Review

Pore stability and gating in voltage-activated calcium channels

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Calcium channel family members activate at different membrane potentials, which enables tissue specific calcium entry. Pore mutations affecting this voltage dependence are associated with channelopathies. In this review we analyze the link between voltage sensitivity and corresponding kinetic phenotypes of calcium channel activation. Systematic changes in hydrophobicity in the lower third of S6 segments gradually shift the activation curve thereby determining the voltage sensitivity. Homology modeling suggests that hydrophobic residues that are located in all four S6 segments close to the inner channel mouth might form adhesion points stabilizing the closed gate. Simulation studies support a scenario where voltage sensors and the pore are essentially independent structural units. We speculate that evolution designed the voltage sensing machinery as robust “all-or-non” device while the variety of voltage sensitivities of different channel types was accomplished by shaping pore stability.

Introduction

Voltage-dependent gating involves the movement of a charged voltage sensor through the transmembrane electric field. Voltage sensor movements were first detected in sodium channels as “gating currents”. 1 Gating currents precede the ionic current and reflect the translocation of charged amino acid residues (mainly arginines) in segments S4 across the membrane. The opening of the channel pore can be considered as a multi-step process consisting of: (i) the movement of the voltage sensor; (ii) a transmission of the conformational change to the pore region and (iii) the opening of the pore itself. 2 These theoretical considerations are supported by structural data indicating that the transmembrane portion of voltage gated channels is composed of structurally distinct domains. Four voltage-sensing domains, each of which is composed of four transmembrane segments called S1–S4, are peripheral to a central pore domain formed by four sets of segments called S5, P and S6. The voltage sensors are essentially independent structural units. 3–5 Semi-independence of the major structural features of voltage sensors and the pore is apparent from crystal structures and from the fact that some K+ channels have a pore domain but no voltage-sensing domain while voltage sensing domains without a pore domain regulate phosphatase activity and proton permeation. 6 Voltage-induced conformational changes within the voltage-sensing unit are transmitted to the pore through a segment linking the two units. Hence, some features of the pore may not involve the voltage sensor. For example, single channel studies indicate that the open probability is far from unity even at high depolarizations. Flickering between open and closed states reflects either fluctuations of the voltage-sensing machinery or, alternatively, fluctuations within the pore domain while the voltage sensors are steadily in an activated position. In the latter scenario, voltage sensor movements during a depolarization would just increase the probability of pore openings but not open the pore directly. 2–7

To understand the voltage dependence of ion channel openings and closures, one must answer several questions: How tightly are gating charge movements coupled to the pore region? What determinants stabilize the pore in the open or closed conformations? Which physico-chemical properties of pore residues stabilise the channel in the closed and/or open states? How do changes in pore stability modulate the kinetics of channel activation? Does the pore have multiple mechanisms by which it opens and closes?

No answers have yet been provided for voltage gated calcium channels (CaV). Calcium channel activation is affected by numerous mutations in the pore region. Diseases caused by mutations in ion channels are termed channelopathies. 10 Many of these structural changes are associated with calcium channelopathies such as hemiplegic migraine, ataxia, stationary night blindness and other diseases. 11 Other residues determining CaV activation have been detected in pore forming S6 segments during structure activity studies. 12–15

In this review we focus on recent progress in analyzing the determinants of pore stability of CaV. Specifically, we analyze the impact of structural changes in the pore region of CaV (point mutations in pore forming S6 segments) on steady state channel activation and kinetics. We use homology modeling to illustrate why hydrophobic interactions in the lower third of S6 segments may have profound effects on the stability of the closed channel gate.

Voltage Dependence of Calcium Channel Activation

Calcium entry through calcium channels during an action potential initiates and controls multiple cascades of intracellular...
events affecting a large variety of cellular functions such as generation and propagation of electrical impulses, sensory processes, muscle contraction, secretion of hormones and neurotransmitters, cell differentiation and gene expression. Mammalian Ca\(^{2+}\) channel \(\alpha_{1}\)-subunits are encoded by at least 10 genes. The potential where voltage gated ion channels first open during a depolarisation (so called “threshold potential”) or, the more commonly estimated voltage where 50% of the channels are activated in steady-state (\(V_{0.5}\)) are hallmarks of the respective channel family members. It appears that the adjustment of the threshold potential during evolution represents an important mechanism for fine-tuning of voltage-dependent Ca\(^{2+}\) entry into cells of different tissues. Low voltage activated calcium channels (Ca\(V_3\)) open after small depolarisations of the plasma membrane and mediate low-threshold Ca\(^{2+}\) spikes.\(^{18}\) The \(V_{0.5}\) of the Ca\(V_3\) family is around -45 mV in 2 mM extracellular calcium.\(^{18}\) Larger membrane depolarisations are required for activation of high voltage-activated Ca\(^{2+}\) channels. Different isoforms of high threshold Ca\(^{2+}\) channels activate at significantly different potentials. The voltage for half-maximal activation of Ca\(V_{1.1}\) lies between 8 and 14 mV (10 mM Ca\(^{2+}\)), \(V_{0.5}\) of Ca\(V_{1.2}\) at -4 mV (15 mM Ba\(^{2+}\)), \(V_{0.5}\) of Ca\(V_{1.3}\) at -18 mV (15 mM Ba\(^{2+}\)), \(V_{0.5}\) of Ca\(V_{1.4}\) between -2.5 and -12 mV (15–20 mM Ba\(^{2+}\)), \(V_{0.5}\) of Ca\(V_{2.1}\) between -5 and -11 mV (5 mM Ba\(^{2+}\)), \(V_{0.5}\) of Ca\(V_{2.2}\) at 8 mV (15 mM Ba\(^{2+}\)), \(V_{0.5}\) of Ca\(V_{2.3}\) at 3.5 mV (15 mM Ba\(^{2+}\)).\(^{17}\) Ca\(V_{1.1}\) channels activate slowly and serve as voltage sensors for the SR ryanodine receptor in skeletal muscle. Ca\(V_{1.2}\) and Ca\(V_{1.3}\) are distributed in neurons, sensory cells of the retina and the inner ear\(^{19}\) whereas the Ca\(V_{1.4}\) is mainly found in the retina.\(^{20}\) Ca\(V_{2.1}\), Ca\(V_{2.2}\) and Ca\(V_{2.3}\) are predominantly found in presynaptic terminals, dendrites and cell bodies of neurons (Ca\(V_{2.1}\) and Ca\(V_{2.2}\) are also found in heart, testes and pituitary).\(^{17}\)

**Channelopathies Are Associated with Changes in Voltage Dependence of Calcium Channel Activation**

Structural determinants underlying the different voltage sensitivities of different calcium channel family members are currently unknown. The pore-forming \(\alpha_{1}\)-subunits of all calcium channels are composed of four homologous repeats formed by six transmembrane segments (S1–S6) that are linked together on a single polypeptide.\(^{17,18,21}\) In analogy to other voltage gated ion channels, it is assumed that the voltage-sensing machinery of calcium channels is formed by multiple charged residues located in S4 and interacting with surrounding residues. This motif of hydrophobic residues in the lower third of channel activation plays an important role in activation gating. Replacement any of these IIS6 residues with helix breaking prolines induced similar changes in channel activation near footstep of the activation curve in the hyperpolarising direction upon replacement of G369D in segment IS6 of Ca\(V_{1.4}\) that was associated with slowed inactivation and removal of Ca\(^{2+}\)-dependent inactivation.

**Kinetic Hallmarks of Pore Stability**

Substitutions of I781 in \(\alpha_{1.2}\)-subunit by residues of different hydrophobicity, size and polarity\(^{12}\) all shifted channel activation in the hyperpolarising direction with I781P causing the most severe (-37 mV shift) effect (Fig. 2F). Mutations in position I781 slow deactivation at all potentials (Fig. 2C and E). The voltage dependence of the activation time constants of different mutants is illustrated in Figure 2B. The time courses of current activation at large depolarizations (∼20 mV) were similar, while channel activation near the footstep of the activation curve was slower than in wild-type (Fig. 2D, between -60 and -30 mV). Shifts to more hyperpolarized voltages correlated with slower activation. Additional mutational studies revealed an important role of neighboring residues. This motif of hydrophobic residues in the lower third of segment IS6 is conserved in high voltage activated calcium channels (Fig. 2A). It was hypothesized that residues LAIA (779–782)\(^{12}\) play an important role in activation gating. Replacement any of these IS6 residues with helix breaking prolines induced similar changes in channel gating: a shift in the voltage-dependence of activation accompanied by a slowing of the activation kinetics near footstep of the activation curve, a slowing of deactivation at all potentials and decreased inactivation.

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**Figure 1. Functional analysis of I781T, the Ca\(V_{1.2}\) mutant corresponding to I745T Ca\(V_{1.4}\). (A) Representative families of \(I_{\text{Ba}}\) through wild-type (top) and I781T mutant (bottom) channels during depolarizing test pulses from -100 mV (threshold and maximal voltages are indicated, 10-mV increments). Wild-type or I781T mutant Ca\(V_{1.2}\) \(\alpha_{1}\)-subunits were co-expressed together with \(\beta_{1a}\) and \(\alpha_{2,3}\)-subunits. (B) Averaged current-voltage relationships (normalized to maximal current) of the wild-type (n = 8, open circles) and I781T mutant (n = 7, filled circles) channels. Data reproduced from Hohaus et al.\(^{12}\) with permission.
Similar shifts of the activation curve and even more dramatic slowing of the activation and deactivation kinetics were observed in NaChBac. Mutation of the conserved glycine 219 in NaChBac (corresponding to a putative hinge glycine in S6 of most K+ channels, see Fig. 2A) to proline not only shifts the voltage dependence, but also slows deactivation (Fig. 3).15 A comparison of Figures 2 and 3 illustrates the common kinetic phenotypes in different channels: (i) shifted activation curve; (ii) slow activation and (iii) a deceleration of channel deactivation. Obviously these can be induced by structural changes in different positions of the S6 segments. Helix bending in the upper third of S6 in NaChBac induces even stronger effects than structural changes close to the inner channel mouth of L-type (CaV1.2) channels. A locus of conserved hydrophobic residues VAVIM (1718–1722) in pore forming segment IVS6 of CaV2.3 was systematically substituted by flexible glycines.14 Glycine mutations affected channel inactivation kinetics. Slow activation of mutant V1720G (IVS6, Fig. 4A) was accompanied by a -20 mV shift of the curve, suggesting a relative increase in open state stability. Different deactivation kinetics of glycine mutants V349G (IS6), I701G (IIS6), L1420G (IIIS6) and V1720G (IVS6) are illustrated in Figure 3. The most prominent effect in CaV2.3 was, however, induced by mutating I701 (corresponding to the I781 in IIS6 of CaV1.2) to glycine (Fig. 4B) which may indicate a particular role of IIS6 in calcium channel gating (Fig. 4). Different contributions of residues in analogous positions in segments IS6–IVS6 reflect the structural asymmetry of the channel pore.

**Gating Hinges in Calcium Channel S6 Segments?**

In analogy to KcsA30,31 it is assumed that S6 segments of voltage-gated calcium channels line the channel pore with a bundle of S6 segments to form a transmembrane bundle, as is suggested by structural studies of the voltage-gated potassium channel KcsA.4,5 Such a bundle would provide a hydrophobic core for the channel pore and ensure structural stability. The S6 segments are believed to contribute to the selectivity filter, determining the permeation of calcium ions through the channel. Functional evidence for the importance of S6 segments in calcium channel gating comes from site-directed mutagenesis studies, which have shown that mutations in S6 can significantly affect channel function.

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**Figure 2.** Altered Gating of CaV1.2 by Substitution of Threonine and Proline for Isoleucine 781 in Segment II S6. (A) Alignment of S6 segments (TM2 helices) for CaV1.2, CaV2.3, NaChBac and KcsA. Adhesion points are highlighted gray, putative hydrophobic seals in CaV and NaChBac in analogy to KcsA are coloured green. (B and C) Activation (B) and deactivation (C) kinetics of I Ba through wild-type, I781T and I781P mutant channels. (D and E) Voltage dependencies of the time constants of activation (D) and deactivation (E) for wild-type, I781T and I781P mutant channels. (F) Averaged voltage dependences of activation of wild-type (n = 8), I781T (n = 8), I781P (n = 8), I781T (n = 7), I781A (n = 7), I781L (n = 5) and I781N (n = 4) channels. Data reproduced from Hohaus et al.12 with permission.

|      | S6   |      |      |      |      |
|------|------|------|------|------|------|
| Cav1.2 | ELPWVYFVSLVFQGFSFLNVFLVLS | 409–438 |      |      |      |
| Cav2.3 | TWNLHFFIPILISFGSLNVFLVLS | 325–354 |      |      |      |
| Cav1.0 | MELVVCYFIIIPLIGNYILL | 757–766 |      |      |      |
| Cav2.3 | MWSILYFIVLPTLPGNTLNLNVFLAI | 677–706 |      |      |      |
| Cav1.2 | VEISIFIIYIIIIFAFMAMNTFVGC | 1170–1199 |      |      |      |
| Cav2.3 | MESSIYVVFVFFPPFVNFVATLIII | 1396–1425 |      |      |      |
| Cav1.2 | SFVVFNSFSFTML | 1480–1509 |      |      |      |
| Cav2.3 | DLAYVFFSFIIFCSFLML | 1696–1725 |      |      |      |
| NaChBac | PWSLYFVSFLGTTIFNLPIGVTVNNV | 206–235 |      |      |      |
| KcsA  | LWGRCVAVVVMAGITSFGLVPAALTW | 86–115 |      |      |      |
Figure 3. Altered Gating in NaChBac by Substitution of Proline for a Hinge Glycine Residue in the S6 Segment (see Fig. 2A for location of G219). (A and B) Homology model of the proposed change in the preferred position of the S6 segments of NaChBac due to substitution of a proline (in space-filling representation, orange) for glycine (G219P). Model based on Jiang et al. (2002b), as described in Experimental Procedures as the channels move from closed (blue helices) to open (magenta helices) conformations (opening movement shown by arrows). (A) Side view of two channel subunits. (B) View from the cytoplasmic channel surface. (C) Sodium current records from wt (top) and G219P (bottom) in response to a series of depolarized test-pulse potentials from -55 mV (wt) or -115 mV (G219P) with 10 mV steps from a holding potential of -120 mV. (D) Voltage dependence of activation from measurements of tail currents after 50 ms (wt) or 2 s (G219P) depolarization. Voltages to activate half of the maximum current (V0.5) are -24.0 ± 1.6 mV and -75.2 ± 0.9 mV for wild-type (n = 7) and G219P (n = 7), respectively. The slope factors are 10.8 ± 1.1 mV and 6.8 ± 0.7 mV for wild-type and G219P, respectively. (E) Sodium current traces during depolarizations to 0 mV (top) and time constants (τ) of activation (bottom) obtained by fitting the final activation phase of currents with single exponentials for wild-type (open circles, n = 6) and G219P (filled circles, n = 6). (F) Tail currents at -120 mV (top) and time constants of channel closure (bottom) measured during repolarization to the indicated potentials following a test pulse to -10 mV from a holding potential of -120 mV for wild-type (open circles, n = 5) and following a test pulse to -60 mV for G219P (closed circles, n = 8). From Zhao et al.15 with permission.

Figure 4. Deactivation Time Constants for the S6 Residues Facing Val1720 in CaV2.3. (A) representative tail currents for wild-type, V349G, L1420G and V1720G channels. Currents were activated during a 8-ms conditioning depolarization to 0 mV for CaV2.3 wild-type, V349G and L1420G; and -10 mV for V1720G. Deactivation was recorded during subsequent repolarizations with 10 mV increments starting from -120 mV (test potentials). Time constants were estimated by fitting current deactivation to a monoeponential function. (B) representative tail currents for I701G channels. Currents were activated during a 15-ms conditioning depolarization to -40 mV. Deactivation was recorded during subsequent repolarizations with 10 mV increments starting from -120 to -40 mV (test potentials). Time constants were estimated by fitting current deactivation to a mono- or a biexponential function. Monoeponential functions were found to fit reasonably well the tail currents of all channels and allow for a better comparison between the wild-type and other mutants. (C) mean time constants of channel deactivation (monoeponential functions) for CaV2.3 wild-type, V349G, I701G, L1420G and V1720G are plotted versus test potential. At -40 mV, the time constants of deactivation were: 1.7 ± 0.1 ms (n = 8) for wild-type; 2.0 ± 0.2 ms (n = 7) for V349G; 3.3 ± 0.06 ms (n = 6) for L1420G; 5 ± 1 ms (n = 6) for V1720G; and 130 ± 20 ms (n = 6) for I701G such that deactivation kinetics decreased from wild-type ≅ V349G < L1420G < V1720G << I701G. The pulse protocol is shown in the inset. From Raybaud et al.14 with permission.

crossing region forming the channel gate. In Kv, the inner part of S6 rotates about a glycine “gating hinge” during the closed to open transition.32 This glycine residue, in position G89 of the MthK and G99 in KcsA potassium channels,33 is highly conserved in many ion channels. Mutating the analogous glycine to proline in NaChBac drastically alters gating properties (Fig. 3). An analogous glycine 770 in the corresponding position of segment IIS6 of CaV1.2 was mutated to proline by Hohaus et al.12 G770P had, however, neither significant effects on the current kinetics nor on the voltage-dependence of channel activation and inactivation. This finding suggests that the mechanism of CaV1.2 activation is different from NaChBac and MthK. Furthermore, in CaV1 and CaV2 the analogous S6 glycine is present only in IS6 and IIS6 (Fig. 2A) suggesting different structural changes during activation than in Kv and NaChBac. It is interesting to note that lethal arrhythmias are associated with mutations of glycine residues (G406R) in the human CaV1.2.34,35

Sealing Points and Adhesion Points

From the crystal structure of the KcsA it was concluded that three amino acids in the pore lining transmembrane helices (TM2)
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(Thr107, Ala111 and Val115)\(^3\) are likely to form “hydrophobic seals” that prevent permeation through closed channels. A “sealing point” is characterized by convergence of the methylene groups from all amino acids of the symmetric TM2 segments. The methylene groups seal tight enough to prevent the passage of dehydrated potassium ions.\(^3\) It is currently unknown, however, whether hydrophobic interactions in these positions contribute to stabilization of KcsA in the closed state.

In calcium channels changes in hydrophobicity in the inner pore region induce major changes in pore stability. Examples of how these changes in selected positions of S6 segments affect the kinetics and voltage sensitivity of CaV1.2 are shown in Figure 2 (see ref. 14 for CaV2.3).

Plotting the shifts of the activation curves (\(\Delta V_{0.5}\)) versus the changes in hydrophobicity in position 781 reveals a strong correlation (Fig. 5B), whereas no correlation was observed for changes in molar mass and residue size (\(\Delta\)Van der Waals volume). A similar approach for analysing the mutational data of Raybaud et al.,\(^1\) for substitutions in positions 1701 (IIIS6), 1742 (IIIS6) and V1720 (IVS6) revealed a similar strong correlation between \(\Delta V_{0.5}\) and changes in hydrophobicity for CaV2.3 (Fig. 6). The positions of these residues in CaV2.3 are highlighted in Figure 2A.

The correlation between activation and hydrophobicity suggests that the side chains are buried in a hydrophobic environment when the channel is closed but exposed to water when it is open. The homology models suggest that these residues interact with neighbouring hydrophobic residues in other S6 segments for the closed conformation. We hypothesise that the interaction between residues L347 (IS6) and V1720 (IVS6), I701 (IIIS6) and V1418 (IIIS6), and L1420 (IIIS6) and V1718 (IVS6), provides stability to the closed channel pore. These interactions significantly contribute to pore stability and may therefore differ from the “sealing points” in KcsA described above. We prefer the term “adhesion point”. An “adhesion point” would thus more efficiently stabilize helix-helix interactions while prevention of calcium ion passage may occur at a different location in the pore.

**Activation Determinants in Calcium Channel Families**

The first attempt to localise structural elements in Ca\(^{2+}\) channel \(\alpha_1\)-subunits involved in channel activation was made by Tanabe et al.,\(^3\) who constructed chimeric channels in which sequence stretches of a slow-activating (“skeletal muscle-like”) CaV1.1 \(\alpha_1\)-subunit were replaced by sequences from a fast-activating (“cardiac-like”) CaV1.2 \(\alpha_1\)-subunit. The chimeras activated slowly if repeat I of the CaV1.2 \(\alpha_1\)-subunit was replaced by the CaV1.1 \(\alpha_1\)-sequence. Later studies of Nakai et al.\(^3\) revealed that segment S3 and the S3-S4 linker of repeat I are critical for the difference in activation kinetics between cardiac (CaV1.2) and skeletal muscle (CaV1.1) calcium channels. An important role of the voltage sensors of domains I and III but not II and IV in voltage-dependence (\(V_{0.5}\)) and time-course of activation were reported by Garcia et al.,\(^3\) who mutated the arginines in the S4 segments of all four domains of a chimeric channel to neutral or negative amino acids. The removal of prolines that are conserved in segments IS4 and IIIS4 of Ca\(^{2+}\) channels resulted in shorter channel open time, whereas introduction of extra prolines to corresponding positions of IIS4 and IVS4 lengthened the channel open time.\(^3\)

Li et al.\(^3\) constructed a series of chimeras between low voltage activated (CaV3.1) and high voltage activated (CaV1.2) channels. Their data suggest that domains I, III and IV (rather than domain II) are apparently critical for channel opening and, therefore, contribute strongly to the difference in voltage dependence of activation between CaV3.1 and CaV1.2. Determinants of the half-activation potential of low voltage activated CaV3 channels were identified in domains I and IV.\(^4\)

Zhen et al.\(^3\) described substantial changes in CaV2.1 activation upon replacement of S6 residues and residues of the adjacent intracellular loops by cysteines. Their study suggests that important activation determinants of this channel type may be localised in intracellular loop segments. Cysteine accessibility by methanethiosulfonate ethyltrimethylammonium (MTSET) of the inner pore region suggests possible differences in the architecture of CaV2.1 compared to K\(^+\) channels.\(^3\)
Modulation of Calcium Channel Activation by Auxiliary Subunits

It is well established that β-subunits modulate the gating of high-voltage-activated Ca\(^{2+}\) channels.\(^{42-44}\) A significant hyperpolarising shift of the activation curve of the ionic current is observed upon coexpression of a β-subunit with the Ca\(^{2+}\) \(\alpha_{1}\)-subunit. The activation curve for the gating current was unaffected, suggesting that the β-subunit modulates activation exclusively by affecting pore stability (Fig. 7). The time-course of channel activation is also modulated in a subunit-specific manner.\(^{42}\) These findings suggest that β-subunits modulate not only the inactivation properties of the channels, but also affect activation by modulating pore stability.\(^{\alpha_{2}-\delta}\) subunit causes a shift in the current-voltage and conductance-voltage curves toward more positive potentials and accelerates activation and deactivation kinetics.\(^{45,46}\) Data of Obermair et al.\(^{47}\) show, however, that \(\alpha_{2}-\delta\) depletion of reconstituted dysgenic \(\alpha_{15}\)-null myotubes significantly accelerated the current kinetics, suggesting a conversion of slowly activating into fast activating Ca\(^{2+}\) channels.

The Link Between Pore Stability and Activation Kinetics

A major challenge for biophysicists and molecular biologists is the interpretation of the kinetic changes shown in Figures 2–4. Yifrach and MacKinnon\(^{48}\) discussed that the leftward shift of the activation curve in “mutational perturbation” studies on Shaker potassium channels could reflect either the stabilization of the open state or the destabilization of the closed state. The authors reasoned, however, that the shifts towards negative potentials are more likely result from a destabilization of the closed channel pore than from a stabilized open state. Some of the “kinetic fingerprints” suggest, however, a stabilization of the open conformation (e.g., slow tail current kinetics, Figs. 2–4).

Changes in pore stability of Ca\(^{2+}\) in terms of rate constants can be simulated making use of a simplified version of the model of Zagotta et al.,\(^{8}\) by assuming that Ca\(^{2+}\) consist of two functionally distinct parts: (i) a voltage-sensing mechanism and (ii) a pore that opens and closes independently of voltage. As illustrated in Figure 8, the voltage sensor may dwell in the resting (locking) and activated (releasing) states and the pore in the open or closed states. The molecule therefore dwells in 2 x 2 = 4 states: locked/closed (R), released/closed (A), open (O) and locked/open (D). Rate constants of the pore opening and closing are assumed to be dependent on the voltage sensor position: rate constants α and β describing the pore opening and closure at an activated voltage sensing machinery (\(A \leftrightarrow O\)) accordingly differ from γ and δ (\(D \leftrightarrow R\)). The rate constants of the voltage sensor transitions between locking and “releasing” positions depend on the membrane potential. All rate constants are linked by the thermodynamic reversibility condition:

\[
\frac{x \cdot \alpha \cdot \omega \cdot \delta}{v \cdot \beta \cdot u \cdot \gamma} = 1
\]

at all potentials.

This approach can be used to analyze kinetic effects of mutations in the calcium channel pore if we lump together possible closed state transitions and described the rate constants of the voltage sensing subsystem (\(R \leftrightarrow A\)) by simple exponential functions with V- membrane voltage, x and y rate constants of voltage sensor movements, xo and yo amplitude coefficients and k\(_{x}\) and k\(_{y}\) the inverses of the steepness of voltage dependences:

\[
x = x_{0} \cdot \exp \left( \frac{V}{k_{x}} \right)
\]
\[
y = y_{0} \cdot \exp \left( -\frac{V}{k_{y}} \right)
\]

It can be easily deduced from this model that the midpoint of the activation curve is:

\[
V_{m} = V_{o} - k_{y} \cdot \ln \left( \frac{1 + \frac{\alpha}{\beta}}{1} \right)
\]

As discussed previously, shifts of the activation curves might be explained in terms of stabilization or destabilization of closed or open states (stabilization/destabilization are defined as decrease/increase of
rate constants of the leaving states). Shifts of the activation curves can be solely described as changes in $\alpha/\beta$ (pore opening and closure) without changes in the slope factor:

$$k_S = \frac{k_x k_y}{k_x + k_y}$$

and midpoint $V_x = k \ln(y_0/x_0)$ of the $R \leftrightarrow A$ transition.

The transition from the locked-closed state ($R$) into the open state ($O$) under a depolarization occurs predominantly via the $A$ state. Transitions $R \leftrightarrow D \leftrightarrow O$ are very rare because the first transition $R \leftrightarrow D$ (Fig. 8) is slow and negligible, which enabled the analysis of a simplified activation pathway:

$$R \xrightarrow{\alpha/\beta} A \xrightarrow{k_x/k_y} O$$

This scheme reflects voltage sensor activation (transition $R \leftrightarrow A$) with the voltage-dependent rate constants $x$ and $y$ and the subsequent pore opening (transition $A \leftrightarrow O$) with the voltage-independent rate constants $\alpha$ and $\beta$. During deactivation a strong hyperpolarization will first induce a movement of the voltage sensors from the active to the “locking” position. In other words, the transition from open ($O$) to the resting state ($R$) under a hyperpolarization occurs predominantly via a deactivated but still open conformation ($D$), which enabled the analysis of the simplified model:

$$O \xrightarrow{k_x/k_y} D \xrightarrow{\delta/\gamma} R$$

The scheme describes the voltage sensor deactivation (transition $O \leftrightarrow D$) with the voltage-dependent rate constants $\delta$ and $\gamma$ and the subsequent pore closure (transition $D \leftrightarrow R$) with the voltage-independent rate constants $\alpha$ and $\beta$.

Time constants of activation and deactivation of wild-type CaV1.2 and mutants I781P and I781T are given in Figure 2. Traces in Figure 9A represent a simulation with the rate constants given in Tables 1 and 2. It appears that the description of the activation processes with unchanged $x(V)$ and $y(V)$ (see also Fig. 7) requires simultaneous changes in $\alpha$ and $\beta$. In other words, in such a scenario an increase in $\alpha$ (destabilization of the open conformation) goes in parallel with a decrease in $\beta$ (stabilization of the open conformation). The activation time constant for wild-type channels appears to be almost voltage-independent, suggesting that the pore opening is the rate-limiting stage at all potentials. Destabilization of the pore accentuated voltage dependence of activation gating in CaV1.2 (Fig. 4).

Conclusions and Outlook

Different calcium channel family members expressed in different tissues open at different membrane voltages. Their activation thresholds enable calcium entry to be fine-tuned with respect to resting potential and action potential firing for specific tissues. The molecular basis for differences in channel activation between calcium channel family members (e.g., CaV1 and CaV3) remains obscure.

Progress has been made, however, in understanding the impact of individual amino acids (e.g., mutations associated with channelopathies). Replacement of single amino acid may shift the activation threshold by more than 30 mV, as illustrated in Figures 2 and 3. The evidence shows that changes in hydrophobicity play an essential role (Figs. 5 and 6) for mutations within the activation gate, while other amino acid properties such as bulkiness (van der Waals volume)
pair of S6 helices in addition to the one corresponding to the I781 data described here. Future studies will reveal the relative impact of these interactions in closed state stability and/or pore occlusion. The molecular mechanism of the particular strong effect of mutations in position I781 of CaV1.2 (I701 in CaV2.3, Figs. 2 and 4) remains, however, to be elucidated. Putative hinge points known from potassium channels have yet to be identified in voltage-gated calcium channels. Figure 5 illustrates that changes in voltage sensitivity induced by mutations to flexible glycines show no particular phenotype and reflect at least partially changes in hydrophobicity. Other open questions concern the correlation between the shifted activation and inactivation curves observed in Hohaus et al. (reviewed in ref. 49).

It is tempting to speculate that evolution designed the voltage sensing machinery as robust “all-or-non” device while the verity of voltage sensitivities was accomplished by shaping pore stability. The pore of α subunits of voltage gated calcium channels is asymmetric. Mutations of amino acids in different S6 segments (I–IV) will correspondingly differentially affect pore stability and kinetics. In NaChBac a single point mutation (e.g., G219P) induces changes in all four pore lining helices, which may explain the stronger modulation. Progress in understanding of calcium channel activation will depend on the quantification of pore stability in terms of rate constants (e.g., Figs. 8 and 9). Previous attempts revealed that
steady-state and/or kinetic changes can be described by changes in the rate constants of pore transitions without affecting rate constants of voltage sensor movements. A similar scenario is illustrated in Figure 9 for CaV_{1.2}. The best simulation was obtained by simultaneous changes in \( \alpha \) and \( \beta \), suggesting that changes in current kinetics and steady state activation reflect a concurrent destabilization of the closed conformation (increase in \( \alpha \)) and a stabilization of the open conformation (decrease in \( \beta \), see Table 1). Analyzing a larger set of mutations may help to understand this intriguing interrelationship.

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