Skeletal muscle DHP receptor mutations alter calcium currents in human hypokalaemic periodic paralysis myotubes

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1. Mutations in the gene encoding the α1-subunit of the skeletal muscle dihydropyridine (DHP) receptor are responsible for familial hypokalaemic periodic paralysis (HypoPP), an autosomal dominant muscle disease. We investigated myotubes cultured from muscle of patients with arginine-to-histidine substitutions in putative voltage sensors, IIS4 (R528H) and IVS4 (R1239H), of the DHP receptor α1-subunit.

2. Analysis of the messenger ribonucleic acid (mRNA) in the myotubes from such patients indicated transcription from both the normal and mutant genes.

3. In control myotubes, the existence of the slow L-type current and of two rapidly activating and inactivating calcium current components (T-type with a maximum at about −20 mV and 'third type' with a maximum at +10 to +20 mV) was confirmed. In the myotubes from patients with either mutation, the third-type current component was seen more frequently and, on average, with larger amplitude.

4. In myotubes with the IVS4 mutation (R1239H) the maximum L-type current density was smaller than control ($-0{\cdot}53 \pm 0{\cdot}31$ *vs.* $-1{\cdot}41 \pm 0{\cdot}71$ pA pF^{-1}). The voltage dependence of activation was normal, and hyperpolarizing prepulses to −120 mV for 20 s did not increase the reduced current amplitude during test pulses.

5. In myotubes with the IIS4 mutation (R528H) the L-type current–voltage relation, determined at a holding potential of −90 mV, was normal. However, the voltage dependence of inactivation was shifted by about 40 mV to more negative potentials (voltage at half-maximum inactivation, $V_{½} = -41{\cdot}5 \pm 8{\cdot}2$ *vs.* $-4{\cdot}9 \pm 4{\cdot}3$ mV in normal controls).

6. We conclude that the arginine-to-histidine exchanges enhance inactivation of the L-type calcium channel and do not alter its voltage-dependent activation.

The transverse tubular membrane of skeletal muscle contains high density dihydropyridine receptor proteins (DHP receptors) which are thought to function both as voltage-dependent calcium channels and as voltage sensors for the control of calcium release from the sarcoplasmic reticulum (see Ríos & Pizarro, 1991). The protein consists of five subunits: α1, α2/δ, β and γ (Hofmann, Biel & Flockerzi, 1994). The α1-subunit contains the putative voltage-sensing structures, receptors for calcium channel-modulating drugs, as well as the calcium-conducting pore (Catterall & Striessnig, 1992). The understanding of the structure–function relations for this polypeptide has been much advanced by studies of the mouse mutant mdg (muscular dysgenesis) which does not express the α1-subunit (Tanabe, Mikami, Niidome, Numa, Adams & Beam, 1993). Muscle cells which are

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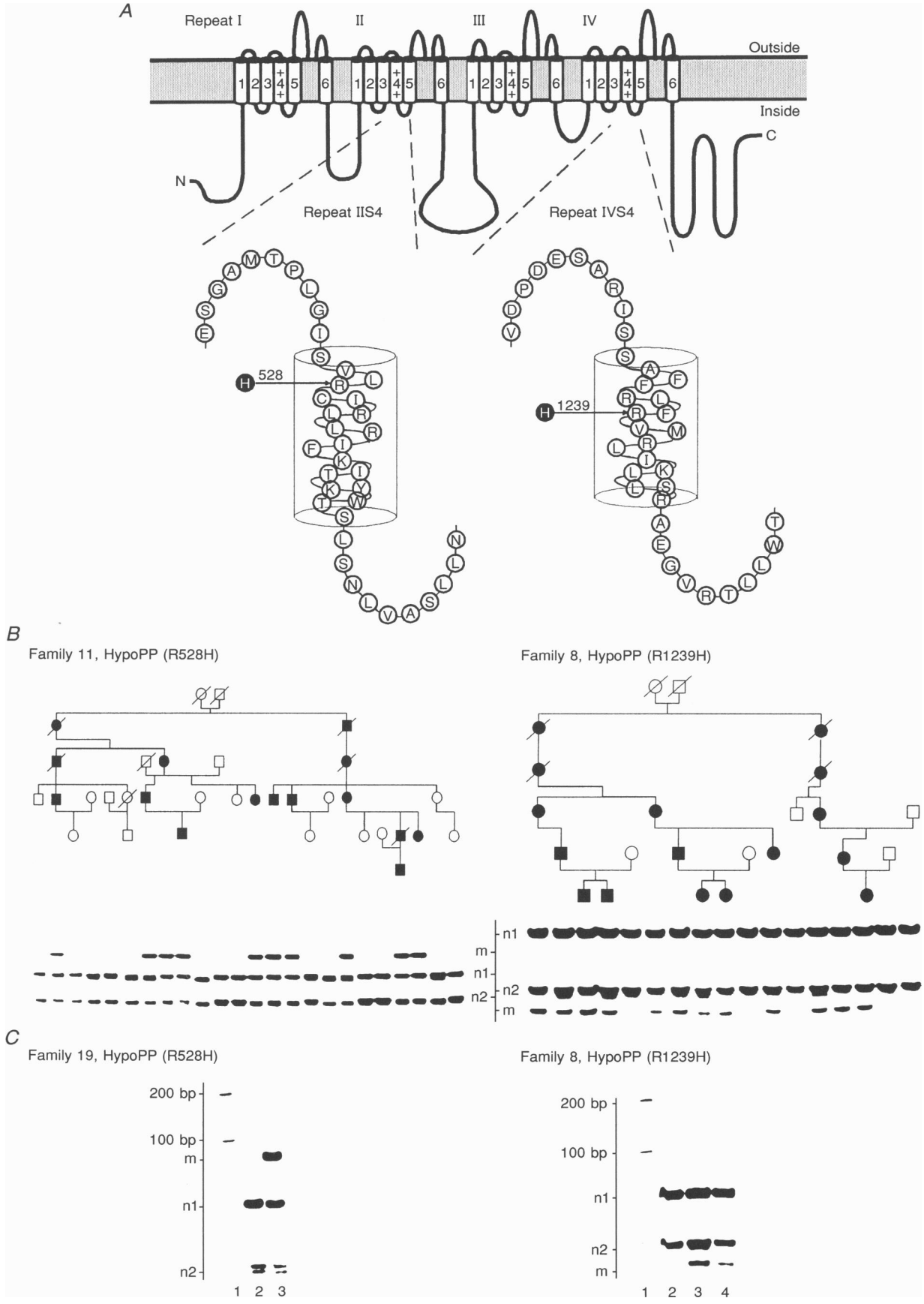

Figure 1. For legend see facing page.

homozygous for the mdg mutation lack both DHP-sensitive inward current and excitation–contraction coupling (Tanabe, Beam, Powell & Numa, 1988).

For a long time, the mdg mutation was the only known genetic disorder linked to the α1-subunit of the DHP receptor. Recently, a genome-wide search established that the human muscle disease hypokalaemic periodic paralysis (HypoPP) is also linked to the homologous human gene (CACLN1A3) located on chromosome 1q32 (Fontaine *et al.* 1994). Sequencing of the CACLN1A3 complementary DNA (cDNA) from various patients with this dominantly inherited disease revealed base exchanges predicting either a substitution of histidine for arginine^{528}, the outermost positively charged amino acid located in the transmembrane segment IIS4 (Jurkat-Rott *et al.* 1994), or a substitution of histidine or glycine for arginine^{1239} located in IVS4 (Jurkat-Rott *et al.* 1994; Ptáček *et al.* 1994).

Functional studies of the DHP receptor in HypoPP have not been published until now. Here we report voltage clamp measurements on cultured myotubes derived from biopsies of patients which contain the mutant RNA, and describe alterations in the calcium currents.

METHODS

Families

Members of twenty-four hypokalaemic periodic paralysis families gave informed consent for a thorough examination. The families were chronologically numbered for easier cross-referencing with our other publications. Of the nine families in which mutations could be found, clinical findings and electrophysiological recordings or myograms of excised native muscle fibres have been reported for families 1 and 2 (Rüdel, Lehmann-Horn, Ricker & Küther, 1984) and families 8, 11 and 16 (Iaizzo, Quasthoff & Lehmann-Horn, 1994). Genetic linkage and mutation data have been presented for family 8 (family B in Fontaine *et al.* 1994 and Jurkat-Rott *et al.* 1994) and families 11, 16, 19 and 20 (Jurkat-Rott *et al.* 1994).

Preparations

Genomic DNA was extracted from anticoagulated blood with the informed consent of each individual. Muscle specimens were obtained from four patients with HypoPP (three members of family 8 and one of family 19) and from twenty-one individuals who underwent muscle biopsy for the test of susceptibility to malignant hyperthermia; their muscles served as controls after susceptibility was excluded. The muscle specimens were taken from the vastus lateralis muscle. All procedures were in accordance with the Helsinki convention and were approved by the Ethics Committee of the University of Ulm.

Molecular biology

The genomic DNA of nineteen families had been screened for the IIS4 mutation with positive results in four families (Jurkat-Rott *et al.* 1994). The remaining fifteen families were tested for the IVS4 mutation in the following manner: short (94 bp) fragments of genomic DNA were amplified by polymerase chain reaction (PCR) with primers derived from the cDNA sequence (Hogan, Powers & Gregg, 1995) of the skeletal muscle DHP-sensitive calcium channel using 5′-CGCATCTCCAGCGCCTTCTTC-3′ as the forward primer and 5′-ACGTCCACAGGAGGGTTCGCACT-3′ as the reverse primer (annealing temperature 68 °C, for reaction mixtures and amplification conditions see Jurkat-Rott *et al.* 1994). After the total PCR product was precipitated in 300 μl ethanol and redissolved in 30 μl distilled water, it was digested by 5 units of the restriction endonuclease NlaIII (New England Biolabs) at 37 °C for 8 h. The reaction was stopped with 3 μl blue sucrose (30% sucrose, 50 mM ethylene diamine tetraacetate (EDTA), 0·25% Bromophenol Blue, 0·1% sodium dodecyl sulphate (SDS)). Six microlitres of the product were loaded on a 15% acrylamide gel and run at 300 V for 3–4 h in a buffer containing 89 mM tris(hydroxymethyl)aminomethane, 89 mM boric acid and 2 mM ethylenediamine tetraacetic acid (TBE buffer). Following electrophoresis, the gels were stained with 0·5 μg ml^{-1} ethidium bromide. The DNA of the carriers was directly PCR sequenced as described by Heine, Pika & Lehmann-Horn (1993).

For the characterization of the DHP receptor alleles expressed in the patients' muscles, RNA was extracted from cultured

Figure 1. Localization and detection of DHP receptor mutations

A, schematic diagram of the DHP receptor α1-subunit consisting of four regions of internal homology (repeats). Segments IIS4 and IVS4 are enlarged in the insets showing their amino acid sequence and the position of mutations R528H and R1239H (indicated by arrows). *B*, pedigrees of hypokalaemic periodic paralysis families 11 (left) and 8 (right). Patients are represented by filled symbols and unaffected individuals by open symbols. Below the pedigree, in alignment with the corresponding family members, the results of polyacrylamide gel electrophoresis are shown displaying PCR-amplified genomic DNA fragments followed by complete digestion with the endonucleases BbvI (left) and NlaIII (right). The gels of the unaffected individuals in both families show two bands of 44 and 33 bp (n1 and n2, respectively, left), and 55 and 39 bp (n1 and n2, respectively, right). Complete digestion of DNA from patients of family 11 (left) yields an additional band of 77 bp (m, left) resulting from loss of the restriction site by the base exchange. DNA of patients from family 8 (right) shows an additional band of 34 bp (m, right) due to the introduction of a new restriction site. The second resulting band of 5 bp is not detectable on the gel. *C*, presence of mRNA of normal and mutated DHP receptors in the myotubes used for electrophysiological experiments. The same procedure as used in *B* was applied to PCR products of myotube cDNA. The gels show the resulting bands following digestion of one control myotube (2) and one or two patients' myotubes (3 and 4; one R528H on the left and two R1239H on the right) next to an 100 bp ladder (1).

myotubes which were at the same stage of differentiation as those used for electrophysiology. The RNA was isolated with TRIzol (Gibco). Using reverse transcriptase PCR with appropriate screening primers (Jurkat-Rott *et al.* 1994), the PCR products were then screened for the two above-mentioned mutations.

Cell culture

The procedure of growing myotubes from human satellite cells followed the description by Brinkmeier, Mutz, Seewald, Melzner & Rüdel (1993). Briefly, the biopsy material was enzymatically dissociated at 37 °C in phosphate-buffered saline (PBS; Biochrom) containing collagenase (250 U ml^{-1}, Type II, Sigma) and bactotrypsin (3%, Difco). The cells were cultured in a mixture (1:1) of Ham's F-12 and CMRL media (Biochrom) containing 5% horse serum (HS: Gibco), 5% fetal calf serum (FCS; Gibco) in a 5% CO_2 atmosphere at 37 °C. After 1–2 days, the serum content of the culture medium was reduced to 2% FCS and 2% HS to induce fusion.

Calcium current recording and data analysis

Whole-cell recordings were performed on ca. 11-day-old myotubes using an EPC-7 (List) or an Axopatch 200A (Axon Instruments) patch-clamp amplifier. After partial series resistance and capacitance compensation, currents were sampled at 2–10 kHz using commercial data acquisition hard and software (TL1; Digidata 2000; pCLAMP 5.5.1 and 6.0.1; Axon Instruments) on IBM-compatible microcomputers. Calcium currents were elicited by depolarizing the cells from a holding potential of −90 mV while they were kept in a solution in which the major cations, potassium and sodium, were replaced by ions incapable of permeating the myotube membrane (Rivet, Cognard, Imbert, Rideau, Duport & Raymond, 1992). Currents were leak-corrected and normalized by the linear capacitance of the cell. For the determination of the voltage dependence of steady-state inactivation of the slowly activating and inactivating L-type current, 15 s prepulses to various potentials (V_{prep}) between −80 and +40 mV were applied, followed by a 200 ms repolarization step to −90 mV and a 4·6 s test pulse (V_{test}) to +20 mV (see Fig. 3). The voltage dependence of steady-state inactivation of the fast activating and inactivating third-type current (for definition see below) was determined by applying 1 s prepulses to potentials between −80 and 0 mV followed by a 100 ms test pulse to +10 mV (without any intermediate repolarization).

The bathing solution contained (mM): TEA-Cl, 120; $CaCl_2$, 10; $MgCl_2$, 1; Hepes, 10; glucose, 5; TTX, 0·02; EGTA, 0·1; pH 7·4 (adjusted with TEA-OH). The patch pipettes were filled with (mM): CsCl, 130; $MgCl_2$, 0·5; Hepes, 10; EGTA, 1; Mg-ATP, 5; phosphocreatine, 5; pH 7·2 (adjusted with CsOH). All measurements were performed at room temperature. In some experiments, the dihydropyridine drug nifedipine was added from a 10 mM stock solution in dimethyl sulphoxide (DMSO) to obtain a final concentration of 5 μM. Nifedipine was photolysed using intense light flashes of about 1 ms duration generated by a Xenon arc lamp (JML; G. Rapp, Dossenheim, Germany) and filtered with an UG11 (Schott) filter (see Feldmeyer, Melzer, Pohl & Zöllner, 1992).

The data are presented as means ± standard deviation. Error bars in the figures denote the standard error of the mean. Statistical significance of differences in averaged data was determined using Student's paired *t* test.

RESULTS AND DISCUSSION

Presence of mutations in genomic DNA and RNA of myotubes

Of the HypoPP families tested, families 11, 16, 19 and 20 showed the IIS4 mutation and families 1, 2, 8, 23 and 24 the IVS4 mutation. The positions of the predicted amino acid substitutions R528H and R1239H in the putative voltage-sensing structures of the channel are illustrated in Fig. 1*A*. Examples of the cosegregation of the mutations with the disease are given in Fig. 1*B*. Either mutation was also found (in addition to the normal sequence) in the RNA isolated from myotubes derived from the muscle specimens of three of the patients (examples in Fig. 1*C*). Since the RNA isolation occurred at the same culture stage used for the electrophysiogical studies, the presence of both the mutated and the normal channel protein in the myotubes could be expected.

Calcium currents in control myotubes

In agreemeent with a report in the literature (Rivet *et al.* 1992), three types of voltage-gated inward currents could be recorded in control myotubes. (i) In 43% of the cells only the slow L-type current could be found which became activated at potentials less negative than −20 mV and reached a peak of $-1{\cdot}41 \pm 0{\cdot}71$ pA pF^{-1} at +20 mV (Fig. 2*A*). This current originates from DHP receptors since it could be blocked completely by 5 μM of the dihydropyridine drug nifedipine (not shown). The block could be largely reversed by flash illumination of the cells (see Methods) thus photolysing the ultraviolet light-sensitive drug. The voltage at half-maximum inactivation ($V_{½}$) of the L-type current was $-4{\cdot}9 \pm 4{\cdot}3$ mV (Fig. 3*A*). (ii) The T-type current, detected in addition to the L-type current in the remaining 57% of the cells, was characterized by a voltage threshold of about −50 mV and reached a maximum of $-0{\cdot}43 \pm 0{\cdot}68$ pA pF^{-1} at −20 mV (Fig. 2*A*). (iii) In 34% of the total number of cells, a second type of fast calcium inward current ('third type' according to Rivet *et al.* 1992) was recorded which revealed kinetics similar to the T-type. Its maximum appeared at −20 mV, i.e. 30 mV more positive than the maximum of the T-type current. Since this current was found only in a third of the cells with generally small amplitude, it did not lead to a distinct peak in the average current–voltage relation of the fast currents (Fig. 2*A*). It could, however, be more easily detected in the patients' myotubes (Fig. 2*B* and *C*). $V_{½}$ of the third-type current was $-45{\cdot}0 \pm 4{\cdot}8$ mV (Fig. 3*A*), about 10 mV more positive than reported by Rivet *et al.* (1992). Of these three channel types, only the L-type is present in adult muscle fibres and sensitive to dihydropyridines.

Calcium currents in myotubes with the R528H mutations

The current–voltage relation of the L-type current in myotubes of the patient with the R528H mutation

(family 19) was not different from controls (Fig. 2*B*), exhibiting its threshold at −20 mV and its current maximum at +20 mV with an average density of $-1{\cdot}47 \pm 0{\cdot}52$ pA pF^{-1}. However, a strong increase in the fraction of cells conducting the third-type current from 34 to 60% lead to a pronounced second phase of the voltage dependence of fast calcium currents with peak values of $-0{\cdot}80 \pm 0{\cdot}72$ pA pF^{-1} at +20 mV (Fig. 2*B*).

Whereas the inactivation curve of the third type was not different from controls, the inactivation curve of the L-type current was markedly shifted to more negative potentials (Fig. 3*B*). Half-maximum inactivation was obtained at $-44{\cdot}8 \pm 3{\cdot}7$ mV for the third-type current and at $-41{\cdot}5 \pm 8{\cdot}2$ mV for the L-type current. Thus, inactivation of the slow current takes place at about 40 mV more negative potentials in the myotubes carrying the R528H mutation than in controls. The effect resembles that of phenylalkylamine drugs such as gallopamil (D600) and devapamil (D888) which bind to the intracellular side of segment IVS6 of the DHP receptor, selectively stabilizing a voltage-dependent inactive state (Erdmann & Lüttgau, 1989).

Since both the wild-type and the mutant channels are expressed, two L-type current components, a normal and a modified one, should be expected. However, the presence of a monophasic inactivation curve suggests a

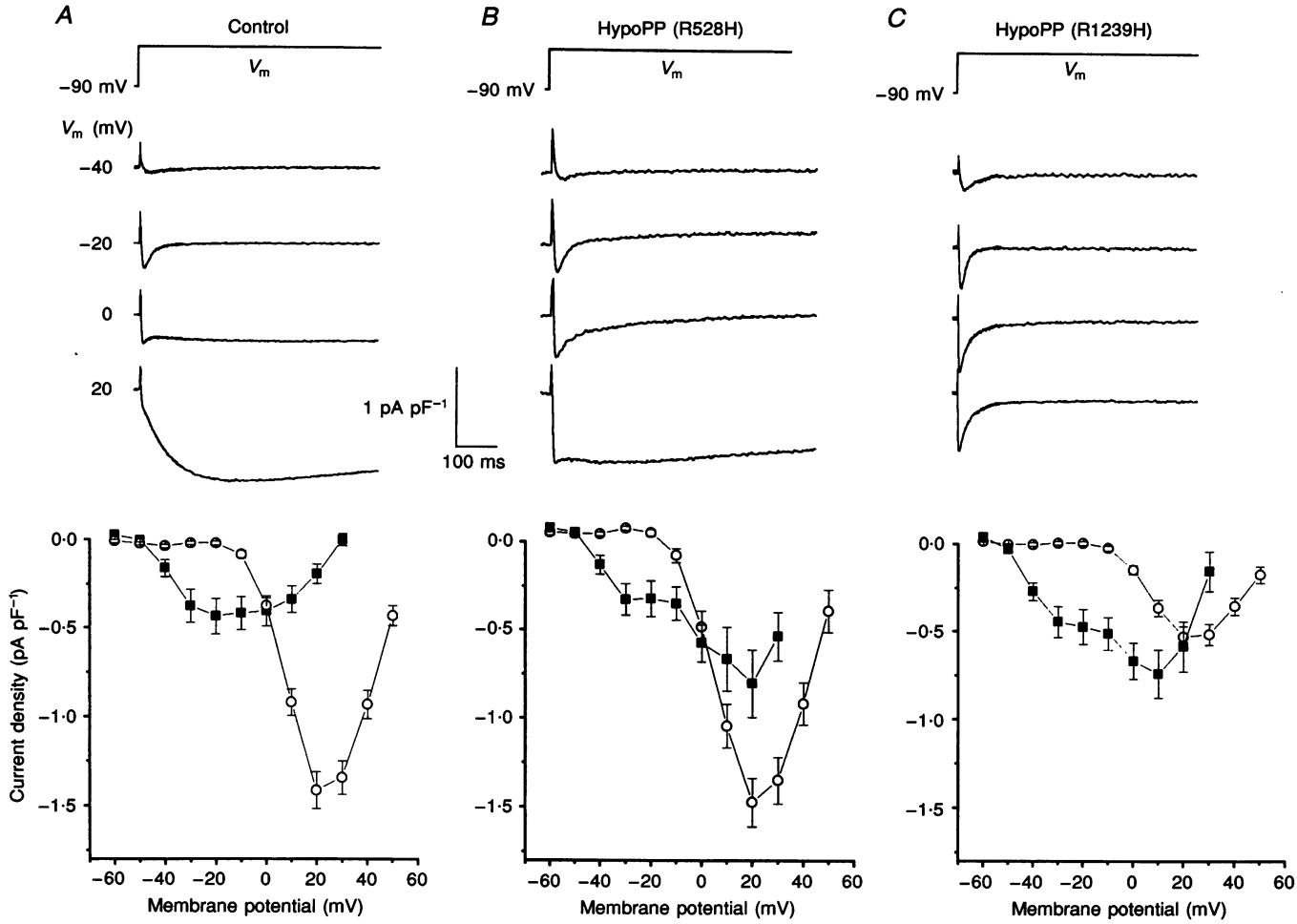

Figure 2. Voltage-dependent activation of calcium inward currents in normal and HypoPP myotubes

Voltage steps were applied from a holding potential of −90 mV to various membrane voltages (V_m, −60 to +50 mV). Selected current traces (upper panels) and current density–voltage relations (lower panels) are illustrated. Symbols represent: ■, peaks of the rapid currents (T-type and 'third type', for definition see Rivet *et al.* 1992); ○, maximum amplitudes of the slow (L-type) current. *A*, controls ($n = 47$). *B*, mutation R528H ($n = 15$). *C*, mutation R1239H ($n = 28$). The density of the DHP-sensitive L-type current is significantly reduced in the 0 to +50 mV range for the R1239H mutation (Student's paired *t* test, $P = 0{\cdot}02$) while it is unaltered in R528H. The amplitudes of the third-type current are increased for both mutations. The differences between controls and HypoPP are statistically significant in the +10 to +30 mV range ($P = 0{\cdot}02$).

homogeneous population of channels with abnormal inactivation. It might be explained by an oligomeric arrangement of the ion-conducting channels forming a functional unit in which one mutant monomer is sufficient to produce malfunction of the entire complex. Such a functional unit might be constituted by the transverse tubular tetrads (Block, Imagawa, Campbell & Franzini-Armstrong, 1988; Lamb, 1992).

Calcium currents in myotubes with the R1239H mutation

In the myotubes derived from three related patients (family 8), the peak density of the L-type current was reduced to about one-third of the control values, i.e. $-0{\cdot}53 \pm 0{\cdot}31$ pA pF^{-1} at +20 mV (Fig. 2*C*). The reduction of the average current density was statistically significant in the 0 to +50 mV range ($P = 0{\cdot}02$). Similar to the finding for R528H myotubes, the fraction of cells showing the third-type current was increased from 34 to 75% leading to a pronounced second phase of the current–voltage relation with a peak value of $-0{\cdot}74 \pm 0{\cdot}72$ pA pF^{-1} at +10 mV (Fig. 2*C*). The inactivation curve of the third-type current showed no significant deviation from the control (data see legend of Fig. 3*C*).

Since strongly reduced amplitudes made the determination of the inactivation curve of the L-type current difficult, the measurements were performed on selected myotubes with larger amplitudes. The inactivation curve obtained from these myotubes revealed a normal $V_{½}$ value ($-4{\cdot}5 \pm 2{\cdot}9$ mV) when fitted with a single Boltzmann function (Fig. 3*C*). The least-squares fit of the inactivation curve deviated from the data points due to a region of reduced slope between −50 and −20 mV. This deviation might result from the contribution of a channel population with a left-shifted inactivation curve as seen in the R528H mutation (Fig. 3*B*). To test whether the reduced

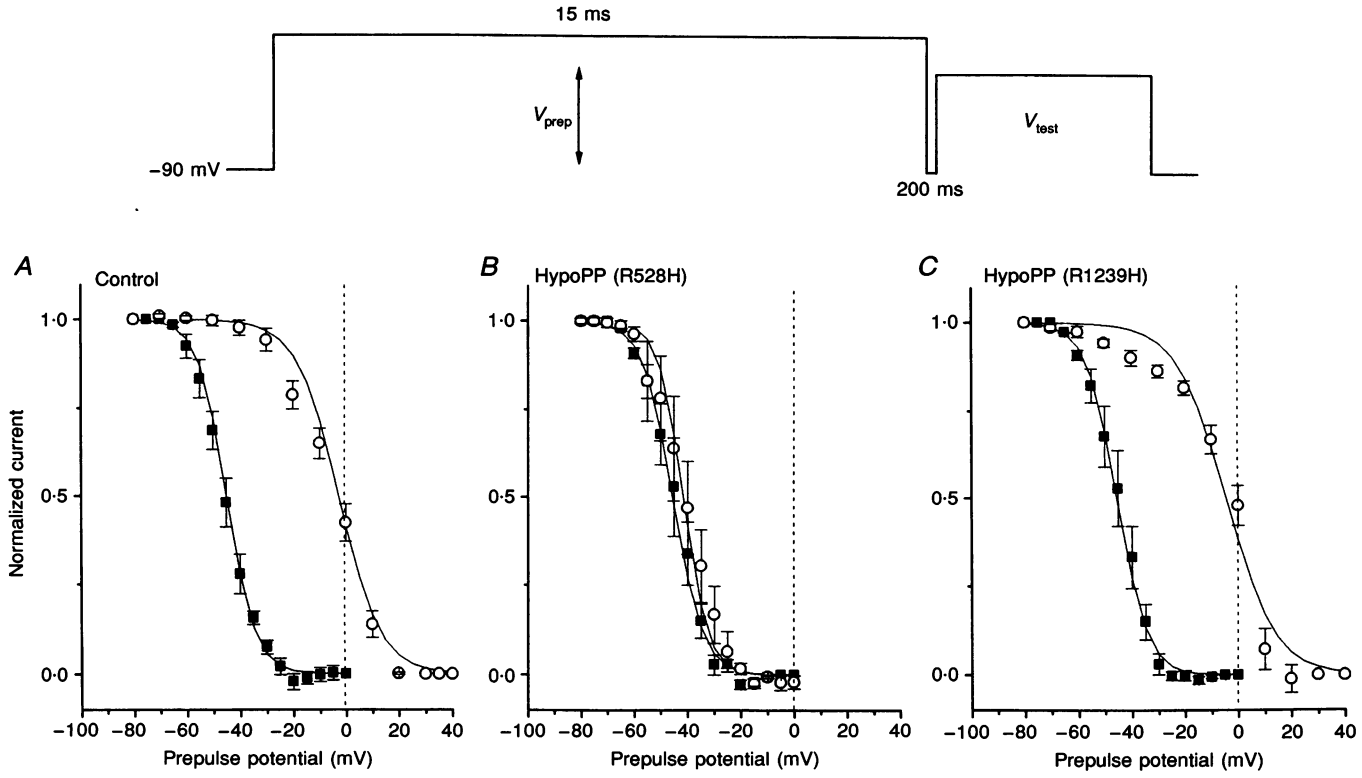

Figure 3. Voltage dependence of steady-state inactivation of calcium currents in normal and HypoPP myotubes

For the inactivation curves, the current values obtained at the test pulses with a conditioning prepulse (I_{TP}) were normalized by the test pulse response without a prepulse (I_T). Note the scheme at the top which shows the pulse protocol for the L-type current (see Methods for further information). Symbols represent: ■, third-type current; ○, L-type current. Boltzmann functions of the form $I_{TP}/I_T = 1/\{1 + \exp((V - V_{½})/k)\}$ were fitted to the data points of each experiment, where $V_{½}$ and the steepness constant k were free parameters. The continuous lines were drawn by using the mean values of the fit parameters. *A*, controls. L-type: $V_{½} = -4{\cdot}9 \pm 4{\cdot}3$ mV, $k = 8{\cdot}8 \pm 1{\cdot}3$ mV ($n = 5$); third type: $V_{½} = -45{\cdot}0 \pm 4{\cdot}8$ mV, $k = 5{\cdot}3 \pm 1{\cdot}2$ mV ($n = 3$). *B*, mutation R528H. L-type: $V_{½} = -41{\cdot}5 \pm 8{\cdot}2$ mV, $k = 4{\cdot}6 \pm 1{\cdot}5$ mV ($n = 5$); third type: $V_{½} = -44{\cdot}8 \pm 3{\cdot}7$ mV, $k = 5{\cdot}4 \pm 0{\cdot}3$ mV ($n = 3$). *C*, mutation R1239H. L-type: $V_{½} = -4{\cdot}5 \pm 2{\cdot}9$ mV, $k = 9{\cdot}1 \pm 1{\cdot}6$ mV ($n = 4$); third type: $V_{½} = -45{\cdot}5 \pm 3{\cdot}3$ mV, $k = 5{\cdot}6 \pm 0{\cdot}3$ mV ($n = 4$).

current density results from partial inactivation at the holding potential of −90 mV, we applied strong hyperpolarizing prepulses to −120 mV for 20 s. This procedure did not lead to an increase in the L-type current; thus, the reduction of the current density seems to be voltage independent. It seems possible that the channels carrying the IVS4 mutation are completely nonfunctional or permanently inactivated so that the reduced current density results from the normal channels only.

Functional implications of the DHP receptor mutations

Presence of messenger ribonucleic acid (mRNA) of both normal and mutant DHP receptors and clear deviations from the normal calcium current pattern in these myotubes, suggest the expression of modified channels in the membrane. It seems unlikely that the enhanced third-type current reflects mutant L-type calcium channels, because this current can also be found in control myotubes. Its increased average amplitude may be due to a delay in the normal downregulation of the corresponding channel. On the other hand, it is likely that the changes observed in the L-type current are direct consequences of the mutations in the α1-subunit. As this current is the main calcium current component of adult skeletal muscle fibres, the myotubes of patients can be regarded as a valuable model for the elucidation of the pathogenesis of hypokalaemic periodic paralysis.

The mutations reduce the positive charge in regions supposedly involved in voltage sensing. Our results point to an enhanced inactivation rather than an effect on voltage-dependent activation of the L-type current. Parallels to results on analogous R1448H/C mutations of the sodium channel α-subunit causing paramyotonia congenita by modifying inactivation (Chahine *et al.* 1994) can be noted. These mutations do not affect channel activation although they are also located in the IVS4 putative voltage sensor region.

How inactivation of the L-type calcium current is related to hypokalaemia-induced attacks of muscle weakness which characterize familial hypokalaemic periodic paralysis can so far only be speculated upon. The DHP receptor has been proposed to act as a calcium channel and as a control device for internal calcium release (Ríos & Pizarro, 1991); therefore, both functions may be affected. Reduced calcium release is equivalent to muscle weakness. The hypokalaemia-induced membrane depolarization observed in excised muscle fibres (Rüdel *et al.* 1984) might reduce calcium release by inactivating sodium channels and therefore suppressing action potentials as well as by a direct effect on its voltage control.

A cation binding site on the external side of the DHP receptor has been postulated, the occupancy of which is necessary for restoration of calcium release from inactivation (Ríos & Pizarro, 1991). Reduced cation binding to this site affects excitation–contraction coupling by causing a left-shift of the voltage dependence of inactivation to more negative potentials (Schnier, Lüttgau & Melzer, 1993) similar to the R528H mutation. However, small changes in monovalent cations like potassium should not cause a significant shift of the inactivation curve as long as calcium is present in millimolar concentrations. Therefore, only a major change in ion selectivity of the cation binding site would explain the high potassium sensitivity in HypoPP. Further research is required to test selectivity of the cation binding site of the mutant DHP receptors. Measurements of both calcium currents and calcium transients will be necessary in order to find out whether both functions of the DHP receptor are modified in an analogous way. We expect that continuing work on hypokalaemic periodic paralysis will clarify the function of the L-type current in skeletal muscle and refine the model for excitation–contraction coupling.

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