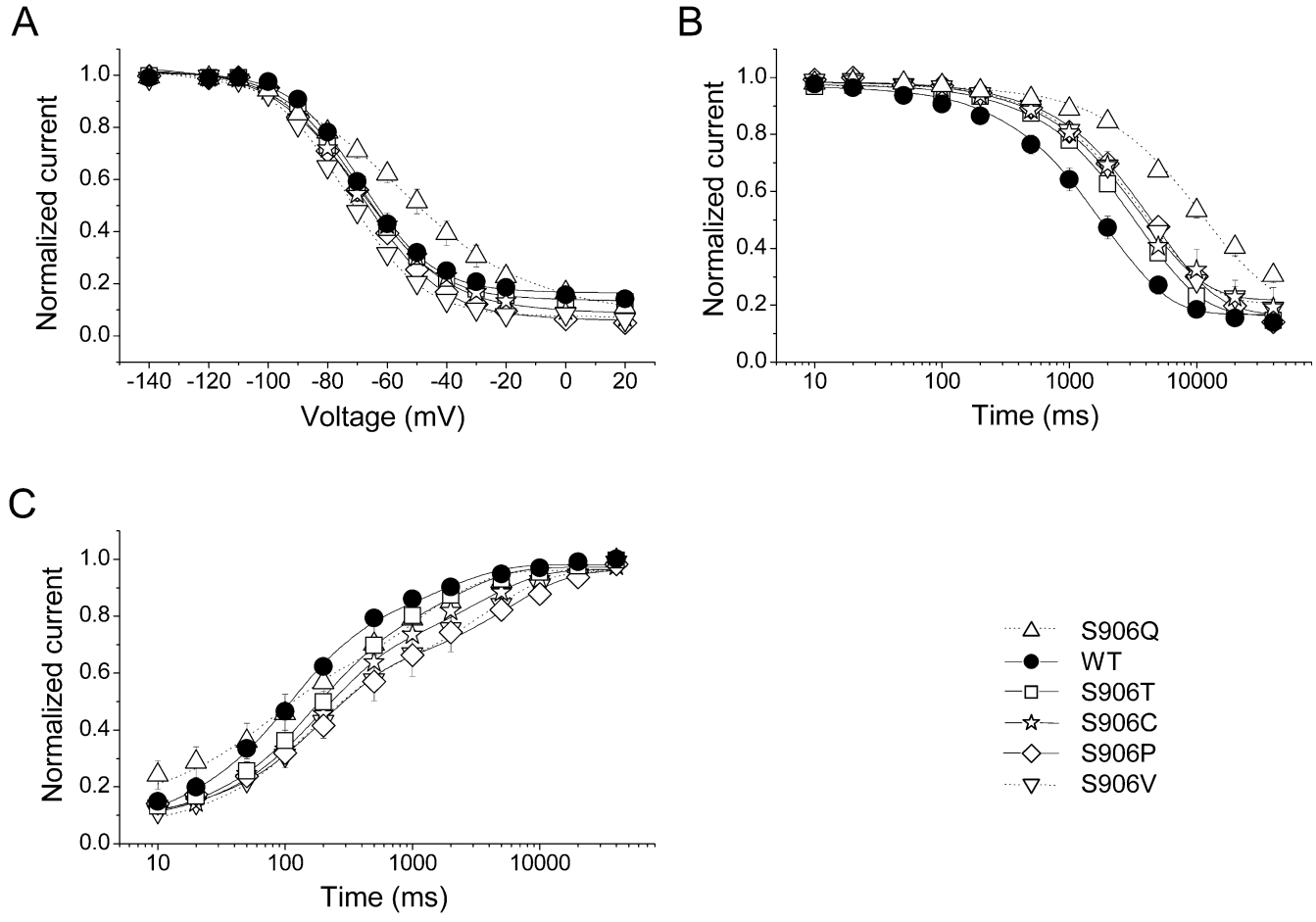

Fig. 3A–C **A**Cumulative steady-state slow inactivation was determined from a holding potential of –140 mV using a series of 30-s prepulses from –140 to +20 mV. We let channels recover from fast inactivation for 20 ms at –140 mV prior to the test pulse to -20 mV. Between two episodes channels did not recover from slow inactivation. Obtained data were normalized to the maximum and were fitted with a Boltzmann function. **B** Entry into the slow inactivated state for all six constructs. Voltage was stepped from the holding potential of –140 mV to –20 mV for various times (1 ms to 40 s), stepped to –140 mV for 20 ms to let the channels recover from fast inactivation and then stepped to –20 mV (10 ms) to record sodium current. Obtained data were normalized to the maximum and were fitted with a monoexponential function. The time at the holding potential between episode starts was long enough to allow complete recovery from fast and slow inactivation. **C** Recovery from slow inactivation for a holding potential of –140 mV was determined by a 30-s depolarization to –20 mV followed by a variable-duration return to –140 mV. The voltage was then stepped to –20 mV for 10 ms to record sodium current. The data were normalized to the currents recorded with a short control pulse to –20 mV before each episode. Obtained data were then fitted with a double-exponential function

modify slow channel gating, either reduced entry into slow inactivation is accompanied by accelerated recovery, suggesting a destabilization of the slow inactivated state and thus a "gain-of-function" effect (e.g. [15]), or entry is enhanced and recovery is slowed, meaning a stabilization of slow inactivation representing a "loss-of-function" feature (e.g. [2, 34]). On the contrary, for an S906T polymorphism as well as for other channel mutants studied, except for S906Q, both entry into and recovery from slow inactivation were slowed, reflecting a "change-of-function" mechanism seemingly caused by elevated free energy barriers for transitions into and from the slow inactivated state.

Role of II-III loop in slow inactivation

The II-III loop is a region not known to affect channel gating much. The loop is highly conserved among species, but less conserved among the various members of the sodium channel family [23]. It contains four clusters of acidic residues that may be important for channel gating [22, 23], but their role has not been clarified. The II-III loop was shown to alter the voltage dependence of channel activation in an isoform-specific manner; however, the mechanism of this effect remains a mystery [4]. Although the II-III loop contains potential phosphorylation sites [6] and voltage-gated sodium channels were found to be modulated by phosphorylation, the effects on sodium channels are restricted to PKA and PKC consensus sites located in the cytoplasmic linkers

connecting domains I-II and III-IV of the channels [3, 8, 11, 20, 21, 26, 27, 30, 31, 32, 36]. Mutations at potential PKA consensus sites in the linker between domains II and III of the rat cardiac sodium channel did not relieve the observed effect of PKA channel modification, suggesting that the examined consensus sites are not phosphorylated in vivo [28]. Thus, there is no evidence that the intracellular loop between the second and the third domains plays a role in the phosphorylation of the voltage-gated sodium channels. As position S906 is not located within confirmed PKA or PKC consensus sites [6], we suggest that modulation of the channel by PKA or PKC cannot occur at this place. The only kinase which may potentially phosphorylate S906 is caseine kinase II (CKII), as determined by protein kinase target site analysis [6]. Considering the low phosphorylation probability predicted [6] as well as unknown effects of this kinase on sodium channels, it is unlikely that CKII-dependent phosphorylation occurs at this place.

The pioneering study by Stühmer and colleagues [35] proposed that the II-III loop in rat voltage-gated sodium channel type II is of less functional importance, because cleaving of it as well as a large deletion in this region did not cause appreciable effects. However, the authors took into account only activation and fast inactivation as well as the kinetics of these processes, but not slow inactivation. Considering the fact that the differences among the channels in our study mainly affected the slow gating mode and that there were only subtle effects on fast inactivation, there are no inconsistencies between our data.

Moreover, later studies revealed the link between the II-III loop missense mutations in various voltage-gated sodium channel isoforms and hereditary diseases, underlining the importance of this region for physiological channel functioning. These are mutations in the cardiac sodium channel α-subunit gene *SCN5A* causing LQT-3 (S941N [29], A997S [1], D1114N [33]) and in the brain sodium channel α-subunit gene *SCN1A* causing GEFS+ (W1204R [10]), or ICEGTC (N1011I [12]). Two amino acid substitutions in the II-III loop of the human brain sodium channel α-subunit could also be predisposing mutations either for juvenile absence epilepsy (G1081R [10]) or for juvenile myoclonic epilepsy (T1174S [10]). Although S941N, A997S and W1204R mutations have already been expressed in frog oocytes or in a mammalian cell line and characterized by a slower decay and an increase in late sodium current conferring a "gain-of-function" sodium channel phenotype [1, 18, 29], it is still unclear how these mutations affect channel gating.

Observed marginal changes in the fast gating kinetics as well as a mild stabilization of the fast inactivated state for the S906C and S906P mutants seem to:(1) reflect disruption of a highly specific molecular conformation of this region by substituting different amino acids for a native serine, and (2) have a non-specific character. In contrast, the effects on slow inactivation caused by various substitutions at position 906 have a residue-specific character: the more hydrophobic the amino acid side chain at position 906, the more stabilized is the slow inactivated state of the mutant. Thus, the physiological nature of these residue-specific effects appears to be the most likely explanation.

Many hydrophobicity scales have been predicted to date. For amino acid residues, there are two major scale types that take into account whether an amino acid residue is buried within a protein. For buried residues, Cys was evaluated to have the greatest hydrophobicity, because it is involved in disulphide bonds that occur inside a protein [16, 25]. In these scales, hydrophobicity was examined in proteins with known 3-D structure and was defined as the tendency for a residue to be found inside a protein, rather than on its surface. In contrast, other scales are derived from the physicochemical properties of amino acid side chains and, therefore, more precisely follow the trend that would be expected when an amino acid residue faces a water environment. In this case, Cys appears to be moderately hydrophobic, whereas Val is the most hydrophobic residue of all the amino acid residues at position 906 we studied [5, 17, 37]. To choose the appropriate scale, we examined the accessibility of the cysteine residue at position 906 for methanethiosulfonate reagents. Adding of MTSES [sodium (2-sulfonatoethyl) methanethiosulfonate] resulted in up to 1.6-fold slowed kinetics of fast inactivation, 1.6-fold accelerated recovery from fast inactivation and a 4-mV right-shift of the steady-state fast inactivation curve, compared with S906C (data not shown). Hence, Cys at position 906 can be modified with methanethiosulfonate reagents; it faces a water environment and, consequently, is of moderate hydrophobicity.

As we have seen, changes in hydrophobicity at position 906 result in conspicuous effects on slow inactivation. These effects probably occur because of modification of the specific protein conformation required for normal slow channel gating. Dependence of stability of the slow inactivated state on the hydrophobic features of the amino acid side chain is the novel finding that emphasizes the functional importance of the II-III loop for channel gating. We suggest that position S906 in $Na_v1.4$ plays an important role in slow channel gating and that molecular rearrangement may take place at or near residue 906 in the II-III loop during slow inactivation.

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