Modeling

Molecular dynamics and mutational analysis of a channelopathy mutation in the IIS6 helix of CaV1.2

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A channelopathy mutation in segment IIS6 of CaV1.4 (I745T) has been shown to cause severe visual impairment by shifting the activation and inactivation curves to more hyperpolarised voltages and slowing activation and inactivation kinetics. A similar gating phenotype is caused by the corresponding mutation, I781T, in CaV1.2 (midpoint of activation curve (V0.5) shifted to -37.7 ± 1.2 mV). We show here that wild-type gating can partially be restored by a helix stabilising rescue mutation N785A. V0.5 of I781T/N785A (V0.5 = -21.5 ± 0.6 mV) was shifted back towards wild-type (V0.5 = -9.9 ± 1.1 mV). Homology models developed in our group (see accompanying article for details) were used to perform MD-simulations on wild-type and mutant channels. Systematic changes in segment IIS6 (M1187-F1194) and in helix IIS6 (N785-L786) were observed. The simulated structural changes in S6 segments of I781T/N785A were less pronounced than in I781T. A delicate balance between helix flexibility and stability enabling the formation of hydrophobic seals at the inner channel mouth appears to be important for wild-type CaV1.2 gating. Our study illustrates that effects of mutations in the lower part of IIS6 may not be localized to the residue or even segment being mutated, but may affect conformations of interacting segments.

Introduction

Mutations inducing channelopathies cause changes in electrical excitability and calcium entry, thereby increasing susceptibility to migraine, ataxia, periodic paralysis, cardiac arrhythmias, hyperglycemia and incomplete congenital stationary night blindness (reviewed in refs. 1–4). Point mutations in CaV1 and CaV2 have been shown for example to accelerate or decelerate inactivation kinetics, to shift the threshold of channel activation, to decelerate activation and to change recovery from inactivation.5,8

We have recently analyzed the consequences of a point mutation (I745T) in segment IIS6 of the CaV1.4 α1-subunit that causes a retinal disorder by shifting the voltage-dependence of CaV1.4 channel activation and inactivation by about -30 mV with slowed activation, deactivation and inactivation kinetics.5,8 Affected males and females of a large New Zealand family suffer from severe visual impairment.5

Latter studies revealed that substitutions of I781, the CaV1.2 residue corresponding to I745 in CaV1.4, by residues of different hydrophobicity, size and polarity shifted activation and steady state inactivation in the hyperpolarising direction. The changes in channel gating indicated that amino acid substitutions for I781 and adjacent residues destabilize the closed conformation and/or stabilize the open conformation of CaV1.2.9

In the present study we analysed possible consequences of I781T and other amino acid substitutions in segment IIS6, using Molecular Dynamics simulations, which suggest systematic changes in segment IIS6 and distortions in the neighboring segment IIS6. The “allosterically” affected region in segment IIS6 (starting at residue M1187-F1194) has previously been shown to play a crucial role in Ca2+ channel inactivation (Hering et al.,10 J Physiol 2000; 528:237–49 for review). A rescue mutation (I781T/N785A) substantially reduced the gating changes caused by I781T in both segments and, remarkably, also diminished the helix distortions in both S6 segments in MD simulations.

Results

Interactions of I781 with the environment. Based on our homology model of the open conformation (see accompanying article), I781 is located at the lower third of the IIS6 segment and faces helix IIS5. The L4-5 helix of domain II is located in close proximity. I781 is surrounded by hydrophobic amino acids, and interacts with highly conserved amino acids L684 and L687 of helix IIS5. The backbone nitrogen atom of I781 forms a hydrogen bond to the backbone oxygen of F778 of the same helix, and the backbone oxygen atom of I781 bonds to the backbone nitrogen atom of residue N785, which is located one helical turn below. Additionally, the side chain of N785 forms hydrogen bonds with two conserved serines from helix IIS5 (Fig. 1).

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MD simulations of I781T and I781P suggest helix distortions in segments IIIS6 and IIIS6. Replacement of I781 by various amino acids severely influences channel gating. Polar or charged amino acid replacements induce the strongest changes in channel gating (with the exception of proline), while hydrophobic residues such as alanine or leucine have less severe consequences on channel activation and inactivation.9

The static structure models only partially disclosed the molecular mechanism of the gating distortions. Therefore, the dynamics of calcium channel mutants were analyzed, using molecular dynamics simulations. The stability of the mutant channels, which can be measured by calculating the RMSD (root mean square displacement) for, was only slightly increased compared to wild-type channels (~0.5 Å, data not shown).

Detailed analysis of the trajectories revealed distortions in the pore-forming helices IIIS6 and IIIS6 for the threonine and proline mutant compared to wild-type, where no such distortions could be detected. Surprisingly, effects associated with replacement of the medium size hydrophobic isoleucine with a small polar threonine, extend beyond domain II. We consistently observed an unwinding of helix IIIS6 at the helix crossing interface. To analyse this phenomenon in more detail, backbone angles of the corresponding region in helix IIIS6 and IIIS6 were studied (Fig. 2A, B and E). Amino acid in the middle of helix IIIS6 (M1187 to F1191) lost their helical conformation within the first 300 ps (Fig. 3C and D). During the rest of the 10 ns (6 ns) simulation runs distortions in this region remained, while the rest of the structure stayed intact. Interestingly, the overall helical structure of IIIS6, where mutations have been introduced, remained intact during the whole simulation. Detailed analysis of the backbone angles of IIIS6 segment revealed unchanged behaviour of the backbone angles ($\phi$/$\psi$) for residue 781, irrespective of the amino acid at this position (Fig. 2A). However, one helical turn below, the backbone angles for mutant channels change. The backbone angles of residue 785 vary only slightly (data not shown), but considerable changes are observed in the next residue, the highly conserved L786 (Fig. 2B). Interestingly, in the wild-type channel this amino acid is near (within 3–5 Å) the distorted amino acids in helix IIIS6 (Fig. 3B). Simultaneously, the interacting amino acid from IIIS6, I1190 looses its hydrophobic contact with L786 from IIIS6 (Fig. 3D). As a consequence large fluctuations of the adjacent backbone dihedral angle occurred (see Fig. 2E).

Our simulations suggest changes in hydrophobic-hydrophobic interactions between neighboring S6 segments as a consequence of distortions in segment IIIS6. In the wild-type channel hydrophobic interactions between helix IIIS6 and IVS6 are formed between residues V1192, I1196 and F1199 from IIIS6 and F1495, L1496, N1499 and L1500 from helix IVS6 (Fig. 5A). In the threonine mutant hydrophobic interactions between these two helices are formed by I1190, F1191, F1194, V1195 and F1199 from segment IIIS6 and residues M1491, L1492, F1495 and L1496 from segment IVS6 (Fig. 5B).

Rescue mutation N785A. If a threonine or proline (data not shown) in position I781 induces structural changes in IIIS6 one helical turn below, as suggested by MD simulations (see changes in backbone angles of residue L786 in Fig. 2B), we reasoned that introducing a helix stabilizing alanine might rescue the gating of wild-type channels. Since we did not want to abolish hydrophobic interactions between L786 (IIIS6) and I1190 (IIIS6), we choose to mutate the neighboring residue 785 (I781 + 4, to force $\alpha$-helical configuration). The double mutant (I781T/N785A) was created and tsA-201 cells were co-transfected with mutant $\alpha$1 and $\beta_{14}$, and $\alpha_{1}$-, $\delta$-, subunits (see Methods for details). Remarkably, the activation curve of the corresponding double mutant ($V_{0.5} = -21.5 \pm 0.6$ mV, $n = 6$) was shifted back towards the position of wild-type Ca$_{\alpha}$1.2 ($V_{0.5} = -9.9 \pm 1.1$ mV) compared to I781T ($V_{0.5} = -37.7 \pm 1.2$ mV, Fig. 6A). The kinetics of activation and deactivation of the double mutant were faster than in I781T, at negative voltages, slightly slower than in wild-type (Fig. 6C).9 Thus, our functional analysis of double mutant I781T/N785A suggests a partial restoration of wild-type kinetics. Substitution of N785 by glycine, which introduces greater flexibility in the $\alpha$-helix, did not rescue wild-type gating. Instead, the position of the activation curve remained shifted (I781T/N785G 41.2 ± 0.7 mV), and activation and deactivation kinetics at hyperpolarised voltages remained slow, as observed for mutants I781T/P (Fig. 6D and E).

In order to elucidate the rescue mechanism we analysed the gating properties of the corresponding single point mutants. N785G slightly shifted the voltage dependence of channel activation in the depolarising direction (Fig. 6A), while N785A was not functional (Table 1).

To elucidate the possible structural implications, we performed MD simulations. The double mutants I781T/N785A and I781/N785G were built using the program WHAT IF.14 The mutant channels were energy minimized using 10000 steps of steepest descent with the minimization tool in Gromacs,15 as described above. As shown in Figure 5A, the double mutant I781T/N785A prevents unwinding of residues M1187 to F1191 in segment IIIS6. Detailed analysis of backbone angles, in agreement with the above described threonine mutant, revealed unchanged behaviour at position 781.
Figure 2. For figure legend, see page 3.
Structural changes at the inner channel mouth

The C-terminal backbone angle at position L786 is still more flexible than wild-type, but does not lead to conformational changes in IIIS6 (Figs. 2C and 4). Consequently, the interacting amino acid I1190 does not show any different behaviour compared to wild-type (see Fig. 2F). In agreement with experiments, the double mutant I781T/N785G did not rescue wild-type gating, and MD simulations reveal similar behaviour of I781T/N785G and I781T single mutant channel, with analogouse distortions of segments IIIS6 and IIIS6, as described above (Figs. 2D, G and 4B).

These data may suggest that higher helix stability induced by the additional mutation N785A can shift the activation and inactivation curves back towards the positions of wild-type CaV1.2 (Fig. 6A and B), whereas higher flexibility facilitated by the additional N785G prevents efficient channel closure. Interestingly, mutating the hydrophilic N785 to the hydrophobic leucine did not significantly rescue wild-type gating ($V_{0.5}(I781T/N785L) = -34.4 \pm 0.9 \text{ mV}$, $p > 0.05$, not significantly different from I781T, Table 1). The activation curve of the single mutant N785L ($V_{0.5} = -12.3 \pm 1.1 \text{ mV}$, Table 1, not significantly different from wild-type, $p > 0.05$) was also not shifted compared to wild-type, suggesting that hydrophobic interactions in position 785 play no major role.
Evidence for membrane incorporation of none conducting mutant channel N785A. Mutant N785A did not conduct Ba\(^{2+}\) currents. We have therefore examined the subcellular distribution of the GFP-tagged mutant N785A by confocal microscopy in order to analyze plasma membrane targeting. N781A GFP-tagged Ca\(_V\)1.2 \(\alpha_1\)-subunits were co-expressed together with \(\beta_1\alpha_2\delta_1\) subunits in tsA-201 cells. The plasma membrane was visualized by staining with FM4-64 (Fig. 7, see also ref. 9).

As previously shown for wild-type Ca\(_V\)1.2 \(\alpha_1\)-subunits, we observed significant localization of N785A at the plasma membrane, suggesting that the lack of current observed for these mutants can not be attributed to a failure of the mutant Ca\(_V\)1.2 \(\alpha_1\)-subunits to reach the plasma membrane.

**Discussion**

Mutational effects on closed and open channel conformations. Studies on \(K_V\) have shown that the closed state in voltage gated ion channels may be stabilised by hydrophobic interactions between amino acids in the lower third of the “bundle crossing” region of S6 segments.\(^{17,18}\) In such a scenario fast closure of Ca\(_V\)1.2 is likely to require intact S6 helical conformations enabling efficient hydrophobic interactions in the lower third of the channel pore. Helix deformations caused by I781T/P in IIS6, with possible allosteric effects on IIIS6, lower the probability of a tight fit closed conformation during repolarization, which could explain the deceleration of tail currents (Fig. 6). Furthermore, hydrophobic interactions in the open channel conformation of mutant channels I781T, I781P and I781T/N785G are changed, as a consequence of the structural rearrangements, caused by the increased flexibility of residues M1187-F1194 in segment IIIS6. Residues following F1194 remain in \(\alpha\)-helical conformation, are rotated. This leads to different helix-helix contacts, as described in the Results section.
segment IIIS6 and IIIS6 remain unchanged. Obviously, replacements in position I781, with the more flexible but less hydrophobic threonine, or other less hydrophobic residues, leads to pronounced changes in helix-helix interactions, which might stabilize the open conformation as suggested previously.

MD simulations suggest changes in segments IIIS6 and IIIS6 by I781T/P. Our models, together with MD simulations, suggest complex conformational changes caused by mutation I781T (Figs. 2A, B and E and 3C and D). Similar changes were observed for I781P (data not shown) and I781T/N785G (Figs. 2D and G and 4B). Proline may, however, induce additional effects caused by the lack of the backbone hydrogen bond and steric constrains, leading to bending and destabilization of helices.

The functional properties of the partial rescue mutant I781T/N785A (Fig. 6) can be explained by a helix stabilizing effect of alanine (Figs. 2C and F and 4A). I781T/N785G, or I781T/N785L, did not rescue wild-type current kinetics (Fig. 6D and E and Table 1). It is therefore unlikely that the partial rescue of I781T/N785A is based on additional hydrophobic interactions. The different effects of the single mutants (N785G shifted the activation curve to the right, N785A did not conduct barium currents and N785L induced no significant shift) suggest that other properties than flexibility and hydrophobicity in this position play a role. The elucidation of the rescue mechanism warrants further research. Molecular dynamics simulations revealed additional information, which could never be obtained from static models. The inserted threonine seems to affect the conformation of helix IIIS6 via disruption of hydrophobic helix-helix interactions between the two domains in the open channel conformation (L786 interacting with I1190, see Fig. 3B and D). As a consequence the stability of helix IIIS6 is decreased, which leads to partial unwinding at residues M1187-F1194 (Figs. 2B and E and 3C and D). Interestingly, key inactivation determinants have been identified in the lower part of segment IIIS6 (reviewed in ref. 10). It is tempting to speculate that structural changes in the adjacent segment IIIS6 contribute to simultaneous changes in channel activation and inactivation caused by point mutations in the lower part of IIIS6. Hence, key residues affecting CaV1.2 inactivation (IFV motif, reviewed in ref. 10) have previously been identified in the allosterically affected region of IIIS6.

Summarizing, our MD simulations reveal significant effects of mutations in position 781 on sequentially distant residues, especially on the adjacent IIIS6 helix. The assumption that mutational effects are due solely to localized changes does not appear to be valid in this case. More complex conformational changes, involving neighboring segments, should also be considered. Our results suggest that molecular modeling combined with molecular dynamics simulations may prove effective in identifying and analyzing these more complicated situations.

Materials and Methods

Experimental procedures. Mutagenesis. The CaV1.2 α1-subunit coding sequence (GenBankTM X15539) in-frame 3' to the coding region of a modified green fluorescent protein (GFP) was kindly donated by Dr. M. Grabner. For electrophysiological studies we
used the plasmid lacking the GFP tag. Mutations in segment II S6 of the Ca\textsubscript{\textalpha}1.2 \alpha\textsubscript{1}-subunit were introduced by the “gene SOEing” technique.\textsuperscript{12}

The mutated fragments were cloned into a BamHI-AflII-cassette (nt 1265 and 2689, numbering according to the Ca\textsubscript{\textalpha}1.2 \alpha\textsubscript{1}-subunit coding sequence). All constructs were checked by restriction site mapping and sequencing.

Cell culture and transient transfection. Human embryonic kidney tsA-201 cells were grown at 5% CO\textsubscript{2} and 37°C to 80% confluence in Dulbecco’s modified Eagle’s/F-12 medium supplemented with 10% foetal-calf serum and 100 units/ml penicillin/streptomycin. Cells were split via trypsin EDTA and plated on 35 mm Petri dishes (Falcon) at 30–50% confluence ~16 h before transfection. The extracellular bath solution contained (in mM): CsCl 145, MgCl\textsubscript{2} 3, HEPES 10, EGTA 10, titrated to pH 7.25 with CsOH. All data were digitised using a DIGIDATA 1200 interface (Axon Instruments, Foster City). The voltage-dependence of inactivation (inactivation curve) was measured using a multi-step protocol to account for run-down. The pulse sequence was applied every 40 seconds from a holding potential of -100 mV. Inactivation curves were drawn according to a Boltzmann equation:

\[
I(t) = A \cdot \exp \left( \frac{t}{\tau} \right) + C
\]

where \(I(t)\), current at time \(t\); \(A\), the amplitude coefficient; \(\tau\), time constant; \(C\) a constant \(C\). The voltage-dependence of \(I_{\text{Ba}}\) inactivation (inactivation curve) was measured using a multi-step protocol to account for run-down. The pulse sequence was applied every 40 seconds from a holding potential of -100 mV. Inactivation curves were drawn according to a Boltzmann equation:

\[
I_{\text{Ba, inactivation}} = I_{\text{gs}} + \frac{1 - I_{\text{gs}}}{1 + \exp \left( \frac{V - V_{\text{0.5, inact}}}{k} \right)}
\]

where \(V_{\text{reV}}\), extrapolated reversal potential; \(V\), membrane potential; \(I\), peak current; \(G_{\text{max}}\), maximum membrane conductance; \(V_{0.5, \text{act}}\), voltage for half-maximal activation; and \(k_{\text{act}}\), slope factor. The time course of current activation was fitted to a mono-exponential function:

\[
I = \frac{G_{\text{max}} \cdot (V - V_{\text{reV}})}{1 + \exp \left( \frac{V_{0.5, \text{act}} - V}{k_{\text{act}}} \right)}
\]

where \(V_{\text{reV}}\), extrapolated reversal potential; \(V\), membrane potential; \(I\), peak current; \(G_{\text{max}}\), maximum membrane conductance; \(V_{0.5, \text{act}}\), voltage for half-maximal activation; and \(k_{\text{act}}\), slope factor. The voltage-dependence of activation was determined from current-voltage (I–V) curves that were fitted according to the following modified Boltzmann term:

\[
I = \frac{G_{\text{max}} \cdot (V - V_{\text{reV}})}{1 + \exp \left( \frac{V_{0.5, \text{act}} - V}{k_{\text{act}}} \right)}
\]

where \(V_{\text{reV}}\), extrapolated reversal potential; \(V\), membrane potential; \(I\), peak current; \(G_{\text{max}}\), maximum membrane conductance; \(V_{0.5, \text{act}}\), voltage for half-maximal activation; and \(k_{\text{act}}\), slope factor. The time course of current activation was fitted to a mono-exponential function:

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I(t) = A \cdot \exp \left( \frac{t}{\tau} \right) + C
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I_{\text{Ba, inactivation}} = I_{\text{gs}} + \frac{1 - I_{\text{gs}}}{1 + \exp \left( \frac{V - V_{\text{0.5, inact}}}{k} \right)}
\]
where $V$, membrane potential; $V_{0.5, \text{inact}}$, midpoint voltage; $k$, slope factor and $I_{\text{n}}$, fraction of non-inactivating current. Data are given as Mean ± S.E. Statistical significance was assessed with the Student’s unpaired t-test.

Confocal imaging. Membrane localisation of the non-conducting channel construct N785A was analysed by means of confocal microscopy. Confocal images were obtained ~30 h after transfection and acquired with a Zeiss LSM-510 confocal laser scanning microscope, using a x63 (1.4 NA) oil immersion objective. Data illustrated are representative for 15–20 tsA-201 cells from three independent experiments. The plasma membrane was stained with 1 μM FM4-64 (amphiphilic styril dye, Molecular Probes). Images were acquired using an argon laser (excitation, 488 nm; emission BP505-530 nm) for the GFP-tagged Cav1.2 α1-subunits and a He-Ne laser (excitation, 543 nm; emission filter, LP650 nm) for the GFP-tagged Cav1.2 α1 subunits and a He-Ne laser (excitation, 543 nm; emission filter, LP650 nm) for the GFP-tagged Cav1.2 α1-subunits and a He-Ne laser (excitation, 543 nm; emission filter, LP650 nm) for the GFP-tagged Cav1.2 α1-subunits and a He-Ne laser (excitation, 543 nm; emission filter, LP650 nm) for the GFP-tagged Cav1.2 α1-subunits.

Molecular dynamics simulations of the open Cav1.2 mutants. To gain information about structural effects of mutations at position 781, we generated mutants I781T and I781P9 using the WHAT IF software.14 The mutant channels were energy minimized using 10000 steps of steepest descent with the minimization tool in Gromacs.15 Mutant channels were embedded in a lipid bilayer of POP:POPE, and simulated using the same parameters as for the wild-type Cav1.2, as described in the accompanying paper. Default protonation states, based on calculations with the software Propka 1.0.1,16 which showed default values for all residues in the region of the 781 hotspot, were used. Channels were simulated 5 times with different random starting velocities for 10 ns for the first run of each channel, followed by 4 repeating runs for 6 ns each, resulting in a total simulation time of 34 ns for each channel.

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