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A novel SCN4A mutation causing myotonia aggravated by cold and potassium

Roland Heine; Ursula Pika and Frank Lehmann-Horn*

Department of Applied Physiology, University of Ulm, D-89069 Ulm, Germany

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The single strand conformation polymorphism (SSCP) technique was used to screen genomic DNA of a family with myotonia aggravated by cold, potassium loading and suxamethonium, but without muscle weakness. An aberrant band was found in exon 24 of SCN4A, the gene encoding the adult skeletal muscle sodium channel α-subunit. DNA sequencing led to the detection of a G-to-A transition of cDNA nucleotide 4765 predicting a substitution of methionine for valine at position 1589 of the protein sequence. This amino acid is located within transmembrane segment S6 of channel repeat IV close to the cytoplasmic surface, a region which is supposed to act as acceptor of the inactivation gate of the channel. Four lines of evidence indicate that this mutation causes the disease: (i) the transition was only found for affected family members; (ii) no mutations were found in all other SCN4A exons; (iii) the affected gene region is conserved among various species; and (iv) an increase in the number of non-inactivating sodium channels had been revealed in earlier electrophysiological studies on an excised muscle specimen from the index patient. In addition, the close-by occurring substitution of valine for methionine at position 1592 known to cause hyperkalemic periodic paralysis was deduced for six families with the myotonic, non-dystrophic form of this disease.

INTRODUCTION

Different mutations in SCN4A, the gene encoding the adult skeletal muscle sodium channel α-subunit have been shown to cause the human hereditary disorders hyperkalemic periodic paralysis and paramyotonia congenita.[1–8] As a consequence, the long-standing question as to whether these two diseases are directly related was finally settled: thus the term 'sodium channel disease'[7] was introduced to encompass the different allelic syndromes caused by SCN4A gene mutations.

According to this definition, muscle sodium channel diseases encompass: i) classical paramyotonia congenita (PC) as characterized by the key symptoms of paradoxical myotonia (muscle stiffness aggravated by exercise) and cold-induced muscle stiffness followed by weakness,[9,10] and its variants such as: PC with myotonic signs present even in a warm environment; PC without weakness, not even during strenuous exertion of the cooled muscles; and PC with spontaneous and potassium-induced attacks of weakness; ii) hyperkalemic periodic paralysis (HyperPP) characterized by the symptoms of spontaneous and potassium-induced attacks of weakness[11] and associated with or without myotonia or paramyotoni.[12] The relationship between these diseases and another condition characterized by muscle stiffness responsive to acetazolamide,[13] a potassium-lowering drug, has not been clarified.

We recently classified a third group of muscle sodium channel diseases, namely sodium channel myotonia.[8,14] This disease is characterized by a dominant mode of inheritance, and has as its key symptom muscle stiffness without muscle weakness. Moreover, in the families examined the stiffness was not substantially aggravated by cold. In some of them myotonia is mild and considerably variable from day to day (myotonia fluctuans[15,8]). In other families the myotonia is very severe and continually present (permanent myotonia[8]).

The molecular biological findings of different defects in the gene encoding the muscle sodium channel had been anticipated by electrophysiological studies on excised muscle specimens from such patients. Experiments on native muscle fiber segments performed with the three-microelectrode voltage clamp and, later, also with patch-clamp methods have revealed that the sodium currents underlying the repetitive firing of muscle fiber action potentials show a peculiar failure to inactivate properly.[8,16–18]

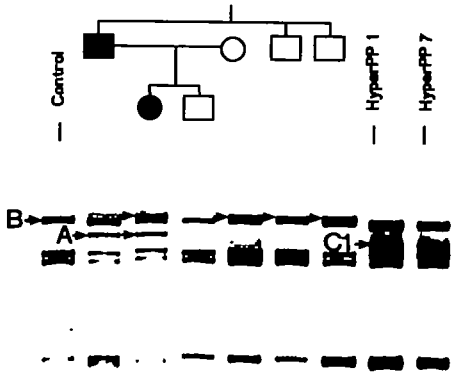
In the present study we investigate the genetic defect in a family with dominant myotonia for which an earlier electrophysiologic study had revealed abnormal sodium currents.[19] Based on the dominant inheritance and the clinical signs we had then misdiagnosed the disease as myotonia congenita (Thomsen), a disease now known to be caused by a defect in the muscle chloride channel.[20] Now we are able to show that the symptoms are caused by a previously unidentified mutation in SCN4A, and thus provide further evidence for the existence of sodium channel myotonia. Some of the data were presented at the 17th ENMC Workshop on 'Non-dystrophic myotonias and periodic paralyses'.[7]

* To whom correspondence should be addressed

RESULTS

Abnormalities in the mobility of single-strand DNA fragments of the SCN4A transcript were found for several members of family SCM8. All abnormalities were detected in the region of exon 24 which encodes the transmembrane segment S6 of repeat IV. For the affected family members, the index patient and his affected daughter, a peculiar aberrant band (A in Figure 1) was discovered which was not present in the DNA of the parents, the brothers and the son of the index patient. A second aberrant band was found for the daughter (Figure 1, band B). This band was also found for her mother and several other non-affected family members and also for a normal control. In the same exon, an aberrant band different from the two former ones was detected in six out of 25 HyperPP families (two examples in Figure 1, band C). No exon 24 abnormalities were detected for 76 normal controls. For the index patient and his daughter, no abnormal polymorphisms were detected in exons 1–23. For some normal controls, aberrant bands were found in exons previously described and, in addition, in exons 3 and 10 (not shown).

By sequencing the DNA eluted from the aberrant band (A) which was found for the index patient and his daughter, a G-to-A transition at position 4765 of the adult skeletal muscle sodium channel α-subunit cDNA was discovered, predicting a substitution of methionine for valine-1589 (Figure 2); no mutation was found in exons 1–23 of the genomic DNA from the index patient. The aberrant band (B) found for the index patient's daughter and her mother was caused by a C/A polymorphism at cDNA nucleotide 4536 which has no effect on the amino acid. For the affected members of the six families with HyperPP, a previously described A-to-G transition at position 4774 was detected, predicting the substitution of valine for methionine-1592 (Figure 1; Rojas *et al.* 1991). All non-affected family members revealed neither the val-to-met nor the met-to-val substitution (data not shown). Valine-1589 as well as methionine-1592 are highly conserved in all sodium channels sequenced to date (Figure 3). The aberrant bands in exons 3 and 10 were caused by polymorphisms: 403 A/C (135 val/met) and 1570 G/A (524 gly/ser).



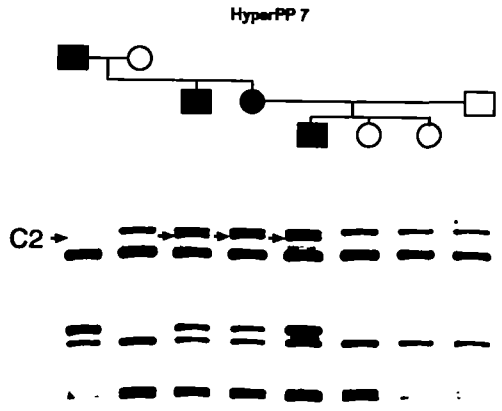
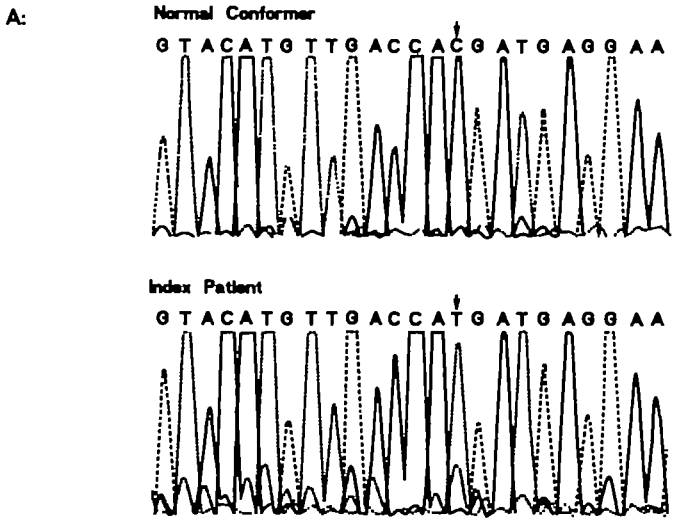


Figure 1. Polyacrylamide gels showing single strand conformational polymorphisms for the fragment of exon 24 of the muscle sodium channel α-subunit gene (SCN4A) which encodes the transmembrane segment S6 of channel repeat IV. Aberrant bands were found for the index patient (band A) and his affected daughter (bands A, B), her non-affected mother and several other non-affected members (open symbols) of family SCM8 and a normal control (band B); no aberrant bands were detected for the index patient's parents (not shown since paternity and maternity were not tested without agreement). In the same SCN4A fragment, another aberrant band (C1) different from A and B was detected in the affected members of families with the myotonic form of HyperPP: shown are the bands for index patients of families HyperPP1 and HyperPP7. For the latter, family pedigree and polymorphisms of several affected and non-affected family members are shown in the lower panel. Here, the aberrant band (C2) is different from C1 since the PCR products were made smaller by digestion (for details see Methods section).



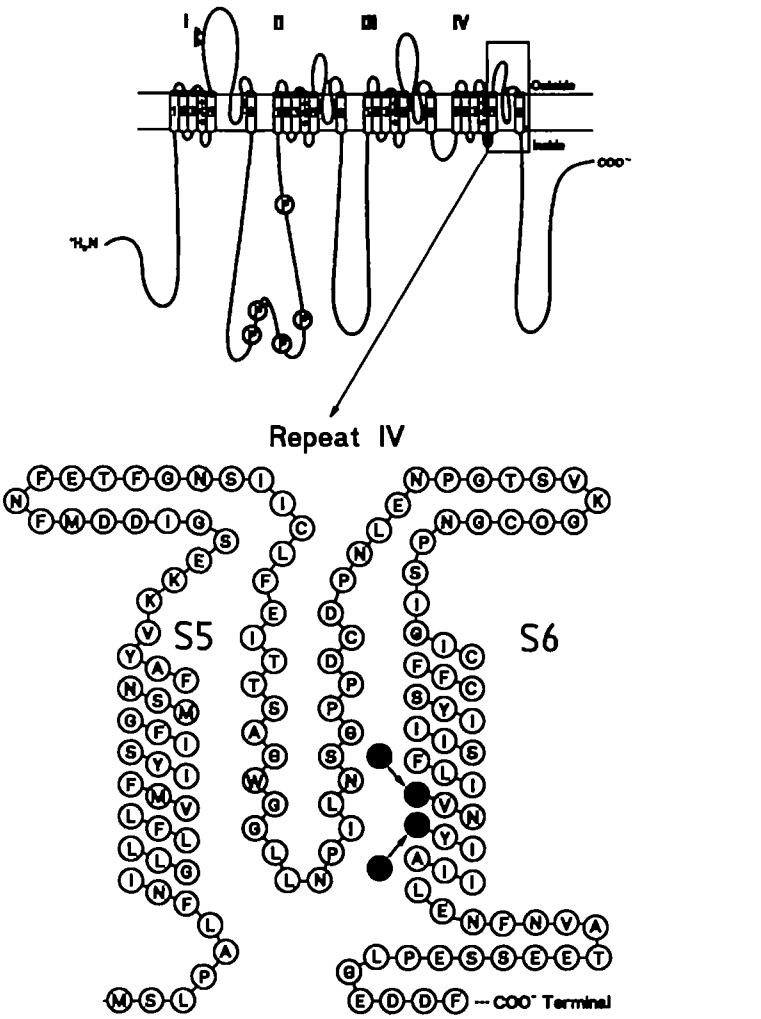
B:

Normal Conformer :	Phe TTC	Leu CTC	Ile ATC	Val GTG	Val GTC	Asn AAC	Met ATG	Tyr TAC
Index Patient :	TTC Phe	CTC Leu	ATC Ile	ATG Met	GTC Val	AAC Asn	ATG Met	TAC Tyr

Figure 2. A. A comparison of the wild-type and the mutant SCN4A sequence of cDNA nucleotides 4756 to 4779 is shown.30 The C to T base exchange (arrows) found in the antisense DNA sequence of the index patient of family SCM8 corresponds to a G to A transition in the sense DNA sequence (not shown). B. The mutation predicts a substitution of methionine for valine at position 1589.

DISCUSSION

The novel mutation within the gene encoding the muscle sodium channel α subunit is present in two related patients with myotonia markedly aggravated in the cold, after oral intake of potassium or administration of suxamethonium. Muscle weakness did not occur, even under provocative test conditions that induce weakness in congenital paramyotonia patients. In the past, patients showing such symptoms were therefore not classified as having PC but a special form of 'myotonia congenita with cold-sensitivity', by Becker[12]. Accordingly, this was the diagnosis when the family in question was first studied[19]. Meanwhile, it has been shown by molecular genetics that myotonia congenita is a chloride channel disease.[20–22] On the other hand, the results of the present study corroborate our earlier postulate for the existence of a 'sodium channel myotonia' as third group of muscle



	1589	1592
Human adult skeletal muscle	S Y I I I S F L i V	V N M Y I A i I L E
Human fetal/cardiac muscle	t Y I I I S F L i V	V N M Y I A i I L E
Rat adult skeletal muscle	S Y I I I S F L i V	V N M Y I A i I L E
Rat fetal/cardiac muscle	t Y I I I S F L i V	V N M Y I A i I L E
Rat brain type 1-3	S Y I I I S F L v V	V N M Y I A v I L E
Electroplax Eel	S Y I I I S F L v V	V N M Y I A i I L E
Drosophila para	S Y I v I S F L i V	I N M Y I A v I L E
Drosophila DSC1	S f I I I S y m i V	I N M Y I A i I L E

Figure 3. A. Schematic of the sodium channel α-subunit consisting of four regions of internal homology, so-called repeats, connected by intracellular loops. Each repeat contains six hydrophobic segments (S1 to S6), putative transmembrane helices. Between segments S5 and S6 of each repeat, an interlinker is found consisting of an extracellular part and a sequence which dips into the membrane. The four intra-membrane loops are thought to form the lining of the channel pore. The orifice on the intracellular side of the pore or its surrounding protein parts are supposed to act as acceptor of the inactivation gate. **B.** Amino acid sequence of segments S5–S6 of repeat IV: the substitution of methionine for valine predicted for the affected members of family SCM8 is close to the substitution of valine for methionine-1592 causing myotonic HyperPP. **C.** Alignment of predicted sodium channel sequence from various species over a part of IV-S6 (modified after[1,31]).

sodium channel diseases.[8,14] Sodium channel myotonia seems now to become a larger group of allelic syndromes which have in common a dominant mode of inheritance, and as key symptom muscle stiffness aggravated by oral potassium load but without muscle weakness. In contrast to the affected members of the family reported here, the families with the various substitutes for glycine-1306 did not reveal substantial aggravation of their myotonia in the cold.[8,14] The condition characterized by muscle stiffness responsive to acetazolamide may also belong to this group since linkage to the SCN4A gene has been reported.[13]

Obviously, the symptoms of sodium channel myotonia overlap considerably with those of chloride channel myotonia, and for an unexperienced examiner clinical differentiation may be impossible. We expect that more diagnoses of 'dominant myotonia congenita' will have to be converted to sodium channel myotonia after molecular biological investigations similar to the present study have been performed.

The symptoms of sodium channel myotonia also overlap with those of PC. For all PC families that were studied with molecular biological methods, point mutations were detected in distinct regions of SCN4A: (i) a C3938T transition causing a substitution of threonine for methionine-1313 was discovered in the gene fragment encoding the cytoplasmic loop between repeats III and IV, the putative inactivation gate;[4,23] (ii) two mutations at the adjacent nucleotide sites 4342/4343 were detected in a gene region encoding the transmembrane segment S4 of repeat IV;[3] (iii) another point mutation was discovered in the transmembrane segment S3 of repeat IV. The transversion T4298G predicts the amino acid substitute arginine for leucine at position 1433 located in segment S3 of repeat IV.[6]

The novel G4765A transition causing sodium channel myotonia predicts the substitution of methionine for valine-1589 in segment S6 of repeat IV. It is located right next to the helical position of the inverse substitution for methionine-1592 that causes hyperkalemic periodic paralysis.[1] Both substitutions have in common i) that they affect highly conserved amino acids, ii) that the amino acids both are close to the cytoplasmic surface and iii) that both induce an increase in the number of non-inactivating sodium channels.[19,24,25] This result indicates that this region of the channel is important for normal fast inactivation of the sodium channel and lends support to the view that it is this region that acts as the acceptor of the inactivation gate.[26] Not surprisingly, both mutations are associated with potassium sensitivity (potassium-induced weakness in the met-to-val substitution; potassium-induced stiffness in the val-to-met substitution). The difference between the two phenotypes, weakness versus stiffness, could be due to a different degree of sustained membrane depolarization resulting from the disturbed sodium channel inactivation: slight sustained depolarization to potentials at which the muscle fiber is still hyperexcitable (stiffness); or larger sustained depolarization to values at which most of the genetically normal sodium channels are already inactivated and the membrane is therefore inexcitable (weakness).[8] The second difference pertains to the temperature sensitivity which is only found for the mutation at the 1589 location.

It must be admitted, however, that the clinical phenotypes of sodium channel myotonia and hyperkalemic periodic paralysis are so different that we must discuss the possibility that the described mutation is not responsible for the disease. In particular the fact that several SCN4A polymorphisms have been detected makes this alternative possible: including the two polymorphisms discovered in this study, eight polymorphisms causing 'benign' amino acid substitutions are now known (reviewed by[14]). On the other hand, several arguments support our contention that it is the described point mutation that causes the disease: (i) the G4765A transition was only found in the DNA of the two affected family members; (ii) no other SCN4A mutation was detected for these two patients; (iii) the affected gene region is highly conserved; and (iv) the mutation is associated with disturbed sodium channel inactivation as reported earlier from electrophysiological studies performed on an excised muscle specimen obtained from the index patient of this family.[19]

MATERIALS AND METHODS

Patients

The family pedigree is shown in Figure 1 (SCM8). In a study performed at a time when SCN4A had not yet been located, the family was diagnosed as having autosomal dominant myotonia congenita with aggravation of myotonia in cold environment and by potassium load.[19] The index patient (who had given informed consent for a thorough clinical and genetic investigation as well as for a vastus medialis muscle biopsy) was consequently labeled as myotonia congenita patient 2 (MyC2). This diagnosis is now corrected to muscle sodium channel myotonia (SCM) aggravated in cold, by potassium and suxamethonium.

History: In 1986, the index patient (then at the age of 32 years) had a generalized myotonic reaction and bronchial spasms after initiation of general anaesthesia with thiopental and suxamethonium (100 mg; the drug is known to aggravate myotonic reaction).

Clinical findings: At room temperature, the elbow flexor and the elbow and knee extensor muscles were able to produce normal force, but the knee flexor muscles showed initial weakness that gradually disappeared with repetitive activity. The lid lag phenomenon was present and aggravated by repetitive closure and opening of the eyes. Percussion myotonia was elicitable in almost all muscles. Grip myotonia was present, slightly after rest and becoming better with repetitive movements (warm-up phenomenon), but increased after strenuous work. Muscle mass was normal except for a slight atrophy of the right pectoralis major muscle, perhaps as part of a C-7 syndrome. Myotonic cataract was excluded.

Additional findings: At room temperature, the EMG of the right biceps brachii revealed myotonic runs. A CT scan showed normal thigh and leg muscles. Creatine kinase (CK) was always elevated to approximately 200 U/l. The vastus medialis muscle showed focal fiber type I atrophy. The presence of prolonged CTG repeats within the myotonic dystrophy gene was excluded. Abnormal single strand conformational polymorphisms were not found in the fragments of the muscle chloride channel gene which primer sequences are available for.[20]

Provocative tests: Cooling of the forearm muscles in water of 15°C for 45 minutes aggravated the stiffness to a degree that the patient had never experienced before. When the muscles were cooled, the stiffness became worse with continued exercise, but muscle weakness did not occur even when the exertion was strenuous. Also, the muscles became very stiff 30 minutes after oral potassium loading (80 mmol). Because of this extreme muscle stiffness the patient was then unable to rise and walk.

Family history: In 1987, the patient's daughter (then at the age of 5 years) was found one night cyanotic and with severe spasms of the facial, temporalis and masseter muscles. In 1988, she had a severe myotonic reaction after initiation of general anaesthesia with thiopental and suxamethonium. Since the age of 1 year she has had clinical and electrical myotonia and a persistently elevated CK of approximately 300 U/l. All other family members (including the parents and the older and younger brothers and sisters of the index patient) are not affected.

In addition, six HyperPP families with a met-to-val mutation at position 1592 were included in this study (some of them were reported before molecular genetic data have been available): HyperPP1 (case 1[27]), HyperPP3,[16] HyperPP7, HyperPP17 (Kunath et al., unpublished results), HyperPP23, and HyperPP25. They allowed us to compare their single strand conformational polymorphisms with those of the affected members of family SCM8 which were detected in the same gene fragment (Figure 1). All affected members of the six families (age of years: 4 to 72) presented with the myotonic form of HyperPP. None revealed permanent weakness.

Molecular genetics

Genomic DNA was extracted from anticoagulated blood obtained from patients, their relatives and controls with informed consent.

Polymerase chain reaction (PCR): Samples of genomic DNA were amplified by PCR with primers specific for all exons (1–24) encoding the α subunit of the sodium channel protein.[28] In particular, the primer sequences used for the part of exon 24 which encodes the transmembrane segment S6 of repeat IV were: 5′ CCT CCT CCT CCT CCT GGT CAT 3′ and 5′ GGG CTC GCT GCT CTC CTC TGT 3′.[3] The experimental conditions were optimized for each primer. In addition, to get small fragments for SSCP analysis, some PCR products were digested with restriction endonucleases MspI (exons 4, 5), PstI (exons 6, 14, 15), BglI (exon 13), ApaI (exon 24), and AluI (exons 23–24).

Single strand conformational polymorphism (SSCP) analysis: PCR products and digested DNA were diluted in 30 μl of a mixture containing 0.05% Na+ dodecyl sulfate and 5 mM EDTA to which 7.5 μl of a mixture of 95% formamide, 20 mM EDTA, 0.05 % bromophenol blue and 0.05% xylene xyanol was 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 at 4°C and were subsequently stained with 0.5 μg/ml ethidium bromide following the protocol of Yap & McGee.[29]

Direct PCR sequencing: 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 Ptáček and co-workers.[3] 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 a 373A DNA sequencer (Applied Biosystems, Foster, CA).

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