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HUMAN SODIUM CHANNEL MYOTONIA: SLOWED CHANNEL INACTIVATION DUE TO SUBSTITUTIONS FOR A GLYCINE WITHIN THE III–IV LINKER

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SUMMARY

1. Three families with a form of myotonia (muscle stiffness due to membrane hyperexcitability) clinically distinct from previously classified myotonias were examined. The severity of the disease greatly differed among the families.

2. Three dominant point mutations were discovered at the same nucleotide position of the *SCN4A* gene encoding the adult skeletal muscle Na^+ channel α-subunit. They predict the substitution of either glutamic acid, valine or alanine for glycine[1306], a highly conserved residue within the supposed inactivation gate. Additional *SCN4A* mutations were excluded.

3. Electrophysiological studies were performed on biopsied muscle specimens obtained for each mutation. Patch clamp recordings on sarcolemmal blebs revealed an increase in the time constant of fast Na^+ channel inactivation, τ_h, and in late channel openings as compared to normal controls. τ_h was increased from 1·2 to 1·6–2·1 ms and the average late currents from 0·4 to 1–6 % of the peak early current.

4. Intracellular recordings on resealed fibre segments revealed an abnormal tetrodotoxin-sensitive steady-state inward current, and repetitive action potentials. Since K^+ and Cl^- conductances were normal, only the increase in the number of non-inactivating Na^+ channels has to be responsible for the membrane hyperexcitability.

5. Length, ramification and charge of the side-chains of the substitutions correlated well with the Na^+ channel dysfunction and the severity of myotonia, with alanine as the most benign and glutamic acid as the substitution with a major steric effect.

6. Our electrophysiological and molecular genetic studies strongly suggest that these Na^+ channel mutations cause myotonia. The naturally occurring mutants allowed us to gain further insight into the mechanism of Na^+ channel inactivation.

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INTRODUCTION

The symptom myotonia is characterized by an uncontrollable muscle stiffness caused by muscle fibre membrane hyperexcitability. Since myotonia was found, by *in vitro* electrophysiological studies, to be caused by either Na^+ or Cl^- channel dysfunction, the group of hereditary non-dystrophic diseases characterized by myotonia can be divided into two major subgroups; the muscle Na^+ and the muscle Cl^- channel diseases. Hyperkalaemic periodic paralysis (HyperPP) and paramyotonia congenita (PC) have been shown to be caused by a non-inactivating Na^+ current (Lehmann-Horn, Rüdel & Ricker, 1987; Lehmann-Horn, Iaizzo, Hatt & Franke, 1991; Cannon & Strittmater, 1993; Cummins *et al.* 1993) whereas a reduced Cl^- conductance was found to be responsible for congenital myotonia (reviewed by Rüdel & Lehmann-Horn, 1985). Linkage of HyperPP to the *SCN4A* gene encoding the adult skeletal muscle Na^+ channel α-subunit was found (Fontaine *et al.* 1990). Subsequently, allelic mutations were discovered in HyperPP and PC within this gene (Rojas, Wang, Schwartz, Hoffman, Powell & Brown, 1991; McClatchey *et al.* 1992; Ptácek *et al.* 1993); in congenital myotonia the muscle Cl^- channel gene was found to be affected (Koch *et al.* 1992).

The aim of our study was to identify *SCN4A* mutations in patients with a putative third form of myotonia – besides HyperPP and PC – and to correlate these naturally occurring mutations with both the electrophysiological channel dysfunction and the clinical symptoms.

METHODS

Patients

PM1, this sporadic patient has been suffering from such a severe, permanent myotonia (PM) that, without medication, stiffness of her ventilatory muscles sometimes caused hypoxia and acidosis. The patient has been continuously taking the antimyotonic drug tocainide, 3 × 400 mg per day (Tocainid®, Astra, Wedel, Germany), for several years except for 3 days prior to muscle biopsy. EM1A and B, two members of a family have been affected with muscle stiffness aggravated by exercise (exercise-induced myotonia, EM); compared with PM1, the myotonia was milder and did not require medication. FM2, affected members of this family experienced muscle stiffness subject to daily fluctuations (fluctuant myotonia, FM) similar to the family reported by Ricker, Lehmann-Horn & Moxley (1990).

A common feature of these patients is a non-dystrophic, generalized myotonia. In contrast to HyperPP and PC, muscle weakness did not occur and muscle stiffness was not influenced by cold but aggravated by oral intake of K^+.

Molecular genetics

Genomic DNA was extracted from anticoagulated blood obtained with informed consent from patients, their relatives and normal controls. Samples of genomic DNA were amplified by the polymerase chain reaction (PCR) with primers specific for all exons (1–24) encoding the α-subunit of the Na^+ channel protein (George, Iyer, Kleinfield, Kallen & Barchi, 1993). The experimental conditions were optimized for each primer.

The DNA was analysed using single strand conformational polymorphism (SSCP) analysis. PCR products were diluted in 30 μl of a mixture containing 0·05% sodium dodecyl sulphate, 5 mM EDTA and then 7·5 μl of 95% formamide, 20 mM EDTA, 0·05% Bromophenol Blue and 0·05% xylene xyanol were added. A 6 μl aliquot of the products was denatured for 5 min at 95 °C and kept on ice until loading onto 5% polyacrylamide gels (200 × 200 × 1·0 mm, 45 mM Tris borate–1 mM EDTA buffer, pH 8·3). Gels were allowed to run for 4–6 h at 20–30 V cm^{-1} at 4 °C and were subsequently stained with 0·5 μg ml^{-1} ethidium bromide following the protocol of Yap & McGee (1992).

Single SSCP bands were cut directly from the gel under UV light and placed for 1 h in 100 μl of distilled water at 37 °C. A 10 μl aliquot was used for PCR amplification with 5′ extended universal primers as described by Ptácek *et al.* (1993). Amplified products were resolved on a 3% agarose gel and isolated from the gel with Qiaex (Diagen, Düsseldorf, Germany). Samples were sequenced with the dideoxy termination method using Taq polymerase, M13 universal and reverse sequencing primers, and fluorescently tagged dideoxynucleoside triphosphates on an 373A DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Muscle preparation

The four patients gave informed consent for a quadriceps muscle biopsy. Further probes were obtained from individuals who had undergone muscle biopsy for exclusion of malignant hyperthermia susceptibility; these probes served as controls if susceptibility was excluded. All muscle specimens were removed under local or regional anaesthesia and consisted of approximately 3 cm long fibre segments. All procedures were in accordance with the Helsinki convention and were approved by the Ethical Committee of the Technical University of Munich.

The standard solution (except for patch clamp recordings) contained (mM): NaCl, 108; KCl, 3·5; $CaCl_2$, 1·5; $MgSO_4$, 0·7; $NaHCO_3$, 26·2; NaH_2PO_4, 1·7; sodium gluconate, 9·6; glucose, 5·5; sucrose, 7·6; pH 7·4, 315 mosmol l^{-1}, at 37 °C. The Cl^--free solution was made by replacing Cl^- with methane sulphonate (MeS). Some solutions contained 1 μM TTX (Roth, Karlsruhe, Germany) or 100 μM tocainide.

For the determination of the steady-state relationship between membrane current density and membrane potential, resealed fibre segments (Lehmann-Horn *et al.* 1991) were impaled midway between the ends with three capacity-compensated microelectrodes. The voltage-clamp set-up and the experimental protocol were as described in Lehmann-Horn *et al.* (1987).

Patch-clamp experiments

Sarcolemmal blebs formed mechanically without enzyme treatment (Quasthoff, Franke, Hatt & Richter-Turtur, 1990) were used for patch-clamp recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) in the 'bleb attached' mode. The bathing solution contained (mM): CsMeS, 130; NaCl, 5; $CaCl_2$, 1; $MgCl_2$, 2; Hepes, 10; pH at 7·2; temperature was kept at 21 °C. The pipette solution contained (mM): sodium gluconate, 130; KCl, 3·5; $CaCl_2$, 1; $MgCl_2$, 2; Hepes, 10 at pH 7·4. Data were recorded by means of an EPC7 amplifier (List, Darmstadt, Germany), stored on a modified DAT recorder (48 kHz digitization rate, Sony) and evaluated off-line. They were low-pass filtered at 1·5 kHz, digitized at a rate of 10 kHz and analysed using pCLAMP (Axon Instruments, Foster City, CA, USA). The capacity transients were eliminated by subtracting averaged and scaled records without channel activity.

RESULTS

Molecular genetics

A difference in the mobility of single-stranded DNA fragments was found for each of our three patients in exon 22 encoding the cytoplasmic loop connecting repeats III and IV. Different aberrant bands were found for each family (Fig. 1*A*). No aberrant exon 22 bands were detected in all non-affected family members studied (n = 12) and seventy-six normal controls. No abnormal polymorphisms were found for all other exons (1–21, 23, 24).

By sequencing the DNA eluted from the aberrant bands, three different mutations at nucleotide position 3917 of the adult skeletal muscle Na^+ channel α-subunit cDNA were discovered: (i) a guanine-to-adenosine transition for PM1 resulting in a glutamic acid substitution for glycine; (ii) a previously described guanine-to-thymidine transversion for EM1A and B predicting a valine substitution (McClatchey *et al.* 1992) and (iii) a guanine-to-cytosine transversion for FM2 resulting in an alanine substitution (Fig. 1*B*). Glycine^{1306} is one of two adjacent glycines that are highly conserved in all Na^+ channels sequenced to date (Fig. 1*C*).

Patch-clamp measurements

Data were obtained in fifty-five bleb attached patches from eleven control muscles and in twenty nine bleb attached patches from our four patients' muscles. Na^+ currents in a patch activated by depolarizing voltage steps from a holding potential of −100 mV ranged from 0 to 60 pA. Figure 2*A* shows current traces from a patch of

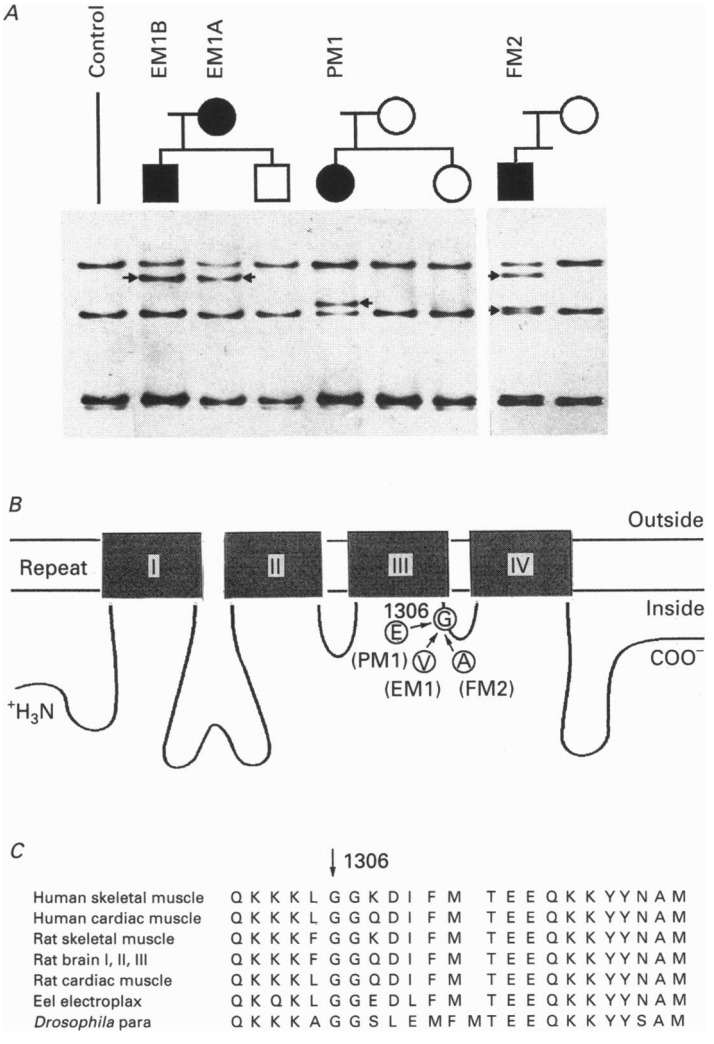

Fig. 1. Molecular genetics. *A*, polyacrylamide gel showing SSCP products for exon 22 (by use of primers 22F and 22R) which encodes the cytoplasmic loop connecting repeats III and IV of the muscle Na^+ channel α-subunit. Patients PM1, EM1A and B and FM2 revealed aberrant bands (arrows) in contrast to non-affected family members and normal controls (shown are a few examples). Note that the position of the aberrant bands is different in each family. *B* presents a model of the Na^+ channel α-subunit. Glycine^{1306} and its different substitutions are located in exon 22. *C*, amino acid sequences of a part of exon 22 from various species (modified after McClatchey *et al.* 1992). Glycine^{1306} is completely conserved among all Na^+ channel α-subunits.

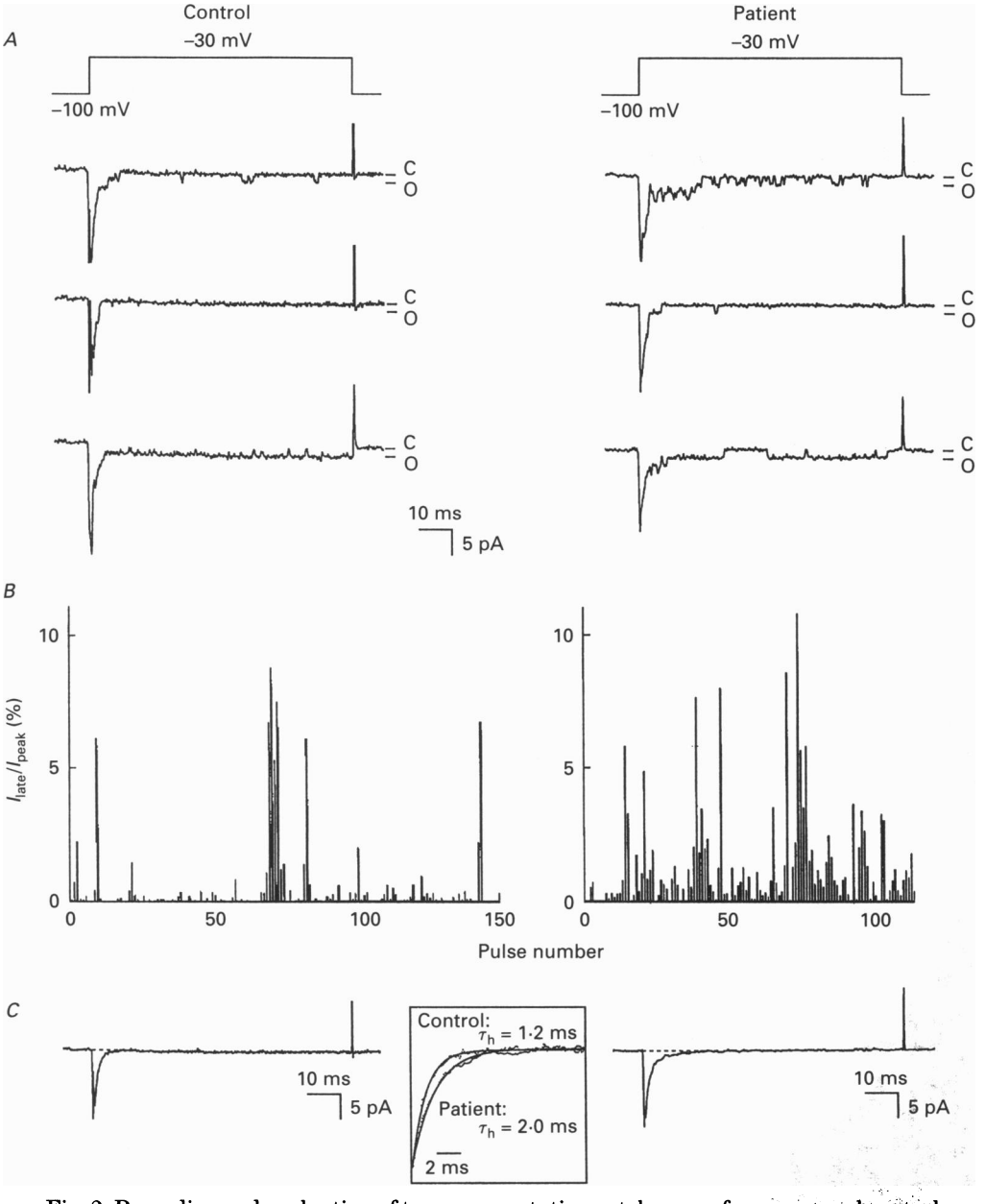

Fig. 2. Recording and evaluation of two representative patches, one from a normal control and one from patient EM1B. *A*, original current traces from Na^+ channel openings, elicited by depolarization steps from −100 to −30 mV. Low-pass filter was set at 1·5 kHz (C, closed; O, open channel). *B*, the current induced by late channel openings beginning 15 ms after the onset of the depolarization was calculated for each of the up to 150 consecutive depolarization steps (1 s^{-1}) using idealization with a 50 % amplitude criterion. The data were then normalized to the average peak early current shown in *C*. The mean values are 0·5 % (control) and 1·2 % (patient) respectively. *C*, reconstructed 'macroscopic' currents show slowing of the decay of the peak early current. Monoexponential fits superimpose the decay (inset). The late channel openings are too rare to produce a 'macroscopic' late current visible in the averaged records.

a control muscle (*A*) in comparison to those of patient EM1B. Following the decay of the peak current carried by early Na^+ channel openings, late channel openings were recorded which occurred either as short single events, as seen in the first traces, or in bursts as in the third traces of Fig. 2*A* and *B*.

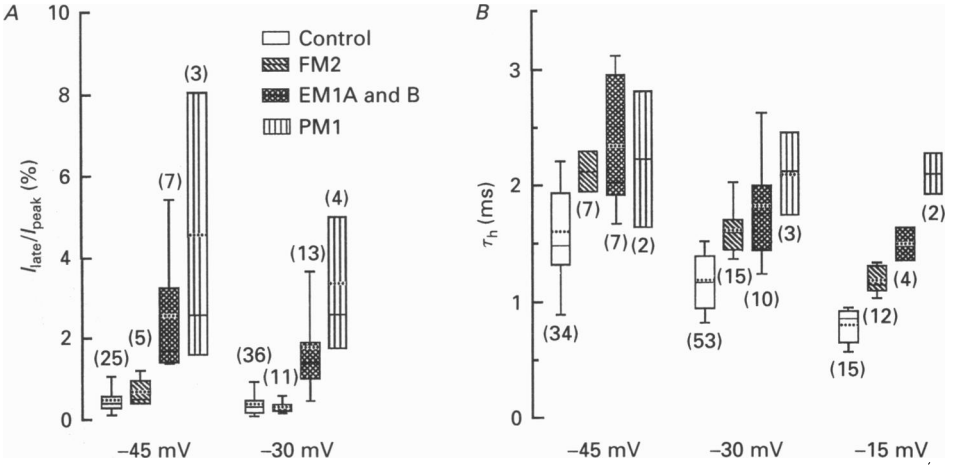

Fig. 3. Box plots of all evaluated averages of I_{late}/I_{peak} (*A*) and τ_h values (*B*) for several voltage steps. The boxes represent the 50th, 25th and 75th percentiles, the error bars the 10th and 90th percentiles; means are plotted as dotted horizontal lines, and numbers of patches are given in parentheses. All values of the patients differed significantly from those of the controls (*t* test with $\alpha = 0{\cdot}05$), except the two for τ_h at −45 mV of PM1 and the values for I_{late}/I_{peak} of FM2.

Late Na^+ currents were calculated for each pulse and normalized with respect to the average peak early current (I_{late}/I_{peak}, Fig. 2*B*). In these plots the higher peaks correspond to a burst of late openings, whereas the lower peaks represent a correspondingly high number of short single events of late openings. Means of I_{late}/I_{peak}, taken 15 ms after onset of depolarization to the end of the steps (50–180 per patch), were significantly increased for all patients (*t* test with $\alpha = 0{\cdot}05$), except for FM2, when compared to controls, e.g. at −30 mV, the means for all patches were 0·4 % for controls, 3·5 % for PM1, 1·8 % for EM1A and B and 0·4 % for FM2. The data for −30 and −45 mV are given in detail in Fig. 3*A*.

The time constants, τ_h, for the fast inactivation of the 'macroscopic' Na^+ currents were deduced from the averages of thirty to one hundred depolarization steps per patch. As in Fig. 2*C*, the patient's Na^+ currents inactivate significantly more slowly than those in control muscles (*t* test with $\alpha = 0{\cdot}05$). Means for all τ_h values at −30 mV are 1·2 ms for normal controls, 2·1 ms for PM1, 1·8 ms for EM1A and B and 1·6 ms for FM2. All values are listed in Fig. 3*B*.

Single-channel conductance determined by evaluation of the late Na^+ channel openings was the same for control and myotonic muscles (15 pS). When 100 nM TTX was added to the pipette solution no channel activity was recorded.

Intracellular microelectrode measurements

In muscle preparations from patients EM1B and FM2, fibres had membrane potentials either at approximately −80 mV or between −65 and −55 mV. When fibres of −80 mV were impaled, long lasting runs of repetitive action potentials occurred immediately or after a delay (Fig. 4*A*). Twitching ceased at the lower potential range (−65 to −55 mV, right panel of Fig. 4*A*), at which the fibres were

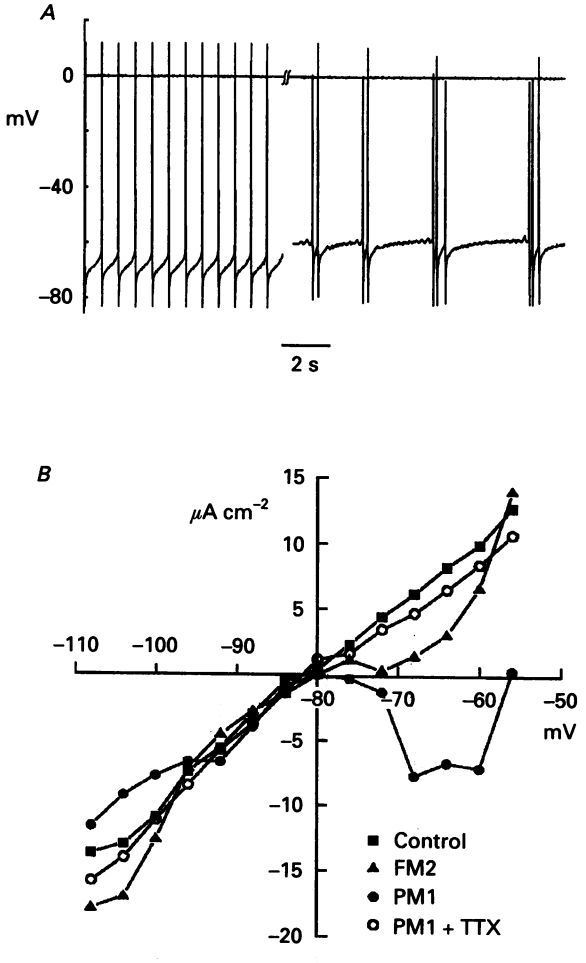

Fig. 4. *A*, intracellular recording of a spontaneous train of action potentials from a resealed fibre segment of patient EM1B. In the left panel discharges of the train are shown measured at 37 °C, 10 min after the recording microelectrode was impaled at a resting potential of −82 mV. The train lasted half an hour and ceased at −64 mV revealing doublets and triplets at the end. *B*, steady-state current–voltage relationships of resealed fibre segments from patients PM1 and FM2 determined in standard solution without (FM2, 7 fibres; PM1, 4 fibres) and with TTX (PM1, 8 fibres). Note the abnormal inward current of the fibres, in particular for PM1, at voltages less negative than −75 mV and its abolition by TTX. For comparison, the current–voltage relationship from normal controls (15 fibres) is given.

not spontaneously active but still excitable. In a muscle bundle from PM1, resting membrane potentials were approximately −80 mV in TTX-containing solution (1 μM). After wash-out of TTX, almost all fibres started to twitch continuously for several minutes and the resting potential dropped to approximately −60 mV.

The steady-state current–voltage relationship was determined in fibres from patients PM1 and FM2. This relationship differed from controls by the presence of an abnormal TTX-sensitive inward current at voltages less negative than −75 mV (Fig. 4*B*). In other words, TTX normalized the *I–V* relationship. Whereas, for FM2, I_{late}/I_{peak} was not clearly increased in the potential range investigated, a macroscopic inward current was detected in the *I–V* relationship.

The K^+ conductance, measured in TTX-containing Cl^--free bathing solution, was approximately 50 μS cm^{-2} and the Cl^- conductance, calculated as the difference between the conductance values in TTX-containing solutions with and without Cl^-, was about three-quarters of the total conductance, i.e. both were normal.

Mechanography

Muscle bundles obtained from patient PM1 showed intense spontaneous twitches at 37 °C and to a lesser extent at 27 °C. The activity was enhanced by increasing the K^+ concentration in the bath to 7 mM. Addition of 100 μM tocainide lowered the activity. Similar responses were found for muscle bundles from EM1A and B and FM2 but the spontaneous activity and the K^+ effects were markedly smaller than for PM1.

DISCUSSION

Native skeletal muscle preparations of human 'mutants' have allowed us to correlate molecular genetic data, electrophysiological data at the single-channel and cellular level, and clinical data. Our results link the substitutions for glycine[1306] clearly to the phenotype and further suggest that glycine[1306] is an important site for Na^+ channel inactivation. The electrophysiological results are complementary to the report that the substitution of glutamine for a phenylalanine in the same region of the rat Na^+ channel (corresponding to codon 1311, cf. Fig. 1*C*) removes fast Na^+ channel inactivation nearly completely (West, Patton, Scheuer, Wang, Goldin & Catterall, 1992). As our clinical data demonstrate, the substitution of glutamic acid for glycine[1306] constitutes a severe disease although its effects on channel inactivation are minimal when compared to the substitution of glutamine for phenylalanine. Replacement of glycine with valine or alanine has minor effects.

Pathogenesis of myotonia

The shift in the inactivation pattern towards an increase in both the fast inactivation time constant and the more frequent occurrence of late Na^+ channel openings (I_{late}/I_{peak} increased from 0·4 to up to 6%) can explain the pathological steady-state inward current (see Fig. 4*B*), the sustained membrane depolarization and the muscle fibre hyperexcitability. This is supported by observations made in anemone toxin-treated rat muscle. In this experimental preparation, spontaneous

activity was produced when only 2% of Na^+ channels failed to inactivate (Cannon & Corey, 1992) corresponding to I_{late}/I_{peak} of 5% (40% of the channels were activated during the peak early current in this study).

Genotype–phenotype correlations

In earlier reports on PC patients with cold-induced weakness and HyperPP patients with K^+-induced weakness, repetitive action potentials ceased at a membrane potential (about −40 mV) at which the fibres were inexcitable (Lehmann-Horn *et al.* 1987, 1991). Such patients carry substitutions in the S4 segment of repeat IV (PC) and in S5/II or S6/IV (HyperPP) respectively (Rojas *et al.* 1991; Ptácek *et al.* 1993; authors' unpublished observations). In contrast, spontaneous activity of fibre segments from the patients studied here ceased at −60 mV and fibres were still excitable. The patients never experienced weakness. Because of these electrophysiological differences and the clinical deviations from HyperPP and PC (see Methods), we suggest that the patients reported here belong to a third type of Na^+ channel diseases; the descriptive terms 'fluctuating' (Ricker *et al.* 1990), 'exercise-induced' and 'permanent' myotonia may be appropriate in regard to the different degree of the disease corresponding also to the different side-chain properties (length, ramification and charge) of the substitutions for glycine^{1306}.

McClatchey *et al.* (1992) found one of the three substitutions at location 1306 (glycine-to-valine) in a family characterized by 'chronic myotonia' who reported no temperature dependence and in a second family with cold-induced myotonia; neither have experienced weakness.

Evidence that the substitutions cause the disease

Including the families reported by both McClatchey *et al.* (1992) and us, five families carry base changes at the same nucleotide position 3917 predicting substitutions for glycine^{1306}. All affected family members carry a mutation at this and no other site, whereas for all non-affected family members and a large number of normal controls from our laboratory and another (McClatchey *et al.* 1992) a mutation at this site was excluded.

Further evidence for the importance of glycine^{1306} is its preservation in all Na^+ channels sequenced up to date (some of them shown in Fig. 1*C*) and its location in a functionally important protein region. West *et al.* (1992) proposed a 'hinged-lid model' for Na^+ channel inactivation in which glycines$^{1306/7}$ act as the hinge of the lid occluding the channel pore. The good correlation of side-chain properties of the substitutions for glycine^{1306} (no side-chain) with the severity of myotonia *in vivo* and *in vitro* supports the hypothesis that the two adjacent glycines confer a high flexibility of the hinge that could be restricted by side-chains. A possible hindrance of flexibility by the valine substitution has already been suggested by McClatchey *et al.* (1992).

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