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Electrophysiological properties of the hypokalaemic periodic paralysis mutation (R528H) of the skeletal muscle α_{1S} subunit as expressed in mouse L cells.

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Abstract Hypokalaemic periodic paralysis (HypoPP) is an autosomal dominant muscle disease which has been linked to point mutations in the skeletal muscle L-type calcium channel α_1 subunit (α_{1S}). Here, we have introduced one of the point mutations causing HypoPP (R528H) into cDNA of the rabbit α_{1S}. Expression of either the wild-type α_{1S}or the mutant R528H α_{1S} ($\alpha_{1S-R528H}$) subunits was obtained in mouse Ltk$^-$ cells using a selectable expression vector. The $\alpha_{1S-R528H}$ subunit led to the expression of functional L-type Ca^{2+} channels. Corresponding whole-cell Ba^{2+} currents exhibit very slow activation and inactivation kinetics, typical for recombinant skeletal Ca^{2+} channel currents. Voltage-dependent activation and inactivation properties were similar for α_{1S}-and $\alpha_{1S-R528H}$, as well as their sensitivity to the dihydropyridine agonist Bay K 8644. Differences in α_{1S}-and $\alpha_{1S-R528H}$-directed channels reside in the Ba^{2+} current density, which was significantly reduced 3.2 fold in cells expressing $\alpha_{1S-R528H}$. It was concluded that the R528H mutation of α_{1S} results in minor differences in the electrophysiological properties but significantly reduces the whole-cell Ca^{2+} channel current in its amplitude.

Key words: Hypokalaemic periodic paralysis; L-type Ca^{2+} channel; Mouse L cell; Skeletal α_1 subunit; Transfection

1. Introduction

Hypokalaemic periodic paralysis (HypoPP) is a genetic muscle disorder of autosomal dominant inheritance characterized by acute attacks of muscle weakness concomitant with a decrease in the blood potassium level. Linkage studies have shown that the HypoPP gene maps to chromosome 1q31–32, and colocalizes with the gene encoding the α_1 subunit (CACNL1A3) of the skeletal muscle L-type Ca^{2+} channel [1]. The L-type Ca^{2+} channel in skeletal muscle is located in the membrane of transverse tubules and consists of five subunits, α_1, α_2/δ, β and γ. It mediates Ca^{2+} entry and acts as a voltage sensor for the control of calcium release from the sarcoplasmic reticulum [2]. Three point mutations resulting in non-conservative changes were found within the coding sequence of CACNL1A3 [3,4], establishing it as the HypoPP gene. These mutations are responsible for arginine-to-histidine (R528H, R1239H) and arginine-to-glycine (R1239G) substitutions. These mutations occur within the IIS4 and IVS4 regions of the DHP receptor α_1 subunit, that are likely to serve as the voltage sensor of this ion-conducting subunit [5].

The functional consequences of HypoPP mutations are just being explored and pioneer studies have indicated that myotubes cultured from HypoPP patients exhibit abnormal Ca^{2+} channel activity [6,7]. The mutations would result in very distinct Ca^{2+} channel behaviour: a strong reduction of current amplitude with the R1239H mutation [6,7]; and a large hyperpolarizing shift of 40 mV in the voltage-dependent inactivation with the R528H mutation [7]. These preliminary studies suggest a loss of function of HypoPP mutated Ca^{2+} channels which has now to be probed at the molecular level.

The functional consequences of HypoPP mutations can be studied at the molecular level since the cDNA encoding rabbit skeletal muscle α_1 subunit (α_{1S}) has been cloned [8,9] and expressed in mammalian cells [10]. Using muscular dysgenesis (mdg) myotubes that are specifically defective in the functional α_{1S} subunit but express the other subunits, α_2/δ, β and γ, as well as the cardiac isoform, α_{1C} [11], recomplementation with recombinant α_{1S} restored Ca^{2+} channel activity as well as excitation-contraction coupling [12]. Using mouse Ltk$^-$ cells (L cells), a cell line which is devoid of the expression of any Ca channel subunit [13], expression experiments have revealed that α_{1S} encodes by itself functional Ca^{2+} channels with typical kinetics properties of skeletal muscle L-type Ca^{2+} channels [13–16].

The purpose of our study was to characterize the channel activity of the R528H-mutated α_{1S} subunit. Because this mutation takes place inside the voltage sensor segment IIS4 (for review, see [5]), it was hypothesized that activity of the α_{1S} channel might be altered [3]. To avoid putative regulation by auxiliary subunits [10,13], we have expressed the $\alpha_{1S-R528H}$ subunit in L cells. Previous functional studies of recombinant skeletal muscle L-type Ca^{2+} channels in L cells [13,16] were performed using a cell line named LCa.11, stably transfected with rabbit α_{1S} [14]. Here we have optimized a transfection/selection procedure, using a selectable expression vector. We present evidence that the R528H mutation results in minor changes in the electrophysiological properties of the α_{1S} subunit but significantly affects current density.

2. Materials and methods

2.1. Molecular biology

The wild-type rabbit α_{1S} cDNA subcloned into the pCEP4 expression vector containing a selectable marker (Invitrogen) was generously provided by Dr. L. Garcia (Paris). Transfection with this construct (pCEP4α_{1S}) confers resistance to cells cultivated in the presence of hygromycin B [17]. The G1583A mutation in the α_{1S} cDNA, which results in R528H substitution in the α_{1S} protein ($\alpha_{1S-R528H}$), was introduced using a site-directed mutagenesis procedure (Muta-gene, Bio-Rad) using the phosphorylated reverse oligonucleotide 5′-CGGATGCAGTGCAACACGG-3′ and verified by sequencing (Sequenase, USB). The final construct that contains the mutation was

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named pCEP4$\alpha_{1S-R528H}$. Plasmid DNA for mammalian transfection was purified by adsorption to macroporous silica gel anion exchange columns (Qiagen). The expression of either α_{1S} or $\alpha_{1S-R528H}$ mRNA in transfected cells was verified using RT-PCR. Total RNA from the transfected cells was prepared as described earlier [18]. Reverse transcription was performed using Superscript II (Gibco), according to the manufacturer's instruction. The PCR amplification was performed as described earlier [3]. The presence of the G1583A mutation in $\alpha_{1S-R528H}$ transfected cells was verified by the loss of a *Bbv*I restriction site in the corresponding sequence [3].

2.2. Cell culture and transfection

The Ltk$^-$ cells (mouse L cells) were grown in DMEM supplemented with 10% fetal calf serum, 1.5 mM glutamine, 0.1 μg/ml streptomycin and 100 UI/ml penicillin (Eurobio). The day before transfection, cells were plated to 40–50% confluency on glass coverslips. The transfection was performed using Lipofectamine (Gibco), according to the manufacturer's instructions. The day after transfection, culture medium was supplemented with 200 μg/ml hygromycin B (Sigma), and maintained for up to 5 days in this culture condition, prior to electrophysiology.

2.3. Electrophysiology and data analysis

Barium (Ba^{2+}) currents were recorded in the whole cell configuration as described earlier [16,19]. The bathing solution was (in mM): $Ba(OH)_2$ 40; glutamate, 40; *N*-methyl-D-glutamine, 80; HEPES, 10; $MgCl_2$, 2; pH adjusted to 7.4 with CH_3SO_3H. Pipettes were filled with (in mM): *N*-methyl-D-glutamine, 110; EGTA, 15; HEPES, 10; $MgCl_2$, 2 pH adjusted to 7.3 with CH_3SO_3H. Pipettes had resistances between 2 and 5 MΩ. Capacitive transients were minimized using the analog circuitry of the amplifier (Axopatch 200A, Axon Instruments, CA). Ba^{2+} currents were recorded at various digitizing rates and filtered at 500 Hz using a four-pole Bessel filter. Stimulation of cells, data acquisition and analysis were performed using the pCLAMP package (version 5.5; Axon Instruments) and Excel (version 5; Microsoft). Inactivation curves were fitted with the Boltzmann equation ($I = 1 - 1/[1+\exp((V-V_{0.5})/k)]$), where $V_{0.5}$ represents the potential for half-inactivation. Activation curves were deduced from the current-voltage relationships according to a modified Boltzmann equation ($I = g(V-V_{rev})/[1+\exp((V-V_{0.5})/k)]$), where $V_{0.5}$ represents the potential for half-activation. Values were expressed as mean ± S.E.M. Statistical comparisons between groups of values were made using Student's unpaired *t*-test, where $P < 0.05$ was considered significant.

3. Results and discussion

To test whether the altered behaviour of L-type skeletal muscle Ca^{2+} channels in HypoPP myotubes [6,7] was specifically related to abnormal activity of the α_{1S} subunit, it was necessary to study the functional properties of $\alpha_{1S-R528H}$ recombinant channels in a suitable expression system. Mouse L cells that dit not present any Ca^{2+} channel activity ($n = 42$; see Fig. 1A) even in the presence of 1 μM Bay K 8644 ($n = 15$; not shown) were chosen. For the purpose of our study, α_{1S} and $\alpha_{1S-R528H}$ cDNAs were subcloned in a selectable expression vector (pCEP4) containing the gene encoding hygromycin-B-phosphotransferase (see section 2). Following transfection with pCEP4α_{1S} or pCEP4$\alpha_{1S-R528H}$, cells surviving in the presence of hygromycin B (200 μg/ml) for up to 5 days (less than 5%) were studied for their expression of Ca^{2+} channels. Ba^{2+} current recordings clearly indicated that expression of α_{1S} or $\alpha_{1S-R528H}$ leads to functional Ca^{2+} channels (Fig. 1B and C, respectively). This result rules out the hypothesis that HypoPP Ca^{2+} channels might be silent. Ba^{2+} currents were detectable in 100% of the tested cells ($n = 58$), demonstrating the reliability of the transfection/selection procedure.

Here we show that both α_{1S}-and $\alpha_{1S-R528H}$-directed currents exhibit very slow activation kinetics (Fig. 1B,C). The time-to-peak was 3.6 ± 0.8 s ($n = 5$) and 3.3 ± 0.7 s ($n = 7$) for α_{1S} and

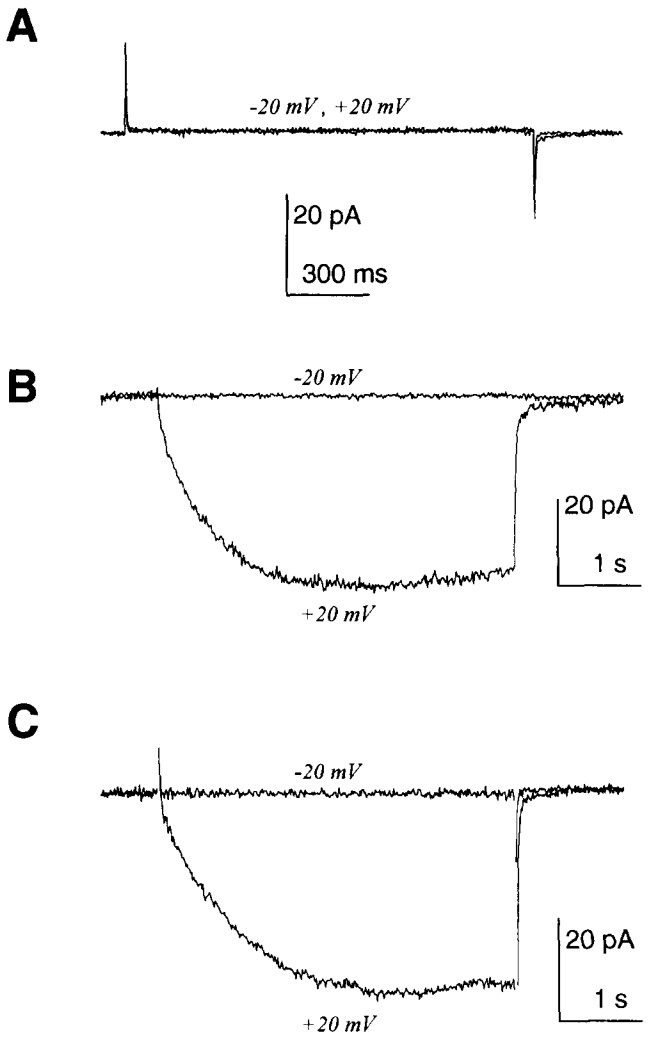

Fig. 1. Ba^{2+} currents in mouse L cells expressing α_{1S} or $\alpha_{1S-R528H}$. Holding potential (HP) was −80 mV. The presented traces were recorded for two depolarizing test pulses (−20 and +20 mV). (A) No inward current was recorded in a non-transfected cell using a 1.5 s pulse. The membrane capacitance of this cell was 61 pF. (B) Inward Ba^{2+} current recorded in a cell transfected with pCEP4α_{1S}, using a 5 s pulse. The membrane capacitance of this cell was 48 pF. (C) Similar to B, for a cell transfected with pCEP4$\alpha_{1S-R528H}$. The membrane capacitance of this cell was 79 pF.

$\alpha_{1S-R528H}$, respectively. The current voltage (*I/V*) relationships for α_{1S}-and $\alpha_{1S-R528H}$-directed Ba^{2+} currents can be superimposed (Fig. 2A). Activation occurred near −10 mV and the peak of the *I/V* curves was obtained at +20 mV. Inactivation kinetics were determined using a 1 min depolarizing pulse, which is required to reach complete inactivation (see also [13]), both for α_{1S}-and $\alpha_{1S-R528H}$-directed Ba^{2+} currents (Fig. 2B). Time constants of inactivation were found to be similar for α_{1S}-directed Ba^{2+} currents ($\tau = 8.3 \pm 2.4$ s; $n = 3$) and for $\alpha_{1S-R528H}$-directed Ba^{2+} currents ($\tau = 9.0 \pm 1.2$ s; $n = 6$) recorded at +20 mV. Voltage-dependent activation and inactivation curves were constructed for α_{1S}-directed Ba^{2+} currents (Fig. 2C) and for $\alpha_{1S-R528H}$-directed Ba^{2+} currents (Fig. 2D). The potential for half-activation ($V_{0.5}$) was −14 ± 3 mV ($n = 6$) for α_{1S}, and −9 ± 3 mV ($n = 5$) for $\alpha_{1S-R528H}$. These values were not significantly different when compared using Student's unpaired *t*-test ($P > 0.05$). Particular attention was given to determine steady-state inactivation

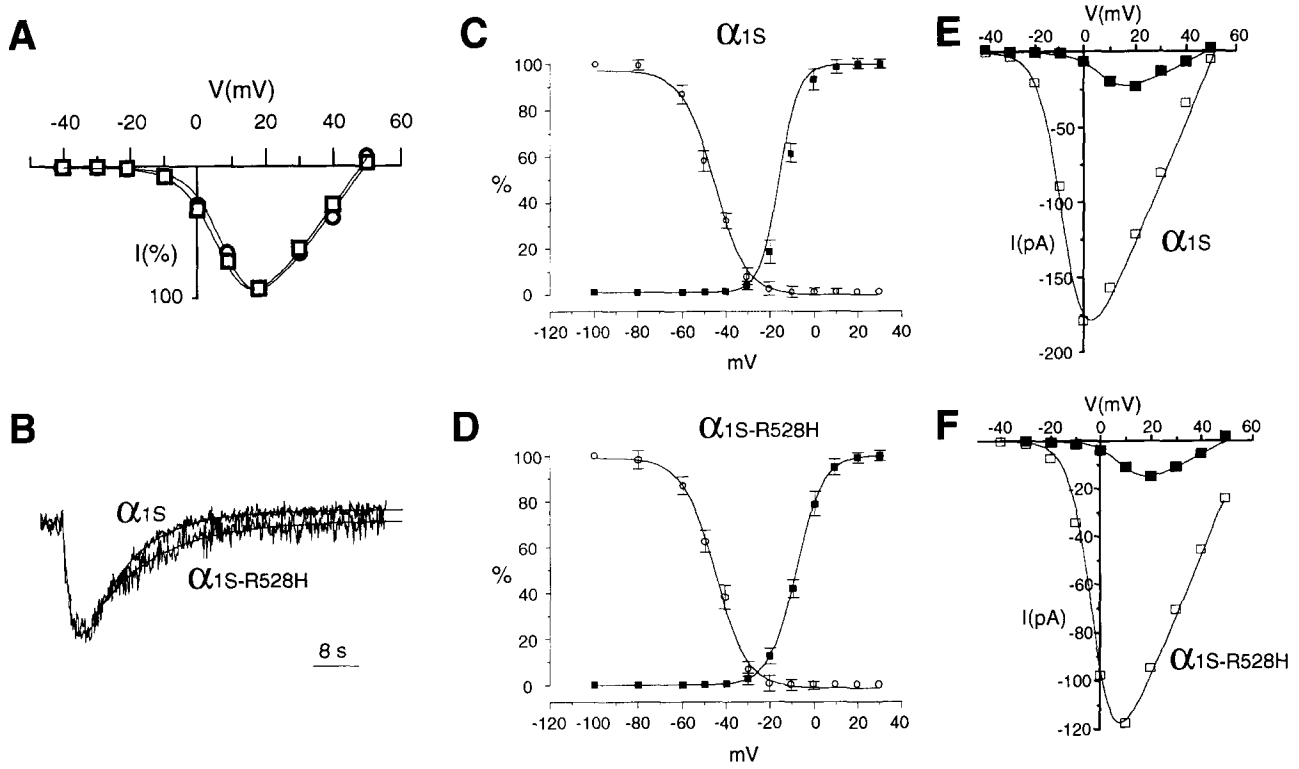

Fig. 2. Electrophysiological properties of α_{1S}-and $\alpha_{1S-R528H}$-directed Ba^{2+} currents. (A) Normalized current-voltage relationships for α_{1S}(squares, $n=3$), and $\alpha_{1S-R528H}$ (circles, $n=4$). (B) Superimposition of traces recorded during 1 min pulses to +20 mV (HP −80 mV) in a cell transfected with α_{1S} (upper trace), and a cell transfected with $\alpha_{1S-R528H}$ (lower trace). (C) Voltage-dependent activation (filled squares) and inactivation (open circles) curves for α_{1S}-directed Ba^{2+} currents. (D) Voltage-dependent activation (filled squares) and inactivation curves (open circles) for $\alpha_{1S-R528H}$-directed Ba^{2+} currents. (E) *I/V* curve for control (filled squares) and Bay K 8644-stimulated (open squares) currents in a cell transfected with α_{1S}. (F) *I/V* curve for control (filled squares) and Bay K 8644-stimulated (open squares) currents in a cell transfected $\alpha_{1S-R528H}$.

properties precisely. The prepulse duration was 90 s, and the cells were stimulated every 4 min to allow total recovery of the maximum of current amplitude between two episodes. Indeed, the potential values for half-inactivation were similar (-46 ± 3 mV, $n=7$ and -45 ± 4 mV, $n=9$; for α_{1S} and $\alpha_{1S-R528H}$, respectively). Both for α_{1S}-and $\alpha_{1S-R528H}$-directed Ba^{2+} currents were sensitive to a dihydropyridine agonist, Bay K 8644 (Fig. 2E,F), and a dihydropyridine antagonist, PN 200-110 (not shown). Following Bay K 8644 application (1 μM), the currents were enhanced 6–7 fold in average for α_{1S} ($n=6$) and for $\alpha_{1S-R528H}$ ($n=5$). A leftward shift of the *I/V* curve (10 mV) was observed following Bay K 8644 application, as previously described [13]. Altogether, our data indicate that α_{1S}-and $\alpha_{1S-R528H}$-directed Ba^{2+} currents are similar in their electrophysiological parameters.

The most striking effect related to the expression of recombinant $\alpha_{1S-R528H}$ channels was observed on current density (Fig. 3). To analyze this parameter more precisely, the transfection procedure was carefully controlled and the current recordings were performed using a double blind strategy. The Ba^{2+} current density was 3.2 fold lower in L cells transfected wit $\alpha_{1S-R528H}$ (0.24 ± 0.06 pA/pF, $n=15$), compared to the cells transfected with wild-type α_{1S} (0.78 ± 0.26 pA/pF, $n=14$). This result, obtained from 3 independent experiments, is illustrated in figure 3.

Thus, an important finding of our study is that the point mutation R528H significantly reduces the Ba^{2+} current density in L cells. Under our experimental conditions, we can postulate that the amplitude of Ba^{2+} currents is a good index of the α_1-directed Ca^{2+} channel function. The decreased activity of $\alpha_{1S-R528H}$ channels can be due to either abnormal electrophysiological properties or to alterations at the protein level, such as maturation or traffic. To date, three point mutations (R528H, R1239H and R1239G) of α_{1S}, linked to the HypoPP disease, have been described [3,4]. The relationship between various genotypes (R528H and R1239H) and specific phenotypes is not immediately obvious, since no clinical signs preferentially associated with either mutation has been found [20]. In human myotubes, several types of Ca^{2+} channels coexist [21], which makes their electrophysiological dissection difficult. Nevertheless, recent studies by Lehmann-Horn and co-workers [6,7] have described a strong reduction in the amplitude of the slow L-type Ca^{2+} current in cultured myotubes from patients with the R1239H mutation. Unexpectedly, no such reduction in current amplitude was observed in myotubes from a patient with the R528H mutation [7]. Because HypoPP myotubes also express the wild type isoform, α_{1S}, as well as the auxiliary subunits, α_2/δ, β and γ, it is possible that some compensatory mechanisms mask the mere biophysical consequence of the HypoPP point mutation identified in our study.

The decrease in Ba^{2+} current density with $\alpha_{1S-R528H}$ is unlikely to be caused by a change in the macroscopic electrophysiological properties of $\alpha_{1S-R528H}$-directed channels, since we did not observe any significant differences in the Ba^{2+} current parameters, such as kinetics, voltage-dependent acti-

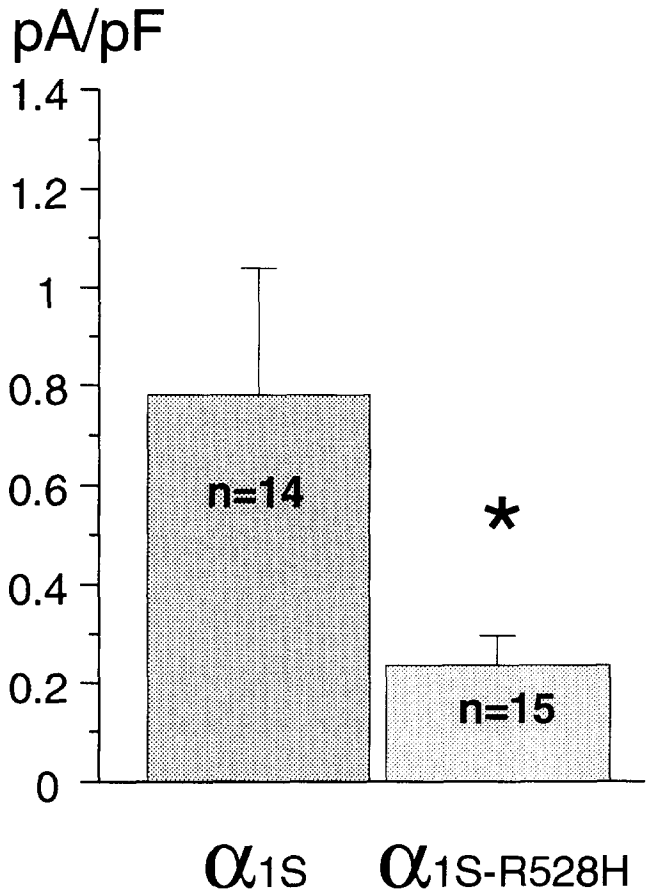

Fig. 3. Histograms representing averaged current density in cells transfected with α_{1S} (left) and cells transfected with $\alpha_{1S-R528H}$ (right). The difference was found significantly different ($P < 0.05$) using unpaired Student's t-test (asterisk).

vation and inactivation, when compared to α_{1S}-directed channels. Moreover, our data regarding wild-type α_{1S} recombinant channels clearly match with previous studies performed in L cells, and refering to the LCa.11 cell line [13–16,19]. Surprisingly, Sipos et al. [7] reported that R528H mutation, but not R1239H, resulted in a profound change in the voltage-dependence of inactivation, since the potential for half-inactivation was shifted by 40 mV towards negative potentials. This latter result, was obtained in cultured myotubes of only one patient and should be substantiated. Replacement of arginine, a positively charged residue, into histidine corresponds to a neutralizing mutation. Comparatively, a similar mutation (R1448H) occurs in the IVS4 segment of the α subunit of Na^+ channels (hSkM1) of patients with paramyotonia congenita and is responsible for only a slight hyperpolarizing shift in steady-state inactivation: less than 5 mV [22]. This behaviour was observed with recombinant Na^+ channels when the R1448H mutation was introduced either into the rat or the human SkM1 sequences [22]. Whether the R528H mutation is directly responsible for a large hyperpolarizing shift (40 mV) in the steady-state inactivation should have been observed in our experiments. Another possibility is that auxiliary subunits, which are missing in Ltk^- cells, may exert a distinct modulatory role on α_{1S}-and $\alpha_{1S-R528H}$-directed Ba^{2+} currents. It is unlikely that the discrepancies between our study and Sipos et al. [7] are related to the use of α_{1S} cDNA from rabbit in our experiments. Indeed, the full length cDNA encoding for the human α_{1S} has recently been cloned [23] and shows 92% of homology with its rabbit counterpart [8,9]. Within the IIS4 segment, the homology reaches 95% and only a conservative amino-acid substitution can be found. Therefore, from the model of the R1448H mutation of the Na^+ channel described above, it is tempting to speculate that the R528H mutation is equivalent in terms of function when introduced within the rabbit α_{1S} sequence.

The preliminary electrophysiological studies of the HypoPP mutations have suggested a loss of function as the major alteration of the mutated Ca^{2+} channels (for a recent review, see [24,25]). Our study indicates that this phenomenon relies directly to a reduced Ca^{2+} channel activity of $\alpha_{1S-R528H}$, independently of a substantial change in electrophysiological parameters. How a decrease in Ca^{2+} current amplitude might interfere with proper excitation-contraction coupling is still unclear. Moreover, the origin of muscle paralysis and decrease in blood potassium [26] remains unexplained. Consequently, further studies of the mutated $\alpha_{1S-R528H}$ protein should also probe additional parameters, such as subunit interaction, protein quantitation or cellular localization, that would provide a better understanding of the HypoPP Ca^{2+} channel defect.

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