Brain (2001), **124,** 1091–1099

Hypokalaemic periodic paralysis type 2 caused by mutations at codon 672 in the muscle sodium channel gene *SCN4A*

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Summary

Hypokalaemic periodic paralysis (hypoPP) is an autosomal dominant muscle disorder characterized by episodic attacks of muscle weakness associated with a decrease in blood potassium levels. Mutations in the gene encoding the skeletal muscle voltage-gated calcium channel α-1 subunit (CACNL1A3) account for the majority of cases. Recently, mutations in the gene coding for the skeletal muscle voltage-gated sodium channel α subunit (SCN4A) have been reported in a small number of hypoPP families. In order to determine the relative frequency of the CANCL1A3 and SCN4A mutations in a large population of hypoPP patients, and to specify the clinical and pathological features associated with each of them, we searched for mutations in 58 independent hypoPP index cases. We detected the causative mutation in 45 cases: 40 were linked to the *CACNL1A3* gene and five to the *SCN4A* gene. One mutation has not been described before. Some remarkable clinical features were observed in a large hypoPP family carrying an SCN4A mutation: a complete penetrance in men and women, an early age at onset, postcritic myalgias and an increased number and severity of attacks induced by acetazolamide. A muscle biopsy, performed in two members of this family, revealed a peculiar myopathy characterized by tubular aggregates. In contrast, vacuoles were predominant in muscles from hypoPP patients carrying CACNL1A3 mutations. Our findings point to the usefulness of a molecular characterization of hypoPP patients in clinical practice. They also provide new clues for understanding the mechanisms behind functional and structural alterations of the skeletal muscle in hypoPP.

Keywords: hypokalaemic periodic paralysis; calcium channel; sodium channel; tubular aggregates

Abbreviations: CACNL1A3 = α-1 subunit of adult skeletal muscle voltage-dependent calcium channel; hypoPP = hypokalaemic periodic paralysis; hypoPP-1 = hypoPP linked to CACNL1A3; hypoPP-2 = hypoPP linked to SCN4A; PCR = polymerase chain reaction; RFLP = restriction fragment length polymorphism; SCN4A = α subunit of adult skeletal muscle voltage-dependent sodium channel; SSCA = single strand conformation analysis.

Introduction

Hypokalaemic periodic paralysis (hypoPP) is an autosomal dominant muscle disorder characterized by episodic attacks of muscle weakness accompanied by a decrease in blood potassium levels. The age at onset of recurrent attacks is usually within the first or second decade. Attacks become rarer or disappear after 40 years of age. The frequency of attacks varies from patient to patient, even within the same family. The expression of the phenotype is usually less intense in women, who may experience only a small number of attacks. Provocative factors include carbohydrate-rich meals, rest after exercise, sudden exposure to heat or cold, glucose perfusion and acute stress. A progressive muscle weakness may also develop, independently of the number of attacks, starting in most cases after 40 years of age. Histopathological studies, performed before the discovery of the genes involved in hypoPP, have shown myopathic

alterations characterized by the presence of vacuoles or tubular aggregates in the muscle fibres (Bekeny, 1961; Engel *et al*., 1970).

The majority of hypoPP families previously were linked to hypoPP-1 on chromosome 1q32 (Fontaine *et al*., 1994). Subsequent gene sequencing demonstrated that mutations in the gene encoding the α-1 subunit of the skeletal muscle calcium channel (CACNL1A3) caused hypoPP-1 (Jurkat-Rott *et al*., 1994; Ptacek *et al*., 1994*a*). This α-1 subunit is composed of four homologous domains (DI–DIV), each of them made of six transmembrane segments (S1–S6) (Catterall, 1996; Lehmann-Horn and Jurkat-Rott, 1999). Three point mutations affecting an arginine have been found in segment S4 of domains II and IV (R528H, R1239H and R1239G) (Jurkat-Rott *et al*., 1994; Ptacek *et al*., 1994*a*). The R528H and R1239H mutations are responsible for most cases of hypoPP-1 and their relative incidence varies from one population to another (Elbaz *et al*., 1995; Fouad *et al*., 1997; Sillen *et al*., 1997). An incomplete penetrance in women and a later onset were observed for the R528H mutation (Elbaz *et al*., 1995; Fouad *et al*., 1997). Although the majority of hypoPP families were linked to the hypoPP-1 gene, at least one large family was not, suggesting the existence of a second hypoPP locus (hypoPP-2) (Plassart *et al*., 1994*a*). Furthermore, an extensive search for CACNL1A3 mutations in large samples of hypoPP families did not disclose mutations for a number of them (Fouad *et al*., 1997). Recently, linkage analysis, gene sequencing and *in vitro* expression studies demonstrated that the gene encoding the α subunit of the skeletal muscle sodium channel (SCN4A) located on chromosome 17q22–23 was the second locus hypoPP-2 (Bulman *et al*., 1999; Jurkat-Rott *et al*., 2000).

In the present work, we screened 58 hypoPP families for all known mutations in *CACNL1A3* and *SCN4A* genes that were associated with this disease. CACNL1A3 mutations were found in 40 families and SCN4A mutations in five families. We also identified a new hypoPP mutation in the *SCN4A* gene.

Material and methods

Patients

The search for a causal mutation was undertaken in 58 independent probands with a diagnosis of hypoPP. Probands presented episodes of flaccid paralysis with a low percritic potassium level (blood potassium level <3.5 mmol/l during the attacks). Myotonia was neither clinically present nor detectable by electromyography. Forty-four patients were index cases in families with several affected members, compatible with an autosomal dominant mode of inheritance. Fourteen cases were apparently sporadic. Secondary causes of hypoPP were excluded, especially in sporadic cases. Clinical data for probands and related mutation carriers are summarized in Table 1. Blood samples were obtained after the informed consent of each individual according to the European Union and French bioethics laws and the Declaration of Helsinki. DNA was extracted from blood samples by a classical phenol–chloroform protocol or a rapid procedure using Qiagen DNA minikit (Qiagen S.A., Les Ulis, France).

The pedigree of Family 41 is shown in Fig. 1. In this large French family, linkage analysis had already excluded the hypoPP-1/CACNL1A3 locus (Plassart *et al*., 1994*a*). The proband was a 43-year-old man. Paralytic attacks began at age 10 years (one/month) and their frequency and duration increased between ages 13 and 25 years (three/week, lasting 12–24 h, with postcritic myalgias lasting several days). A percritic hypokalaemia was demonstrated (the lowest was 1.4 mmol/l). Attacks occurred frequently in the morning and treatment by potassium chloride salts decreased the frequency of attacks by two-thirds. Triggering factors included carbohydrate-rich meals, sudden rest after exercise, sudden exposure to heat or cold and general anaesthesia. Treatment by acetazolamide was attempted at age 36 years but was interrupted because of the occurrence of subintrant attacks of paralysis with intercritic weakness during several weeks. At age 40 years, a permanent muscle weakness affecting the upper segments of the lower and upper limbs was evidenced. A muscle biopsy was performed at age 42 years. His 18-year-old son experienced the first paralytic attacks at age 10 years. The frequency and duration of these attacks developed in a manner similar to that described for his father. At age 17 years, no permanent muscle weakness was evidenced on clinical examination although the patient complained of muscle fatigue. Acetazolamide had the same deleterious effect as for his father. A muscle biopsy was performed at age 17 years. Clinical information about the proband, his son and nine other affected relatives is summarized in Table 1 and Fig. 1.

Methods for mutation screening

Mutations in the *CACNL1A3* and *SCN4A* genes were searched for in all proband DNAs. The CACNL1A3 R528H mutation resulting from a G $\rightarrow$ A transition at nucleotide 1583 was searched for by performing a polymerase chain reaction (PCR) and analysing the restriction fragment length polymorphisms (RFLPs) of the PCR products. Primers and restriction endonuclease were as described (Jurkat-Rott *et al*., 1994). PCR was performed in 25 µl of standard PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 240 µM of each primer, 0.025 U/µl of *Taq* polymerase Gold (Applied-Biosystems, Foster City, Calif., USA) and 80 ng of DNA. Steps of amplification were 94°C 15 min (94°C 30 s, $T°_{an}$ 30 s, 72°C 1 min) $\times$ 40 cycles, 72°C 3 min. $T°_{an}$ decreased from 60 to 50°C by 1° steps in the first 10 cycles and remained at 50°C for the last 30 cycles. A 5 µl aliquot of each 77 bp PCR product was mixed with 2 IU of *Bbv*I, 1.7 µl of appropriate 10$\times$ buffer and 10 µl of H_2O. Mixes were left under mineral oil at 37°C for 5 h. The resulting digestion products were electrophoresed for 1 h on a 4% agarose gel

Table 1 *Features of hypoPP in groups of patients carrying different mutations of the calcium channel CACNL1A3 and the sodium channel SCN4A*

	R528H (26 families)	R1239H (14 families)	R672G (one family)
Repetitive paralytic attacks			
Penetrance among carriers	0.75 (25/33) M: 0.90 (20/22)[a] F: 0.45 (5/11)[b]	0.84 (22/26) M: 0.91 (11/12)[c] F: 0.71 (10/14)[d]	1.00 (11/11) M: 1.00 (6/6) F: 1.00 (5/5)
Mean age at onset (years)	14 ± 3.1 (n = 23) M: 14.5 ± 1.5 (n = 19) F: 11.8 ± 7.1 (n = 4)	9.6 ± 4.7 (n = 18) M: 10.4 ± 4.8 (n = 9) F: 8.8 ± 4.8 (n = 9)	9.6 ± 2.9(n = 11) M: 9 ± 3 (n = 6) F: 10.2 ± 3.1 (n = 5)
Percritic K^+ level	1.72 ± 0.58 (n = 16) M: 1.69 ± 0.49 (n = 13) F: 1.87 ± 1 (n = 3)	2.17 ± 0.55 (n = 7) M: 2.23 ± 0.86 (n = 3) F: 2.12 ± 0.32 (n = 4)	1.4 (n = 1)
Associated myakgias	5 cases (M: 3; F: 2) Percritic (n = 4) Postcritic (n = 1)	2 cases (M: 1; F: 1) Percritic (n = 2)	10 cases (M: 6; F: 4) Postcritic (n = 10)
Reported acetazolamide treatment	8 cases (M: 8) Beneficial (n = 5) No effect (n = 3)	13 cases (M: 8; F: 5) Beneficial (n = 8) No effect (n = 4) Deleterious (n = 1)	3 cases (M: 3) Deleterious (n = 3)
PMW or histopathological myopathy:			
Frequency of pmw among carriers	8/33 M: 4/22[e] F: 4/11[f]	7/26 M: 3/12[g] F: 4/14[h]	5/11 M: 3/6[i] F: 2/5[j]
Reported muscle histopathology	3 cases (M: 1; F: 2)[k] Vacuoles in all 3 cases	–	2 cases (M: 2)[l] Tubular aggregates in both cases

M = male; F = female; PMW = permanent muscle weakness. Repetitive paralysis attacks were defined as the occurrence of a period of recurrent attacks (>2) in lifetime. PMW was defined by an objective muscle deficiency at clinical examination in an intercritic period. [a]A 79-year-old man had no paralysis attacks in his lifetime; a 65-year-old man had two paralysis attacks at age 41 and 45 years. [b]Four women (76, 45, 43 and 37 years old) had no paralysis attacks in their lifetime. [c]A 10-year old boy had experienced only his first paralysis attack. [d]Four women (65, 34, 33 and 12 years old) experienced only one paralysis attack in their lifetime after general anaesthesia (n = 2), during influenza (n = 1) or after intoxication (n = 1). PMW was noticed at [e]66, 63, 44 and 39 years, [f]40 (n = 2), 37 and 33 years, [g]42, 30 and 16 years, [h]75, 34, 14 and 8 years, [i]67, 47 and 40 years, and [j]55 and 50 years of age. [k]All three patients had PMW. [l]See text for details.

[1% standard agarose, 3% Nusieve (Bio Whittaker Molecular Applications, Rockland, USA), 0.5× TBE, 0.5 µg/µl ethidium bromide], in a 0.5× TBE buffer at 100 V. Bands were visualized by ultraviolet transillumination.

Mutations of CACNL1A3 exon 30 (R1239H and R1239G) were searched for by performing a PCR and analysing the PCR products by means of single strand conformation analysis (SSCA) (Ptacek *et al*., 1994*a*). Primers were 5′-ATGAGAGTGCCCGCATCTCC-3′ (F) and 5′-CTGT-TGCACCTGGAAGGACTTG-3′ (R) (181 bp). PCR was performed in 25 µl of standard PCR buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 240 µM of each primer, 0.025 U/µl of standard *Taq* polymerase (Applied-Biosystems, Foster City, Calif., USA) and 80 ng of DNA. Steps of amplification of the 181 bp product were 94°C 5 min (94°C 30 s, $T°_{an}$ 30 s, 72°C 1 min) × 40 cycles, 72°C 3 min. $T°_{an}$ decreased from 70 to 58°C by 1.2° steps in the first 10 cycles and remained at 58°C for the last 30 cycles. A 3 µl aliquot of each PCR was mixed with 20 µl of 10% saccharose, 0.05% bromophenol blue and xylene 0.05% cyanol (S medium). The mix was denatured for 5 min at 98°C and kept on ice. A 5 µl aliquot was electrophoresed on a 10% polyacrylamide gel at 25°C for 4 h. The gel was then fixed, silver-stained and dried.

Mutations were searched for in exon 12 of SCN4A by PCR-SSCA (Jurkat-Rott *et al*., 2000). Primers were 5′-CTCTGTGACAGGGCCTCATG-3′ (F) and 5′-TCCTC-ACCCCACCCCCATCC-3′ (R) (250 bp). PCR was performed in 25 µl of standard PCR buffer containing 2 mM $MgCl_2$, 0.2 mM of each dNTP, 240 µM of each primer, 0.025 U/µl of *Taq* polymerase Gold and 80 ng of DNA. Steps of amplification were 94°C 15 min (94°C 30 s, 61°C 30 s, 72°C 1 min) × 35 cycles, 72°C 3 min. PCR products were denatured in two different conditions. In the first, 3 µl of each PCR were mixed with 20 µl of S medium. In the second, 4 µl of each PCR were mixed with 8 µl of 100% formamide, 10 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol (FE medium). Both kinds of mixes were denatured for 5 min at 98°C, kept on ice, and 5 µl of each were electrophoresed at two different temperatures (7 and 25°C) on 10% polyacrylamide gels. Gels were then fixed, silver-stained and dried.

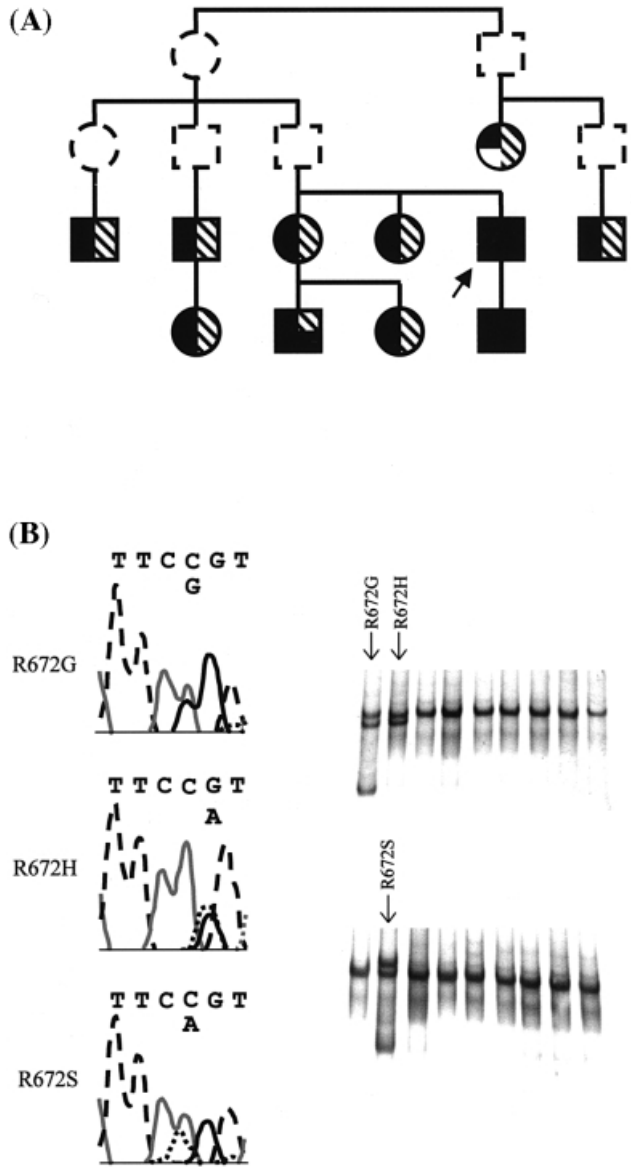

Fig. 1 Sodium channel (SCN4A) mutations in hypoPP-2. (**A**) Segregation of clinical and histopathological traits in 11 individuals of hypoPP-2 Family 41. Full upper left, lower left, upper right and lower right quadrants correspond to episodic paralytic attacks, postcritic myalgias, tubular aggregates and acetazolamide-induced aggravation of attacks, respectively. An empty quadrant corresponds to the absence of the trait. A hatched quadrant means that the trait was not determined (no muscle biopsy or no acetazolamide treatment). Individuals shown as dashed empty symbols are ancestors for whom no data were available. (**B**) SSCA detection and direct sequencing of three different heterozygous mutations at codon 672 in exon 12 of SCN4A. For SSCA, 5 µl of a mix of PCR and S medium were heated at 94°C, ice-cooled and electrophoresed on a 10% polyacrylamide gel at 25°C. Gels were fixed and silver stained.

Direct sequencing was used to sequence variants responsible for each abnormal RFLP or SSCA profile using a fluorescent procedure and a DNA sequencer according to the manufacturer protocol (ABI 377; Applied-Biosystems, Foster City, Calif., USA).

Muscle biopsy and histopathological examination

Open deltoid muscle biopsies were performed under local anaesthaesia. Specimens were frozen and sectioned at a 10 µm thickness. The following histochemical reactions were carried out: haematoxylin–eosin, modified Gomori's trichrome, oil-red O, NADH (nicotinamide adenine dinucleotide)-tetrazolium reductase, periodic acid–Schiff, myophosphorylase, myofibrillar adenosine triphosphatase (pH 4.3, 4.6 and 9.4), succinate dehydrogenase and cytochrome *c* oxidase. Others specimens were paraffin-embedded, serially sectioned and examined by haematoxylin–eosin and Congo red stains.

Results

Calcium (CACNL1A3) and sodium (SCN4A) channel gene mutations in hypoPP

The presence of a CACNL1A3 (hypoPP-1) R528H mutation was suggested by a PCR digestion assay which showed a partial loss of the *Bbv*I restriction site in 26 probands (families 1–26). In all cases, direct sequencing confirmed a heterozygous G → A transition at nucleotide 1583, resulting in a R528H mutation (Fig. 2). A R528H mutation was also demonstrated in 60 relatives (34 men and 26 women).

Fourteen probands (Families 27–40) showed an additional band at CACNL1A3 exon 30 by SSCA analysis, suggesting a R1239H mutation (hypoPP-1). Direct sequencing revealed a heterozygous G → A transition at nucleotide 3716 resulting in a R1239H mutation (Fig. 2). The occurrence of a R1239H mutation was also demonstrated in 22 relatives (nine men and 13 women).

The SSCA screening of SCN4A (hypoPP-2) exon 12 in probands was performed for each PCR sample in four different conditions by combining two different denaturing media (S and FE) and two temperatures of electrophoresis. No abnormal profile was detected after electrophoresis at 7°C, but electrophoresis at 25°C revealed abnormal profiles with additional bands in five probands (Families 41–45). Three different types of abnormal profiles could be clearly distinguished using the S denaturing medium (Fig. 1). Direct sequencing revealed that each type was due to a different base substitution in codon 672 of SCN4A. A C → G transversion at nucleotide 2014 (Family 41), a C → A transversion at nucleotide 2014 (Family 45) and a G → A transition at nucleotide 2015 (Families 42–44) resulted in an arginine to glycine, arginine to serine and arginine to histidine substitution at position 672, respectively (Figs 1 and 2). All were in a heterozygous state. The R672G mutation was searched for in 16 affected (eight men and eight women) and 13 adult unaffected relatives (nine men and four women) of the Family 41 proband. This mutation was found in all affected and in none of the unaffected relatives. The R672H mutation was found in Family 44 proband's father who had only one paralysis attack at age 30 years. Samples from

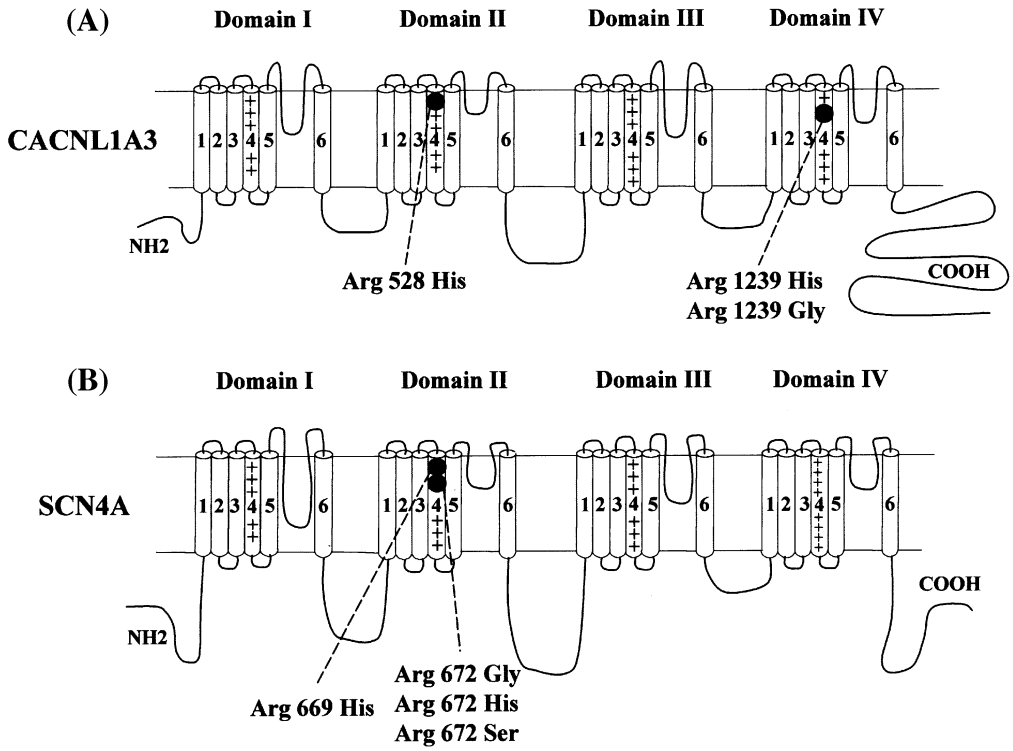

Fig. 2 Arginine substitutions causing hypoPP-1 and hypoPP-2 located in the α-1 subunits of the calcium (CACNL1A3) and sodium (SCN4A) skeletal muscle channels, respectively.

relatives of Families 42, 43 (R672H mutation) and 45 (R672S mutation) were not available. The SCN4A R672S mutation had not been reported before the present study. We confirmed its absence in 50 unrelated normal individuals, i.e. 100 chromosomes (data not shown).

Clinical phenotypes associated with CACNL1A3 (hypoPP-1) and SCN4A (hypoPP-2) mutations

The screening for hypoPP-1 and hypoPP-2 mutations in a population of 58 hypoPP probands yielded the following distribution: 26 cases (45%) carrying CACNL1A3 R528H (23 familial, three sporadic), 14 cases (24%) carrying CACNL1A3 R1239H (10 familial, four sporadic), three cases (5%) with SCN4A R672H (two familial, one sporadic), one case (2%) with SCN4A R672G (familial) and also one (2%) with SCN4A R672S (familial). No mutation was found in 13 index cases (22%).

The penetrance of the R672G mutation was complete since all mutation carriers, both men and women, presented with repetitive paralysis attacks (Table 1). In contrast, six adult R528H female carriers and one adult male carrier never experienced paralytic attacks. Three adult R1239H female carriers had only a single attack which was triggered by an acute stress (influenza, surgery or intoxication). The mean ages at onset of hypoPP for R528H, R1239H and R672G mutations were 14 ± 3 years (n = 22), 10 ± 5 years (n = 18) and 10 ± 3 years (n = 11) (data corrected to nearest 1 year), respectively (Table 1). The observed differences between R528H and R1239H populations, as well as between R528H and R672G populations were statistically significant (U test, $P = 0.003$ and $P = 0.0012$, respectively). Episodes of myalgias were reported for five R528H, two R1239H and 10 R672G patients (Table 1). They were percritic in four out of five R528H and in both R1239H patients, but were postcritic in the remaining R528H and in all R672G patients for whom myalgias could last from one to several days. A prophylactic acetazolamide treatment was administered to eight R528H, 13 R1239H and three R672G patients (Table 1, Fig. 1). This treatment was beneficial in five out of eight R528H and eight out of 13 R1239H patients, ineffective in three out of eight R528H and four out of 13 R1239H patients, and deleterious in one R1239H patient and all three R672G patients.

The development of a progressive myopathy was observed in four male and four female R528H carriers (Table 1). All affected men and one affected woman had previously experienced paralytic attacks, whereas three affected women from the same family never had attacks. A permanent muscle weakness was observed in three men and four women with the R1239H mutation (Table 1). All affected R1239H carriers experienced recurrent paralytic attacks except one woman who experienced a single attack at age 34 years and later developed a progressive myopathy in her seventies. A progressive myopathy was observed in three male and two female R672G carriers of Family 41 (Table 1).

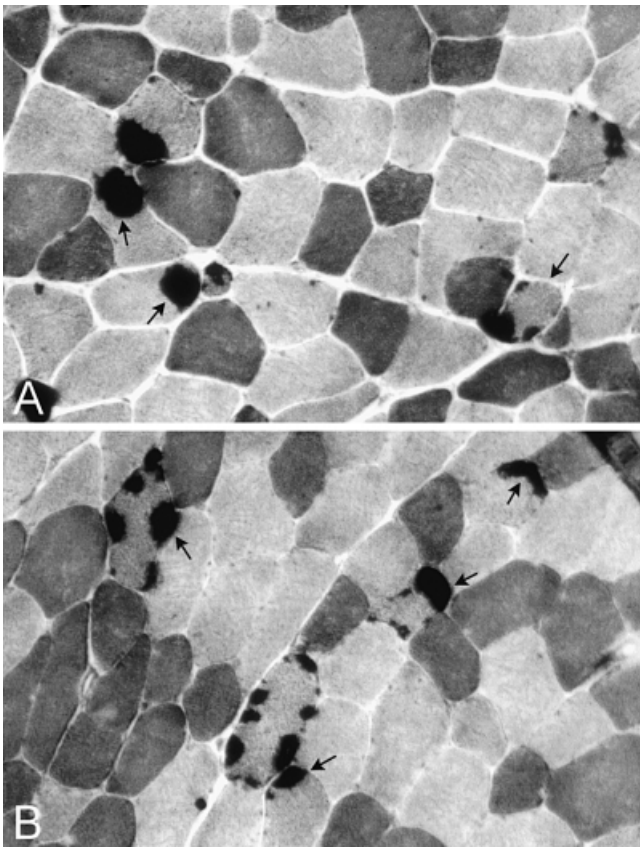

Fig. 3 Histopathological abnormalities observed in muscle biopsies of two patients with hypoPP2-associated myopathy. NADH-tetrazolium reductase stain (×150) of transversal sections from frozen deltoideus muscle biopsies. (**A**) Biopsy of Family 41 proband. (**B**) Biopsy of his son. Both displayed the same pathological abnormalities. The NADH reaction evidenced an intense staining of subsarcolemmal masses along edges of fibres (arrows). The tubular aggregate deposits were selective for type II fibres.

Histopathological characterization of hypoPP-associated myopathy

Histological analysis was performed on muscle biopsies from hypoPP-2 Family 41 proband and his son. Both patients showed the same pathological changes, though in a more severe form for the father (Fig. 3). There was a slight abnormal variability in fibre size, with some isolated atrophic fibres. The distribution of the fibre types was normal and the number of central nuclei remained in the normal range. There were no inflammatory cell exudates, basophilic regenerating fibres, necrotic fibres or vacuoles. Phosphorylase activity was normal. The striking abnormality consisted of multifocal accumulations of a well-demarcated material in type 2 fibres. These subsarcolemmal areas stained markedly with the NADH-tetrazolium reductase and were present in 25 and 12% of type 2 fibres in father and son, respectively (Fig. 3). These aggregates were slightly basophilic in haematoxylin–eosin, red in Gomori's trichrome, darkly reactive in NADH-tetrazolium reductase, normal in succinate dehydrogenase and unreactive in periodic acid–Schiff and in myosin ATPase preparations. Succinate dehydrogenase histochemical reaction showed no mitochondrial proliferation and there was no cytochrome *c* oxidase-deficient fibre. This pattern corresponded to tubular aggregates.

The finding of a tubular aggregate myopathy in two R672G individuals contrasted with the vacuolar myopathy observed in patients carrying the R528H mutation (two women from the same family and the proband from another family). Published cases on the histological changes observed in muscle biopsies of hypoPP patients are summarized in Table 2.

Discussion

Although mutations in the voltage-gated calcium channel (CACNL1A3) remain the major causes of hypoPP (i.e. hypoPP-1), we clearly confirm in the present study that mutations at amino acid 672 of the sodium channel (SCN4A) also cause hypoPP (i.e. hypoPP-2). The screening of a large population of 58 independent patients yielded 69% (40 families) of hypoPP-1 and 9% (five families) of hypoPP-2 cases.

The evidence of hypoPP2-related mutations of SCN4A expands the spectrum of diseases due to mutations in this gene. To date, >20 SCN4A mutations have been identified in normo- or hyperkalaemic periodic paralysis (Ptacek *et al*., 1991, 1994*b*; Rojas *et al*., 1991; Plassart *et al*., 1994*b*), paramyotonia congenita (Ptacek *et al*., 1993, 1994*b*; Plassart *et al*., 1994*b*, 1996), potassium-aggravated myotonia (Heine *et al*., 1993), myotonia fluctuans (McClatchey *et al*., 1992; Ricker *et al*., 1994), myotonia permanens (Lerche *et al*., 1993) and acetazolamide-responsive myotonia (Ptacek *et al*., 1994*b*). It is worth noting that some of the mutations causing paramyotonia congenita affect the outermost arginine of the S4 segment of domain IV, similarly to the mutations in the S4 region of domain II causing hypoPP-2. These paramyotonia-associated mutations uncouple inactivation from activation, which is not the case for hypoPP-2 mutations (Chahine *et al*., 1994; Jurkat-Rott *et al*., 2000). This confirms that DIIS4 and DIVS4 segments have different functions in the gating of the sodium channel α-subunit, as previously demonstrated by *in vitro* mutagenesis studies. It also provides some clues for understanding why mutations which affect a similar amino acid in different S4 segments lead to distinct phenotypes (Kontis and Goldin, 1997; Kontis *et al*., 1997).

HypoPP due to the SCN4A R672G mutation might be associated with peculiar clinical features. The age at onset for SCN4A R672G carriers differs significantly from that observed in CACNL1A3 R528H carriers, but is reminiscent of that reported for the patients carrying the CACNL1A3 R1239H mutation. The complete penetrance of the disease at age 20 years in Family 41 stands in contrast to the incomplete penetrance at the same age displayed by CACNL1A3 R528H and R1239H families. Postcritic myalgias and cramps were reported by the proband and nine other affected relatives in Family 41, whereas only one

Table 2 *Muscle histological abnormalities reported in hypokalaemic periodic paralysis*

Reference	No. of patients	Sex	Age at biopsy (years)	Clinical features and age at onset	Optic microscopy observations	Electron microscopy observations	Mutation
Pearson *et al*. (1964)	1	M	47	pa 3, pmw 42	vac		nd
	1	M	65	pa 29, pmw 60	vac		nd
	1	F	43	pa 5, pmw 40	nsp		nd
De Fine Olivarius and	1	M	57	pa 14, pmw 50 vac, nsp	nd		nd
Christensen(1965)	1	M	34	pa 14, no pmw	vac		nd
	1	M	57	pa 7, no pmw	nl		nd
	1	M	75	pa 19, pmw 55	vac		nd
Howes *et al*. (1966)	1	M	47	pa 3, pmw 43	vac	vac	nd
Odor *et al*. (1967)	1	M	39	pa 13, pmw 20	rare vac, nsp	ta, vac	nd
Buruma and Bots (1978)	1	F	9	pa 8, pmw 8	vac	vac	nd
	1	M	18	no pa, no pmw	nl	vac, mito	nd
Gerard *et al*. (1978)	1	M	20	pa 13, no pmw	vac	vac	R528H
Faugere *et al*. (1981)	1		20	pa 13, no pmw	vac	vac	nd
	1		26	pa 12, no pmw	ta	ta	nd
	1		41	pa 15, no pmw	ta	ta	nd
	1		13	pa 5, pmw	ta, nsp	ta, nsp	nd
	1		24	pa 4, pmw	ta, nsp	ta, nsp	nd
Martin *et al*. (1984)	1	M	51	pa 14, pmw 44	vac		R528H*
Links *et al*. (1990)	1	F	33	pa 13, no pmw	vac	rare ta	R528H*
	1	F	34	pa 17, no pmw	vac, rare ta	rare ta	R528H*
	1	F	53	pa 15, pmw	vac		R528H*
	1	M	70	no pa, pmw 55	vac, rare ta, nsp		R528H*
Gold *et al*. (1992)	1	M	12/29	pa 11, pmw 29	rare vac, ta		nd
Fouad *et al*. (1997)	4				vac		R1239H
	1				ta		R1239G
	3				vac		R528H
	2				nsp		R528H
	2				nsp		nf
	1				vac		nf
	1				ta		nf

M = male; F = female; pa = age (years) at onset of paralysis attacks; pmw = age (years) at reported permanent muscle weakness; vac =vacuoles; ta = tubular aggregates; nsp = non-specific myopathic changes; nl = normal aspect; mito = mitochondrial changes; nf = not found; nd = not searched for. *The mutation was determined after histological findings were published.

CACNL1A3 R528H and no R1239H patient complained of this symptom. Acetazolamide is a carbonic anhydrase inhibitor, which has been shown to decrease the number of attacks in hypoPP patients (Griggs *et al*., 1970; Vroom *et al*., 1975; Johnsen, 1977; Goulon *et al*., 1978). However, sporadic hypoPP cases were reported in which acetazolamide precipitated muscle weakness (Torres *et al*., 1981; Vern *et al*., 1987). In one of these cases, a tubular aggregate myopathy was found, similar to that observed for patients of Family 41. Treatment by acetazolamide increased the frequency and the severity of attacks in the three tested patients of Family 41, and only in one out of the 13 acetazolamide-treated CACNL1A3 R1239H patients. No deleterious effect of this treatment was observed in any of eight CACNL1A3 R528H patients. We therefore suggest that hypoPP patients with sodium channel mutations might not benefit from acetazolamide treatment, and that genetic testing in hypoPP should be undertaken before starting the treatment.

The hypoPP-associated myopathy caused by the SCN4A R672G mutation might have distinctive histopathological characteristics. In the three patients with the CACNL1A3 R528H mutation in whom a muscle biopsy was performed, as well as in at least six other reported R528H patients (Tables 1 and 2), a pure vacuolar myopathy was observed with no tubular aggregates. In contrast, a major tubular aggregate myopathy exclusive to type II fibres, with no vacuoles, was observed in two patients with the SCN4A R672G mutation. The greater severity of the lesions observed in the father and the specific involvement of type II fibres may suggest that the extent of tubular aggregates increases with age and is related to exercise. Tubular aggregates consist of densely packed double-walled tubules originating from the terminal cisterns of the sarcoplasmic reticulum (Engel *et al*., 1970; van Engelen and Ter Laak, 1999). They are found as a minor feature in skeletal muscle of normal patients but also in subacute alcoholic myopathy, drug myopathy, malignant hyperthermia and metabolic or inflammatory myopathies (Morgan-Hughes, 1998). Tubular aggregates are major features of some inherited or sporadic disorders such as the familial limb-girdle myasthenia or weakness, the exercise-related muscle pain and cramp syndrome and the gyrate atrophy of the choroid and retina (Morgan-Hughes,

1998). HypoPP patients, with no vacuoles but with a major tubular aggregate myopathy, have been reported previously using both light and electronic microscopy (Vern *et al*., 1987; Gold and Reichmann, 1992; Fouad *et al*., 1997). Nonetheless, vacuoles remain the morphological hallmark of hypoPP at the myopathic stage, and may be observed in patients with no clinical permanent muscle weakness (Bekeny, 1961; Howes *et al*., 1966; Odor *et al*., 1967; Buruma and Bots, 1978; Gerard *et al*., 1978). In some hypoPP cases, tubular aggregates could also be detected among predominant vacuoles using electronic microscopy (Odor *et al*., 1967; Links *et al*., 1990). Unfortunately, most of these reported cases, as shown in Table 2, did not undergo a molecular characterization of the gene defect. It is worth noting that the co-existence of vacuoles and tubular aggregates has been reported in hyperkalaemic periodic paralysis, a muscle sodium channel disorder as mentioned above (Bradley *et al*., 1990). Overall, these data suggest that the presence of vacuoles or tubular aggregates in the muscle fibres might originate from a persistent sarcolemmal dysfunction and might be dependent on the type of ionic channel affected by the genetic mutation.

Thirteen families remained without mutation. This suggests that allelic and/or genetic heterogeneity of hypoPP may be greater than what is now known. We have already searched for mutations in other S4 segment-encoding regions of *CACNL1A3* and *SCN4A*, with negative results (data not shown). An exhaustive screening of *CACNL1A3* and *SCN4A* is underway in probands of this group of 13 families, and other candidate genes will be screened for mutations if no mutation is found in either *CACNL1A3* or *SCN4A*.

In conclusion, the present study demonstrates that genetic characterization of hypoPP patients is important to decipher the clinical and histopathological features of the disease, and to predict the response to therapy. We suggest that mutations in the *SCN4A* gene should be systematically sought in hypoPP patients suffering from paralytic attacks followed by myalgias or worsened by acetazolamide, and when muscle biopsies reveal the presence of tubular aggregates.

Acknowledgements

We wish to thank the families and their physicians for their help and support. This work was financially supported by INSERM, AFM, PROGRES (INSERM), AP-HP, IFR of Neurosciences, RESOCANAUX and the German Ministry of Research. S.N. received a post-doctoral fellowship from AFM.

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Received August 14, 2000. Revised January 3, 2001.
Accepted January 24, 2001