

# Altered Calcium Currents in Human Hypokalemic Periodic Paralysis Myotubes Expressing Mutant L-type Calcium Channels

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In a genome-wide search, linkage of hypokalemic periodic paralysis (HypoPP), a muscle disease with autosomal dominant inheritance, to chromosome 1q31-32 and cosegregation with the gene encoding the L-type calcium channel/DHP receptor  $\alpha_1$  subunit has been reported (Fontaine et al., 1994). Here we show the extended haplotypes of a large HypoPP family who made the detection of the gene product possible. Sequencing of cDNA synthesized from RNA isolated from muscle specimens of two affected family members revealed a G-to-A transition of nucleotide 3716. This base exchange predicts a substitution of histidine for arginine 1239 located in segment IVS4 of the channel protein. By restriction fragment analysis, the mutation was detected in the genomic DNA of all affected family members. Myotubes cultured from the muscle specimens also revealed the mutation suggesting the expression of mutant L-type calcium channel/DHP receptors. Whole-cell recordings of 20 such myotubes showed a strong reduction of the DHP sensitive, slowly activating and inactivating L-type current density to 30% of the current in normal controls. A rapidly activating and inactivating current component (third-type), which is distinct from the also occurring T-type current, was increased. We conclude that HypoPP is a disease of the skeletal muscle DHP receptor. The point mutation in repeat IV of the protein may have a similar effect as drugs which downregulate the channel activity by binding to this domain.

## Introduction

Dihydropyridine (DHP) receptors are located in the transverse tubular system of skeletal muscle fibers and consist of five subunits:  $\alpha_1$ ,  $\alpha_2/\delta$ ,  $\beta$ , and  $\gamma$  (Catterall, 1988). The  $\alpha_1$  subunit (Tanabe, Takeshima, Mikami, Flockerzi, Takahashi, Kangawa, Kojima, Matsuo, Hirose, and Numa, 1987) contains the putative voltage sensing structures (the S4 segments in the four homologous repeats), receptors for calcium channel agonists and antagonists such as dihydropyridines and phenylalkamines, as well as the calcium conducting pore (Tanabe, Mikami, Niidome, Numa, Adams, and Beam, 1993; Mori et al., 1993; Hofmann, Biel, and Flockerzi, 1994). The receptor is thought to fulfill a dual function as calcium channel and as voltage sensor for the

control of calcium release from the sarcoplasmic reticulum. Both functions are eliminated in mouse skeletal muscle fibers homozygous for the mdg mutation (muscular dysgenesis) which disables the  $\alpha_1$  subunit of the L-type calcium channel/DHP receptor (Beam et al., 1986; Chaudhari, 1992). They could be recovered by injecting the cDNA of the rabbit  $\alpha_1$  subunit (Tanabe, Beam, Powell, and Numa, 1988). The mdg mouse was the only example so far of an abnormal phenotype linked to a mutation in a voltage-gated calcium channel.

Recently, evidence was presented that the hereditary human muscle disease hypokalemic periodic paralysis (HypoPP) is linked to the gene encoding the L-type calcium channel/DHP receptor  $\alpha_1$  subunit of skeletal muscle (Fontaine et al., 1994; Jurkat-Rott et al., 1994) which has been localized to region q32.1–q32.2 on chromosome 1 (Drouet, Garcia, Simon-Chazottes, Mattei, Guénet, Schwartz, Varadi, and Pinçon-Raymond, 1993). The linkage data induced successful searches for mutations in this gene predicting arginine to histidine substitutions in IIS4 and IVS4 and in addition a substitution of glycine for the same arginine in IVS4 (Jurkat-Rott et al., 1994; Ptácek et al., 1994).

HypoPP is the most common of the primary periodic paralyses. It is an autosomal dominant disease characterized by attacks of flaccid weakness which are provoked by excessive intake of carbohydrates, rest after strenuous exercise or mental stress. A stimulation of the sodium-potassium pump by insulin is assumed to be the mechanism by which potassium ions are transported from the extracellular into the intracellular compartment causing hypokalemia (for review see Lehmann-Horn, Engel, Rüdel, and Ricker, 1994). Next to a fall of serum potassium, other factors influencing frequency and severity of clinical symptoms might be other hormones, as onset is usually during puberty, as penetrance in women is incomplete, as attacks become predominant during pregnancy, and as adrenaline is a specific triggering agent.

Here we report more evidence for tight genetic linkage between HypoPP in a large pedigree and the gene of the L-type calcium channel/DHP receptor  $\alpha_1$  subunit. Furthermore, we demonstrate the presence of a IVS4 mutation in all affected members. For the study of the functional consequences of the mutation on the gene product we measured calcium currents on myotubes derived from two patients of this family as partially described by Sipos, Szücs, Struk, Lehmann-Horn, and Melzer (1994).

## **Material and Methods**

Genomic DNA was extracted from anticoagulated blood of all individuals with their informed consent. Muscle specimens were obtained from two patients and 15 individuals who had undergone muscle biopsy for exclusion of malignant hyperthermia susceptibility; these specimens served as controls if susceptibility was excluded. All procedures were in accordance with the Helsinki convention and were approved by the Ethical Committee of the University of Ulm.

### **Genetic Linkage**

Genotyping of all microsatellites was performed by the polymerase chain reaction (PCR) according to Généthon standard protocols. Dinucleotide repeats (Gyapay et al., 1994) and three additional microsatellites developed at Généthon were used:

AFM136xa7; AFM337xd5; AFM312yb5 (Jurkat-Rott et al., 1994). The likelihood of the order of the loci, genetic distances, and localization of the DHP-receptor were established with odds over 10<sup>3</sup>:1 after genotyping the microsatellites in eight C.E.P.H. families using the program ILINK of the LINKAGE package (version 5.1). Recombination fractions were converted to map distances using the Haldane mapping function.

### Molecular Biology

As sequencing of cDNA had shown a G-to-A transition at position 3716 of the human  $\alpha_1$  subunit, a method was developed for screening of the genomic DNA of the family members. Short fragments (length of PCR product 94 bp) of genomic DNA were amplified by PCR with primers derived from the human cDNA sequence (Hogan, K., P. Powers, and R. Gregg, 1994): as forward primer 5'-CGCATCTCCAGC-GCCTTCTTC-3' and 5'ACGTCCACAGGAGGGTTCGCACT-3' as reverse primer.

The reaction mixture with a final volume of 100  $\mu$ l contained: 50 ng DNA, 50 pmol of each PCR primer, 50  $\mu$ M of each deoxynucleotide triphosphate, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl<sub>2</sub> and 1.5 U of Taq polymerase. Amplification conditions: 10 min at 96°C, followed by 30 cycles of 94°C for 30 s, 66°C for 45 s, and 72°C for 1 min. After precipitation of the total volume of the PCR product in 300  $\mu$ l ethanol and redissolving in 30  $\mu$ l distilled water, a digestion with 1 U of NlaIII (New England Biolabs, Beverly, MA) was performed at 37°C for 8 h. The reaction was stopped with 3  $\mu$ l of blue sucrose (30% sucrose, 50 mM EDTA, 0.25% bromophenol blue, 0.1% SDS). 6  $\mu$ l of the product were loaded on a 15% acrylamide gel and run at 300 V for 3–4 h in TBE buffer. After electrophoresis, the gels were stained with 0.5  $\mu$ g/ml ethidium bromide. Direct sequencing was performed as earlier described (Heine, Pika, and Lehmann-Horn, 1993).

### Cell Culture

Biopsies were obtained from the vastus lateralis muscle. The procedure of growing myotubes from human satellite cells followed closely the description by Brinkmeier, Mutz, Seewald, Melzner, and Rüdel (1993). Briefly, the biopsy material was dissociated enzymatically at 37°C in PBS solution (Biochrom) containing collagenase (250 U/ml, Type II, Sigma Chemical Co., St. Louis, MO) and bactotrypsin (3%, Difco Laboratories, Inc., Detroit, MI). The reaction was stopped with Hank's solution containing 10% FCS (fetal calf serum). After filtering and centrifugation (100 g, 10 min) the pellet was resuspended in a culture medium consisting of a mixture (1:1) of Ham's F-12 and CMRL medium (Biochrom, Berlin, Germany) containing 5% FCS, 5% HS (both GIBCO-BRL, Gaithersburg, MD), 2.5 mg/ml glucose, 0.3 mg/ml glutamine (Biochrom) and 1.2 mg/ml NaHCO<sub>3</sub> and kept in a 5% CO<sub>2</sub> atmosphere. After 1–2 d, the serum content of the culture medium was reduced to 2% FCS and 2% HS. Fusion of myotubes sufficient for electrophysiological studies was observed after 10–14 d.

### Screening of the RNA of Cultured Cells

RNA was isolated from the cultured cells using TRIzol (GIBCO-BRL) and reverse transcriptase PCR with the screening primers mentioned above was performed according to (Jurkat-Rott et al., 1994). PCR products were tested for the mutation as described above in Molecular Biology.

## Electrophysiology

Whole-cell recordings were performed by use of an EPC-7 patch clamp amplifier (List Biological Laboratories, Inc., Campbell, CA). The currents were sampled at 2–10 kHz using commercial data acquisition hard and software (TL1 and Pclamp 5.51, Axon Instruments, Inc., Foster City, CA) on an IBM AT486 compatible microcomputer. The linear capacitance of the myotubes was determined by the integral of the transient current divided by the corresponding voltage step amplitude (+5 mV). The series resistance was calculated by the ratio of the time constant of the transient current decay and the capacitance. Statistics were performed by use of the two-tailed *t* test. Values are given as mean  $\pm$  SD.

Calcium currents were studied in a solution in which the major cations, potassium and sodium, were replaced by ions incapable of permeating the membrane of the myotubes. The bathing solution contained (in millimolar): TEA-Cl 120, CaCl<sub>2</sub> 10, MgCl<sub>2</sub> 1, HEPES 10, Glucose 5, TTX 0.02, EGTA 0.1; pH at 7.4). The patch pipettes were filled with CsCl 130, MgCl<sub>2</sub> 0.5, HEPES 10, EGTA 1, Mg-ATP 5, creatine phosphate 5; pH at 7.2. All measurements were performed at room temperature.

## Results

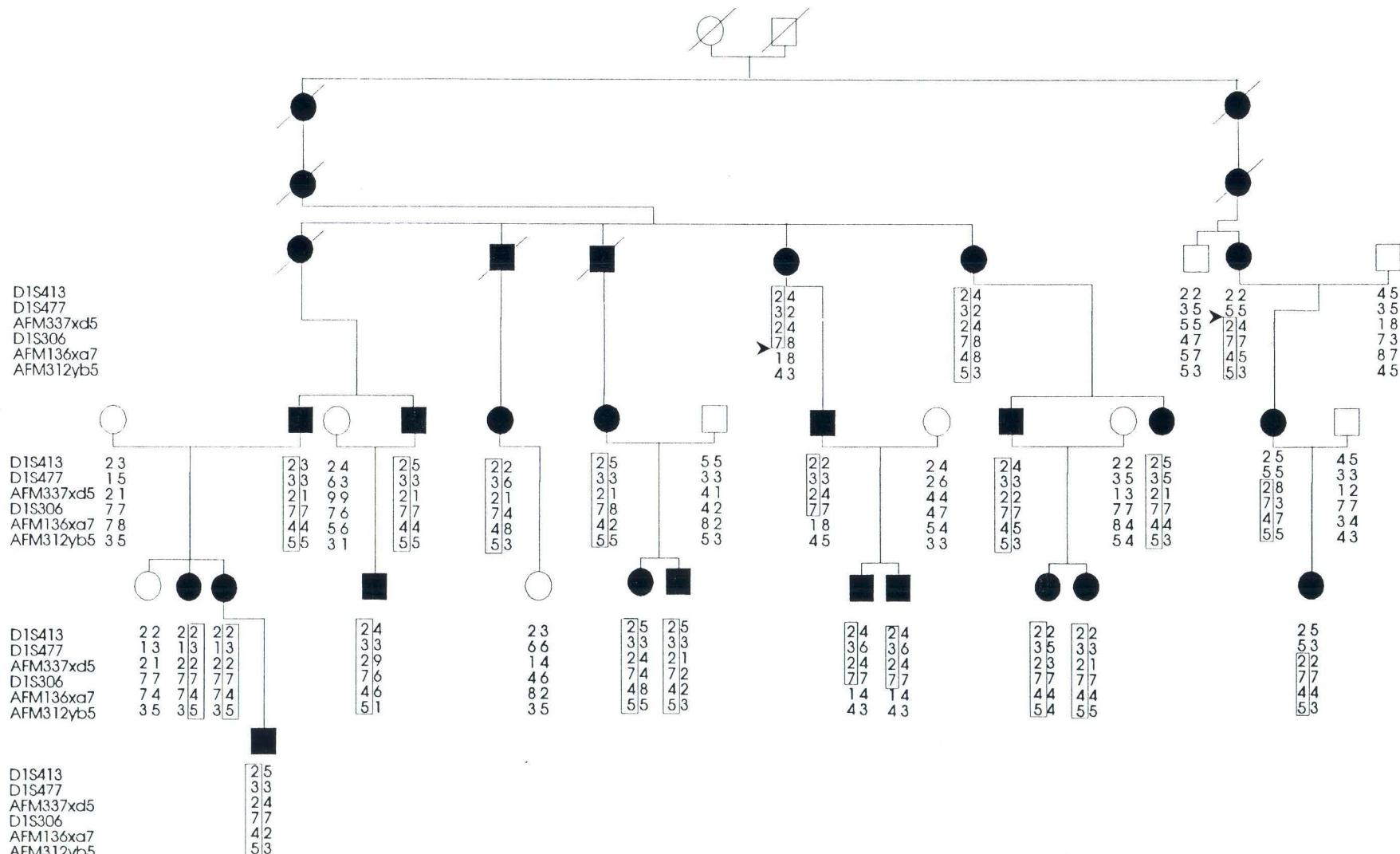
### HypoPP Family Pedigree, Haplotypes, and Genetic Map

For a more precise mapping of the HypoPP locus and to yield a higher lod score, we extended the linkage study for our family (already partially published as family B by Fontaine et al. [1994] and Jurkat-Rott et al. [1994]) by inclusion of additional family members and the use of three AFM microsatellites located between D1S413 and D1S510. Because an affected member of our family is recombinant for both AFM312yb5 and AFM136xa7 but is not recombinant for the other markers, we deduced that these markers are located telomerically to AFM337xd5 (Fig. 1). Because an affected member of another pedigree (family A in Jurkat-Rott et al. [1994]) is recombinant for both D1S413 and AFM337xd5 (not shown), the respective position of AFM337xd5 and AFM136xa7 is confirmed. Due to the uninformative nature of D1S306 for the two crucial recombinants, the HypoPP locus could not be placed relative to this microsatellite. Therefore, the HypoPP locus is flanked by AFM337xd5 and AFM136xa7 (Fig. 2) which are separated by a genetic distance of 0 cM as calculated in the eight C.E.P.H. families used to type the Généthon markers for chromosome 1. The highest two-point LOD scores at a recombination fraction of zero were found for AFM337xd5 ( $Z_{\max} = 5.26$ ).

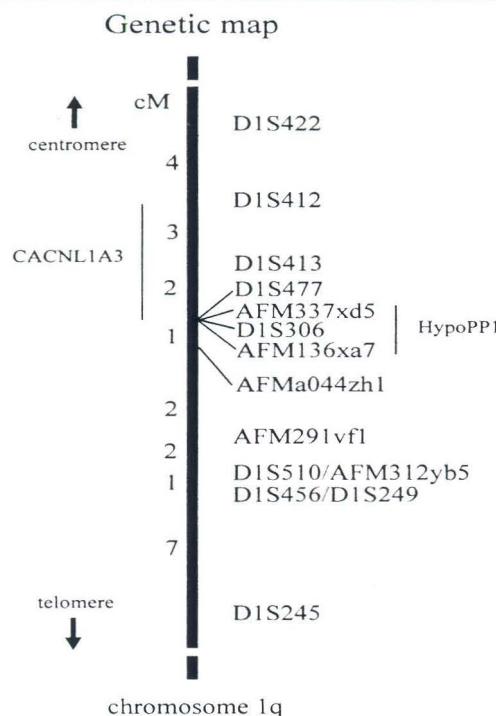
### Search for the Mutation in cDNA

Total RNA was isolated from muscle specimens obtained from one patient of our family and from four controls. RNA was reverse transcribed and regions of the CACNL1A3 cDNA were amplified by use of PCR primers derived from human cDNA sequence (Hogan, Powers, and Gregg, 1994). Amplified DNA fragments obtained by asymmetrical PCR were purified and directly sequenced. Of the full-length cDNA (~5,600 bp), 4,100 bp encoding the four (highly conserved) domains were sequenced. We identified a G-to-A transition of nucleotide 3716 which results in an arginine to histidine substitution at position 1239 (Fig. 3A). This amino

pedigree of hypopp family 8



**Figure 1.** HypoPP family pedigree and haplotypes. (*Filled symbols*) Patients; (*open symbols*) unaffected individuals. The haplotypes segregating with the disease is boxed. (*Arrow heads*) Recombination events.



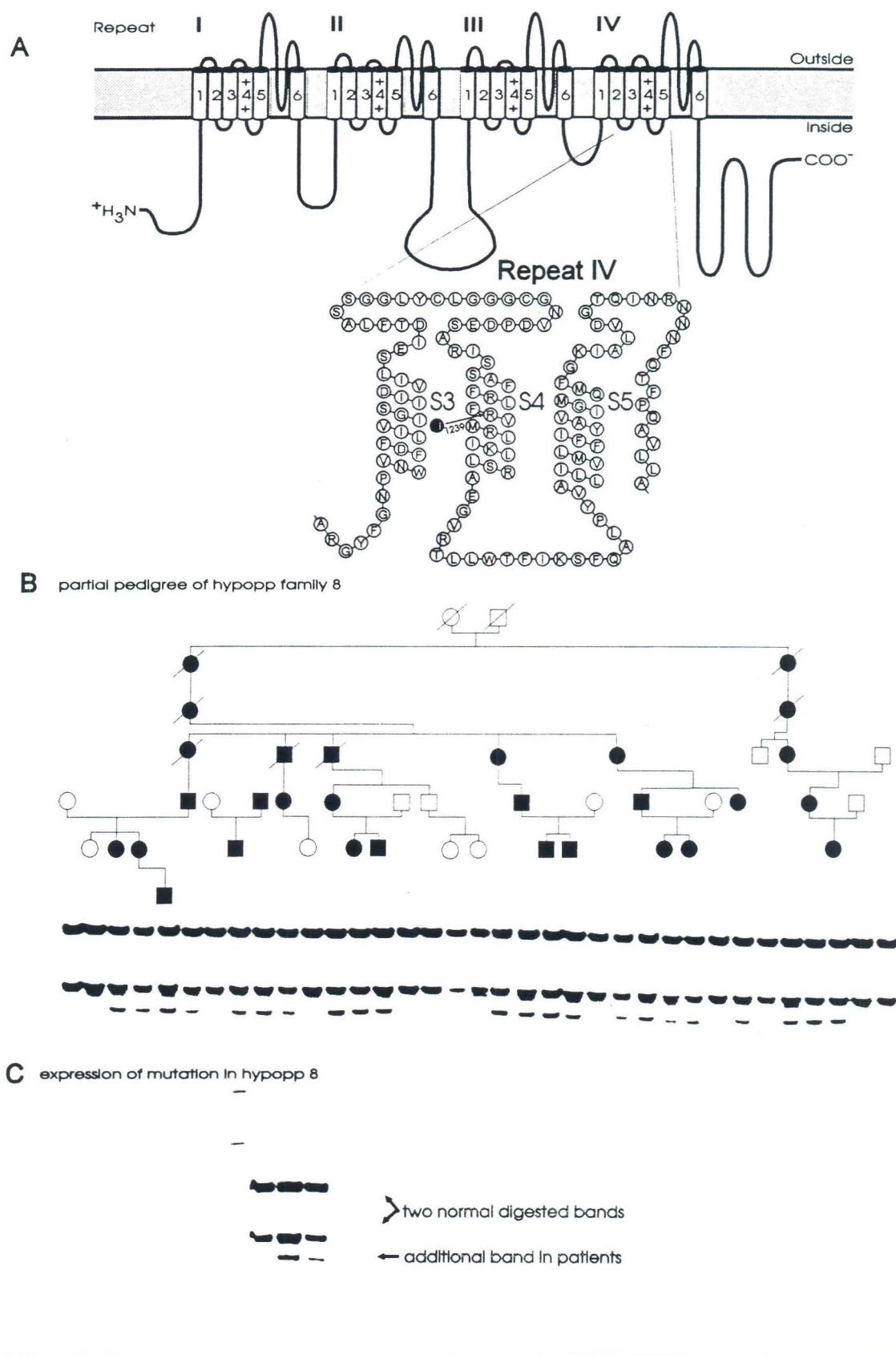
**Figure 2.** Genetic map of region q32 of chromosome 1 including the localization of the HypoPP locus between AFM337xd5 and AFM136xa7. The locus of CACNL1A3 (which could not be localized more precisely than between D1S412 and the cluster of D1S477/AFM337xd5/D1S306/AFM136xa7 due to the uninformative nature of the intragenic markers available) is also indicated. Note that the overlapping region is equivalent to the HypoPP locus and is likely to contain at least part of CACNL1A3.

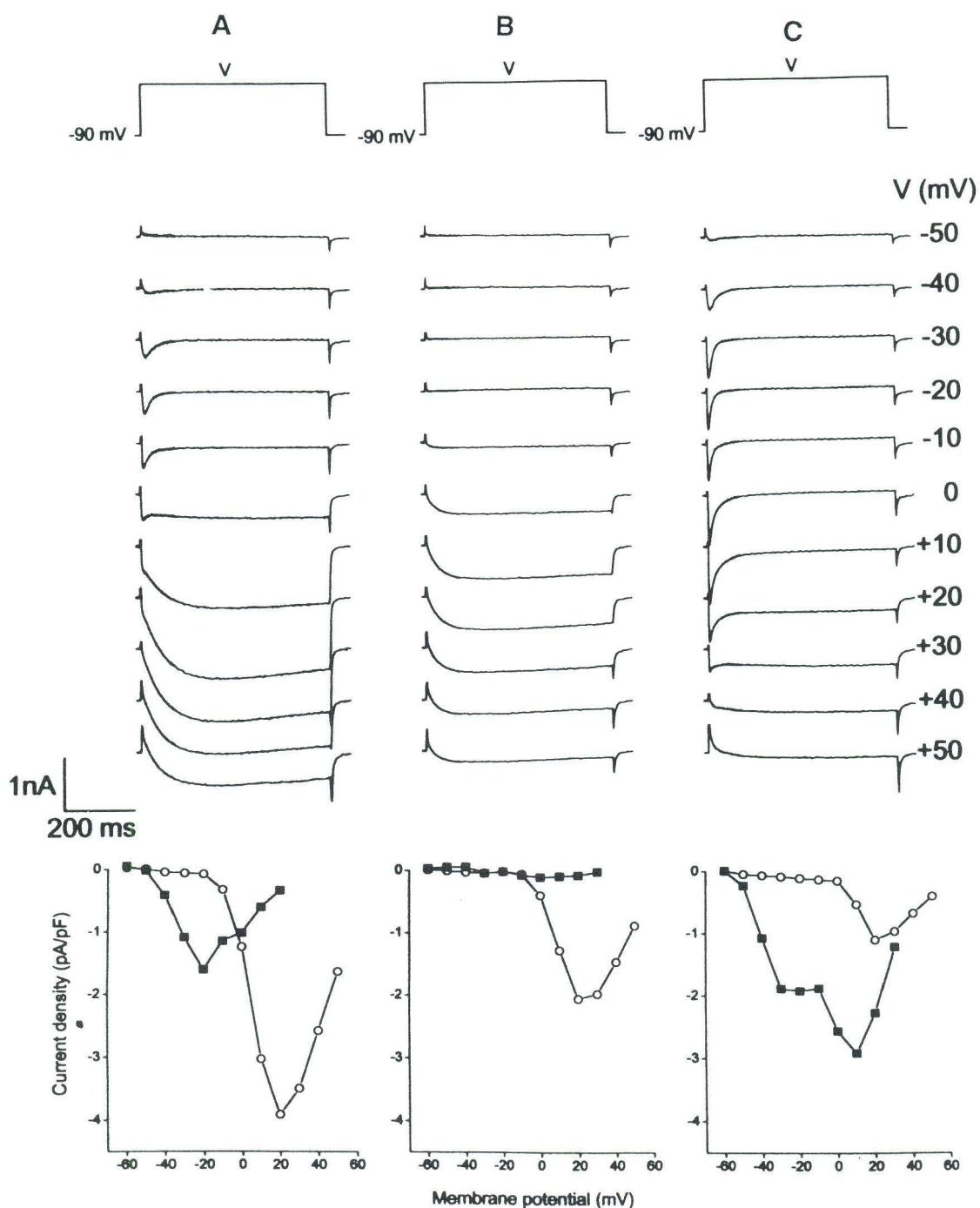
acid is located in repeat IV within an  $\alpha$  helix containing a positively charged amino acid at every third position, a putative voltage sensor of the channel protein.

### Presence of the Mutation in Genomic DNA and RNA of Myotubes

Introduction of a restriction site by the base exchange and design of adequate exon primers, made it possible to screen genomic DNA in all family members. The mutation segregated perfectly with the disease (Fig. 3 B) and was not present in DNA of 100 control subjects (200 chromosomes). In addition to the normal sequence, the mutation was also found in the RNA isolated from myotubes derived from the two patients biopsied (Fig. 3 C). Because the RNA isolation occurred at the same culture stage as the electrophysiological studies, the presence of both the mutated and the normal channel protein in the myotubes could be expected.

**Figure 3.** (A) Schematic diagram of the DHP-receptor  $\alpha_1$  subunit consisting of four regions of internal homology, so-called repeats, connected by intracellular loops. Each repeat contains six hydrophobic segments (S1–S6), putative transmembrane helices. An interlinker is found between segments S5 and S6 of each repeat, consisting of an extracellular loop and a sequence which reemerges into the membrane lining the channel pore. Segment IVS4 containing positive charges at each third position is enlarged in the inset showing the arginine to histidine mutation found in all affected family members. (B) Partial pedigree of the HypoPP family with corresponding polyacrylamide gel displaying PCR-amplified genomic DNA fragments after digestion with NlaIII and staining with ethidium bromide. PCR products of unaffected individuals show complete digestion: two bands of 55 and 39 bp; patients' DNA shows an additional band of 34 bp resulting from the mutated PCR product due to introduction of a new restriction site in the mutant DNA. (C) Presence of the mutation in myotubes. For details see Methods and Fig. 3 B.

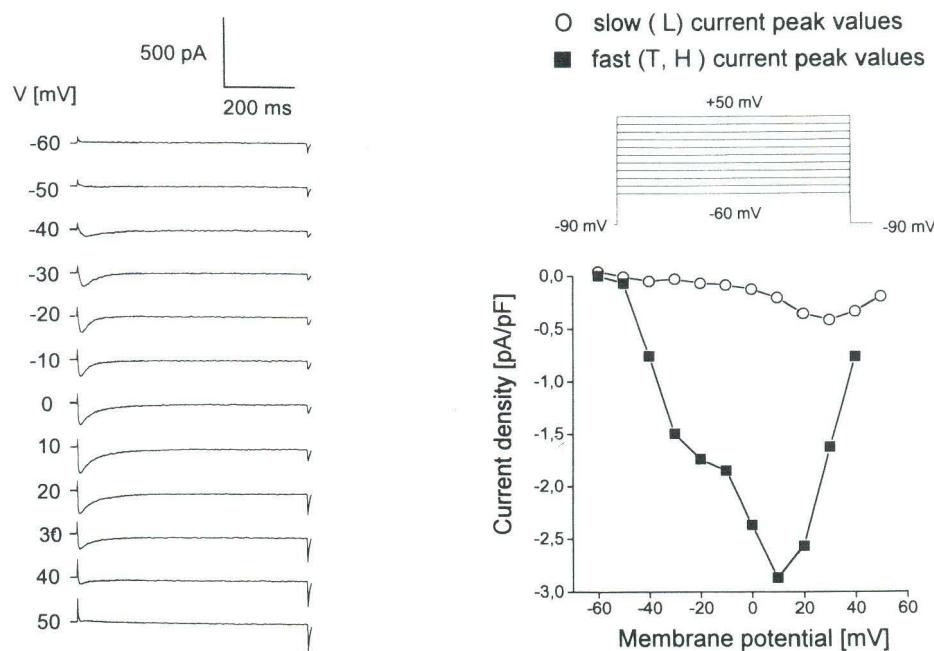




**Figure 4.** Whole-cell currents recorded on human myotubes cultured from muscle specimens obtained from normal controls (*top*) and corresponding current-voltage relations showing the peak current normalized by the linear capacitance of the given myotube (*bottom*; fast currents: *filled symbols*; slow currents: *open symbols*). The myotubes revealed a rapidly activating and inactivating calcium current with a voltage threshold at  $\sim -50$  mV (T-type current; *A*) and a slowly activating and inactivating (L-type; *A* and *B*) current. Some myotubes showed a second type of fast current exhibiting maximum inward current at more positive potential (third type; *C*).

### Voltage-gated Calcium Currents in Normal Human Myotubes

The majority of 57% of the 47 cells studied exhibited a rapidly activating and inactivating calcium current component with a voltage threshold at  $\sim -50$  mV (T-type current) in addition to the slow (L-type) current which started to activate at  $\sim -20$  mV (Fig. 4 A). In the remaining myotubes, the fast current was missing and only the L-type current could be activated (Fig. 4 B). In 34% of the total number of cells, a second type of fast current was recorded which revealed kinetics similar to the T-type but exhibited a maximum inward current at  $\sim 30$  mV more positive potential (third-type according to Rivet, Cognard, Imbert, Rideau, Duport, and Raymond, 1992). To show this component clearly, one cell was selected which had a large amplitude of the third-type current and an unusually small L-type current (Fig. 4 C).



**Figure 5.** Whole-cell currents recorded on human myotubes cultured from muscle specimens obtained from a patient (left) and a representative current-voltage relationships which shows the peak current density (right; fast currents: filled symbols; slow currents: open symbols).

Average current-voltage relationships showed that the third-type current is too small so that it could not form a distinct peak of the relation combining both fast current types (not shown). The average current density of the L-type current amounted to  $-1.41 \pm 0.71$  pA/pF when activated by a pulse to +20 mV.

### Voltage-gated Calcium Currents in Human Myotubes Expressing the Mutation

In the 20 myotubes expressing the IVS4 mutation, the L-type current density was drastically reduced to 30% of the control value: the peak value was  $-0.42 \pm 0.25$  pA/pF at +20 mV. Original traces and the determined current-voltage relationship of a representative HypoPP myotube is shown in Fig. 5. The percentage of cells expressing the third-type current was considerably increased (from 34 to 75%). Thus, we noted a significant increase in the average peak value of this fast current which exhibited its maximum at 0 to +10 mV. The differences between control and HypoPP

myotubes were statistically significant in the 0–50 mV range for the L-type and in the 10–30 mV range for the third-type current ( $P = 0.02$ ).

Because the reduction of the L-type current could have been due to a voltage-dependent inactivation to more negative potentials, which might have lowered channel availability already at the holding potential of –90 mV, we applied prepulses which hyperpolarized the membrane for 20 s to –120 mV. This did not lead to a restoration of the current amplitude and thus excluded this possible explanation.

## Discussion

CACNL1A3 co-segregates with the HypoPP locus without any recombinations, and the detected G3716A base exchange fulfills all criteria of a causal mutation such as: (a) absence in a large number of controls (100 individuals with no known neuromuscular disease); (b) segregation with the clinical status of all family members examined; (c) presence in a gene primarily or exclusively expressed in the affected cells (the skeletal muscle); (d) location in a functionally important, highly conserved gene region (a positive charge within IVS4); and (e) change of the function of the gene product (reduced L-type current observed in the HypoPP myotubes).

The results on normal human myotubes confirm an earlier investigation on the same preparation in which three different voltage-gated inward currents were found (Rivet et al., 1992). In particular, we confirm the presence of a further rapid calcium current (third-type), activated at more positive potentials than the T-type current. This current has so far only been found in cultured human skeletal myocytes (Rivet et al., 1992). Its average density was markedly increased in the HypoPP myotubes. Because of its presence also in normal myotubes this current component is unlikely to be caused by mutated L-type channels. We could not find a positive correlation between low L-type current amplitude and high third-type current amplitude in normal myotubes. This rules out the possibility that a cellular compensation mechanism generally leads to a strong third-type current whenever the L-type current is low. Yet, the mutation could alter calcium channel expression, e.g., delay downregulation of the third-type channel gene.

The significantly reduced amplitude of the L-type current density may most easily be explained by an elimination of the channel function by the point mutation. This may not be too surprising because the mutation is located in a domain which seems to be extremely important with regard to the modulation by calcium channel antagonistic drugs (see Catterall and Striessnig, 1992; Striessnig, Murphy, and Catterall, 1991). However, if both the normal and the mutant gene were producing  $\alpha_1$ -proteins (normal and nonfunctional) at the same expression rate, a reduction of the current density to no more than 50% should be expected.

A reduction to a considerably lower value might indicate an inhibitory effect of mutant channels on normal channels which could take place if the DHP receptor molecules were arranged in an oligomeric structure. Such an oligomeric arrangement might be the t-tubular tetrads (Block, Imagawa, Campbell, and Franzini-Armstrong, 1988) which are thought to consist of four DHP receptors interacting with one calcium release channel (ryanodine receptor).

If one modified monomer is sufficient to inhibit the function of the tetramer, which would be compatible with the dominant mode of inheritance of the disease (compare Steinmeyer, Lorenz, Pusch, Koch, and Jentsch, 1994), we would expect a

current reduction to 6% of the normal value corresponding to the expected percentage of normally structured tetramers. However, the current was reduced to one third of the control value; this percentage could be explained by two modified monomers needed to destroy the function of the tetramer. Thus, the data would be compatible with models in which an interaction within a tetrad of DHP receptors (Lamb, 1992; Ríos, Karhanek, Ma, and González, 1993) may be necessary for gating the L-type calcium current.

Because of the proposed dual function of the DHP receptor as calcium channel and control device for calcium release, the altered voltage sensor may also affect control of calcium release from the sarcoplasmic reticulum. Alteration of this control mechanism could also be involved in the observed clinical symptoms, i.e., paralytic attacks or permanent muscle weakness. How a fall in extracellular potassium is able to induce or to aggravate the channel dysfunction causing episodes of paralysis (Rüdel et al., 1984), is still unclear. The open questions will be further clarified by studies on both calcium currents and calcium transients in myotubes of patients and on heterologous expression of the mutant channels, contained in cells which the other four subunits of the pentameric complex required for a functional protein.

Calcium release in skeletal muscle is not dependent on calcium influx as is the case in cardiac muscle. The role of the slow L-type calcium inward current for muscle contraction is therefore unknown. HypoPP is an important system for defining the function of this current and refining the model for excitation-contraction coupling.

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