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A calcium channel mutation causing hypokalemic periodic paralysis

Karin Jurkat-Rott, Frank Lehmann-Horn*, Alexis Elbaz[1], Roland Heine, Ronald G.Gregg[2], Kirk Hogan[2], Patricia A.Powers[2], Pascale Lapie[1], José E.Vale-Santos[1], Jean Weissenbach[3] and Bertrand Fontaine[1]

Department of Applied Physiology, University of Ulm, D-89081 Ulm, Germany, [1]INSERM U134, Fédération de Neurologie, Hôpital de la Salpêtrière, 75013 Paris, France, [2]Department of Anesthesiology and the Waisman Center on Mental Retardation and Human Development, University of Wisconsin, Madison, WI 53705, USA and [3]Généthon, 91000 Evry and Pasteur Institute, 75015 Paris, France

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The only calcium channel mutation reported to date is a deletion in the gene for the DHP-receptor α1-subunit resulting in neonatal death in muscular dysgenesis mice (1). In humans, this gene maps to chromosome 1q31–32. An autosomal dominant muscle disease, hypokalemic periodic paralysis (HypoPP), has been mapped to the same region (2). Sequencing of cDNA of two patients revealed a G-to-A base exchange of nucleotide 1583 predicting a substitution of histidine for arginine[528]. This affects the outermost positive charge in the transmembrane segment IIS4 that is considered to participate in voltage sensing. By restriction fragment analysis, the mutation was detected in the affected members of 9 out of 25 HypoPP families. The results indicate that the DHP-receptor α1-subunit mutation causes HypoPP. An altered excitation–contraction coupling may explain the occurrence of muscle weakness.

INTRODUCTION

Hypokalemic periodic paralysis (HypoPP, MIM 170400) (3) is an autosomal dominant disease characterized by episodic attacks of weakness which are associated with a decrement in serum potassium concentration. Attacks of weakness vary in frequency, duration, and severity. Penetrance may be incomplete in females (4). Late onset progressive myopathy develops independently of the occurrence of attacks (5,6). The vital capacity may be reduced in severe attacks and death can occur from ventilatory failure or cardiac arrhythmia due to hypokalemia, but usually patients show a normal life span (7).

Excised HypoPP muscle specimens exhibit a sustained membrane depolarization leading to fiber inexcitability when the extracellular potassium concentration is reduced, in contrast to hyperpolarization observed in normal muscle (8). Although several mechanisms for this abnormal depolarization have been proposed, the suggested candidate genes have been excluded, e.g. the muscle sodium channel α-subunit and the chloride channel (9,10). A systematic approach demonstrated linkage of three HypoPP families to chromosome 1q31–32 and showed co-segregation with the gene encoding the DHP-receptor α1-subunit (CACLN1A3) (2) which has been localized to the same region (11). This protein is found in the membrane of the transverse tubular system and contains the calcium-selective ion pore, the voltage-sensor, and the DHP-binding sites. It appears to be involved in both calcium inward conduction and excitation–contraction coupling (12–15). So far, the only known example of a defect of this gene is a nonsense mutation in the α1-subunit found in muscular dysgenesis mice (mdg) (1). The DHP-sensitive calcium inward current and excitation-contraction coupling are both absent in this autosomal recessive lethal disorder (16). Mice heterozygeous for this mutation show a normal clinical phenotype suggesting that excitation–contraction coupling can be normal despite a reduced number of normal DHP-receptors.

RESULTS

For a more precise mapping of the HypoPP-1 locus, we extended the linkage study for the reported families A and B each showing a recombination event for one of the flanking markers D1S413 and D1S510 (2,17). Five additional AFM microsatellites located between D1S413 and D1S510, of initially undetermined order were used. By reconstructing the haplotypes in 20 HypoPP families for the chromosome 1q31–32 markers shown in Fig. 1, we were able to order all markers in respect to one another (data not shown). Since an affected member of family A (AIII2) is recombinant for both D1S413 and AFM337xd5 (Fig. 1A) but is not recombinant for the other markers, we deduced that this marker is located centromerically to AFM136xa7 (Figs. 1A and 1C). In family B (Fig. 1B), the recombination event for patient BII7 is centromeric to AFM136xa7 confirming the respective position of AFM337xd5 and AFM136xa7. As D1S306 is uninformative for both of the two crucial recombinants, the HypoPP-1 locus could not be placed relative to this microsatellite. Therefore the HypoPP-1 locus is flanked by AFM337xd5 and AFM136xa7 which are separated by a genetic distance of zero cM as calculated in the 8 CEPH families used to type the Généthon markers for chromosome 1. In order to determine physical distances between the cluster of four markers defining the locus region as well as to locate CACNL1A3 physically amongst them, we are currently building YAC contigs of the area.

Total RNA was isolated from muscle specimens obtained from one patient of family B, from two patients of additional HypoPP families also linked to chromosome 1q31–32 (HypoPP 16, 19) and from four controls. RNA was reverse-transcribed and regions of the CACNL1A3 cDNA were amplified by use of polymerase chain reaction (PCR) primers derived from human cDNA

*To whom correspondence should be addressed

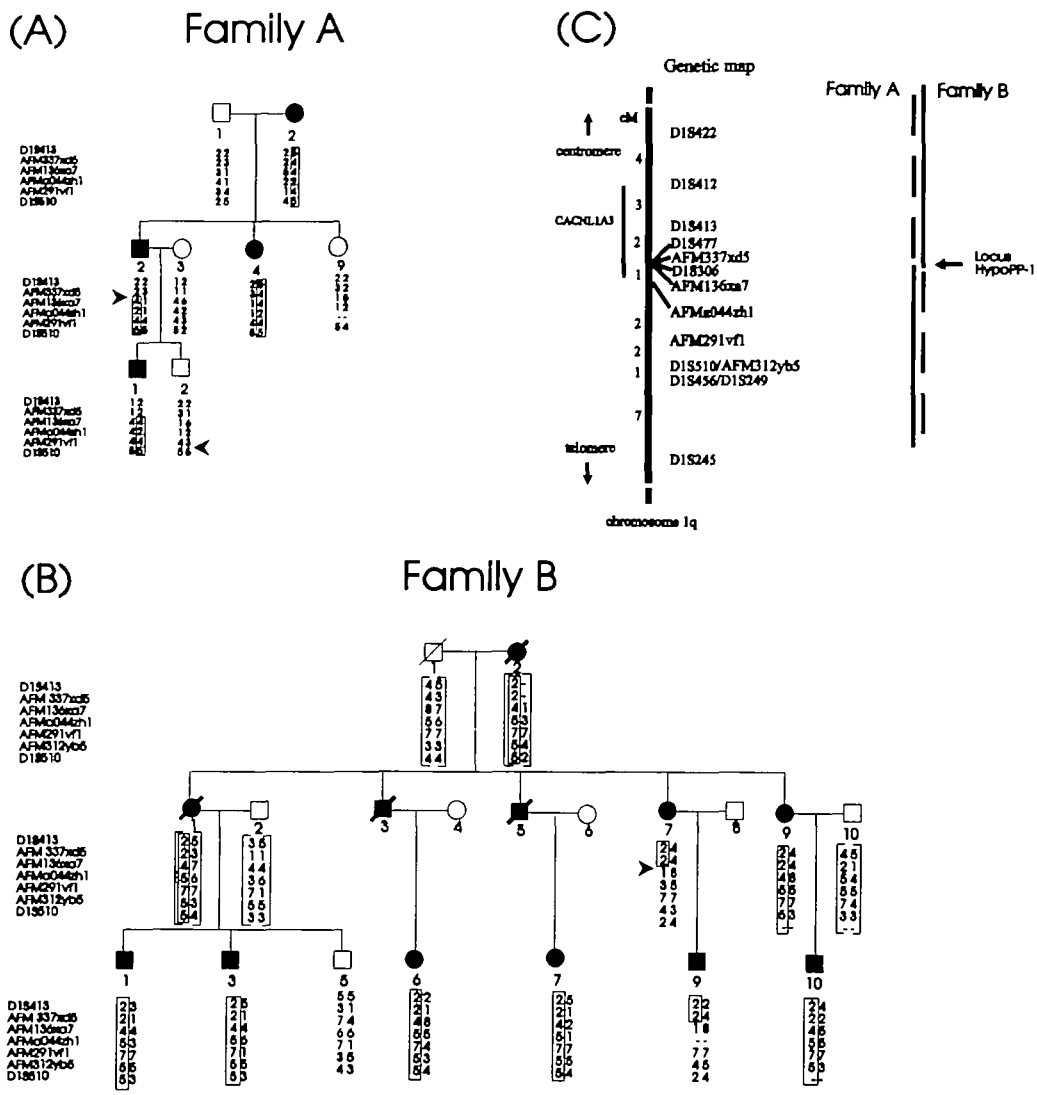

Figure 1. Partial pedigrees of families A and B and genetic map of chromosome 1q31–32. (**A, B**) Patients are represented by filled and unaffected individuals by open symbols. The haplotype segregating with the disease is boxed; recombination events are indicated by arrow-heads and deduced haplotypes by brackets. Marker AFM312yb5 was not typed for family A. The numbering of individuals is the same as in Fontaine *et al* (2) in order to permit comparison. (**C**) Genetic map of chromosome 1q31–32. Additional markers between D1S413 and D1S510 used for establishing haplotypes are shown with their respective genetic distance. Localizations of the HypoPP-1 locus and of CACNL1A3 are indicated. The likelihood of the order of the loci, genetic distances and localization of the DHP-receptor (between D1S412 and D1S477/AFM337xd5/D1S306/AFM136xa7) were established with odds over 10^3:1 after genotyping the microsatellites in 8 CEPH families using the program ILINK of the LINKAGE package (version 5.1). Sex-averaged recombinants and the order of the fixed loci were those determined in the 8 CEPH families. Recombination fractions were converted to map distances using the Haldane mapping function.

sequence (18). Amplified DNA fragments obtained by asymmetrical PCR were purified and directly sequenced. Of the protein-encoding portion of the cDNA (5619 bp), approximately 4100 bp representing the four (highly conserved) domains have been sequenced. We identified a G-to-A transition of nucleotide 1583 for the index patients of families 16 and 19 which results in an arginine to histidine substitution at position 528 (Fig. 2).

Loss of a *Bbv*I restriction site by the base exchange and design of adequate exon primers, made it possible to screen additional genomic DNA. The mutation co-segregated with HypoPP in altogether 9 of 25 families (44 affected individuals, 3 obligate carriers of the HypoPP-1 gene without clinical symptoms and 56 unaffected individuals) but could not be found in any of 102 unrelated control subjects (Fig. 3) nor in the affected members of families A and B.

The G-to-A exchange predicts a substitution of histidine for arginine^{528} located on the outside of the transmembrane segment IIS4 which appears to participate in voltage sensing (Fig. 4). This arginine is highly conserved in all voltage-gated calcium channel α1-subunits and, moreover, in all sodium channel α-subunits sequenced to date (Figure 2c). The substitution of a weakly positive histidine residue for such a conserved, strongly positive charge in the S4 voltage sensor might be important for the function of the gene product.

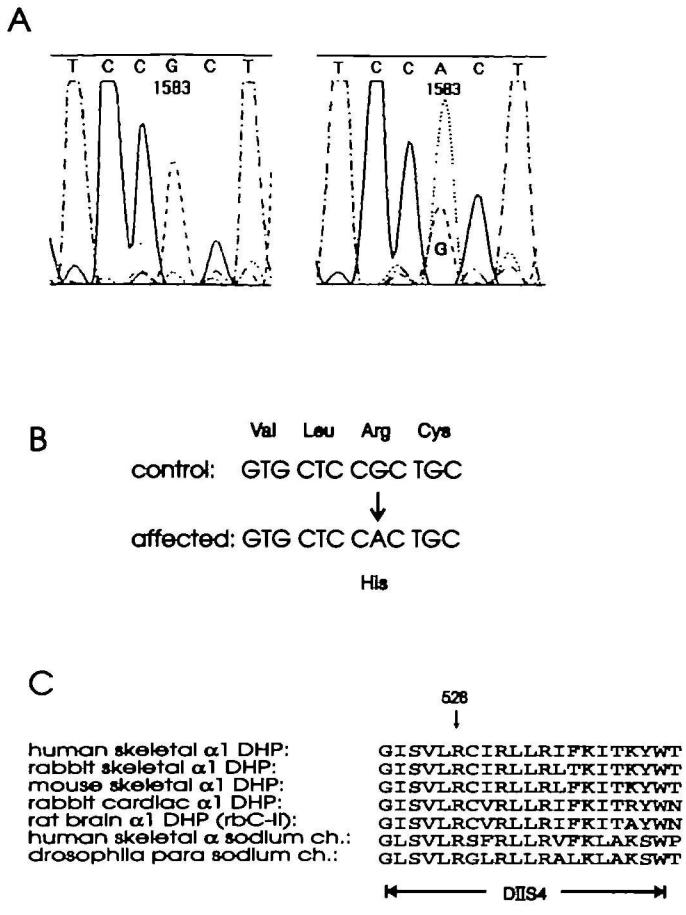

Figure 2. (A, B) Comparison of the wild-type and the mutant CACNL1A3 sequence of cDNA nucleotides 1579 to 1585. The HypoPP patient is heterozygous for a G-to-A base exchange at position 1583. The mutation predicts a substitution of histidine for arginine at position 528. (C) Alignment of deduced calcium and sodium channel sequences from various species over a part of IIS4: human skeletal muscle DHP α1-subunit (18); rabbit skeletal muscle DHP α1-subunit (23); mouse skeletal muscle DHP α1-subunit (1); rabbit cardiac muscle DHP α1-subunit (24); rat brain DHP α1-subunit (25); human skeletal muscle sodium α-subunit (26); drosophila para sodium channel (27).

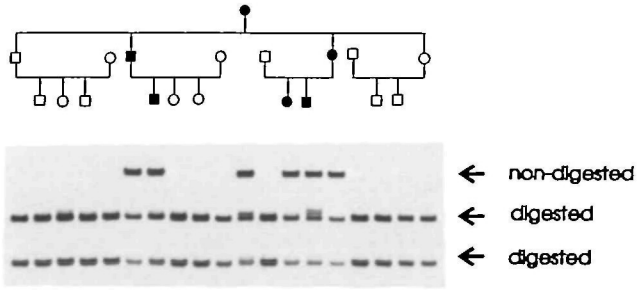

Figure 3. Pedigree of HypoPP family 19 with corresponding polyacrylamide gel displaying PCR-amplified genomic DNA fragments following digestion with *BbvI* and staining with ethidium bromide. PCR products of normal individuals show complete digestion: two bands of 44bp and 33bp; patients' DNA shows an additional band of 77bp resulting from the non-digested PCR product.

DISCUSSION

CACNL1A3 co-segregates with the HypoPP-1 locus without any recombinations, and the detected G1583A base exchange fulfills all criteria of a causal mutation such as: i) absence in a large number of controls (102 individuals with no known neuromuscular disease), ii) segregation with the clinical status (in 47 affected and 56 unaffected family members), iii) presence in a gene primarily or exclusively expressed in the affected cells (the skeletal muscle), iv) location in a functionally important, highly conserved gene region (a positive charge within the voltage sensor). Moreover, the mutation seems to be frequent among HypoPP patients since it is present in 36% of our families.

The reduced clinical penetrance in several women carrying this mutation and the potential onset of attacks during pregnancy confirm former reports (4) and may indicate a dependence of the malfunction of the channel on hormone levels (other than insulin) in addition to the well-known effect of potassium levels on provoking attacks.

How a fall in extracellular potassium is able to induce or to aggravate the channel dysfunction causing episodes of paralysis, remains to be elucidated. However, due to its location, the replaced amino acid could make the protein more sensitive to changes of the extracellular environment, i.e. protons and perhaps other monovalent cations such as potassium. A substitution of histidine for arginine in a voltage sensor of a cation channel similar to the one reported here has been detected for paramyotonia congenita (R1448H) (19). Patch clamp studies on expressed mutant sodium channels have revealed that R1448 has an extracellular position since variation of the extracellular pH influences the coupling between activation and inactivation (20). In spite of the different repeats affected in sodium and calcium channels, the homology of the mutations is striking with regard to the location of the charge-altering sustitution within the voltage sensor and points to a modification of voltage-dependent gating. 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 either mechanism could result in the observed clinical symptoms, i.e. paralytic attacks or permanent muscle weakness. The effects of the mutation on the dual function of the DHP-receptor will be clarified by studies on both calcium currents and calcium transients in myotubes of patients or other adequate expression systems. Furthermore, genetic analysis of the HypoPP family not linked to the DHP-receptor α1-subunit (21) will elucidate whether or not an alteration of another subunit of the pentameric protein can cause the identical phenotype.

MATERIAL AND METHODS

Genomic DNA was extracted from anticoagulated blood of all individuals with their informed consent. Muscle specimens were obtained from three patients and four 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 Technical University of Munich and of the Salpêtrière Hospital (Paris).

Linkage analysis

Genetic markers (17), along with the CACNL1A3 microsatellite (11), and five additional microsatellites developed at Généthon were used: AFM136xa7'(5'-AA-CTGTGTCCAGCAGCAACT-3', 5'-TATGTGCC TGTTGTGTGCAT-3'; PCR

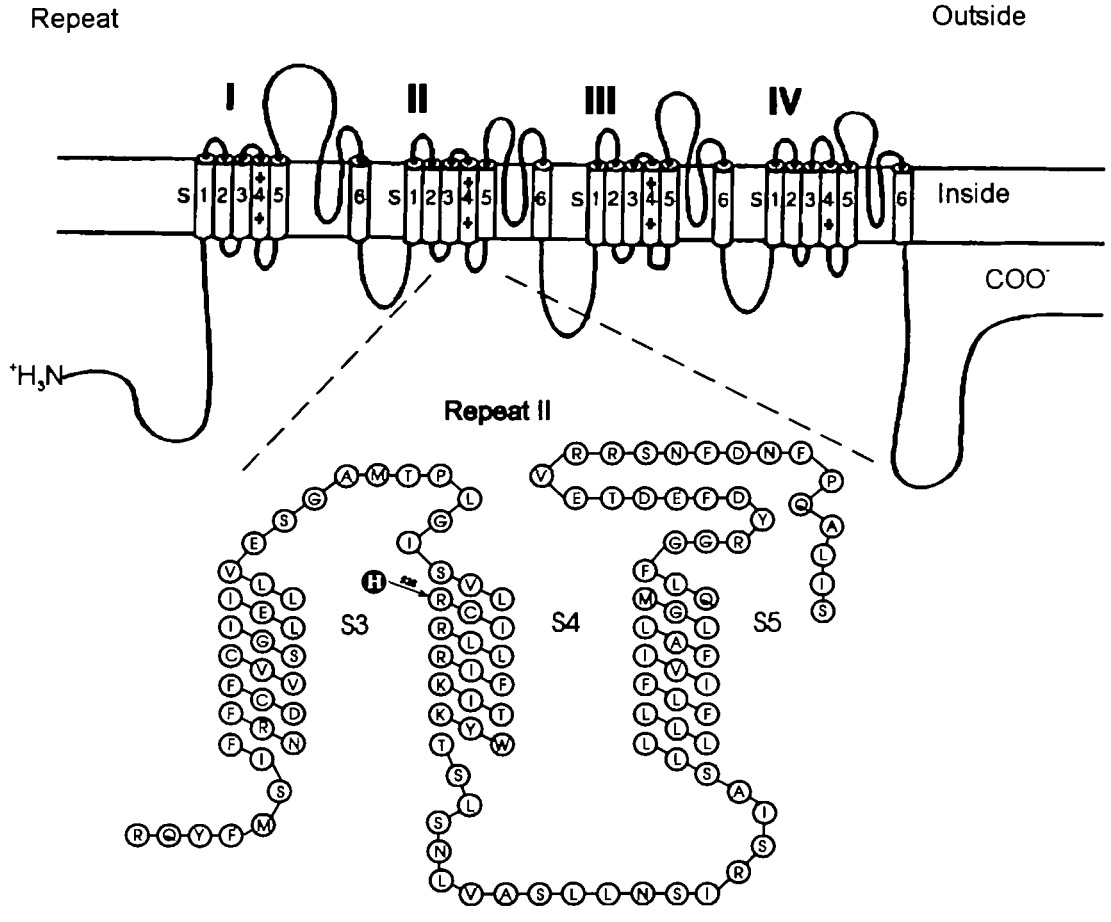

Figure 4. Schematic diagram of the DHP-receptor α1-subunit consisting of four regions of internal homology, so-called repeats, connected by intracellular loops. Each repeat contains six hydrophobic segments (S1 to S6), putative transmembrane helices. Between segments S5 and S6 of each repeat, an interlinker is found consisting of an extracellular loop and a sequence which projects into the membrane. The four intramembrane loops are thought to form the lining of the channel pore. Segment IIS4 containing positive charges at each third position is enlarged in the inset showing the mutation.

product: ≈171 bp; 8 alleles), AFM337xd5 (5'-TGCCAGTTGACCTCAAG-3', 5'-TCCAGTTGCTCCTCACC-3'; PCR product: ≈275 bp; 8 alleles), AFMa044zh1 (5'-AACCCAGCAACTCAACTCAC-3', 5'-TCTGGCTAGGGTAGGGGT-3'; PCR product: ≈111 bp; 6 alleles), AFM291vf1 (5'-AGCTTTGGGGTCTCGC-3', 5'-AGGCTATATCCAGGGACACC-3'; PCR product: ≈106 bp; 7 alleles), AFM312yb5 (5'-TTTGGACTTGAATTTACACCACTTG-3', 5'-ATCAGCCAGAGTTCTCCAGGG-3'; PCR product: ≈257 bp; 7 alleles). Genotyping of all microsatellites was performed by the polymerase chain reaction (PCR).

Reverse transcriptase PCR

Total RNA was isolated from muscle specimens using the isolation reagent TRIzol by Gibco BRL according to their protocols. Complementary DNA was synthesized in a reverse transcriptase reaction using DHP-specific primers derived from the cDNA sequence: forward primer 5'-ATGTCTATCTTCAACCGCTTCG-3', reverse primer 5'-AAGTCATACCTCCCCCCAAAG-3'. The reaction mixture with a final volume of 100 μl contained: 2 μg RNA, 50 pmol of each PCR primer, 50 μM of each deoxynucleotide triphosphate, 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 2 units of reverse transcriptase and 1.5 units of Taq polymerase. Amplification conditions: 30 min at 45°C (synthesis of cDNA), followed by 30 cycles of 94°C for 30 s, 52°C for 45 s, and 72°C for 1 min (length of PCR product: 287 bp). Direct PCR sequencing was performed as described by Heine *et al.* (22).

Screening of genomic DNA

Short fragments (length of PCR product: 77bp) of genomic DNA were amplified by PCR with primers derived from the cDNA sequence of the skeletal muscle DHP-sensitive calcium channel: forward primer 5'-GGAGATCCTGCTGGTGGAGTCG-3', reverse primer 5'-TCCTCAGGAGGCGGATGCAG-3'. The reaction mixture is similar to the one described above. 50 ng DNA were used. Amplification conditions: 10 min at 96°C, followed by 30 cycles of 94°C for 30 s, 58°C for 45 s, and 72°C for 1 min. After precipitation of the total volume of the PCR in 300 μl ethanol and redissolving in 30 μl distilled water, a digestion with 1 unit of *BbvI* (New England Biolabs) was performed at 37°C for 8 hours. The reaction was stopped with 3 μl of blue sucrose (30% sucrose, 50 mM EDTA, 0.25% bromophenol blue, 0.1% SDS). Six microlitres of the product were loaded on a 15% acrylamide gel and run at 300 V for 3–4 hours in an 1×TBE buffer. Following electrophoresis the gels were stained with 0.5 μg/ml ethidium bromide.

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Note added in proof

After submission of this paper, we detected the mutation in the affected members of family B, a G-to-A transition of nucleotide 3716 predicting a substitution of histidine for arginine 1239 located in the S4-voltage sensor of repeat IV.