Rapid Report

A sodium channel mutation causing epilepsy in man exhibits subtle defects in fast inactivation and activation *in vitro*

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1. Generalized epilepsy with febrile seizures plus ($GEFS^+$) is a benign epileptic syndrome of humans. It is characterized by febrile and afebrile generalized seizures that occur predominantly in childhood and respond well to standard antiepileptic therapy. A mutation in the β_1-subunit of the voltage-gated sodium channel, linked to chromosome 19q13 ($GEFS^+$ type 1) has been found in one family. For four other families, linkage was found to chromosome 2q21–33 ($GEFS^+$ type 2) where three genes encoding neuronal sodium channel α-subunits are located (*SCN1–3A*). Recently, the first two mutations were identified in *SCN1A*.

2. We introduced one of these mutations, which is highly conserved to *SCN1A*, into the cDNA of the gene *SCN4A* encoding the α-subunit of the human skeletal muscle sodium channel (hSkm1). The mutation is located in the S4 voltage sensor of domain IV, predicting substitution of histidine for the fifth of eight arginines (R1460H in hSkm1). Functional studies were performed by expressing the α-subunit alone in the mammalian tsA201 cell line using the whole-cell patch clamp technique.

3. Compared to wild-type (WT), mutant R1460H channels showed small defects in fast inactivation. The time course of inactivation was slightly (1.5-fold) slowed and its voltage dependence reduced, and recovery from inactivation was accelerated 3-fold. However, there was no increase in persistent sodium current as observed for *SCN4A* mutations causing myotonia or periodic paralysis. The activation time course of R1460H channels was slightly accelerated. Slow inactivation was slightly but significantly stabilized, confirming the importance of this region for slow inactivation.

4. The combination of activation and fast inactivation defects can explain the occurrence of epileptic seizures, but the effects were much more subtle than the inactivation defects described previously for mutations in *SCN4A* causing disease in skeletal muscle. Hence, with regard to pathological excitability, our results suggest a greater vulnerability of the central nervous system compared to muscle tissue.

Voltage-gated Na^+ 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 the channels recover from inactivation. 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 (*SCN1A–11A*) that are expressed specifically in skeletal muscle (*SCN4A*), heart muscle (*SCN5A*) and neuronal tissue; four subunits (encoded by *SCN1A*, *SCN2A*, *SCN3A* and *SCN8A*) are considered to be responsible for the sodium current in brain. There are three genes for the auxiliary β-subunits (*SCN1B–3B*), which 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).

Ion channel disorders are rare inherited diseases providing interesting models to study dysfunction of excitability *in vivo* and *in vitro*. The first so-called 'channelopathies' identified were skeletal muscle diseases,

the myotonias and hyperkalaemic periodic paralysis, which are sodium and chloride channel disorders. For about 20 known mutations in *SCN4A*, a gain of function mechanism causes hyper- or hypoexcitability in the sodium channel diseases through a defect in channel inactivation resulting in an increase in the sodium inward current, which depolarizes the sarcolemma. A small depolarization will increase whereas a large depolarization will decrease excitability. 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).

Three forms of idiopathic epilepsies have been identified as arising from ion channel disorders. Autosomal dominant nocturnal frontal lobe epilepsy (ADNFLE) is caused by point mutations in the α_4-subunit of a neuronal nicotinic ACh receptor (Steinlein *et al.* 1995), benign familial neonatal convulsions (BFNC) are caused by mutations in two voltage-gated potassium channels (Biervert *et al.* 1998; Charlier *et al.* 1998; Singh *et al.* 1998) and generalized epilepsy with febrile seizures plus ($GEFS^+$) is a sodium channel disorder (Wallace *et al.* 1998; Escayg *et al.* 2000). $GEFS^+$ is a benign childhood-onset epileptic syndrome featuring different forms of febrile and afebrile seizures (Scheffer & Berkovic, 1997; Singh *et al.* 1999). Five large families with autosomal dominant inheritance of $GEFS^+$ have been described so far, showing linkage to chromosome 19q13 in one case ($GEFS^+$ type 1; Wallace *et al.* 1998) and to chromosome 2q21–33 in the other four ($GEFS^+$ type 2; Baulac *et al.* 1999; Moulard *et al.* 1999; Pfeiffer *et al.* 1999; Lopez-Cendes *et al.* 2000). For the chromosome 19-linked $GEFS^+$ family, a mutation has been found in *SCN1B* predicting substitution of tryptophan for one of two cysteine residues (C121W) that stabilize the secondary structure of the functionally important extracellular loop of the β_1-subunit. Although this auxiliary subunit is also expressed in skeletal muscle, only epileptic seizures but no myotonia were reported for affected individuals. Functional studies in *Xenopus* oocytes revealed a loss of β_1-subunit function, resulting in a decreased rate of inactivation, which increased the sodium inward current (Wallace *et al.* 1999).

The neuronal sodium channel α-subunit genes *SCN1–3A* are located at the $GEFS^+$ locus on chromosome 2q21–33. Recently, two mutations have been identified in *SCN1A* in two of the linked families (Escayg *et al.* 2000). Both mutations are located in functionally important regions, the S4 voltage sensors in domains II and IV (T875M in II/S4 and R1648H in IV/S4). The voltage sensor IV/S4 has been shown previously to be important for channel inactivation and mutations therein cause paramyotonia congenita, one of the sodium channel disorders in skeletal muscle (Chahine *et al.* 1994; Lerche *et al.* 1996; Lehmann-Horn & Jurkat-Rott, 1999). The sodium channel α-subunits expressed in skeletal muscle and brain are highly conserved in the functionally important regions of the channel proteins and their kinetic behaviour, known from various functional studies of the human and rat isoforms, is very similar (Goldin, 1999), but the human *SCN1A* gene has not been cloned and functionally expressed so far. The kinetic behaviour of the rat brain type I sodium channel (encoded by the rat *SCN1A* gene) has been described recently (Smith & Goldin, 1998).

In order to study the functional consequences of a mutation causing epilepsy and also to compare the defects to mutations found in the myotonias, we introduced the *SCN1A* mutation R1648H in the voltage sensor IV/S4 into the same conserved region of *SCN4A* (R1460H) and expressed wild-type (WT) and mutant α-subunits in tsA201 cells. As expected, the gain of function was much smaller than that found for the myotonia-causing mutations located within the same channel region.

METHODS

Mutagenesis and transfection

Site-directed mutagenesis to introduce the mutation R1460H was performed using a PCR-based strategy. The mutants were reassembled in the pRC/CMV plasmid (Invitrogen) for transfection into the mammalian cell line tsA201 using a standard calcium phosphate transfection method. A CD8 cDNA-containing plasmid was cotransfected in order to allow identification of transfected cells using anti-CD8 antibody-coated microbeads (Dynabeads M450, Dynal; Lerche *et al.* 1997).

Electrophysiology and data analysis

Standard whole-cell recording was performed using an EPC-7 amplifier (EPC7, List). The pipette solution contained (mM): 105 CsF, 35 NaCl, 10 EGTA and 10 Hepes (pH 7.4). The bath solution contained (mM): 150 NaCl, 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$ and 10 Hepes (pH 7.4). For some experiments, solutions with internal CsCl instead of CsF were used (Lerche *et al.* 1997). Sodium currents in transfected cells for WT and mutant channels ranged between 2.5 and 15 nA. The maximal voltage error due to residual series resistance was < 5 mV. Leakage and capacitative 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 software (Axon Instruments). 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) and ORIGIN software (MicroCal). For statistical evaluation, Student's t test was applied. All data are shown as means ± S.E.M.

RESULTS

The mutation R1460H in the skeletal muscle sodium channel α-subunit predicts the substitution of histidine for the fifth of eight positively charged amino acids in the S4 voltage sensor of domain IV (IV/S4). The almost complete conservation of IV/S4 and R1460 among the *SCN1A* and *SCN4A* gene products and other known voltage-gated sodium channel α-subunits is shown in Fig. 1*A*. The R1460H mutation was engineered into the cDNA of the *SCN4A* gene, and WT or mutant plasmids were transfected into tsA201 cells. Families of normalized whole-cell sodium currents for WT and mutant channels

elicited by various depolarizing voltage steps from a holding potential of −140 mV are shown in Fig. 1*B*. In order to look for differences in gating between WT and R1460H channels that may explain the occurrence of epileptic seizures, the kinetics and voltage dependence of activation, deactivation, fast and slow inactivation were determined.

Fast inactivation

The time course of fast inactivation was fitted to a second order exponential function. The fast time constant, τ_h, accounted for > 95% of the current amplitude for both WT and R1460H. There was a small but significant slowing of fast inactivation at depolarized potentials and a marked decrease in its voltage dependence in the R1460H mutant (Fig. 2*A*). The loss of voltage dependence has already been described for other mutations in IV/S4 and may indicate an uncoupling of inactivation from activation (Chahine *et al.* 1994; Lerche *et al.* 1996; Mitrovic *et al.* 1999). The most distinct difference between WT and R1460H channels was found in the recovery from inactivation, measured at −100, −120 and

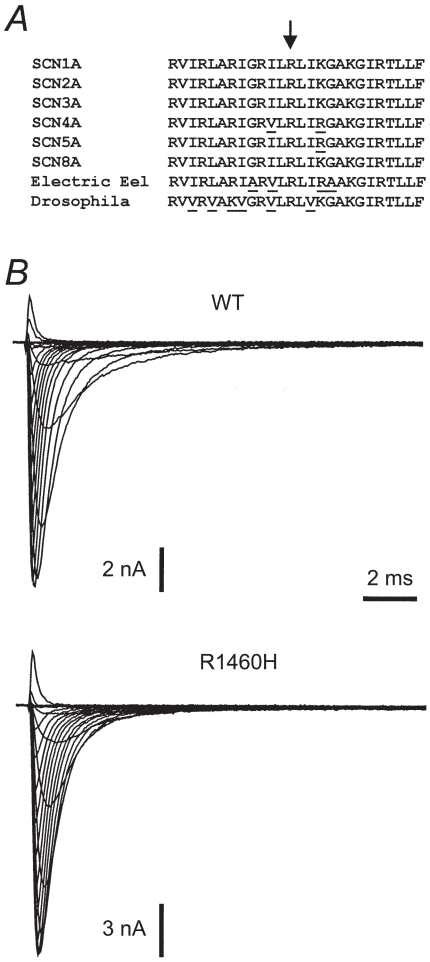

Figure 1. Mutation R1460H in segment IV/S4

A, amino acid sequence comparison of the IV/S4 segment of various sodium channel α-subunits derived from different genes. Sequence changes are underlined. Residue R1648 (*SCN1A* numbering) corresponding to R1460 (*SCN4A* numbering) is marked by an arrow. *B*, representative whole-cell sodium current families recorded from cells transfected with either WT or mutant channel cDNA.

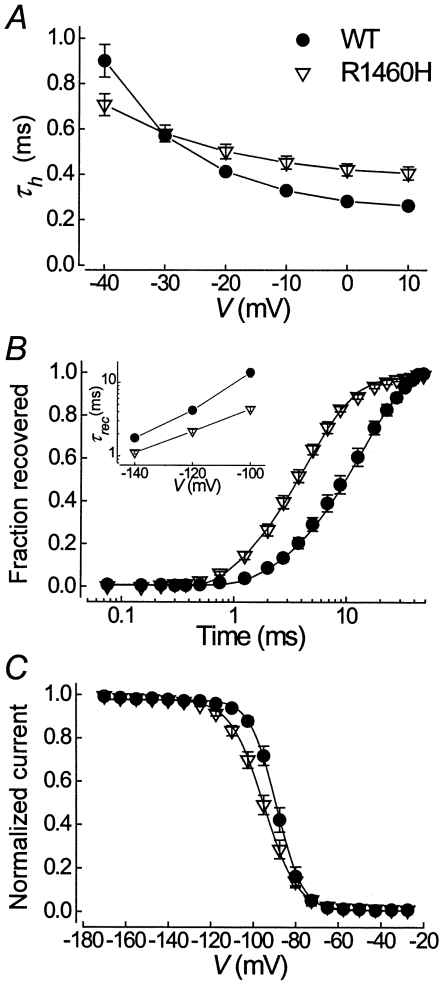

Figure 2. Parameters of fast inactivation

A, voltage dependence of the inactivation time constant, τ_h. Values at 0 mV were 0.28 ± 0.01 for WT *vs.* 0.42 ± 0.03 ms for R1460H; $n = 8, 14$; $P < 0.001$. *B*, recovery from inactivation at −100 mV. Lines are fits to a first order exponential function with recovery time constants (τ_{rec}) of 13.7 ± 1.9 *vs.* 4.4 ± 0.3 ms; $n = 7, 11$; $P < 0.001$; and an initial delay of 1.0 ± 0.1 *vs.* 0.59 ± 0.04 ms, $P < 0.001$, for WT and R1460H, respectively. The inset shows the voltage dependence of τ_{rec}, $n = 4$–11. *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: $I/I_{max} = 1/(1 + \exp[(V - V_{0.5})/k_V])$, where $V_{0.5}$ is the voltage of half-maximal inactivation and k_V is a slope factor. $V_{0.5}$ was −89.0 ± 1.4 *vs.* −96.0 ± 1.6 mV; $n = 7, 16$; $P < 0.02$; k_V was 5.5 ± 0.3 *vs.* 7.2 ± 0.2 mV, $P < 0.001$, for WT and R1460H, respectively.

−140 mV after a 100 ms depolarization to 0 mV. Its time course was well fitted to a first order exponential function, yielding the time constant for recovery, τ_{rec}. For R1460H, τ_{rec} was decreased by about 3-fold at −100 mV (Fig. 2*B*). The steady-state fast inactivation was shifted slightly towards more hyperpolarized potentials (Fig. 2*C*).

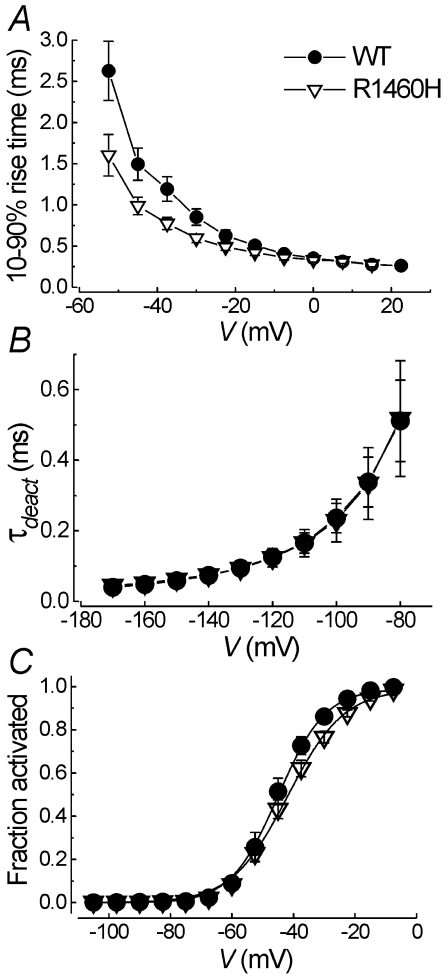

Figure 3. Activation and deactivation parameters

A, 10–90% rise time of the sodium current as a function of test potential. The differences between mutant and WT channels over the range −52.5 to −30 mV are statistically significant at $P < 0.05$; $n = 7, 8$; $T = 15$°C. *B*, in order to measure deactivation, a short depolarizing pulse (0.5 to −10 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; $n = 4$, $P > 0.05$, $T = 15$°C. *C*, 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.* −44.4 ± 2.1 mV; $n = 7, 6$; $P > 0.05$; k_V was 6.2 ± 0.3 *vs.* 7.8 ± 0.2 mV, $P < 0.01$, for WT and R1460H, respectively; $T = 22$°C.

Both the slowing of fast inactivation and the acceleration of its recovery increased excitability, as has previously been shown for *SCN4A* mutations causing myotonia. However, the most important finding with *SCN4A* mutations causing disease in skeletal muscle was an increase in the persistent sodium current depolarizing the muscle fibre membrane (Cannon, 1997; Lehmann-Horn & Jurkat-Rott, 1999). As can be seen in Fig. 1*B*, there was no increase in persistent current for R1460H compared to WT channels. We determined the persistent sodium current (I_{ss}) at the end of a 70 ms depolarizing test pulse to −20 mV relative to the peak current (I_{peak}) using both a CsF and a CsCl intracellular solution (the latter was used previously to analyse myotonia-causing mutations, e.g. Mitrovic *et al.* 1999). There was no significant difference between WT and R1460H channels in either solution (I_{ss}/I_{peak} at −20 mV for WT *vs.* R1460H in CsF: 0.6 ± 0.2 *vs.* 0.7 ± 0.3%; $n = 7, 16$; in CsCl: 0.7 ± 0.1 *vs.* 0.6 ± 0.4%; $n = 7, 3$).

Activation and deactivation

For a better time resolution, the kinetics of activation and deactivation were determined at 15°C. For quantification of the activation time course, the 10–90% rise time was calculated. There was a significant acceleration of the activation time course for R1460H channels between −52.5 and −30 mV (Fig. 3*A*). In contrast, the deactivation time course was almost identical for the two clones (Fig. 3*B*). For the steady-state activation curve we found a significant difference in slope (Fig. 3*C*).

Slow inactivation

Entry into, recovery from and steady-state slow inactivation were determined as shown in Fig. 4. Slow inactivation was slightly stabilized for R1460H channels, as revealed by a hyperpolarizing shift in steady-state slow inactivation, a more complete slow inactivation and a decreased rate of its recovery.

Effects of pH on R1460H

Protonation of the substituted histidine residue in R1460H is pH dependent. For another arginine to histidine mutation in IV/S4 causing paramyotonia congenita (R1448H), protonation at low pH restores normal gating (Chahine *et al.* 1994). Since residue 1460 is only accessible from the intracellular side of the membrane (Yang *et al.* 1996), we examined the gating of R1460H at intracellular pH 6.2 and 8.5. There were no significant differences in comparison to pH 7.4 in all the parameters presented in Figs 2–4. Hence, the functional alterations of R1460H are caused by steric effects of the histidine side chain rather than by the decrease in charge.

DISCUSSION

Subtle differences in activation and fast inactivation of the voltage-gated sodium channel were identified for a mutation causing a benign form of human epilepsy.

According to the genetic data (Escayg *et al.* 2000), there is no doubt that this mutation in *SCN1A* is causative for the disease since it cosegregates perfectly with the phenotype and is localized in an important functional region of a gene that is essential for excitability in nervous tissue. Here, we demonstrate that this mutation has functional consequences that can account for the occurrence of hyperexcitability. One might argue that we did not use the right gene for our functional studies. However, there are three important arguments that prompted us to perform the studies with the same mutation in *SCN4A*. First, all functionally important regions of *SCN1A* and *SCN4A* are highly conserved, as is the voltage sensor IV/S4 (Fig. 1). Second, known functional studies of neuronal and skeletal muscle channels only showed small differences in gating (Goldin, 1999). Third, and most important for us, the use of an established expression system, *SCN4A* and tsA201 cells, had the advantage that we could directly compare our results to those of previous studies concerning sodium channelopathies of skeletal muscle from our and other laboratories, in particular to those for mutations also located in IV/S4 (Chahine *et al.* 1994; Lerche *et al.* 1996; Cannon, 1997; Mitrovic *et al.* 1999; Lehmann-Horn & Jurkat-Rott, 1999). Nevertheless, the data presented here must be interpreted with some caution until they have been confirmed in the human *SCN1A* gene in studies including coexpression of the β_1- and β_2-subunits, which have considerable effects on inactivation of the brain sodium channels (Smith & Goldin, 1998; see also the discussion about different expression systems and the β-subunit below).

The effects of R1460H on fast inactivation presented here are very small compared to those described for other mutations in *SCN4A* or *SCN5A* causing disease in skeletal or heart muscle (Cannon, 1997; Lehmann-Horn & Jurkat-Rott, 1999). For example, the inactivation time constant was found to be increased 3- to 6-fold for other mutations in IV/S4 causing paramyotonia congenita (R1448H/C/P: Chahine *et al.* 1994; Mitrovic *et al.* 1999). Here, τ_h was increased only 1.5-fold. Moreover, the most important and the most consistent finding for almost all known mutations in *SCN4A* and *SCN5A* is an increased persistent sodium current leading to a permanent sodium inward current, which depolarizes the cell membrane. This will decrease the threshold to elicit an action potential and therefore cause hyperexcitability. A very large persistent current in combination with a defect in slow inactivation will cause an even larger depolarization that will inactivate the sodium channels and therefore result in paralysis (Cannon, 1997). For R1460H in contrast, there was definitely no increase in persistent sodium current.

The main mechanism of the R1460H mutation causing hyperexcitability may be a combination of the faster activation time course and the 3-fold acceleration of recovery from inactivation. This will shorten both the period of depolarization needed to elicit an action potential and the refractory period after an action

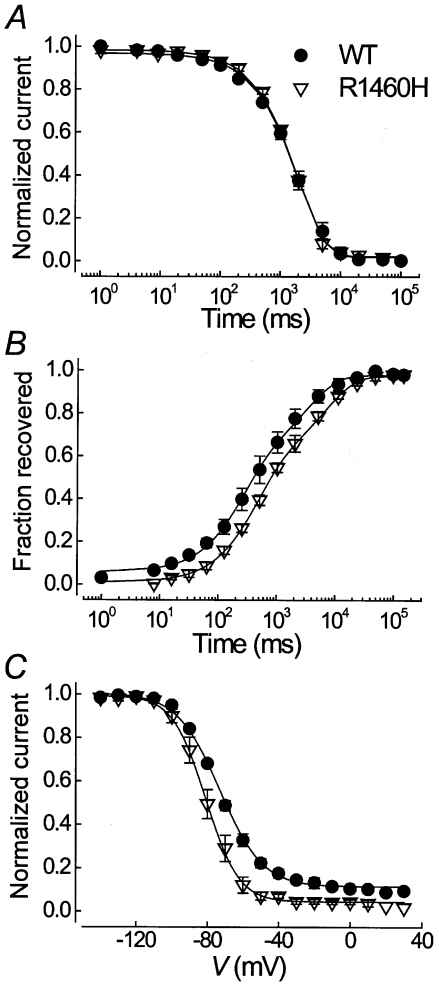

Figure 4. Parameters of slow inactivation

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: 1.9 ± 0.3 for WT *vs.* 2.2 ± 0.1 s for R1460H; $n = 3, 6$; $P > 0.05$. *B*, recovery from slow inactivation measured at −100 mV after a 30 s conditioning pulse to 0 mV. Curves were best fitted to a second order exponential function with the following slow recovery time constants: $\tau_{srec1} = 0.31 \pm 0.11$ *vs.* 0.46 ± 0.06 s, $\tau_{srec2} = 4.2 \pm 1.8$ *vs.* 7.5 ± 1.3 s; relative amplitude of $\tau_{srec1} = 54 \pm 1$ *vs.* 56 ± 4%; $n = 3, 5$; $P > 0.05$, for WT and R1460H, respectively. *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 a short test pulse to −20 mV. The data were fitted to a standard Boltzmann function: $V_{0.5} = -73.0 \pm 1.1$ for WT *vs.* −80.9 ± 2.1 mV for R1460H, $n = 4$, $P < 0.02$.

potential. In contrast to a persistent sodium current, neither mechanism should influence the resting membrane potential, which probably would be fatal when occurring in neurons in contrast to skeletal or heart muscle fibres. An acceleration of sodium channel activation has not been described as a disease-causing mechanism so far.

The subtle alterations in channel gating found in this study for the R1460H mutation are in line with two previous findings concerning sodium channel gating and epilepsy. First, the β_1-subunit mutation described by Wallace and colleagues (Wallace *et al.* 1998) also shows a small defect in inactivation and causes epilepsy but no myotonia, although *SCN1B* is also expressed in skeletal muscle. Second, a transgenic model introducing a *SCN2A* mutation in the mouse yields severe status epilepticus, although the inactivation defect was also very small compared to other known mutations (Kearny *et al.* 1998).

The corresponding mutation to R1460H has already been studied in the rat brain IIa sodium channel for other purposes, before it was known that this mutation can cause epilepsy (R1638H; Kühn & Greeff, 1999). The results are difficult to compare to those presented here, since in the other study the α-subunit was expressed alone in *Xenopus* oocytes. Isolated expression of brain or skeletal muscle sodium channel α-subunits in oocytes results in a large decrease in the rate of inactivation. This is due to the occurrence of a second slow gating mode of inactivation that is not seen upon coexpression of the β_1-subunit. In contrast, isolated expression of α-subunits in HEK293 or tsA201 cells does not significantly alter channel kinetics. The difference between the two expression systems is probably due to an endogenous β_1-subunit in HEK cells (Moran *et al.* 2000). The slow gating mode observed in oocytes seemed to be favoured by R1638H, resulting in a slowing of the inactivation time course.

From a biophysical point of view, our results confirm the importance of the voltage sensor in domain IV for fast channel inactivation (Chahine *et al.* 1994; Chen *et al.* 1996; Yang *et al.* 1996; Lerche *et al.* 1996; Mitrovic *et al.* 1999; Kühn & Greeff, 1999; Cha *et al.* 1999; Horn *et al.* 2000). The effects on activation have not been described so far for other mutations in IV/S4 and suggest a definite role of IV/S4 in channel activation. However, the faster inactivation time course at these potentials may also influence the current rise time. The stabilization of slow inactivation observed for R1460H channels confirms an important role of IV/S4 in slow inactivation and extends recent results of Mitrovic *et al.* (2000). The authors proposed a model in which mutations near the midpoint on one side of the putative IV/S4 α-helix enhance slow inactivation. R1460 is located on the same side of the helix just below the important region between residues A1453 and V1458.

We expect that more mutations in different sodium channel genes associated with epilepsy will be found in the future. It will be interesting to study these defects in comparison with disease-causing mutations in other sodium channel genes. This might establish the view presented here that such subtle alterations in gating are sufficient to cause hyperexcitability in the brain, whereas larger defects are needed to cause disease in skeletal or heart muscle.

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