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\textsubscript{1S} 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} (α_{1S-R528H}) subunits was obtained in mouse Ltk\textsuperscript{-} cells using a selectable expression vector. The α_{1S-R528H} subunit led to the expression of functional L-type Ca\textsuperscript{2+} channels. Corresponding whole-cell Ba\textsuperscript{2+} currents exhibited very slow activation and inactivation kinetics, typical for recombinant skeletal Ca\textsuperscript{2+} channel currents. Voltage-dependent activation and inactivation properties were similar for α_{1S}- and α_{1S-R528H}, as well as their sensitivity to the dihydropyridine agonist Bay K 8644. Differences in α_{1S}- and α_{1S-R528H}-directed channels reside in the Ba\textsuperscript{2+} current density, which was significantly reduced 3.2 fold in cells expressing α_{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\textsuperscript{2+} channel current in its amplitude.

Key words: Hypokalaemic periodic paralysis; L-type Ca\textsuperscript{2+} channel; Mouse L cell; Skeletal α_{1S} 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_{1S} subunit (CACNL1A3) of the skeletal muscle L-type Ca\textsuperscript{2+} channel [1]. The L-type Ca\textsuperscript{2+} channel in skeletal muscle is located in the membrane of transverse tubules and consists of five subunits, α_{1}, α_{2δ}, β, and γ. It mediates Ca\textsuperscript{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_{1S} 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\textsuperscript{2+} channel activity [6,7]. The mutations would result in very distinct Ca\textsuperscript{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\textsuperscript{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_{1S} subunit (α_{1S}) has been cloned [8,9] and expressed in mammalian cells [10]. Using muscular dystrophy (mdg) myotubes that are specifically defective in the functional α_{1S} subunit but express the other subunits, α_{2δ}, β, and γ, as well as the cardiac isoform, α_{1C} [11], recombination with recombinant α_{1S} restored Ca\textsuperscript{2+} channel activity as well as excitation-contraction coupling [12]. Using mouse Ltk\textsuperscript{-} 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\textsuperscript{2+} channels with typical kinetics properties of skeletal muscle L-type Ca\textsuperscript{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 α_{1S-R528H} subunit in L cells. Previous functional studies of recombinant skeletal muscle L-type Ca\textsuperscript{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 (α_{1S-R528H}), was introduced using a site-directed mutagenesis procedure (Muta-gene, Bio-Rad) using the phosphorylated reverse oligonucleotide 5'CGGATGCAGTGCAACACGG-Y and verified by sequencing (Se-quence, USB). The final construct that contains the mutation was
named pCEP4αS-R528H. Plasmid DNA for mammalian transfection was purified by adsorption to macroporous silica gel anion exchange columns (Qagen). The expression of either α1S or α1S-R528H mRNA in transfected cells was verified using RT-PCR. Total RNA from the transfected cells was prepared as described earlier [19]. Reverse transcription was performed using Superscript II (Gibco), according to the manufacturer’s instructions. The PCR amplification was performed as described earlier [3]. The presence of the GI583A mutation in α1S-R528H transfected cells was verified by the loss of a BbVI 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 mM 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 (Ba2+) 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; MgCl2, 2; pH adjusted to 7.4 with CH3SO3H. Pipettes were filled with (in mM): N-methyl-D-glutamine, 110; EGTA, 15; HEPES, 10; MgCl2, 2; pH adjusted to 7.3 with CH3SO3H. Pipettes had resistances between 2 and 5 MΩ. Capacitive transients were minimized using the analog circuitry of the amplifier (Axopatch 200A, Axon Instruments, CA). Ba2+ 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 and maintained for up to 5 days (less than 5%) were studied for their expression of Ca2+ channels. Mouse L cells that did not present any Ca2+ 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, α1S and α1S-R528H cDNAs were subcloned in a selectable expression system. Mouse L cells which were transfected with pCEP4αS-R528H were used to study the functional properties of α1S and α1S-R528H recombinant channels in a suitable expression system. Mouse L cells that did not present any Ca2+ 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, α1S and α1S-R528H cDNAs were subcloned in a selectable expression vector (pCEP4) containing the gene encoding hygromycin-B-phosphotransferase (see section 2). Following transfection with pCEP4αS or pCEP4αS-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 Ca2+ channels. Ba2+ current recordings clearly indicated that expression of α1S or α1S-R528H leads to functional Ca2+ channels (Fig. 1B and C, respectively). This result rules out the hypothesis that HypoPP Ca2+ channels might be silent. Ba2+ currents were detectable in 100% of the tested cells (n = 58), demonstrating the reliability of the transfection/selection procedure.

Here we show that both α1S- and α1S-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 α1S and α1S-R528H, respectively. The current voltage (IV) relationships for α1S- and α1S-R528H-directed Ba2+ currents can be superimposed (Fig. 2A). Activation occurred near −10 mV and the peak of the IV 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 α1S- and α1S-R528H-directed Ba2+ currents (Fig. 2B). Time constants of inactivation were found to be similar for α1S-directed Ba2+ currents (τ = 8.3 ± 2.4 s; n = 3) and for α1S-R528H-directed Ba2+ currents (τ = 9.0 ± 1.2 s; n = 6) recorded at +20 mV. Voltage-dependent activation and inactivation curves were constructed for α1S-directed Ba2+ currents (Fig. 2C) and for α1S-R528H-directed Ba2+ currents (Fig. 2D). The potential for half-activation (V0.5) was −14 ± 3 mV (n = 6) for α1S, and −9 ± 3 mV (n = 5) for α1S-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

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**Fig. 1.** Ba2+ currents in mouse L cells expressing α1S or α1S-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 Ba2+ current recorded in a cell transfected with pCEP4αS, using a 5 s pulse. The membrane capacitance of this cell was 48 pF. (C) Similar to B, for a cell transfected with pCEP4αS-R528H. The membrane capacitance of this cell was 70 pF.

α1S-R528H, respectively. The current voltage (IV) relationships for α1S- and α1S-R528H-directed Ba2+ currents can be superimposed (Fig. 2A). Activation occurred near −10 mV and the peak of the IV 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 α1S- and α1S-R528H-directed Ba2+ currents (Fig. 2B). Time constants of inactivation were found to be similar for α1S-directed Ba2+ currents (τ = 8.3 ± 2.4 s; n = 3) and for α1S-R528H-directed Ba2+ currents (τ = 9.0 ± 1.2 s; n = 6) recorded at +20 mV. Voltage-dependent activation and inactivation curves were constructed for α1S-directed Ba2+ currents (Fig. 2C) and for α1S-R528H-directed Ba2+ currents (Fig. 2D). The potential for half-activation (V0.5) was −14 ± 3 mV (n = 6) for α1S, and −9 ± 3 mV (n = 5) for α1S-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
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 α1S and α1S-R528H, respectively). Both for α1S- and α1S-R528H-directed Ba²⁺ 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 α1S (n = 6) and for α1S-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 α1S- and α1S-R528H-directed Ba²⁺ currents are similar in their electrophysiological parameters.

The most striking effect related to the expression of recombinant α1S-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 Ba²⁺ current density was 3.2 fold lower in L cells transfection with α1S-R528H (0.24 ± 0.06 pA/pF, n = 15), compared to the cells transfected with wild-type α1S (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 Ba²⁺ current density in L cells. Under our experimental conditions, we can postulate that the amplitude of Ba²⁺ currents is a good index of the α1-directed Ca²⁺ channel function. The decreased activity of α1S-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 α1S, 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 Ca²⁺ 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 Ca²⁺ 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, α1S, as well as the auxiliary subunits, αδ, 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 Ba²⁺ current density with α1S-R528H is unlikely to be caused by a change in the macroscopic electrophysiological properties of α1S-R528H-directed channels, since we did not observe any significant differences in the Ba²⁺ current parameters, such as kinetics, voltage-dependent acti-
cells, and referring to the LCa.ll cell line [13, 16, 19]. Surprising and inactivation, when compared to its-directed channels, using unpaired Student's t-test (asterisk).

et al. [7] are related to the use of aS eDNA from rabbit in our experiments. Another possibility is that auxiliary subunits, which are missing in Ltk- cells, may exert a distinct modulatory role on a1S- and a1S-R528H-directed Ba2+ currents. It is unlikely that the discrepancies between our study and Sipos et al. [7] are related to the use of a1S CDNA from rabbit in our experiments. Indeed, the full length cDNA encoding for the human a1S has recently been cloned [23] and shows 92% of homology with its rabbit counterpart [8, 9]. Within the II54 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 a1S sequence.

The preliminary electrophysiological studies of the HypoPP mutations have suggested a loss of function as the major alteration of the mutated Ca2+ channels (for a recent review, see [24, 25]). Our study indicates that this phenomenon relies directly to a reduced Ca2+ channel activity of a1S-R528H, independently of a substantial change in electrophysiological parameters. How a decrease in Ca2+ 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 a1S-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 Ca2+ channel defect.

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