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SHORT COMMUNICATION

H. Lerche · N. Klugbauer · F. Lehmann-Horn
F. Hofmann · W. Melzer

Expression and functional characterization of the cardiac L-type calcium channel carrying a skeletal muscle DHP-receptor mutation causing hypokalaemic periodic paralysis

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Abstract A histidine substitution for the outermost arginine in II/S4 of the α_1 subunit of the human skeletal muscle dihydropyridine (DHP) receptor has been reported to cause hypokalaemic periodic paralysis (HypoPP). This mutation shifts the voltage dependence of L-type Ca current inactivation in myotubes from HypoPP patients by -40 mV without affecting activation. Based on the strong homology of II/S4 in cardiac and skeletal muscle α_1, we introduced the corresponding mutation into the rabbit cardiac α_1 subunit (R650H). Wild type (WT) and mutant constructs were transiently transfected in HEK cells together with β and $\alpha_2\delta$ subunits and Ca and Ba currents were studied using the whole-cell patch-clamp technique. In contrast to the results obtained from human myotubes, R650H produced a small (-5 mV) but significant shift of both the steady-state activation and inactivation curves. When external pH was increased from 7.4 to 8.4 in order to favour deprotonization of H650, the only difference between WT and mutant channels was a slightly reduced steepness of the inactivation curve. Additional cotransfection of the γ subunit which is only found in skeletal but not in heart muscle, shifted the inactivation curves of both WT and R650H by -20 mV. We conclude that R650 plays a different role in voltage-dependent gating of the cardiac L-type Ca channel than the corresponding residue in the human skeletal muscle L-type channel, since a distinct and selective effect on the midpoint voltage of steady-state inactivation could not be found for R650H.

Keywords dihydropyridine receptor, L-type calcium channel, voltage sensor, subunits, patch clamp, site-directed mutagenesis

Introduction

The binding proteins for 1,4-dihydropyridine drugs (DHP-receptors) play a crucial part in excitation contraction coupling in vertebrate striated muscle. In heart and skeletal muscle, DHP-receptors form voltage-dependent calcium channels (L-type) which differ in their kinetics and mode of interaction with calcium release channels in the sarcoplasmic reticulum (reviewed in [4]). The differences arise from distinct structures of the α_1 subunits which are encoded by separate genes. However, certain regions of these two α_1 polypeptides are strongly conserved. Among the conserved regions are the S4 segments in the four homologous domains which are thought to confer voltage-dependence to the molecule. A point mutation in the S4 segment of domain II replacing a positively charged arginine residue for histidine (R528H), which causes the human skeletal muscle disease hypokalaemic periodic paralysis (HypoPP, [3]), was found to produce a -40 mV shift in the voltage dependence of L-type current inactivation in human myotubes from HypoPP patients [7]. This observation points to a crucial role of R528, the outermost arginine of skeletal muscle II/S4, in voltage-dependent inactivation.

The purpose of this investigation was to further characterize the role of this arginine residue in L-type Ca channels and to establish a model for studying the pathogenesis of hypokalaemic periodic paralysis. Therefore the Arg-to-His mutation was introduced at the corresponding location in the highly homologous rabbit cardiac S4 segment, and expressed in human embryonic kidney (HEK293) cells. The cardiac α_1 subunit is known to be a much better subject for heterologous expression than the homologous skeletal muscle polypeptide.

N. Klugbauer · F. Hofmann
Institut für Pharmakologie und Toxikologie,
Technische Universität München, D-80802 München, Germany

H. Lerche · F. Lehmann-Horn · W. Melzer (✉)
Abteilung für Angewandte Physiologie,
Universität Ulm, D-89069 Ulm, Germany

Materials and methods

Mutagenesis and Transfection. The arginine residue R650 of the rabbit cardiac Ca channel α_1 subunit ($\alpha_{1C\text{-}A}$) was replaced by histidine using an "overlap" polymerase chain reaction (PCR) approach. The following mutagenic primers were used for the construction of the R650H mutation in the full-length form of $\alpha_{1C\text{-}A}$ and in $\alpha_{1C\text{-}A}$1733 (carboxy terminus of $\alpha_{1C\text{-}A}$ truncated at aa1733, [5]): 5'-TGT GCT GCA CTG CGT GCG GCT CCT G-3' as forward primer and 5'-GCA CGC AGT GCA GCA CAG AGA TGC CCA-3' as reverse primer. After the second round of PCR, the DNA fragment was ligated into the corresponding sites of a BamHI-EcoRI calcium channel subclone and sequenced on both strands. This BamHI-EcoRI fragment was used to construct the plasmids pcDNA3HK1(R650H) and pcDNA3HK1733(R650H). Human embryonic kidney cells (HEK293) were transiently transfected using either the calcium phosphate or the lipofection method (Lipofectamine, GibcoBRL). Wild type (WT) and mutant $\alpha_{1C\text{-}A}$ constructs were coexpressed alone (truncated form) or together (full length) with other subunits (β_2 or β_1, $\alpha_2\delta$, γ, [9]).

Electrophysiology. Standard whole-cell patch clamp recording was employed to study Ca and Ba currents of the transiently transfected HEK293 cells. The bathing and pipette solutions contained (in mM): bath: 10 $CaCl_2$ or $BaCl_2$, 110 NaCl, 20 TEACl, 1 $MgCl_2$, 0.1 EGTA, 10 Glucose, 5 Hepes, pH 7.4; pipette: 102 CsCl, 5 NaCl, 10 TEACl, 5 MgATP, 10 EGTA, 5 Hepes, pH 7.4; 22-24°C. Peak Ba or Ca currents were on average 350 pA when $\alpha_{1C\text{-}A}$1733 was transfected alone, and 1 nA when $\alpha_{1C\text{-}A}$, β_2 and $\alpha_2\delta$ were cotransfected. The cell capacitance ranged between 20 and 100 pF. Series resistance (R_S) was 3-8 MΩ, R_S compensation 50-85 % and the voltage error due to R_S was maximally 3 mV. E.g. after cotransfection of $\alpha_{1C\text{-}A}$, β_2 and $\alpha_2\delta$ the Ca currents showed the following values for R650H (n=10) vs. WT (n=7): peak current: 0.9±0.2 vs. 1.2±0.4 nA; current density: 13±3 vs. 21±8 A/F; voltage error due to R_S: 1.4±0.3 vs. 1.7±0.4 mV. WT and mutant channels were always transfected and measured on the same day. Currents were recorded using an EPC9 patch clamp amplifier and the appropriate software (HEKA elektronic). The holding potential was -80 mV. Leak and remaining capacitive currents were subtracted by means of a P/4 protocol. Currents were filtered at 3 and sampled at 20 kHz. The data were analysed using a combination of HEKA, Excel (Microsoft) and Sigma Plot (Jandel scientific) software. All values are given as means±SEM. For statistic evaluation, Students t-test was applied (p=0.05 or 0.01).

Results

The R650H mutation, corresponding to R528H in II/S4 of human skeletal muscle (Fig. 1A), was introduced into the α_1 subunit of the rabbit cardiac calcium channel (see Materials and methods).

Calcium currents. When WT or mutant α_1 subunits were coexpressed with β_2 and $\alpha_2\delta$ subunits in HEK293 cells, inward Ca currents exhibiting kinetics and voltage-dependence of typical cardiac L-type channels could be recorded (Fig.1B). Assuming a linear open channel I-V relation and voltage-dependent gating according to a Boltzmann function, the voltage dependence of the peak inward currents I(V) was fitted to the following equation:

(1) $I(V)/I_{max} = g*(V-V_{rev}) / (1+\exp((V_{1/2}-V)/k))$,

where g is the maximum conductance obtained at large voltages, V_{rev} the reversal potential, $V_{1/2}$ the voltage of half-maximal activation and k a parameter determining the voltage sensitivity. The voltage dependence of Ca current activation was shifted by -4.5 mV for R650H vs. WT (Fig 1C and Table 1: lines 1,2).

In order to search for changes in voltage-dependent inactivation, the

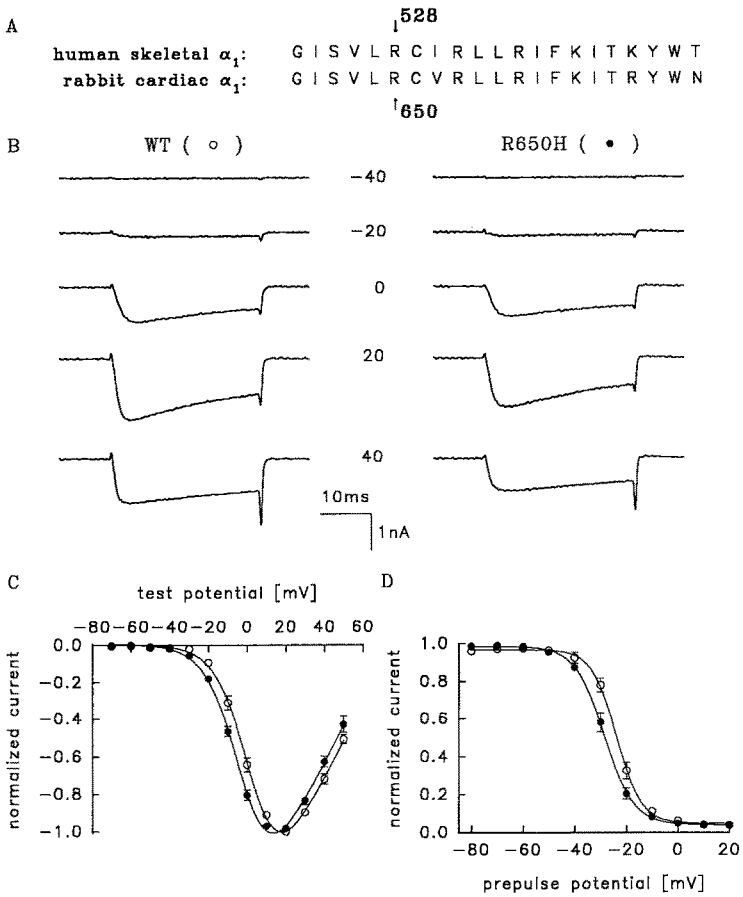

Fig.1. A Amino acid sequences of the II/S4 segment of the human skeletal muscle and the rabbit cardiac α_1 subunit of the DHP-receptor; the homologous outermost arginines at positions 528 and 650 are indicated by arrows.
B Representative Ca currents measured in two HEK cells expressing either wild-type (left) or mutant (right) cardiac α_1 subunits together with β_2 and $\alpha_2\delta$ subunits.
C Average current-voltage relation (means±SEM) for WT (n=6 cells, open symbols) and R650H (n=8 cells, filled symbols). The lines are fits according to equation (1) with parameters given in Table 1, lines 1 and 2.
D Average fractional inactivation (means±SEM) for WT (n=7 cells, open symbols) and R650H (n=6 cells, filled symbols). The lines are fits according to equation (2) with parameters given in Table 1, lines 1 and 2.

Table 1: Boltzmann parameters of steady-state activation and inactivation ($V_{1/2}$: voltage of half-maximal activation/inactivation, k: slope factors), and time constants of activation (τ_m) and inactivation (τ_h or τ_{fast}, τ_{slow}, respectively) for all evaluated Ca and Ba currents (for details see text). All cotransfected subunits are indicated in the first column (in $\alpha_1$1733 the carboxy-terminus is truncated at aa 1733, [5]). Statistically significant differences between R650H and WT are marked as follows: * p = 0.05, ** p = 0.01 (Student's t-test).

		steady-state activation			steady-state inactivation			time course of activation/inactivation		
	calcium currents:	n	$V_{1/2}$ [mV]	k [mV]	n	$V_{1/2}$ [mV]	k [mV]	n	τ_m [ms]	τ_h [ms]
1	α_1R650H + $\alpha_2\delta$ + β_2	8	-1.3±1.1*	-8.6±0.5	6	-28.5±0.9**	5.5±0.5	7	1.08±0.06	21.8±1.2
2	α_1WT + $\alpha_2\delta$ + β_2	6	3.2±1.2	-8.3±0.4	7	-23.6±1.0	5.1±0.4	8	1.03±0.04	19.2±0.7
3	α_1R650H + α_1WT + $\alpha_2\delta$ + β_2	5	2.0±1.7	-8.6±0.5	8	-26.7±1.2	6.2±0.3			
4	α_1R650H + $\alpha_2\delta$ + β_1	3	0.6±1.5	-8.3±0.9	3	-25.9±1.8	5.0±0.4			
5	α_1R650H + $\alpha_2\delta$ + β_2 + γ	7	-3.7±1.3*	-7.1±0.3	5	-48.6±0.9	7.9±0.2			
6	α_1WT + $\alpha_2\delta$ + β_2 + γ	6	1.2±1.0	-8.3±0.3	5	-44.4±2.8	8.8±0.6			
7	α_1R650H+ $\alpha_2\delta$ + β_2 , pH 8.4	5	-2.5±2.2	-8.1±0.4	5	-30.5±1.2	6.5±0.3**			
8	α_1WT + $\alpha_2\delta$ + β_2 , pH 8.4	5	-1.1±1.4	-7.5±0.2	4	-29.6±0.6	5.3±0.3			
	barium currents:	n	$V_{1/2}$ [mV]	k [mV]	n	$V_{1/2}$ [mV]	k [mV]	n	τ_{fast} [ms]	τ_{slow} [s]
9	α_1R650H + $\alpha_2\delta$ + β_2	9	-7.5±0.7**	-6.6±0.2	9	-31.3±1.5*	6.4±0.3	8	169±18	40±3
10	α_1WT + $\alpha_2\delta$ + β_2	9	-2.1±1.2	-6.8±0.2	12	-27.4±0.9	6.0±0.4	6	141±20	37±1
11	$\alpha_1$1733R650H	6	-4.1±1.9	-7.8±0.2	3	-24.1±3.0	9.2±1.2			
12	$\alpha_1$1733WT	4	1.1±2.6	-7.7±0.3	2	-22.8±0.5	8.7±0.3			

following pulse protocol was applied every 30s: a 5 s prepulse of different amplitude, V, followed by a 15 ms repolarization interval and a 50 ms fixed test pulse to +10 mV. Inactivation curves of normalized currents were fitted by a Boltzmann relation:

(2) $I(V)/I_{max} = a/(1+\exp((V_{1/2}-V)/k)+c$,

where $V_{1/2}$ and k were defined as in (1); the constant c accounted for a partial recovery from inactivation after the 15 ms of repolarization and was not larger than 5% of the amplitude a. The R650H mutation shifted the voltage dependence of inactivation by -4.9 mV (Fig. 1D and Table 1: lines 1,2), i.e. one order of magnitude less than found for R528H in HypoPP myotubes [7].

Coexpression of WT and R650H. In myotubes from HypoPP patients both WT and mutant channels are present, due to the dominant mode of inheritance of this disease [7]. Since DHP receptors are thought to form tetramers in contact with the calcium release channel (see [4] for references), interaction of mutant and WT channels might be responsible for the altered voltage dependence of inactivation in HypoPP myotubes. In order to simulate this situation, we transfected HEK293 cells with a 1:1 mixture of WT and R650H α_1 subunits. However, neither activation nor inactivation were substantially changed (Table 1, line 3).

Roles of skeletal muscle specific subunits. Heart and skeletal muscle express specific β subunits, and the γ subunit is only expressed in skeletal muscle. It has been reported that both subunits have effects on the voltage dependence of inactivation [6]. However, when the cardiac β_2 was replaced by the skeletal muscle specific β_1 subunit, the activation and inactivation parameters were not significantly changed (Table 1, line 4). When the γ subunit was cotransfected, the steady-state inactivation curves of both WT and R650H were shifted by -20 mV and had a reduced steepness, confirming the results from expression in Xenopus oocytes [6]. Activation was not significantly affected (Table 1, line 5,6). Thus, none of these two skleletal muscle specific subunits uncovered any obvious difference between WT and R650H.

Effect of increased external pH. An arginine-to-histidine mutation may produce its effect through a change of the local charge density. The arginine residue is predominantly positively charged at pH 7.4 due to its high pK value while the imidazole ring of histidine has a much lower affinity for protons. Since R650 is the outermost arginine in II/S4 external pH may affect protonation of H650 as has been shown for a similar mutation in the human muscle sodium channel [1]. In order to augment the dissociation of protons from the histidine residue and therefore enhance the charge difference between arginine and histidine, we carried out experiments at pH 8.4. An increase of the external pH shifted the activation and inactivation curves to more negative potentials, but the differences in $V_{1/2}$ between WT and R650H became smaller. Instead, a small decrease of the steepness of the inactivation curve became noticeable (Table 1, lines 7,8), which might be explained by a reduction of sensor charge or a change in the cooperativity of repeats I-IV in analogy to K channels [8].

Barium currents. The cardiac L-type channel exhibits a Ca-dependent inactivation which is absent in the skeletal muscle L-type channel. To exclude a possible interaction of Ca-dependent inactivation with any effect of the mutation on voltage-dependent inactivation, we replaced Ca by Ba as charge carrier. Under these conditions, the differences between R650H and WT in voltage-dependent gating were very similar to those described above (Table 1, lines 9,10). Use of a truncated form of the α_1 subunit (see Materials and methods, [5]) allowed the measurement of reasonable currents without the coexpression of additional subunits but did not show a significantly different result (Table 1, lines 11,12). Therefore, it is unlikely that the coexpressed β and $\alpha_2\delta$ subunits prevented any effect of the mutation.

Time course of activation and inactivation. Ca currents obtained at 50 ms lasting pulses to +10 mV were fitted using a Hodgkin-Huxley model (m^3h, with m and h beeing independent first order isomerisation reactions causing activation and inactivation respectively; [2]). The time constants of activation, τ_m, and inactivation, τ_h, did not differ for WT and R650H (Table 1, lines 1,2). To evaluate the time course of Ca-independent inactivation, the decay of 5 s lasting Ba currents was fitted to a second order exponential function resulting in two time constants, τ_{fast} and τ_{slow}. There was a small but not significant difference between WT and R650H in τ_{fast} (Table 1, lines 9,10).

In summary, the heterologously expressed rabbit cardiac α_1 subunit containing the Arg-His mutation in II/S4 which causes hypokalaemic periodic paralysis when present in the human skeletal muscle α_1 subunit does not show striking functional differences compared to wild type channel activation and inactivation.

Discussion

The starting point of this study was the idea (i) that the outermost arginine in II/S4 might play an important role in determining the voltage dependence of inactivation, and (ii) that the cardiac α_1 subunit, which is a much better subject for heterologous expression than the homologous skeletal muscle polypeptide, might serve as a model to study the pathogenesis of hypokalaemic periodic paralysis. The present results indicate that the cardiac α_1 subunit expressed in HEK cells behaves differently from the skeletal muscle α_1 subunit in native muscle cells regarding the effect of the R-to-H exchange in II/S4 - despite its almost perfect homology in the II/S4 region. Apart from a small left shift of the steady-state activation and inactivation curves and a slightly reduced steepness of the inactivation curve at higher pH, no clear functional differences could be detected when comparing the mutant construct with the wild type. Therefore this system can be excluded as a valid model system for studies on HypoPP.

Among the reasons which may explain the deviation from the result in myotubes are the following: 1) Even though the S4 segment itself is highly preserved, differences in other regions of the protein which are less homologous in the heart and skeletal muscle isoform may interact with the S4 segment and cause a differential effect of the mutation. Alternatively, a different subunit interaction in both channel types might cause different roles of the outermost arginine in II/S4. 2) The effect on inactivation reported for human myotubes may depend on a specific oligomeric arrangement of the DHP receptors, which might also be the cause for the dominant inheritance of the disease. DHP receptors seem to be arranged in tetrads (see [4]), a form of organization which is most likely lost in HEK cells. It is possible that the physical interaction between the DHP receptors and the ryanodine receptor in skeletal muscle is necessary to create the specific functional change on inactivation observed in human myotubes. This possibility would likely imply that the voltage-dependence of inactivation of calcium release may be similarily affected in HypoPP myocytes as the inactivation of the Ca current.

In any case, it seems necessary to use cellular systems resembling more the native situation in myocytes in order to study the link between DHP receptor mutations and the pathophysiology of hypokalaemic periodic paralysis.

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