

SHORT COMMUNICATION

Enhanced inactivation and acceleration of activation of the sodium channel associated with epilepsy in man

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Abstract

Generalized epilepsy with febrile seizures-plus (GEFS⁺) is a benign Mendelian syndrome characterized by childhood-onset febrile and afebrile seizures. Three point mutations within two voltage-gated sodium channel genes have been identified so far: in GEFS⁺ type 1 a mutation in the β_1 -subunit gene *SCN1B*, and in GEFS⁺ type 2 two mutations within the neuronal α -subunit gene *SCN1A*. Functional expression of the *SCN1B* and one of the *SCN1A* mutations revealed defects in fast channel inactivation which are in line with previous findings on myotonia causing mutations in *SCN4A*, the skeletal muscle sodium channel α -subunit gene, all showing an impaired fast inactivation. We now studied the second GEFS⁺ mutation (T875M in *SCN1A*), using the highly homologous *SCN4A* gene (mutation T685M). Unexpectedly, the experiments revealed a pronounced enhancement of both fast and slow inactivation and a defect of channel activation for T685M compared to wild-type channels. Steady-state fast and slow inactivation curves were shifted in the hyperpolarizing direction, entry into slow inactivation was threefold accelerated, recovery from slow inactivation was slowed by threefold and the time course of activation was slightly but significantly accelerated. In contrast to other disease-causing mutations in *SCN1A*, *SCN1B* and *SCN4A*, the only mechanism that could explain hyperexcitability of the cell membrane would be the acceleration of activation. Because the enhancement of slow inactivation was the most obvious alteration in gating found for T685M, this might be the disease-causing mechanism for that mutation. In this case, the occurrence of epileptic seizures could be explained by a decrease of excitability of inhibitory neurons.

Introduction

Ion channel disorders are rare inherited diseases providing interesting models for studying dysfunction of excitability *in vivo* and *in vitro*. The first so-called ‘channelopathies’ identified were the myotonias and hyperkalemic periodic paralysis, caused by mutations in the skeletal muscle sodium and chloride channels. More than 20 mutations have been described in *SCN4A*, the gene encoding the skeletal muscle voltage-gated sodium channel. They all lead to a disruption of fast channel inactivation that explain the pathological hyperexcitability via an increase of the sodium inward current causing depolarization of the sarcolemma. The same pathophysiological mechanism applies to one form of the long QT syndrome (LQT type 3, mutations in *SCN5A*), an inherited cardiac arrhythmia (reviewed by Lehmann-Horn & Jurkat-Rott, 1999).

Recently, another form of idiopathic epilepsy called ‘generalized epilepsy with febrile seizures-plus’ (GEFS⁺ Scheffer & Berkovic, 1997), has been identified as a sodium channel disorder (Wallace *et al.*, 1998; Escayg *et al.*, 2000a). In five large families with autosomal dominant inheritance of GEFS⁺ described so far, linkage has been found to either chromosome 19q13 (Wallace *et al.*, 1998) or 2q21–33 (Baulac *et al.*, 1999; Moulard *et al.*, 1999; Pfeiffer *et al.*, 1999; Lopes-Cendes *et al.*, 2000). Up to now, one mutation has been

found in *SCN1B* on chromosome 19 encoding the auxiliary β_1 -subunit (GEFS⁺ type 1; Wallace *et al.*, 1998) and two mutations have been identified in the neuronal α -subunit gene, *SCN1A*, on chromosome 2 (GEFS⁺ type 2; Escayg *et al.*, 2000a).

Functional expression in heterologous systems revealed a loss of β_1 -subunit function for the *SCN1B* mutation resulting in a slight slowing of sodium channel fast inactivation (Wallace *et al.*, 1998) and an acceleration of recovery from fast inactivation for one of the *SCN1A* mutations located in the voltage sensor of domain 4 (Alekov *et al.*, 2000) as the main disease-causing mechanisms. Although the β_1 -subunit is also expressed in skeletal muscle, only symptoms of brain dysfunction, i.e. epileptic seizures, but no myotonia were reported for this family (Singh *et al.*, 1999). In addition, the gating alterations found for the two epilepsy-causing mutations were much more subtle than those found for *SCN4A* mutations associated with myotonia, indicating that the brain shows a greater vulnerability to changes in excitability than does muscle tissue. We examined the disease-causing mechanism of the second GEFS⁺ mutation described in *SCN1A* and found that the alterations in channel gating are different from those of the previously mentioned sodium channel mutations.

Voltage-gated sodium channels are membrane-spanning proteins responsible for the initiation and propagation of action potentials in nerve and muscle cells. In response to membrane depolarization the channels open from the resting, closed, state, and then inactivate spontaneously. Upon repolarization they will recover from inactiva-

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tion. The functionally important α -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. There are several genes encoding different α -subunits (*SCN1–11A*) that are expressed specifically in skeletal muscle (*SCN4A*), heart muscle (*SCN5A*) and neuronal tissue; four subunits (*SCN1–3A* and *SCN8A*) are considered to be responsible for the sodium current in brain. There are three genes for auxiliary β -subunits (*SCN1–3B*), that are all expressed in brain; the β_1 -subunit is also expressed in skeletal and heart muscle (reviewed by Goldin, 1999; Lehmann-Horn & Jurkat-Rott, 1999; Catterall, 2000; Morgan *et al.*, 2000).

Materials and methods

Mutagenesis and transfection

Site-directed mutagenesis to introduce a mutation predicting substitution T685M was performed using the QuickChange™ mutagenesis kit (Stratagene, La Jolla, CA, USA). Two clones were sequenced and reassembled in the pRC/CMV plasmid (Invitrogen, Carlsbad, CA, USA) for transfection in the mammalian cell line tsA201 using a standard calcium phosphate transfection method. A CD8-cDNA containing plasmid was cotransfected in order to recognize transfected cells using anti-CD8 antibody-coated microbeads (Dynabeads M450, Dynal Biotech, Oslo, Norway) (Lerche *et al.*, 1997).

Electrophysiology and data analysis

Standard whole-cell recording was performed using an EPC-7 amplifier (EPC7, List Electronics, Darmstadt, Germany). The pipette solution contained (in mM): CsF, 105; NaCl, 35; EGTA, 10; and Hepes, 10 (pH 7.4). The bathing solution contained (in mM): NaCl, 150; KCl, 2; CaCl₂, 1.5; MgCl₂, 1; Hepes, 10 (pH 7.4). Sodium currents in transfected cells for wild-type (WT) and all mutants ranged from 2.5 to 15 nA. The maximal voltage error due to residual series resistance was < 5 mV. Leakage and capacitive currents were automatically subtracted using a prepulse protocol (–P/4). Currents were filtered at 3 or 10 kHz and digitized at 20 or 50 kHz using pCLAMP (Axon instruments, Foster City, CA, USA). Measurements were performed at room temperature (21–23 °C). For some experiments, the temperature was adjusted to 14.5–15.5 °C via a water bath. All data were analysed using a combination of pCLAMP, EXCEL (Microsoft, Redmond, MA, USA) and ORIGIN software (Microcal Software, Northampton, MA, USA). For statistical evaluation, Student's *t*-test was applied. All data are shown as means \pm SEM.

Results

To study the functional consequences of the *SCN1A* mutation predicting substitution of methionine for threonine in the voltage sensor II/S4 (T875M) causing GEFS⁺, and also in order to compare the results to those described previously by our group (i) for *SCN4A* mutations found in the myotonias (Lehmann-Horn & Jurkat-Rott, 1999) and (ii) for the other *SCN1A* mutation in IV/S4 (Alekov *et al.*, 2000), we introduced the mutation into the same conserved region of *SCN4A* (T685M) and expressed WT and mutant α -subunits transiently in tsA201 cells. The almost complete conservation of the deduced amino acid sequence among *SCN1A*, *SCN4A* and other sodium channel genes in II/S4 is shown in Fig. 1A. Families of representative whole-cell sodium currents for mutant T685M and WT channels elicited by voltage steps from a holding potential of

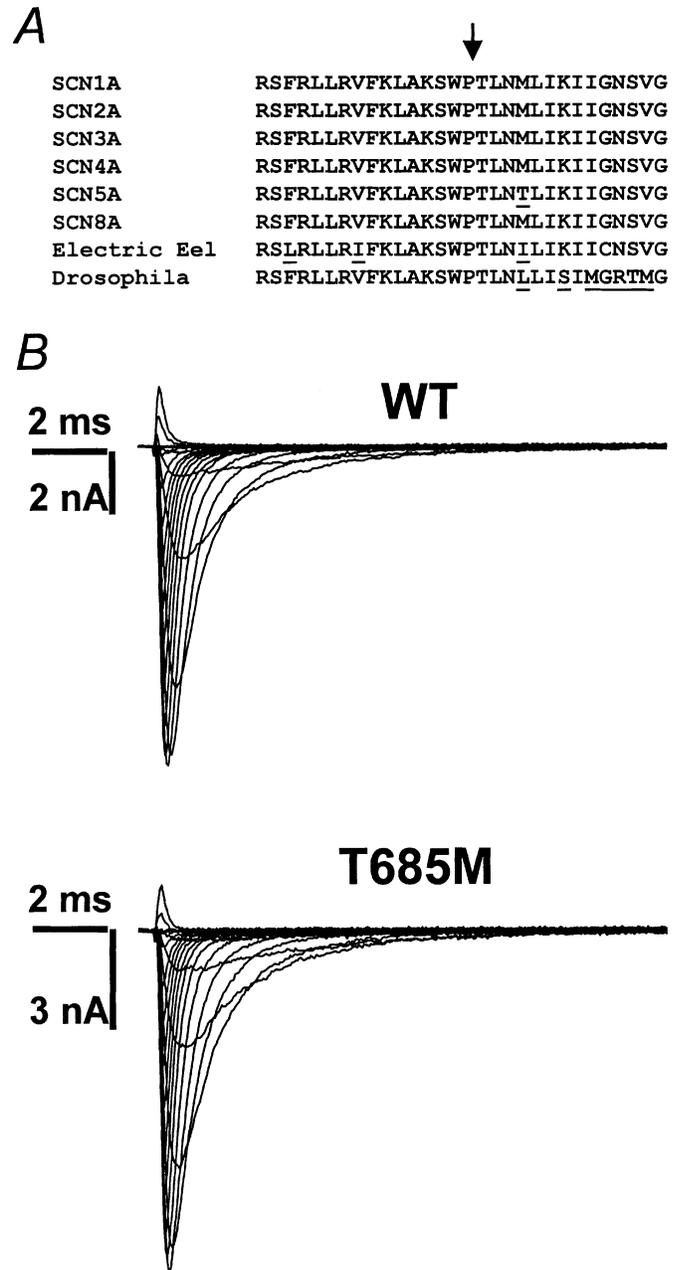


FIG. 1. Mutation T685M in segment II/S4. (A) Amino acid sequence comparison of the II/S4 segment of various sodium channel α -subunits derived from different genes. Sequence changes are highlighted by underlining. Residue T875 (*SCN1A* numbering) corresponding to T685 (*SCN4A* numbering) is marked by an arrow. (B) Representative whole-cell sodium current families recorded from cells transfected with either WT or mutant channels.

–140 mV are shown in Fig. 1B. The absolute sodium current sizes in transfected cells for WT vs. T685M at 0 mV were as follows: 5.3 ± 1.2 vs. 3.8 ± 0.8 nA and current density was 360 ± 80 pA/pF vs. 500 ± 150 pA/pF ($n = 7, 11$; $P > 0.05$). A detailed evaluation of the kinetics and voltage-dependence of channel activation, deactivation and fast and slow inactivation is provided below.

Activation and deactivation

Steady-state activation did not reveal a significant difference in the voltage dependence of mutant and WT channels (Fig. 2A). The time

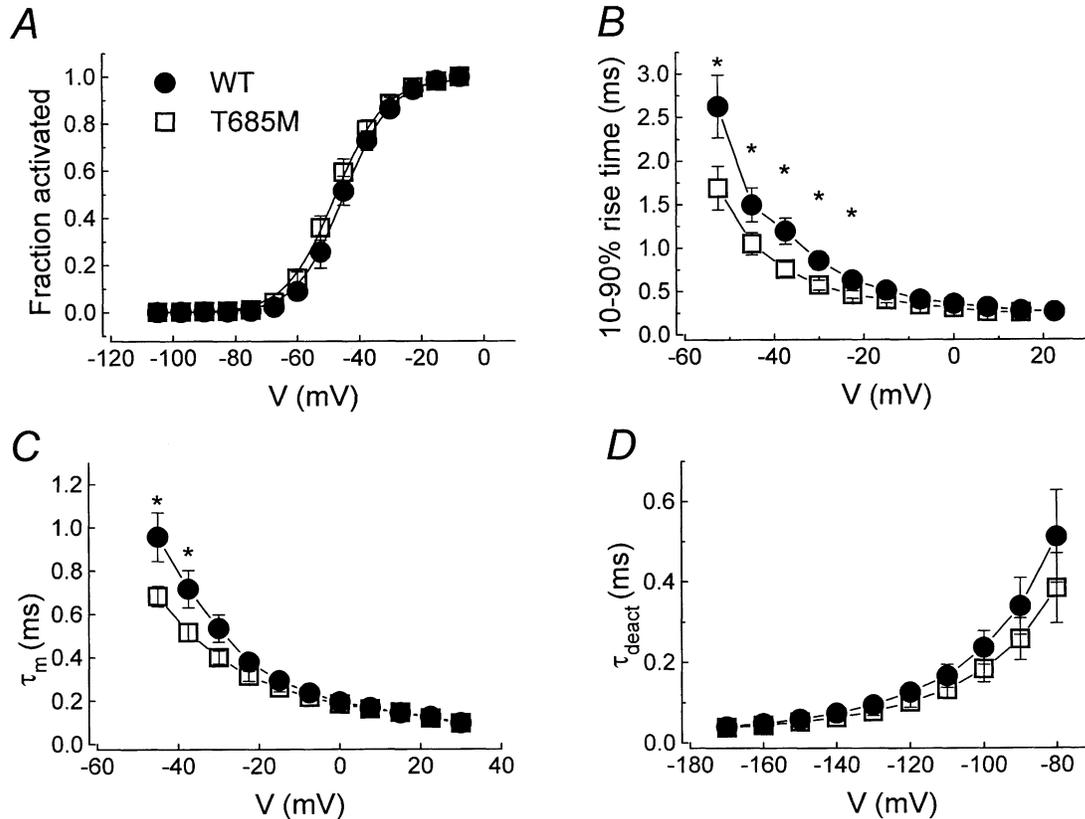


FIG. 2. Activation and deactivation parameters. (A) Voltage dependence of activation for WT and mutant sodium channels, obtained by 25-ms depolarizing pulses to the indicated potentials from a holding potential of -140 mV. Lines are fits to a standard Boltzmann function: $G/G_{max} = 1/\{1 + \exp[(V - V_{0.5})/k_V]\}$. $V_{0.5}$ was -45.3 ± 1.9 vs. -47.6 ± 2.1 mV; k_V was 6.2 ± 0.3 vs. 6.7 ± 0.3 mV, $n = 7, 11$. (B and C) 10–90% rise time and time constant of activation, τ_m , of the sodium current as a function of test potential at 15°C . * $P < 0.05$ for mutant vs. WT channels. Values at -37.5 mV ($n = 8$): 10–90% rise time, 1.19 ± 0.14 vs. 0.75 ± 0.08 ms; τ_m , 0.72 ± 0.08 vs. 0.51 ± 0.04 ms. (D) In order to measure deactivation, a short depolarizing pulse (0.5 ms, to 0 mV) was followed by the test pulse to the indicated potentials. The deactivation time constant, τ_{deact} , was obtained from a first-order exponential fit to the tail currents; $n = 4$. The temperature was 15°C .

course of activation was quantified at 15°C in two different ways. First, the 10–90% rise time was calculated (Fig. 2B) and second, the currents were fitted to a Hodgkin–Huxley function (m^4h ; Hodgkin & Huxley, 1952) to determine the activation time constant τ_m (Fig. 2C). For both methods, a significant acceleration of the activation time course for T685M channels was found at potentials more negative than -20 mV, by a factor of ≈ 0.6 – 0.7 compared to WT. This finding can explain hyperexcitability via a more rapid availability of sodium channels and therefore a decreased threshold for the generation of action potentials upon membrane depolarization. Deactivation was also slightly accelerated but not significantly different from WT (Fig. 2D).

Fast inactivation

The time course of fast inactivation was fitted to a second-order exponential function. The faster time constant, τ_h , accounted for $>95\%$ of the current amplitude. Recovery from inactivation was measured at -100 , -120 and -140 mV after a 100-ms depolarization to 0 mV. Its time course was well fitted to a first order exponential function, yielding τ_{rec} . We did not find a significant difference for either parameter between T685M and WT channels (Fig. 3A and B). We then looked for a putative increase in persistent sodium current as found for almost all mutations causing skeletal muscle sodium channelopathies as a common disease-causing mechanism (Lehmann-Horn & Jurkat-Rott, 1999). As already found for the other *SCN1A*

mutation in segment IV/S4 (Alekov *et al.*, 2000), we could not detect a significant difference in persistent current between mutant and WT channels (for example I_{ss}/I_{peak} for WT vs. T685M determined at 0 mV 50 ms after onset of the depolarization: 0.6 ± 0.4 vs. $0.8 \pm 0.5\%$, $n = 5, 8$). Steady-state fast inactivation was shifted significantly in the hyperpolarizing direction (Fig. 2C). Thus, fast inactivation was enhanced in contrast to other disease-causing sodium channel mutations.

Slow inactivation

Entry into, recovery from and steady-state slow inactivation were determined as shown in Fig. 4. Slow inactivation was considerably stabilized for T685M channels, as revealed by a three-fold acceleration of the entry, a three-fold slowing of the recovery and a -20 mV shift in steady-state slow inactivation. This result shows the most obvious difference between mutant and WT channel gating and therefore may provide a disease-causing mechanism distinct from other mutations described previously. In addition, it indicates an important role of II/S4 for the process of slow sodium channel inactivation.

Discussion

The only difference in gating between WT and T685M channels that can induce the expected hyperexcitability of the cell membrane is the

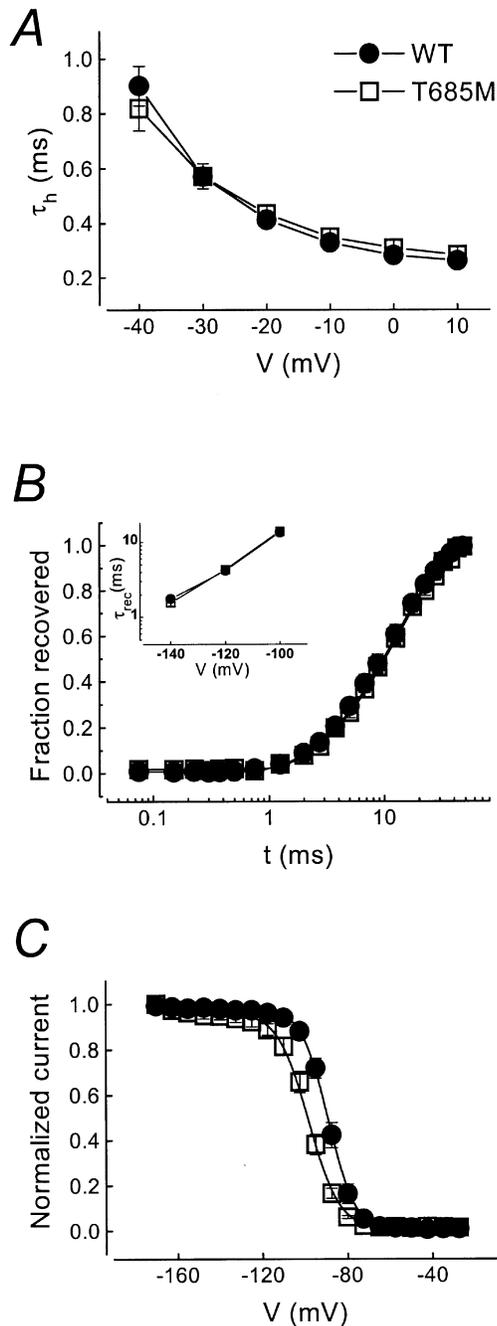


FIG. 3. Parameters of fast inactivation. (A) Voltage dependence of the inactivation time constant, τ_h . Values at 0 mV were 0.28 ± 0.01 vs. 0.31 ± 0.01 ms, $n = 8$. (B) Recovery from inactivation at -100 mV. Lines are fits to a first-order exponential function with time constants $\tau_{rec} = 13.7 \pm 1.9$ vs. 14.0 ± 1.5 ms, $n = 7$. The insert shows the voltage dependence of τ_{rec} , $n = 4-7$. (C) Steady-state inactivation was determined using 300-ms prepulses to the potentials indicated, followed by a short test pulse to -20 mV. Lines are fits to a standard Boltzmann function: $I/I_{max} = 1/[1 + \exp[(V - V_{0.5})/k_V]]$, with $V_{0.5}$ being the voltage of half-maximal inactivation and k_V a slope factor. $V_{0.5}$, -89.0 ± 1.4 vs. -98.3 ± 1.3 mV, $P < 0.001$; k_V , 5.5 ± 0.3 vs. 6.5 ± 0.4 mV; $n = 7, 9$.

observed acceleration of channel activation at voltages more negative than -20 mV. This will increase excitability via a more rapid recruitment of sodium channels for the elicitation of an action potential. In this regard, our results are in contrast to all previously

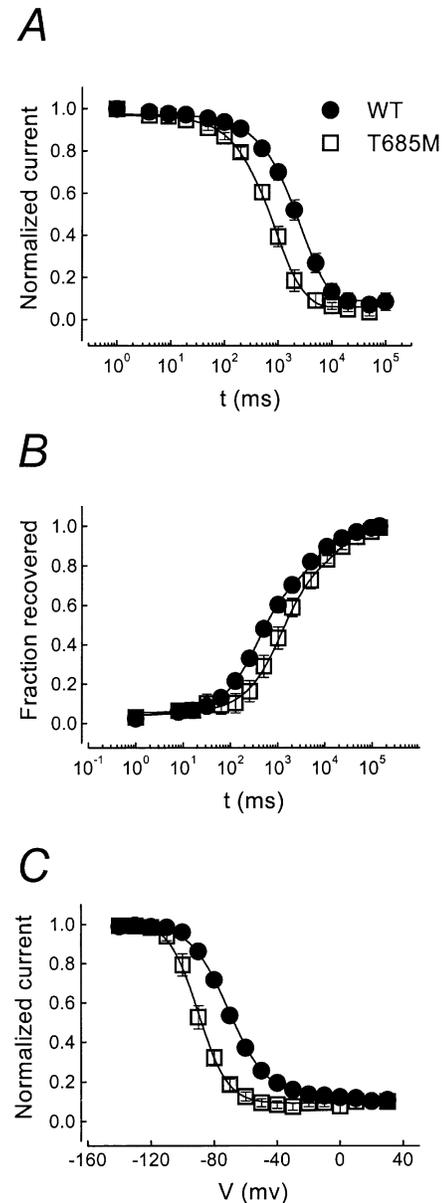


FIG. 4. Parameters of slow inactivation. (A) Entry into slow inactivation at 0 mV. Cells were held at -100 mV, depolarized to 0 mV for increasing durations as indicated on the abscissa, repolarized for 100 ms to -100 mV to let the channels recover from fast inactivation, and then depolarized again to -10 mV to determine the fraction of slow inactivated channels. The lines represent fits to a first-order exponential function with the following time constants: 3.0 ± 0.4 vs. 0.9 ± 0.1 s, $n = 8, 5$, $P < 0.001$. (B) Recovery from slow inactivation measured at -100 mV after a 30-s conditioning pulse to 0 mV. Curves were fitted to a second-order exponential function with the following time constants: $\tau_{srec1} = 0.38 \pm 0.06$ vs. 0.93 ± 0.14 s, $P < 0.05$; $\tau_{srec2} = 9.0 \pm 3.4$ vs. 13.0 ± 1.4 s; relative amplitude of $\tau_{srec1} = 58 \pm 2$ vs. $61 \pm 3\%$, $n = 5, 8$. (C) Steady-state slow inactivation was determined using 30-s prepulses to potentials indicated on the abscissa, followed by a 20-ms repolarizing pulse to the holding potential of -140 mV to let the channels recover from fast inactivation, and the short test pulse to -20 mV. The data were fitted to a standard Boltzmann function (see Fig. 3C): $V_{0.5} = -70.9 \pm 1.5$ vs. -90.1 ± 2.1 mV, $n = 6, 5$, $P < 0.001$.

examined disease-causing sodium channel mutations in *SCN4A* or *SCN5A* going along with hyperexcitability, which exhibit an impaired fast inactivation as the common disease-causing mechanism (Lehmann-Horn & Jurkat-Rott, 1999). If the observed defect in

channel activation is regarded as disease-causing for the T685M mutation, subtle and barely measurable gating defects seem to be sufficient to cause disease in the central nervous system, whereas stronger alterations are needed to induce myotonia or cardiac arrhythmia.

For the other *SCN1A* mutation in the voltage sensor IV/S4 we found the same activation defect as for T685M. However, the IV/S4 mutation in addition showed a three-fold acceleration of recovery from inactivation increasing excitability via a shortening of the refractory period after an action potential as the main disease-causing mechanism (Alekov *et al.*, 2000). Consistent with the *in vitro* results, a more severe phenotype was reported for the family carrying the IV/S4 mutation with 11 of 12 affected members suffering from epilepsy with afebrile seizures. In contrast, afebrile in addition to febrile seizures only occurred in five of 12 affected individuals of the family with the II/S4 mutation (Baulac *et al.*, 1999; Moulard *et al.*, 1999).

On the other hand, the observed stabilization of both fast and slow inactivation are the most pronounced gating defects observed for T685M channels. This will decrease membrane excitability, a mechanism that has been proposed as contributing to paralysis for IV/S4 mutations causing paramyotonia congenita (Lerche *et al.*, 1996; Mitrovic *et al.*, 1999) or more recently for II/S4 mutations causing hypokalemic periodic paralysis type 2 (Jurkat-Rott *et al.*, 2000; Struyk *et al.*, 2000; Kuzmenkin *et al.*, 2001). However, in contrast to skeletal muscle fibers, a decrease of excitability when occurring in inhibitory neurons may be responsible for the generation of synchronous epileptic activity in neuronal circuits and could explain the occurrence of seizures. Hence, there may be distinct pathophysiological mechanisms for different mutations in sodium channels causing epilepsy, because both the other mutations in *SCN1B* or *SCN1A* causing GEFS⁺ clearly lead to an increase of membrane excitability by slowing of fast inactivation or acceleration of recovery from fast inactivation, respectively (Wallace *et al.*, 1998; Alekov *et al.*, 2000). Similarly to these opposite mechanisms observed for different sodium channel mutations, both gain- and loss-function mechanisms were described for distinct mutant nicotinic acetylcholine receptors causing nocturnal frontal lobe epilepsy (Bertrand *et al.*, 1998; De Fusco *et al.*, 2000).

From a genetic point of view, T875M fulfills all criteria for a disease-causing mutation. T875M (i) cosegregates perfectly with the phenotype, (ii) is located in an important functional region within a protein essential for excitability, and (iii) has not been found in a lot of normal controls. However, three sodium channel genes are located in the chromosomal region linked to disease in this family (*SCN1-3A*, Moulard *et al.*, 1999), and only *SCN2A* has been fully sequenced (Escayg *et al.*, 2000a). Thus, it cannot be ruled out with certainty that there is a second mutation in either *SCN1A* or *SCN3A* segregating with the disease in this family.

Another point to discuss is that we used the *SCN4A* instead of the *SCN1A* gene for our functional studies. However, (i) a preliminary study on functional expression of both *SCN1A* mutations in *Xenopus* oocytes yielded similar results with an acceleration of recovery from inactivation for the IV/S4 mutation and no other obvious gating defects for both mutations (Escayg *et al.*, 2000b). The activation defect observed in our studies is below the limit of resolution for two-microelectrode voltage clamp recordings in oocytes and slow inactivation was not examined. (ii) All functionally important regions of *SCN1A* and *SCN4A* are highly conserved, as is the voltage sensor II/S4 (Fig. 1). (iii) Known functional studies of neuronal and skeletal muscle channels only showed small differences in gating (Goldin, 1999). (iv) The use of an established expression system, *SCN4A* and tsA201 cells, had the advantage that we could directly compare our

results with those of previous studies of sodium channelopathies of skeletal muscle from our and other laboratories (Cannon, 1997; Lehmann-Horn & Jurkat-Rott, 1999).

From a biophysical point of view, our results confirm those from earlier studies showing that the voltage sensor II/S4 is more important for activation than for inactivation (Chen *et al.*, 1996; Mitrovic *et al.*, 1998; Cha *et al.*, 1999). In particular, the results extend those by Mitrovic *et al.* (1998) that movement of the II/S4 segment and the kinetics of activation can be influenced by mutations therein. Whereas the mutation used in their study is located at the extracellular end, T685M lies on the cytoplasmic end of the supposed S4 helix. Remarkable are the relatively large effects on slow inactivation, confirming the importance of the II/S4-II/S5 region for this gating process (Cummins & Sigworth, 1996; Hayward *et al.*, 1999; Struyk *et al.*, 2000; unpublished results from our group).

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Abbreviations

GEFS⁺, generalized epilepsy with febrile seizures-plus; WT, wild-type.

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