

## Role in fast inactivation of the IV/S4–S5 loop of the human muscle Na<sup>+</sup> channel probed by cysteine mutagenesis

H. Lerche\*†‡§, W. Peter\*†, R. Fleischhauer\*, U. Pika-Hartlaub\*, T. Malina\*,  
N. Mitrovic\*† and F. Lehmann-Horn\*

*Departments of \*Applied Physiology and †Neurology, University of Ulm, Germany*

1. In order to investigate the role in fast inactivation of the cytoplasmic S4–S5 loop of the fourth domain (IV/S4–S5) within the  $\alpha$ -subunit of the adult human muscle Na<sup>+</sup> channel, every single amino acid from R1469 to G1486 was substituted by a cysteine and the mutants were studied by functional expression in human embryonic kidney cells (tsA201) using whole-cell patch clamping. Effects following intracellular application of the sulfhydryl reagents MTSET and MTSES on the mutants were investigated.
2. Sixteen of eighteen mutants resulted in the formation of functional channels. For P1480C and N1484C, no Na<sup>+</sup> currents could be detected in transfected cells. In the absence of sulfhydryl reagents, F1473C and A1481C slowed fast Na<sup>+</sup> channel inactivation by 2- and 1.5-fold, respectively, and L1482C induced a steady-state Na<sup>+</sup> current ( $I_{ss}$ ) of 3% of peak current ( $I_{peak}$ ) (1% for wild-type).
3. Upon application of MTSET and MTSES, changes in fast inactivation gating occurred for most of the mutants. The most dramatic destabilizing effects on fast inactivation were observed for M1476C (9-fold slowing of inactivation;  $I_{ss}/I_{peak}$ , 3.6%; +15 mV shift in steady-state inactivation; 2- to 3-fold acceleration of recovery from inactivation), A1481C (3-fold; 14%; +20 mV; no change) and F1473C (2.5-fold; 2.4%; +8 mV; 1.5-fold). Less pronounced destabilizing effects were observed for M1477C and L1479C. Strongly stabilizing effects on the inactivated state, that is a 20–30 mV hyperpolarizing shift of the inactivation curve associated with a 3- to 4-fold decrease in the rate of recovery from inactivation, occurred for T1470C, L1471C and A1474C. Almost all effects were independent of the membrane potential; however, A1474C only reacted when cells were depolarized. Significant effects on activation were not observed.
4. We conclude that the IV/S4–S5 loop plays an important role in fast inactivation of the muscle Na<sup>+</sup> channel and may contribute to the formation of a receptor for the putative inactivation particle. The effects of sulfhydryl reagents on the various mutations suggest an  $\alpha$ -helical structure of IV/S4–S5 (up to P1480) with destabilizing effects on inactivation for one cluster of amino acids (1473/76/77/79) and a stabilized inactivation at the opposite side of the helix (1470/71/74).

Voltage-gated Na<sup>+</sup> channels are membrane spanning proteins responsible for the initiation and propagation of action potentials in nerve and muscle cells. In response to voltage changes during action potentials the channels open from the resting, closed state, and then inactivate spontaneously. The functionally important  $\alpha$ -subunit contains four domains (I–IV) of six transmembrane segments each (S1–S6). All S4 segments contain positively charged residues conferring

voltage dependence on the channel protein, the S5–S6 loops contribute to the ion channel pore, and the intracellular loop linking domains III and IV contains the supposed inactivation particle of the channel.

In 1977, Armstrong & Bezanilla proposed a ‘ball and chain’ model for fast Na<sup>+</sup> channel inactivation, in which a cytoplasmic, tethered ball occludes the internal mouth of

‡ These authors contributed equally to this study.

§ To whom correspondence should be addressed.

the pore. In 1992, West and colleagues identified three amino acids (IFM) in the III–IV interlinker which were essential for fast channel inactivation and were supposed to form the inactivation particle (West, Patton, Scheuer, Wang, Goldin & Catterall, 1992). In structurally homologous K<sup>+</sup> channels, the ball-and-chain model has been verified conclusively. The ball is probably formed by twenty amino acids at the cytoplasmic N-terminus (Hoshi, Zagotta & Aldrich, 1990). The receptor catching the ball is probably located in the intracellular S4–S5 loop of the K<sup>+</sup> channel (Isakoff, Jan & Jan, 1991; Holmgren, Jurman & Yellen, 1996). The III–IV interlinker of the Na<sup>+</sup> channel has been shown to confer fast inactivation on a slowly inactivating isoform of the K<sup>+</sup> channel (Patton, West, Catterall & Goldin, 1993), and for both K<sup>+</sup> and Na<sup>+</sup> channels hydrophobic interactions between the inactivation particle and its receptor have been proposed (Hoshi *et al.* 1990; West *et al.* 1992; Holmgren *et al.* 1996; Kellenberger, West, Scheuer & Catterall, 1997). Due to the structural and functional similarities between the two proteins, the S4–S5 loop might play a similar, important role in fast inactivation gating of the Na<sup>+</sup> channel.

Additional evidence for the importance of the S4–S5 loop for Na<sup>+</sup> channel inactivation stems from naturally occurring mutations in the skeletal and heart muscle Na<sup>+</sup> channelopathies. Some of the point mutations found within the muscular Na<sup>+</sup> channel  $\alpha$ -subunit are located in S4–S5 loops (reviewed by Lehmann-Horn & Rüdell, 1997), and in particular one mutation causing paramyotonia congenita, F1473S, is located in IV/S4–S5 (Mitrovic *et al.* 1996). All Na<sup>+</sup> channelopathies have a more or less defective inactivation in common.

Whereas Na<sup>+</sup> channels are composed of four highly homologous but not identical domains, K<sup>+</sup> channels are tetramers of four identical domains. For our experiments, we focused on S4–S5 of the fourth domain of the Na<sup>+</sup> channel, because it seems to play a more important role for inactivation than for activation. In particular, mutations in the voltage sensor IV/S4 have major effects on inactivation (Chahine, Geroge, Zhou, Sun, Barchi & Horn, 1994; Lerche, Mitrovic, Dubowitz & Lehmann-Horn, 1996; Chen, Santarelli, Horn & Kallen, 1996), whereas mutations in S4 of the other domains affect more the activation process (Stühmer *et al.* 1989; Chen *et al.* 1996). Since IV/S4 is directly coupled to IV/S4–S5, mutations in IV/S4 may affect inactivation by influencing the formation of a possible receptor for the inactivation particle in IV/S4–S5.

In our experiments we screened IV/S4–S5 using the cysteine mutagenesis and accessibility method (Stauffer & Karlin, 1994). Cysteine residues were modified by sulfhydryl reagents carrying charges and/or longer side chains yielding further information about their functional importance and their accessibility to reagents.

## METHODS

Site-directed mutagenesis was performed using the 'altered sites' system employing the plasmid vector pSELECT (Promega Corporation, Mitrovic *et al.* 1994). Mutagenic oligonucleotides (antisense strand) were as follows (from 5' to 3'):

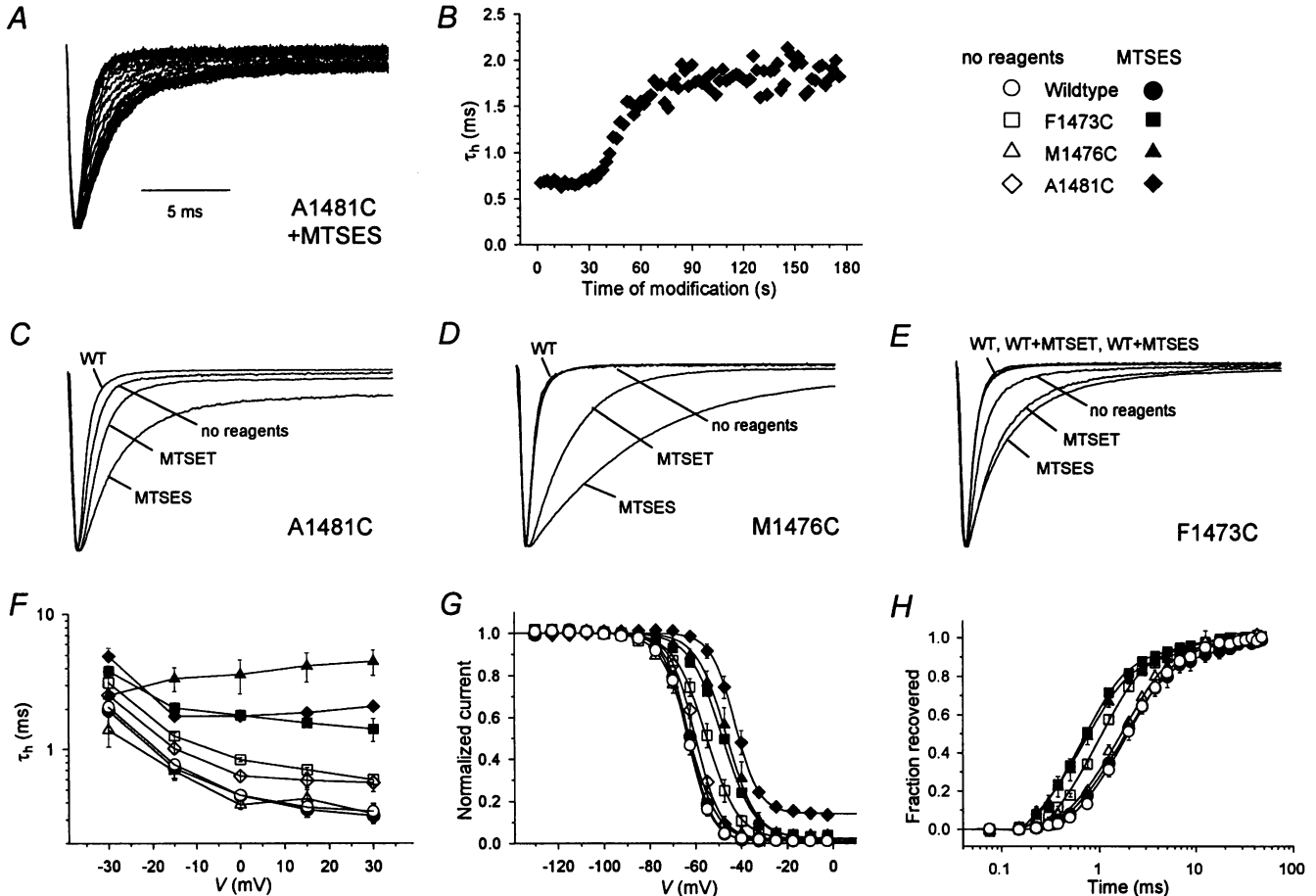
R1469C: GAACAGCAGCGTACAGATGCCCT TGGCCC;  
 T1470C: GCGAACAGCAGACACCGGATGCCCT TG;  
 L1471C: TGAGGGCGAACAGACACGTCCGGATGCC;  
 L1472C: GCGACATCATAAGGGCGAACACAGCG;  
 F1473C: GCGACATCATAAGGGCGCACAGCAGCG;  
 A1474C: GCAGGCAGCGACATCATAAGGCAGAACAG;  
 L1475C: GCAGGCAGCGACATCATAAGGCAGAACAG;  
 M1476C: CAGGCAGCGACATCATAAGGGCGAACAGCG;  
 M1477C: CAGGCAGCGAACACATGAGGGCGAACAG;  
 S1478C: CAGGCAGACACATCATAAGGGCGAACA;  
 L1479C: TGAAGAGGGCAGGGCACGACATCATGAGGG;  
 P1480C: AGAGGGCACACAGCGACATCATAAGGGC;  
 A1481C: GCCGATGT TAAAGAGGCAAGGCAGCGAC;  
 L1482C: CCGATGT TAAAGCAGGCAGGCAGCG;  
 F1483C: GAGGAGGCCTATGT TGCAGAGGGCAG;  
 N1484C: GGAGGAGGCCTATGCAGAAGAGGGCA;  
 I1485C: GAGGAGGAGGCCCGAGT TAAAGAGGGCAGG;  
 G1486C: AAGAGGAGGAGGCATATGT TGAAGAGGG.

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 10  $\mu$ g DNA was transfected transiently into tsA201 cells as described in Chahine *et al.* (1994). A CD8-cDNA-containing plasmid was cotransfected (2  $\mu$ g DNA) so that transfected cells could be recognized using anti-CD8 antibody-coated microbeads (Dynabeads M450; Dynal, Oslo, Norway). Dependent on the expressivity of the mutation, 50–90% of the bead-decorated cells had detectable Na<sup>+</sup> currents. At least two clones of every mutation were investigated.

Standard whole-cell recording was performed using an EPC-7 amplifier (List) and a pCLAMP (Axon Instruments) data acquisition system as described (prepulse protocol  $-P/4$ ; 3 kHz filtering; 20 kHz sampling; Mitrovic *et al.* 1994). Data were analysed by a combination of pCLAMP, Excel (Microsoft) and SigmaPlot software (Jandel Scientific). Currents for WT and all mutants ranged from 1 to 7 nA. Residual series resistance was 0.5–1.5 M $\Omega$  (60–85% compensation) and the maximal voltage error was always  $\leq$  5 mV. The pipette solution contained (mM): 135 CsCl, 5 NaCl, 2 MgCl<sub>2</sub>, 5 EGTA and 10 Hepes (pH 7.4); the bathing solution contained (mM): 140 NaCl, 4 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 4 dextrose and 5 Hepes (pH 7.4). All recordings were performed at a temperature of 21–23 °C which was controlled via a water bath. The sulfhydryl reagents [2-(trimethylammonium)ethyl]methanethio-sulphonate and (2-sulphonatoethyl)methanethiosulphonate (MTSET and MTSES; Toronto Research Chemicals, Toronto, Canada) were kept in a water-based stock solution (100 or 250 mM) on ice and diluted 1:100 to form the pipette solution immediately before the experiments.

To record Na<sup>+</sup> currents, cells were held at -85 mV and depolarized to various test potentials after a 300 ms prepulse to -120 mV. The time course of inactivation was best fitted to a second order exponential function yielding two time constants of inactivation. The weight of the second slower time constant was small (<15%). To describe the slowing of inactivation in the Results section, only the fast time constant, termed  $\tau_h$ , is used. Persistent Na<sup>+</sup> currents, when a steady state was reached ( $I_{ss}$ ), were determined at the end of 100 ms pulses to 0 mV without using leak correction and are given as a percentage of the initial peak current ( $I_{peak}$ ). Steady-state inactivation was determined using 300 ms prepulses to various potentials followed by the test pulse to 0 mV. Steady-state activation and inactivation curves were fitted to the standard

Boltzmann function:  $I/I_{max} = 1/(1 + \exp[(V - V_{0.5})/k])$ , with  $V_{0.5}$  being the voltage of half-maximal activation/inactivation and  $k$  the slope factor. Recovery from inactivation was recorded from a holding potential ( $V_h$ ) of -100 mV. Cells were depolarized to 0 mV for 100 ms to inactivate all Na<sup>+</sup> channels and then repolarized to various recovery potentials for increasing durations. The time course of recovery from inactivation was best fitted to a second order exponential function with an initial delay. For comparison of data from WT, mutant and MTSET/ES-treated cells, only the fast recovery time constant, termed  $\tau_{rec}$ , was used, since the slow one had a relatively small weight (<20%) and did not vary much compared with the fast one. Changes in the delay always accompanied slowing or acceleration of  $\tau_{rec}$ ; thus the delay gave no



**Figure 1. Effects of MTS reagents on F1473C, M1476C and A1481C, destabilizing the inactivated state**

*A*, modification of inactivation of A1481C Na<sup>+</sup> currents by intracellularly applied MTSES (2.5 mM) added to the pipette solution. After whole-cell formation, Na<sup>+</sup> currents were elicited by depolarizing test pulses to 0 mV from  $V_h = -85$  mV at a frequency of 0.5 Hz. *B*, plot of inactivation time constants,  $\tau_h$ , of currents in *A* vs. reaction time; a plateau was reached after 3 min. *C-E*, Na<sup>+</sup> currents for A1481C, M1476C and F1473C superimposed on WT currents and after complete reaction with MTSET and MTSES at a test potential of 0 mV. *E* contains current traces for WT cells treated with MTSET/ES showing that there was no modification of WT currents. *F*, inactivation time constants for WT and all three mutations plotted vs. test potential. *G*, steady-state inactivation curves: normalized currents are plotted vs. prepulse potential. Lines are fits to a standard Boltzmann function. *H*, recovery from inactivation at -100 mV. Lines are fits to a second order exponential function with an initial delay. On a logarithmic time scale, the time constants of recovery from inactivation correspond to the mid-points of the sigmoidal curves. All values are shown as means  $\pm$  s.e.m.,  $n = 3-10$ .

further information and is not provided in the Results section. All data are shown as means  $\pm$  s.e.m. For statistical evaluation, Student's *t* test was applied ( $P < 0.05$ ).

## RESULTS

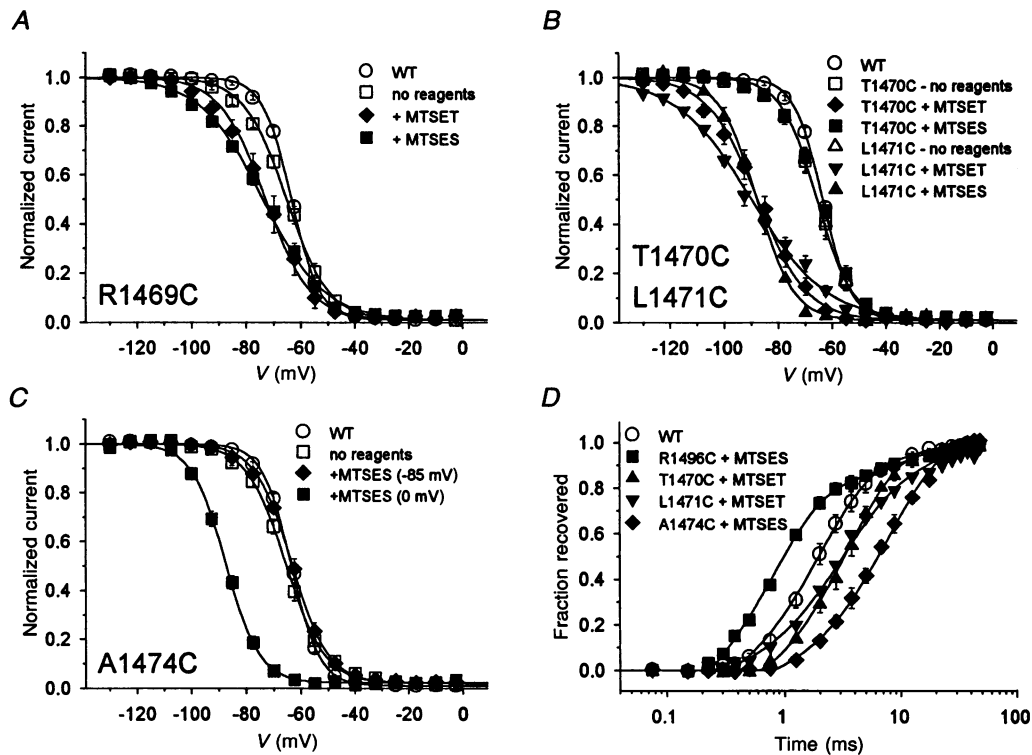
To investigate the role of amino acids in the IV/S4–S5 loop of the human muscle Na<sup>+</sup> channel  $\alpha$ -subunit in the process of fast channel inactivation, all eighteen residues from R1469 to G1486 were individually mutated into a cysteine. The functional consequences of these mutations and the effects of the intracellularly applied sulphhydryl reagents MTSET and MTSES were studied using transient expression in tsA201 cells and the whole-cell patch clamp technique. Sixteen out of eighteen mutations led to functional channels; for P1480C and N1484C no Na<sup>+</sup> currents could be recorded in presumably transfected (bead-decorated) cells.

In the absence of the sulphhydryl reagents, only two mutants exhibited a significant increase in the fast inactivation time constant,  $\tau_h$ , compared with WT: F1473C by 2-fold and A1481C by 1.5-fold (Figs 1C and E and 3A). L1482C induced a significant  $I_{ss}$  of  $3.0 \pm 0.5\%$  of  $I_{peak}$  ( $n = 7$ ; WT:  $0.7 \pm 0.1\%$ ,  $n = 7$ ), and F1473C induced a significant  $I_{ss}$  of

$2.0 \pm 0.4\%$  ( $n = 5$ ). Depolarizing shifts in steady-state inactivation were observed for L1472C and F1473C and were accompanied by an acceleration of recovery from inactivation, indicating a destabilization of the inactivated state. The opposite effect, a hyperpolarizing shift accompanied by a slowing of recovery from inactivation was observed for M1477C, S1478C, L1479C, F1483C, I1485C and G1486C. Significant effects on steady-state activation were not observed for any of the mutations. A mutation predicting the substitution of a serine for F1473 causes paramyotonia congenita by effects on inactivation which are similar to those induced by F1473C (Mitrovic *et al.* 1996).

### Effects of MTSET and MTSES

The sulphhydryl reagents MTSET and MTSES were used to test the accessibility of the cysteines and to look for functional alterations upon their modification. MTSET and MTSES were added to the pipette solution at a concentration of 1 mM and 2.5 mM, respectively. Higher concentrations were also tested but the effects were not different. After rupturing the cell membrane, cells were held at  $-85$  mV and a depolarizing test pulse to 0 mV was applied every few seconds. As already shown in other studies (e.g. Yang,



**Figure 2.** Effects of MTS reagents on T1470C, L1471C and A1474C, stabilizing the inactivated state, and on R1469C

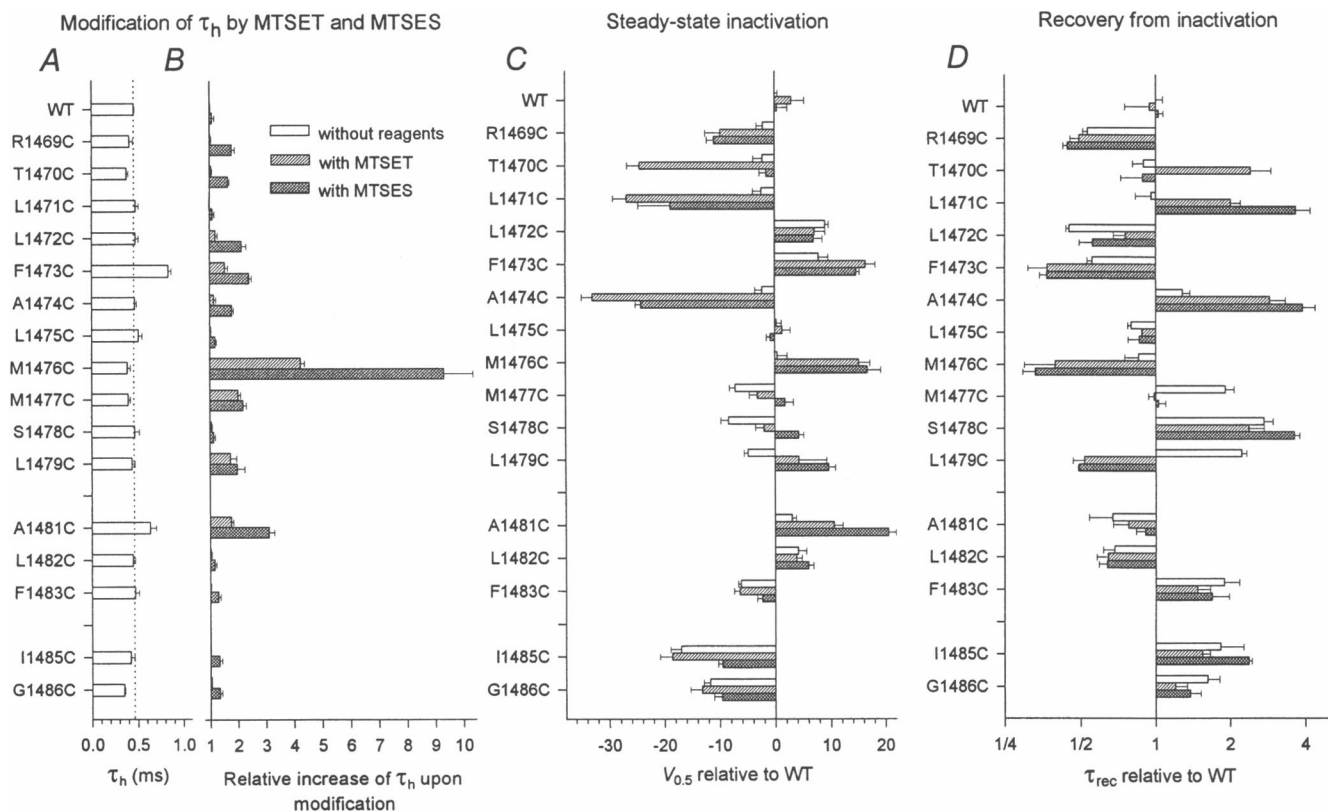
A–C, steady-state inactivation curves in the absence and presence of MTS reagents. Normalized currents are plotted vs. prepulse potential. Lines are fits to a standard Boltzmann function. For MTSET/ES-modified R1469C and MTSET-modified L1471C currents the slope was significantly decreased. C, A1474C reacted with MTSES only when cells were depolarized. Inactivation curves after a 10 min reaction with MTSES at holding potentials of  $-85$  mV and 0 mV are shown. D, recovery from inactivation at  $-100$  mV (see Fig. 1H). The time course of recovery was slowed for T1470C, L1471C and A1474C but accelerated for R1469C. All values are shown as means  $\pm$  s.e.m.,  $n = 3$ –10.

George & Horn, 1996), the reagents had no significant effect on WT channels (Figs 1*E–H* and 3*A–D*). To illustrate the reaction kinetics, modification of A1481C by MTSES is shown in Fig. 1*A* and *B*. After an initial delay, presumably due to diffusion of the reagent into the cell, the rate of inactivation decreased and inactivation became incomplete. The reaction was monitored by the fast inactivation time constant,  $\tau_h$ ; a plateau was reached after a few minutes (Fig. 1*B*). The delay and the time needed for a saturating reaction varied a lot from cell to cell (e.g. with cell size). To ensure complete reaction, all recordings were made after a reaction time of at least 8–10 min, when a plateau was definitely reached. Figures 1 and 2 show individual results for certain mutations and Fig. 3 summarizes the effects of the sulphhydryl reagents on the time course of fast inactivation, steady-state inactivation and recovery from inactivation. Figure 4 shows IV/S4–S5 as a putative  $\alpha$ -helix, which might be useful for orientation while reading the Results section.

The largest decrease in the rate of inactivation upon modification was observed for M1476C (9-fold for MTSES; Figs 1*D* and 3*B*) and less pronounced decreases were

observed for A1481C and F1473C (2.5- to 3-fold; Figs 1*A, C* and *E* and 3*A* and *B*). A striking  $I_{ss}$  of  $13.7 \pm 1.1\%$  ( $n = 4$ ) was recorded in A1481C while  $I_{ss}$  was small in M1476C ( $3.6 \pm 0.5\%$ ,  $n = 3$ ) and F1473C ( $2.4 \pm 0.4\%$ ,  $n = 3$ ) (WT:  $0.7 \pm 0.1\%$ ,  $n = 7$ ). For all three mutations large depolarizing shifts (10–20 mV) in the voltage dependence of steady-state inactivation, and for F1473C and M1476C a significant acceleration of recovery from inactivation, occurred, indicating a severe destabilization of the inactivated state by the modified mutations (Figs 1*G* and *H* and 3*C* and *D*).

A less pronounced destabilization (a +10 to +15 mV shift in steady-state inactivation associated with a 2- and 4-fold acceleration of recovery from inactivation) was observed for M1477C and L1479C (Fig. 3*C* and *D*). Minor slowing of inactivation upon modification was found: by 2- to 2.5-fold for L1472C, M1477C and L1479C, by about 1.7-fold for R1469C, T1470C and A1474C and by 1.3-fold for F1483C, I1485C and G1486C (Fig. 3*A* and *B*). In general, MTSES had larger effects on the rate of inactivation than MTSET, which might be because the negative charge interfered with IV/S4 or other charges in the protein. The inactivation



**Figure 3. Summary of effects of MTS reagents**

*A*, inactivation time constants,  $\tau_h$ , for WT ( $0.46 \pm 0.01$  ms,  $n = 11$ , indicated as a vertical dotted line) and all cysteine mutants in the absence of reagents. Only F1473C and A1481C were significantly different from WT. *B*, modification of  $\tau_h$  by MTSET (1 mM) and MTSES (2.5 mM) was recorded for individual cells as described in Fig. 1*A*; shown are the relative increase factors of  $\tau_h$  after complete reaction. *C*, mid-points of steady-state inactivation relative to WT ( $-63 \pm 1$  mV,  $n = 12$ ). *D*, fast time constants of recovery from inactivation relative to WT ( $1.8 \pm 0.1$  ms,  $n = 7$ ). All values are shown as means  $\pm$  s.e.m.,  $n = 3$ –10.



## DISCUSSION

For almost all of the MTSET/ES-modified mutations in the IV/S4–S5 loop of the human muscle Na<sup>+</sup> channel  $\alpha$ -subunit which we examined, we observed significant changes in fast inactivation gating compared with WT channels, whereas significant changes on activation did not occur. These results underline an important role of this structure in the process of fast inactivation. We will discuss our data with regard to firstly the putative secondary structure of IV/S4–S5, and secondly possible functional implications of our results for this intracellular loop.

### Secondary structure of the IV/S4–S5 loop

The most consistent finding upon modification with MTS reagents was a more or less pronounced slowing of the time course of inactivation for twelve out of sixteen cysteine mutants. Slowing of inactivation for the MTSES-modified mutants compared with WT was most striking for M1476C, F1473C and A1481C, intermediate for L1472C, M1477C and L1479C, little for R1469C, T1470C and A1474C and marginal for F1483C, I1485C and G1486C. No reaction was observed for L1475C and L1482C. For L1471C and S1478C, there was no slowing of inactivation, but a definite reaction with MTSET/ES occurred since we observed changes in steady-state inactivation and/or recovery from inactivation. When IV/S4–S5 is drawn as a helical wheel up to the potential helix breaker proline-1480 (Fig. 4), the two most 'reactive' mutants in terms of slowing and destabilizing inactivation, M1476C and F1473C, are located on one side. The three cysteine mutants without effects on the time course of inactivation, L1471C, L1475C and S1478C, are located on the opposite side, whereas all other mutations are located in between those two distinct centres. A1481C, the third strongly reactive mutant, might also be located on the same side as M1476C and F1473C, if the helix is kinked at P1480. Five modified mutants destabilizing the inactivated state (depolarizing shifts of steady-state inactivation associated with an acceleration of recovery; F1473C, M1476C, M1477C, L1479C, A1481C; diamonds in Fig. 4) are on the opposite side to a cluster of three stabilizing mutants (large hyperpolarizing shifts of steady-state inactivation associated with a slowing of recovery; T1470C, L1471C, A1474C; squares in Fig. 4). These results strongly support an  $\alpha$ -helical structure for IV/S4–S5, which could be separated from the supposedly membrane-spanning helix S5 by a bend caused by amino acids known as helix breakers (P1480, N1484 and G1486).

### Possible role of IV/S4–S5 in fast Na<sup>+</sup> channel inactivation gating

The outward movement of the voltage sensor IV/S4 during activation (Yang *et al.* 1996) may affect the position and/or conformation of the attached IV/S4–S5 segment, initiating the formation of a receptor for the inactivation particle and thereby coupling activation to inactivation (the coupling was abolished for MTSET/ES-modified M1476C). This view is compatible with the effects of mutations in IV/S4 affecting

inactivation (Chahine *et al.* 1994; Chen *et al.* 1996; Lerche *et al.* 1996). If the inactivation particle directly binds to amino acids in IV/S4–S5, they should not be accessible to intracellular agents in the inactivated channel state, that is during depolarization. Regarding the phenylalanine of the suggested inactivation particle IFM within the III–IV linker, Kellenberger, Scheuer & Catterall (1996), who studied an F-C mutant, found it was only accessible on hyperpolarization. In our experiments, all mutants reacting at  $V_h = -85$  mV were also modified at  $V_h = 0$  mV, indicating that these residues are exposed independently of the membrane potential. Hence, our experiments indicate that a direct interaction between the putative inactivation particle and one of the amino acids reported here seems unlikely. However, two of our mutations, P1480C and N1484C, did not yield functional channels, and for a mutation in the rat brain Na<sup>+</sup> channel corresponding to N1484C, N1662A, fast inactivation was almost completely abolished (McPhee, Ragsdale, Scheuer & Catterall, 1996). Our results showing slowed inactivation for almost all mutations proximal to P1480 could be explained by their ability to hinder the outward movement of IV/S4. The receptor might be formed by residues distal to P1480, mainly the asparagine-1484. This hypothesis is supported by the finding that a large persistent current was observed for MTSET/ES-modified A1481C, whereas all mutations preceding P1480 mainly slowed inactivation. Whereas slowing of inactivation indicates altered binding rate constants for the inactivation particle, a persistent current indicates disruption of the binding equilibrium, suggesting that a binding site could be affected.

In conclusion, the  $\alpha$ -helical IV/S4–S5 loop plays a crucial role in fast Na<sup>+</sup> channel inactivation, possibly by forming a receptor for the inactivation particle and coupling inactivation to activation via a direct connection to the voltage sensor IV/S4. Preliminary reports by other groups focusing on S4–S5 loops either in domain III (Smith, Yu & Goldin, 1997) or domain IV (McPhee *et al.* 1996; Tang & Kallen, 1997; Filatov, Kraner & Barchi, 1997) of the Na<sup>+</sup> channel are compatible with our results. The role of other S4–S5 segments and a possible concerted action of all four domains and other regions in the process of fast Na<sup>+</sup> channel inactivation remain to be elucidated.

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### Author's email address

H. Lerche: holger.lerche@medizin.uni-ulm.de

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