A novel sodium channel mutation causing a hyperkalemic paralytic and paramyotonic syndrome with variable clinical expressivity

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Neurology 1997;49;1018

A novel sodium channel mutation causing a hyperkalemic paralytic and paramyotonic syndrome with variable clinical expressivity

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Article abstract—A point mutation A4078G predicting the amino acid exchange Met1360Val in segment IV/S1 of the human muscle sodium channel α-subunit was identified in a family presenting features of hyperkalemic periodic paralysis and paramyotonia congenita with sex-related modification of expression. In this family, only one male member is clinically affected, presenting episodes of flaccid weakness as well as paradoxical myotonia and cold-induced weakness. Three female family members who have the same mutation show only myotonic bursts on EMG. We studied the functional defect caused by this mutation by investigating recombinant wild type (WT) and mutant sodium channels expressed in a mammalian cell line (HEK293) using the patch-clamp technique. With mutant channels, the decay of the sodium currents was two times slower than with WT, the steady-state inactivation curve was shifted by −13 mV, and recovery from inactivation was 1.5 times faster. High extracellular potassium (9 mM) did not affect channel gating. Single-channel measurements revealed prolonged mean open times and an increased number of channel reopenings. The results are remarkable with respect to the lack of complete penetrance usually seen with sodium channelopathies and the site of mutation that was formerly not thought to be involved in channel inactivation.

NEUROLOGY 1997;49:1018–1025

The group of hereditary sodium channelopathies comprises three diseases—hyperkalemic periodic paralysis (HyperPP), paramyotonia congenita (PC), and potassium-aggravated myotonia (PAM)—with some degree of clinical overlap. Each of them may be caused by one of a number of different point mutations in *SCN4A*, the gene encoding the adult human skeletal muscle sodium channel α-subunit (for review, see reference 1). HyperPP is characterized by transient episodes of muscle weakness or paralysis that usually start in the morning and that may last from minutes to hours. Attacks of weakness are often provoked by rest after exercise or by ingestion of potassium-rich food, i.e., by an increased potassium concentration in the extracellular space. Clinical myotonia is not common in HyperPP but may precede an attack. In contrast, PC is characterized by myotonia. The muscle stiffness becomes particularly severe in the cold and may give way to long-lasting weakness if the muscles have to work hard. Typically, in PC the myotonia is "paradoxical" because, contrary to the "warm-up" seen in Thomsen and Becker myotonia congenita (MC), muscle stiffness increases with continued activity. In PAM, the leading symptom is also myotonia and, characteristically, episodes of weakness do not occur. Myotonia in PAM is not temperature-dependent, but is aggravated by intake of potassium. Before the detection of the mutations in *SCN4A* associated with this condition, PAM patients were usually misdiagnosed as having "unusual" forms of dominant MC, which is in fact caused by mutations in the gene encoding the muscle chloride channel. Clinically, PAM may be differentiated from MC by its pronounced potassium dependence.[2] Altogether 20 point mutations have been detected in *SCN4A* to cause sodium channelopathies. In addition, an inherited cardiac arrhythmia was shown to be caused by corresponding mutations in the heart muscle sodium channel gene, *SCN5A*.[3]

Already in "premolecular" days, electrophysiologic studies on muscle fibers obtained at biopsy from HyperPP and PC patients had indicated that these diseases might be caused by an abnormal steady-state sodium current through the muscle fiber membranes.[4,5] Patch-clamp experiments with fibers from patients and on mutant sodium channels that were heterologously expressed in mammalian cell lines revealed that the disease-causing mechanism for all sodium channelopathies is defective sodium channel inactivation (for review, see reference 1). Slowing of

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Supported by the Deutsche Forschungsgemeinschaft (Le 481/3-3), the Deutsche Gesellschaft für Muskelkranke, the Muscular Dystrophy Association, and NIH Grant N532387(ALG).

Received February 21, 1997. Accepted in final form April 21, 1997.

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inactivation, accelerated recovery from inactivation, increased steady-state sodium current, shifts in the voltage-dependence of steady-state inactivation and activation, and uncoupling of inactivation from activation were found to be caused by the various mutations.[6-13]

In this study, we examined the alterations of the sodium currents caused by a natural point mutation in *SCN4A* (A4078G), which we reported at the 19th ENMC Workshop on Non-Dystrophic Myotonias and Periodic Paralyses.[14] Clinically, this mutation is interesting because the predicted amino acid substitution, M1360V, produces symptoms with subclinical expressivity in females (in contrast to the complete penetrance usually observed in sodium channelopathies), whereas in males, it causes an overlapping syndrome of HyperPP and PC. Electrophysiologically, the mutation is of interest because the predicted amino acid substitution is in a channel protein region (IV/S1) that has so far not yet been studied. Our results indicate that this region is involved in channel inactivation.

Methods. *Muscle biopsy.* A small muscle specimen (length about 2.5 cm) was taken from the biceps brachii muscle when the patient had to undergo an orthopedic operation under general (isoflurane) anesthesia. Depolarizing muscle relaxants were avoided as they may cause mus-

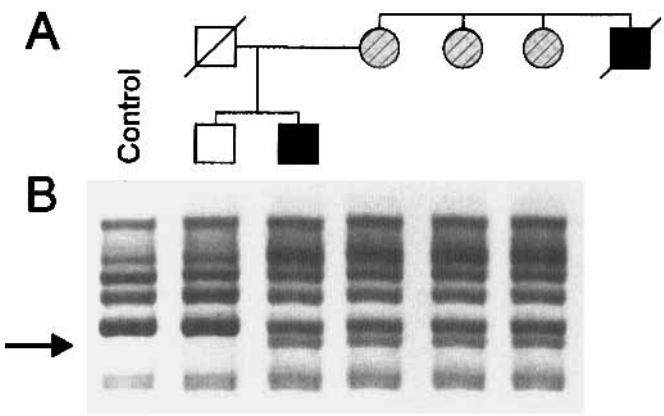
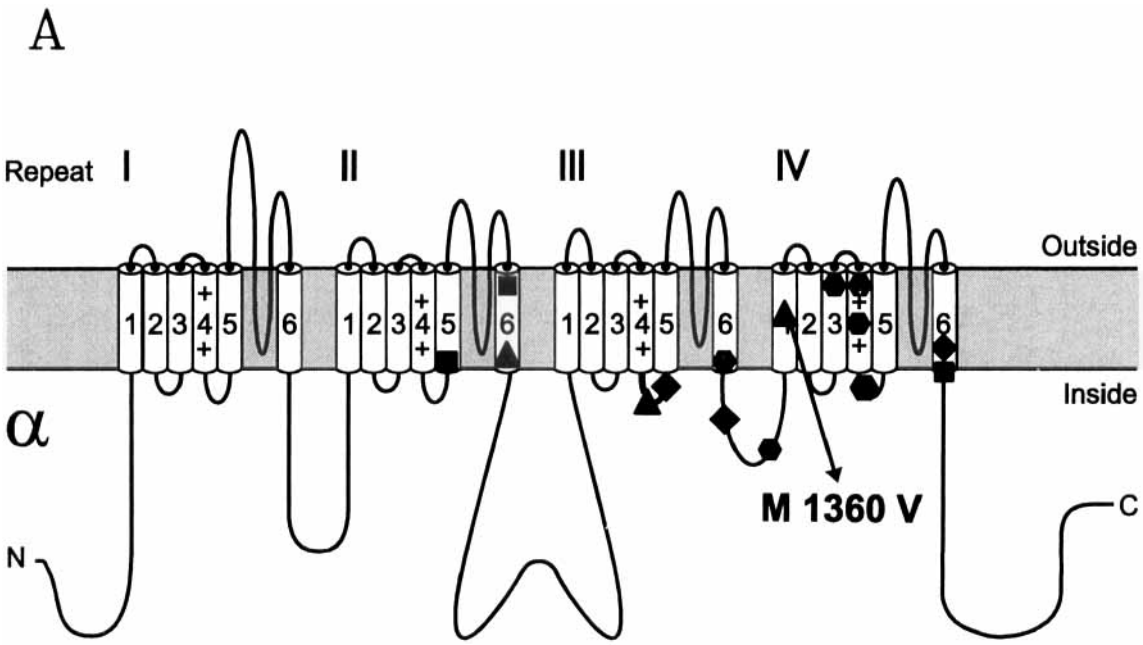


Figure 1. Mutation screening of the family. (A) The pedigree shows the index patient (closed square), his unaffected brother (open square), and his mother and two aunts (hatched circles) who revealed myotonia on EMG, but no clinical symptoms. A deceased uncle was reported to have suffered attacks of weakness (closed square). (B) The ethidium bromide-stained polyacrylamide gel shows an aberrant band (arrow) of single-stranded DNA of the patient and the family members with electric myotonia. Genomic DNA was amplified with primers for exon 23 of SCN4A *encoding the α-subunit of the skeletal muscle sodium channel.*



B

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                    1360
hSkM1:          TKQAFDITIMILICLNMVTMMVET
hSkM2:          TKQAFDVTIMFLICLNMVTMMVET
hNa2.1:         TSQAFNVIVMVLICFQAIAMMIDT
hNaCHII:        TRQVFDISIMILICLNMVTMMVET
rSkM1:          TKQVFDISIMILICLNMVTMMVET
rSkM2:          TKQAFDVTIMFLICLNMVTMMVET
rNaCHII:        TRQVFDISIMILICLNMVTMMVET
Electroporus:   TQPFTDIFIMALICINMVAMMVES
Drosophila Para:TDKKFDIIIMLFIGLNMFNTMLDR
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Figure 2. Model of the α-subunit of the human skeletal muscle sodium channel. (A) The novel mutation, M1360V, is located in the first segment of the fourth domain. Known mutations also causing sodium channelopathies are indicated as a square (HyperPP), diamond (PAM), hexagon (PC), and triangle (overlapping syndromes). (B) This panel indicates conservation of M1360 among all known voltage-gated sodium channels sequenced up to date.

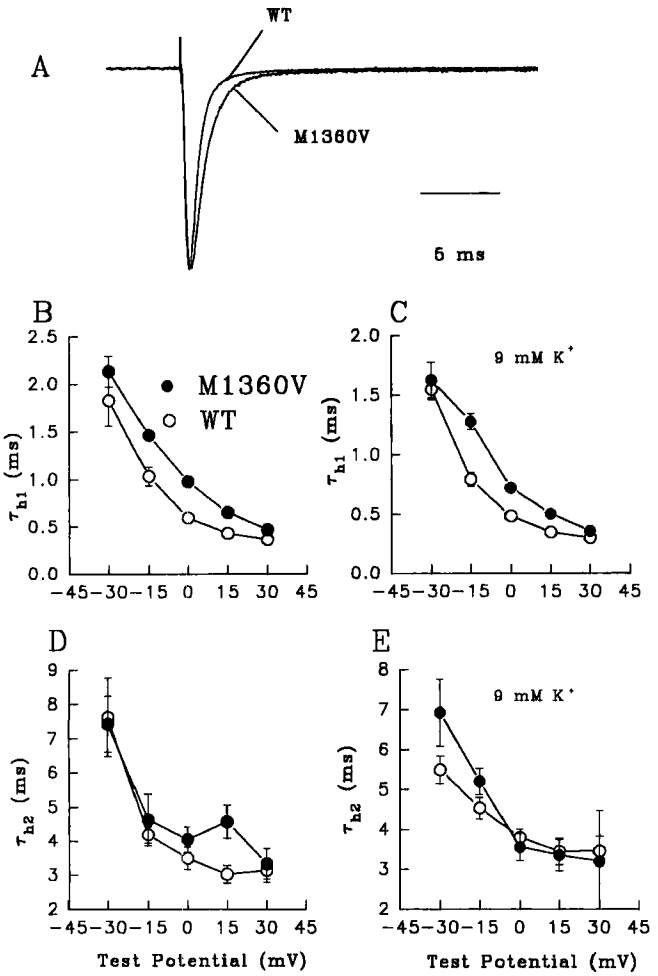

Figure 3. Time course of inactivation of heterologously expressed sodium currents recorded in the whole-cell mode. (A) Currents elicited by a step depolarization from a holding potential of −100 to 0 mV. Currents for M1360V and WT channels are superimposed. (B to E) Fast and slow time constants of inactivation plotted versus test potential with a physiologic (4 mM, B and D) and an increased external potassium concentration (9 mM, C and E). M1360V, closed symbols; WT, open symbols (n = 6 to 12 patches).

cle stiffness or weakness, or both, in PC and HyperPP patients. Likewise, the operating theatre and the recovery room were kept at increased temperature, and the patient was fully covered with blankets during the operation. No complications occurred. The muscle specimen was transported to the laboratory in normal (Bretag) solution kept at 37 °C and bubbled with 95% O_2 and 5% CO_2. Biopsy specimens taken under regional anesthesia from three healthy individuals (tested for malignant hyperthermia susceptibility with negative results) were used as controls. The muscle specimens were dissected in several bundles (diameter 2 to 3 mm) for the measurement of force and for intracellular potential recordings. All procedures were in accordance with the Helsinki Convention and were approved by the Ethics Committee of the University of Ulm.

Mutation screening, site-directed mutagenesis, and transfection. The mutation was detected by single-strand conformation polymorphisms of polymerase chain reaction (PCR)-amplified genomic DNA products obtained with primers specific for all 24 *SCN4A* exons.[15] The primer sequences used for exon 23, which encodes the first three segments of repeat IV of the channel protein, were 5′-CAACTCCTCCACATCCACTCC-3′ (forward) and 5′-GTGCAGGGGCAGGTGTGTCC-3′ (backward). Aberrant bands occurred on electrophoresis gels; corresponding PCR products were sequenced directly upstream and downstream by use of the dideoxy termination method with fluorescently tagged dideoxynucleoside triphosphates on a 373 DNA sequencer (Applied Biosystems, Foster City, CA). The pSELECT mutagenesis system (Promega Corporation, Madison, WI) was used for site-directed mutagenesis. The mutagenic oligonucleotide had the sequence 5′-GATGAGGATCACGATGGTGATGTC-3′ (antisense primer). Wild type (WT) and mutant cDNA were assembled in the mammalian expression vector pRC/CMV and transfected into human embryonic kidney cells (HEK293) using the calcium phosphate method. Stable cell lines were obtained by antibiotic selection[10] and used for patch-clamp measurements.

In vitro force measurements. From each muscle specimen, bundles of resealed muscle fiber segments were mounted in several experimental chambers with one end fixed and the other end fastened to a force transducer (Grass FT03, Quincy, MA). Supramaximal monopolar pulses of 0.1 msec duration were administered as single shocks or in 30-Hz trains of 300 msec duration. Stimulation and recording of the signals of the transducers were performed as described.[16]

Intracellular recordings. Superficial muscle fibers were used for the recording of action potentials with capacity-compensated microelectrodes. After the setting of a variable holding potential for more than 3 minutes, an action potential was elicited by a brief depolarizing current pulse. Recording was performed at 20 kHz sampling rate, and the two data points that were farthest apart during the upstroke were taken for the determination of the maximum rate of rise of the action potential.

Patch-clamp recordings. Standard whole-cell and single-channel patch-clamp recordings[17] were performed at room temperature (21 to 23 °C). Leakage- and capacity-corrected (−P/4) whole-cell sodium currents had amplitudes of 1.5 to 6 nA (60 to 80% series resistance compensation). They were filtered at 5 kHz, digitized at 20 kHz using pCLAMP (Axon Instruments, Foster City, CA), and analyzed using pClamp, Excel (Microsoft), and Sigma Plot software (Jandel Scientific, San Rafael, CA). The pipette solution contained (mM): 130 CsCl, 2 $MgCl_2$, 5 EGTA, and 10 Hepes (pH 7.4). The bathing solution contained (mM): 140 NaCl, 4 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 4 dextrose, and 5 Hepes (pH 7.4). The "high" potassium solution contained 9 mM KCl instead of 4. Single-channel currents were recorded in the cell-attached patch-clamp configuration using pipette and bath containing the inverse solutions. In CsCl-containing bathing solution, the resting potential was permanently at −10 to −15 mV.[11] Voltages in the cell-attached configuration are given in relation to the resting potential. To eliminate leakage and capacity transients we subtracted averaged and scaled records without channel activity. The number of channels present in the patch was determined by inspecting all traces and counting the maximum number of channels that opened simultaneously.[18] All data are shown as means ± SEM. Student's *t* test was applied for statistical evaluation.

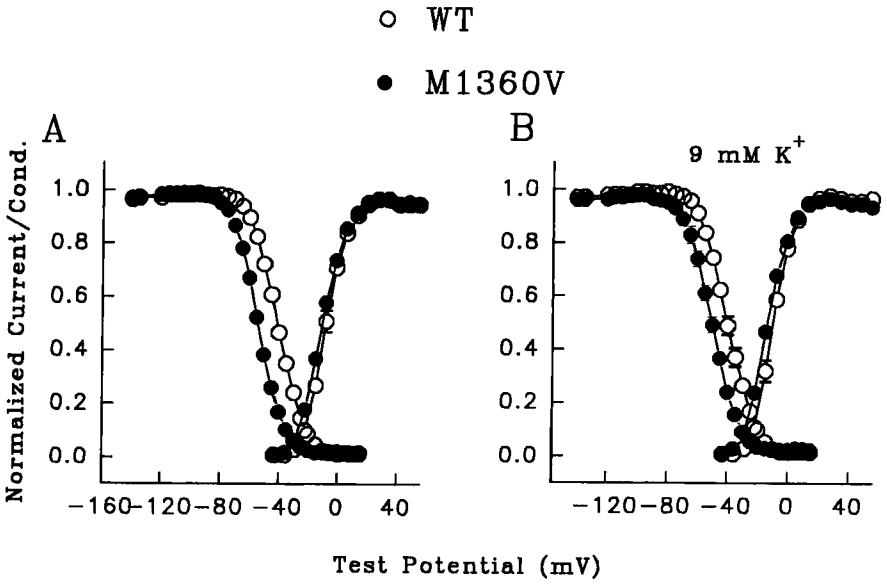

Figure 4. Steady-state activation and inactivation. Voltage dependence of activation and inactivation of M1360V (closed symbols) and WT channels (open symbols) for 4 mM (A) and 9 mM (B) external potassium fitted to standard Boltzmann functions (the values are given in the text, n = 7 to 12 patches).

Whole-cell recordings. The voltage dependence of steady-state activation was determined by step depolarizations to various test potentials from a prepotential of −120 mV (15 msec duration, holding potential −85 mV). Steady-state inactivation was determined by a constant test pulse to 0 mV following a variable prepotential of 15 msec duration (holding potential −85 mV). The corresponding conductance-voltage and current-voltage plots were fitted to standard Boltzmann functions, g/g_{max} or $I/I_{max} = 1/(1+\exp[-(V-V_{0.5})zF/RT])$, where $V_{0.5}$ is the potential for half-maximum activation/inactivation, and z, F, R, and T have the usual denomination.

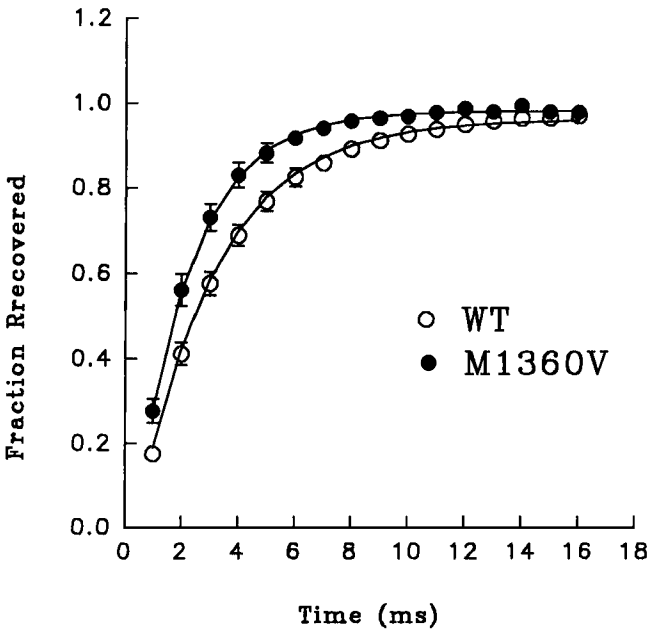

Figure 5. Recovery from inactivation. Sodium current amplitudes were measured at −100 mV following a variable latency after a 12 msec depolarization to 0 mV. The time constants of recovery were 2.0 ± 0.2 msec (n = 8 patches, closed symbols) for M1360V and 2.9 ± 0.2 msec (n = 10 patches, open symbols) for WT channels.

Results. *Case report.* A 56-year-old man reported attacks of weakness occurring about once per month since age 18 years. Such attacks usually took place in the early morning on awakening, and were particularly severe in the legs such that he was unable to walk upstairs. Occasionally, upper extremity weakness was also severe, preventing him from raising his arms. The severe attacks lasted a few hours, whereas slight weakness could last for days. In addition, attacks could be provoked by rest after strenuous exercise. Attacks are preceded by altered sense of taste and nervousness. Since the age of about 45 years, the frequency and the severity of the attacks have decreased, but not the duration. Sometimes the patient is able to prevent or abort attacks by continuing slight exercise and by the frequent ingestion of carbohydrates. After the performance of strenuous work he can immediately take acetazolamide tablets or, even better, inhale fenoterol, a β_2-adrenergic drug. Both drugs reduce severity and duration of the attacks of weakness.

On several clinical examinations the patient showed a distinct lid lag phenomenon and percussion myotonia. The EMG revealed myotonic runs in one of two arm muscles recorded. During rest following a 20-minute exercise at 150 W, maximum hand grip force was decreased to 50% for 15 minutes and recovered slowly during the next few hours. After ingestion of 80 mmol potassium, the leg muscles developed pronounced weakness while the serum potassium concentration increased from 4.6 to 7.3 mM. The patient reported difficulties in swallowing, and the muscle reflexes were clearly diminished.

Cooling of the forearm with cold water caused weakness of the exposed muscles, e.g., grip force decreased to 10% of the pretest value, and the compound action potential of the flexor digitorum superficialis muscle decreased from 7.0 to 2.6 mV. The decrease of force was associated with spontaneous EMG activity consisting of long-lasting series of fibrillation-like potentials that disappeared before the weakness had fully developed. Even after the forearm was quickly warmed, the weakness still persisted for about 10 hours.

No histologic or electron microscopic abnormalities were

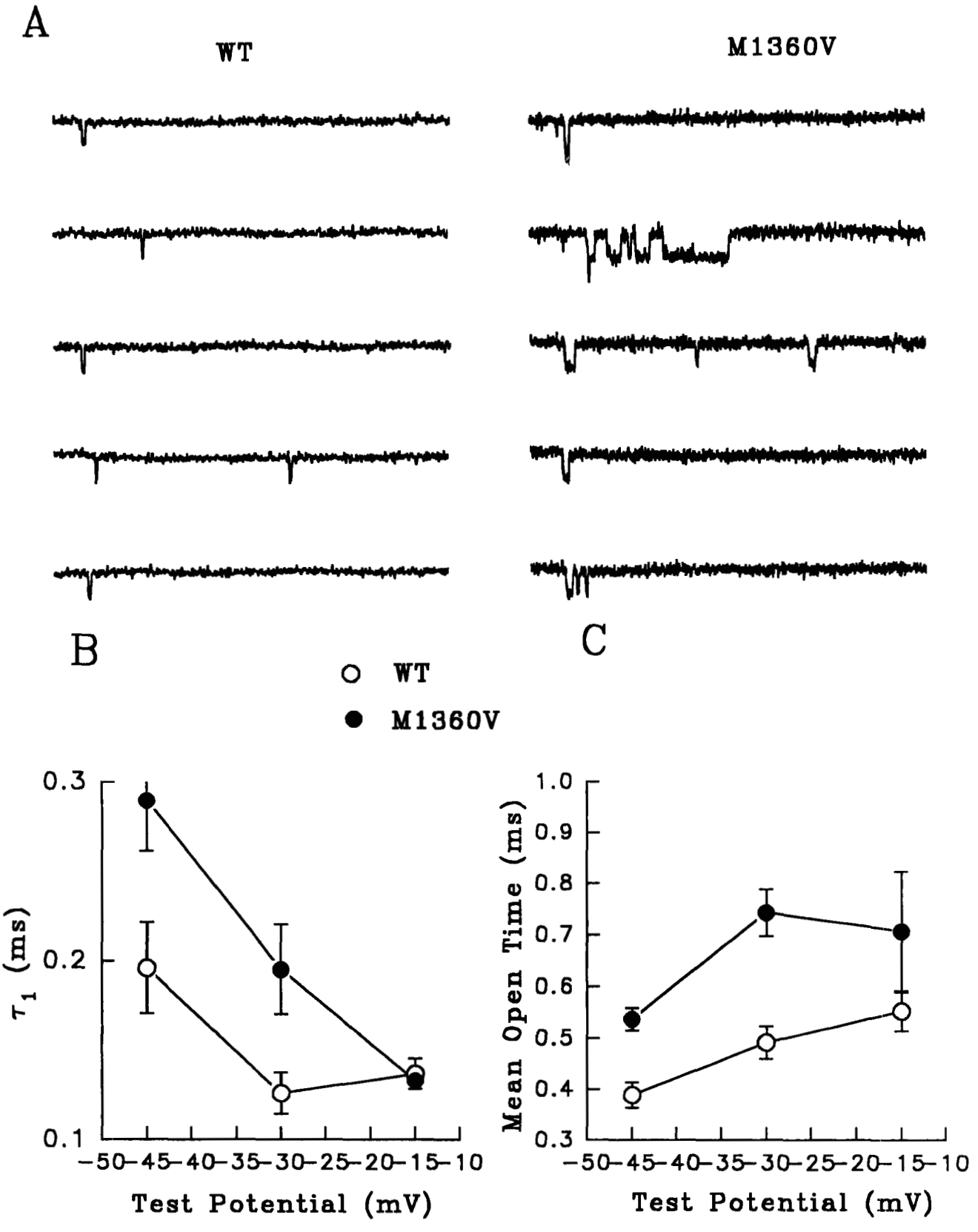

Figure 6. Single-channel recordings. (A) Current traces for M1360V and WT. Channel openings were elicited by step depolarizations from a holding potential of −100 to −30 mV. Reopenings were more frequent for mutant channels. (B) Fast open time constant, τ_1, versus test potential. The differences between mutant (closed symbols) and WT (open symbols) were statistically significant at −45 and −30 mV ($p < 0.01$, $n = 3$ to 8). (C) Mean open times calculated as arithmetic means versus test potential. Statistically significant differences between mutant (closed symbols).

detected in the biopsy specimen of the biceps brachii muscle.

None of the living family members ever experienced muscle stiffness, exercise- or cold-dependent muscle symptoms, or attacks of weakness (see pedigree, figure 1A). One of the patient's deceased uncles was reported to have suffered attacks of weakness. All living family members had neurologic examinations, and none showed clinical signs of myotonia. However, the mother and two maternal aunts, all carriers of the mutation (see below), revealed typical myotonic runs on EMG.

Molecular biology. Mutation screening of *SCN4A* revealed an aberrant DNA band in exon 23, and this was found with the index patient, his mother, and two maternal aunts (figure 1B). The band was absent in the DNA of an unaffected brother and of more than 200 normal individuals, and no aberrant bands were found by screening all other *SCN4A* exons. Sequencing revealed an A to G base exchange in nucleotide 4078 resulting in a valine for methionine substitution at amino acid position 1360. M1360 is located in the first segment of the fourth domain (figure 2A), and all voltage-gated sodium channels sequenced up to date showed this amino acid conserved (figure 2B). The base exchange was introduced into normal human cDNA of *SCN4A* and stably expressed in human embryonic kidney cells (HEK293) for patch-clamp measurements.

In vitro force measurements. Both in standard solution and at elevated potassium concentration (9 mM), the twitch amplitudes were constant as long as the muscle specimens were constantly stimulated at 1 Hz. Following a 10-minute period without stimulation, twitch force was reduced to about 50% when the specimens were exposed to "high" potassium or cooled to room temperature. During series of three brief 30-Hz trains of stimuli, force relaxation became increasingly slower, and the tetanic force increased with each successive response. This paradoxical myotonia was as reported for excised muscle specimens from typical PC patients.[19]

Intracellular recordings. In spite of the precautions taken in the operating theatre (see Methods), the resting membrane potentials were no more than about −40 mV when the fibers, kept in standard solution without tetrodotoxin (TTX), were first examined after preparation. A similar depolarization was observed with muscle fibers from PC patients when their muscle biopsy specimens had also been stored in a TTX-free solution.[5] Constant current applied through a second microelectrode was able to repolarize the fibers to various holding potentials so that action potentials could be stimulated. Maximum rate of rise (717 ± 141 V/sec, n = 7) and peak value (+13.4 ± 8.49 mV, n = 7) of the action potentials were then not different from control (800 ± 255 V/sec, n = 19; −11.3 ± 7.5 mV, n = 22). For a holding potential of −80 mV, the duration of the action potential (90% repolarization) was 1.70 ± 0.35 msec (n = 5) compared with 1.32 ± 0.95 msec (n = 21).

Whole-cell recordings. Sodium currents (figure 3A) were elicited by depolarizing the cells from a holding potential of −100 mV to test potentials ranging from −30 to +30 mV. The time course of inactivation was fit by a second order exponential function, $I(t)/I_{max} = A_1\exp(-t/\tau_{h1}) + A_2\exp(-t/\tau_{h2}) + I_{ss}/I_{peak}$, where τ_{h1} and τ_{h2} are the time constants of inactivation, A_1 and A_2 the relative amplitudes, and I_{ss}/I_{peak} the relative steady-state current amplitude. A significant difference between mutant and WT channels was observed for τ_{h1} (figure 3B), e.g., 0.98 ± 0.05 msec (n = 11) for M1360V versus 0.60 ± 0.05 msec (n = 11) for WT at 0 mV ($p < 0.001$), whereas τ_{h2} and the amplitudes were similar (A_1 0.93 for mutant and WT; and τ_{h2}: 4.1 ± 0.4 versus 3.5 ± 0.3 msec at 0 mV, n = 11). Furthermore the steady-state current, I_{ss}, was 1.5-fold increased, however not at a statistically significant level (1.5 ± 0.3 versus 1.0 ± 0.2% at 0 mV, n = 11, $p > 0.05$) (1.9 ± 0.2 versus 1.3 ± 0.2% at −15 mV, n = 6 to 11).

For M1360V channels the steady-state inactivation curve was shifted by 13 mV in the hyperpolarizing direction ($V_{0.5}$ −64 ± 1 mV and −51 ± 1 mV [n = 10] for M1360V versus WT, $p < 0.001$). Steady-state activation was not significantly different between mutant and WT ($V_{0.5}$ −17 ± 1 mV and −15 ± 1 mV [n = 9–12] for M1360V versus WT). All slopes were similar (z = 3.2 ± 0.1 versus 3.5 ± 0.1 [n = 9–12] for activation and z = −3.0 ± 0.1 versus −2.9 ± 0.1 [n = 10] for inactivation, figure 4A).

Because paralytic attacks are provoked by hyperkalemia, we examined the effects of elevated external potassium on sodium currents. However, an increase of the external potassium concentration to 9 mM did not yield any significant differences (figure 3, C and E, and figure 4B). In particular there was no further slowing of inactivation and no additional increase in the steady-state sodium current.

In order to study recovery from inactivation, cells were held at −100 mV, depolarized for 12 msec to 0 mV to inactivate all channels, repolarized to −100 mV for increasing durations, and again depolarized to the final test pulse of 0 mV. The time course of recovery from inactivation was fit to a second order exponential function (figure 5) with a very small relative amplitude of the second time constant (<5%), which therefore was neglected. Recovery was faster for mutant compared with WT channels: τ_{rec} = 1.98 ± 0.17 msec versus 2.87 ± 0.21 msec (n = 8 to 10) ($p < 0.01$).

Single-channel measurements. Slowing of the sodium current decay may be caused by prolonged channel openings, an increased frequency of reopenings, or by delayed openings (prolonged first latencies). Here, first latencies were normal for mutant channels (0.8 ± 0.1 versus 0.7 ± 0.1 msec for WT, n = 3–6); however, their open times were prolonged and reopenings occurred more frequently (figure 6A). Open time distributions were best fitted by a second order exponential yielding a fast and a slow component. Although the latter was similar for mutant and WT channels, the fast time constant, τ_1, was significantly greater for mutant channels ($p < 0.001$ at −45 and −30 mV, figure 6B). When the mean open times were calculated as arithmetic means, a significant difference was also observed ($p < 0.01$, figure 6C). Reopenings of mutant sodium channels, analyzed for the time interval of 30 to 85 msec after onset of the depolarization, i.e., when a steady state was reached, were more frequent for mutant channels (not shown). The resulting late current, normalized to the average peak current (I_{late}/I_{peak}), showed a statistically significant increase for a test potential for −15 mV (I_{late}/I_{peak} 1.2 ± 0.4 versus 0.2 ± 0.1%, n = 7, $p < 0.05$), whereas a large standard deviation for the mutant at −30 mV increased error probability (1.5 ± 0.7 versus 0.4 ± 0.1%, n = 8 to 12, $p > 0.05$). These values are comparable with

those some of us have found for other sodium channel mutations.[10,11,13,20]

Discussion. The A to G base exchange detected in nucleotide 4078 and resulting in a substitution of valine for methionine at amino acid position 1360 fulfills all criteria of a causal mutation such as (1) absence in a large number of controls (200 individuals with no known neuromuscular disease), (2) segregation with either the clinical or the EMG status, (3) presence in a gene primarily or exclusively expressed in the affected cells (the skeletal muscle), (4) location in a highly conserved gene region, and (5) gain of function (an increased sodium current) in a heterologous expression system. Moreover, the functional alterations can explain the pathophysiology of the disease.

Although sodium channelopathies are usually transmitted as a dominant trait with full penetrance, another mutation with reduced penetrance and normal EMG pattern in females has been reported (A1156T[21]). Thus, the low clinical expressivity in all female family members carrying the M1360V mutation was not too surprising, particularly since the affected phenotype was verified by typical myotonic bursts in the EMG. In families with chloride channel myotonia, females usually are less affected.[22] This similarity may indicate a dependence of muscle fiber excitability on sexual hormones or the expression of an additional membrane-stabilizing channel encoded only by the X chromosome, such as ClC-4 (for review see reference 23).

The common electrophysiologic feature of all sodium channel mutants causing either HyperPP, PC, or PAM is defective inactivation giving rise to an increased sodium influx. As a consequence, the muscle fibers depolarize, and myotonic activity or paralysis ensue. Several mechanisms of incomplete inactivation have been reported: for HyperPP, a persistent sodium current or an increased window sodium current, or both; for PC, pronounced slowing of inactivation in combination with accelerated recovery from inactivation or shifts in steady-state inactivation, or both; and for PAM, a combination of a persistent sodium current, slowing of inactivation, and a right shifted inactivation (for review, see reference 24).

In agreement with the low clinical expressivity, the inactivation of M1360V channels is less affected than in other mutant sodium channels that have been functionally studied, except for the A1156T mutation (see above) that also showed only mild inactivation defects.[9] From the electrophysiologic point of view, the M1360V mutation would fit with a PC phenotype. The presence of a persistent sodium current as well as the hyperpolarizing shift of the inactivation curve may explain the attacks of weakness occurring with this mutation. Since the potential dependency of the steady-state inactivation is shifted by more than 13 mV, particularly weakness might be caused by less sodium channels available for an action potential at a given resting potential and more sodium channels being in the inactivated state from which they can reopen. A similar overlap of PC and HyperPP or PAM on the clinical phenotype and the combination of inactivation defects has been reported for S804F[2,21] and A1156T.[21]

Direct influence of external potassium ions on the gating of homologously expressed mutant sodium channels has been reported;[25,26] however, this effect was never confirmed in more extended studies on heterologous expression systems.[6-8,10,11] In this study, we could neither detect any effect of an increased external potassium concentration. Nevertheless the paralytic effect of hyperkalemia in vivo can well be explained by the persistent sodium current associated with membrane depolarization that is compensated for under voltage clamp conditions.

M1360V is the first mutation in one of the four S1 segments of the sodium channel that has been functionally studied so far, including all engineered mutations. Our results indicate that this protein region of repeat IV is involved in channel inactivation; however, we can only speculate on the mechanism of the substituting valine. According to the commonly accepted model of the sodium channel protein structure, M1360 is located within a membrane spanning segment. The mechanism of how inactivation might be influenced could be an interaction of M1360 with the voltage sensor IV/S4 that is essential for proper inactivation.[8,13,27]

Acknowledgments

We thank Dr. K. Ricker for clinical and neurophysiologic examination of the family, Dr. R. Rüdel for his comments, Drs. D. Pongratz and G. Hübner for the morphologic examinations, and the family for their cooperation.

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