Characterization of the gating brake in the I–II loop of CaV3 T-type calcium channels

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Our interest was drawn to the I–II loop of CaV3 channels for two reasons: (1) transfer of the I–II loop from a high voltage-activated channel (CaV2.2) to a low voltage-activated channel (CaV3.1) unexpectedly produced an ultra-low voltage activated channel; and (2) sequence variants of the I–II loop found in childhood absence epilepsy patients altered channel gating and increased surface expression of CaV3.2 channels. To determine the roles of this loop we have studied the structure of the loop and the biophysical consequences of altering its structure. Deletions localized the gating brake to the first 62 amino acids after IS6 in all three CaV3 channels, establishing the evolutionary conservation of this region and its function. Circular dichroism was performed on a purified fragment of the I–II loop from CaV3.2 to reveal a high α-helical content. De novo computer modeling predicted the gating brake formed a helix-loop-helix structure. This model was tested by replacing the helical regions with poly-proline-glycine (PGPGPG), which introduces kinks and flexibility. These mutations had profound effects on channel gating, shifting both steady-state activation and inactivation curves, as well as accelerating channel kinetics. Mutations designed to preserve the helical structure (poly-alanine, which forms α-helices) had more modest effects. Taken together, we conclude the second helix of the gating brake establishes important contacts with the gating machinery, thereby stabilizing a closed state of T-channels, and that this interaction is disrupted by depolarization, allowing the S6 segments to spread open and Ca2+ ions to flow through.

Chimeric Studies Revealed Important Properties of the I–II Loop

The I–II loop in high voltage-activated Ca2+ channels plays a major role in channel function. It contains binding sites for both its β subunits (CaVβ) and G protein βγ subunits. Binding of CaVβ subunits increases trafficking of the α1 subunits of CaV1 and CaV2 families to the plasma membrane, increases channel Po, and modulates the time- and voltage-dependence of channel gating.1 While binding of G protein βγ subunits to the I–II loop inhibit activity of the family of CaV2 channels.2 The amino acid sequence of the I–II loop differs significantly for the CaV3 family. CaV3 channels have lost the ability to bind β subunits with high affinity, and therefore, they have lost CaVβ regulation. We hypothesized that transfer of the I–II loop from a CaVβ-regulated high voltage-activated channel (CaV2.2) to a CaVβ-independent low voltage-activated channel (CaV3.1) would restore CaVβ regulation. Much to our surprise we created an ultra-low voltage activated channel.3 As predicted the chimera did retain some features of CaVβ regulation, e.g., slowing of inactivation kinetics and shifts in the voltage-dependence of activation. In an effort to improve CaVβ regulation we also transferred the distal part of the IS6 segment from CaV2.2, testing the hypothesis that a glycine hinge (GxxxG3) in α2.2 was important for regulation. Again, to our surprise we nearly abolished open state inactivation and in so doing abolished the transient nature that defines T-currents.3 This finding reveals an important role of the distal IS6 segment in T-channel gating, which is similar to the results found for its IIIIS6 segment.7 Nevertheless, CaVβ regulation was not improved. We next tested the hypothesis that CaVβ regulates channel activity by altering the movement of IS6, an effect mediated by a rigid α-helix. To test this hypothesis we replaced six consecutive residues of this α-helix with glycine residues (G6) that destabilize helices. As a control we replaced six consecutive residues alanine (A6) that preserve α-helical structure and hence function.6,7 Circular dichroism studies confirmed the α-helical structure of the wild-type CaV2.2 IS6 to alpha-interaction domain (AID), its preservation by A6, and its destruction by G6. Notably, CaVβ regulation was abolished by the G6 substitution and maintained in the A6 mutant, thereby providing the first evidence that CaVβ subunits modulate Ca2+ channel gating by direct communication with the IS6 segment. Interestingly, deletion of a single amino acid in this linker abolishes both CaVβ and G protein regulation of CaV2.2 (Bdel ). Bimolecular fluorescence complementation studies confirmed that this deletion altered the orientation of CaVβ with respect to the α1 subunit, strongly suggesting that orientation is critical for aligning multiple points of contact between the two subunits. For a comprehensive review of CaVβ structure and function, see the review written by Buraei and Yang.1
The Role of the I–II Loop in Surface Expression: A Novel Paradigm for Childhood Absence Epilepsy Variants

The three Ca₃.3 channels are expressed throughout the central nervous system, displaying both overlapping and complementary expression. For example in the thalamus, Ca₃.2 and Ca₃.3 mRNAs are expressed in the reticular nucleus, while Ca₃.1 mRNA is abundantly expressed in the relay nuclei. T-channels play important roles as pacemaker currents, depolarizing the plasma membrane enough to trigger bursts of Na-dependent action potentials. Thalamic neurons express the highest T-currents in the body and play a major role in its oscillatory rhythms. Therefore, sequence variations in T-channel genes may increase susceptibility to neurological disorders characterized by thalamocortical dysrhythmia. This hypothesis was tested by Chen and coworkers who sequenced two T-channel genes in Chinese patients with childhood absence epilepsy (CAE). Twelve variants in the gene encoding Ca₃.3 (CACNA1H) were found exclusively in these patients. Many, but not all, of these variants altered the biophysical properties of the recombinant channel expressed in 293 cells. Computer modeling predicts that some of these variants would lead to increased neuronal firing, supporting in part the hypothesis that they contribute to the pathogenesis of this polygenic disorder. For a comprehensive review of Ca₃.2 in epilepsy, see the review written by Khosravani and Zamponi.

Since many channelopathies are due to mutations that alter trafficking, we hypothesized that the CACNA1H variants may also affect trafficking. Notably, seven of the twelve CAE specific variants alter the amino acid sequence of the I–II loop in Ca₃.2. Three of these variants had a large effect on firing in the NEURON model (C456S, P648L and G773D: Vitko et al. 2005), two had little or no effect (G499S, A748V; see also the review written by Khosravani and Zamponi. 17 Supporting this notion are studies in animals in the gene encoding Ca₃.2 (CACNA1H) were found exclusively in these patients. Many, but not all, of these variants altered the biophysical properties of the recombinant channel expressed in 293 cells. Computer modeling predicts that some of these variants would lead to increased neuronal firing, supporting in part the hypothesis that they contribute to the pathogenesis of this polygenic disorder. For a comprehensive review of Ca₃.2 in epilepsy, see the review written by Kho...
While 25% and 34% of CaV3.1 and CaV3.3 channels were expressed at the surface, respectively. It is interesting to speculate that trafficking of CaV3.2 channels is regulated by extrinsic factors, and underlies the increase in T-currents observed in several disease states, such as temporal lobe epilepsy and diabetic neuropathy.

Structure of the Gating Brake

Secondary structure prediction programs suggest the first 62 amino acids that compose the gating brake would form a helix-loop-helix structure similar to that found in fumarase. To test this prediction we purified a fragment of the I–II loop from E. coli, and studied its structure using circular dichroism. The large 10–15 mV shifts in the voltage dependence of gating and accelerated activation and inactivation kinetics. To explore the role of the I–II loop in channel trafficking, we used the same strategy as with CaV3.2, introducing the HA epitope into the extracellular loop connecting IS5 to the pore loop, and measuring surface expression with anti-HA antibodies. Unexpectedly, deletions of the I–II loop that only affected trafficking in CaV3.2 channels only modestly increased trafficking of CaV3.1 yet decreased trafficking of CaV3.3. Notably, amino acid conservation follows a similar pattern, with CaV3.1 and CaV3.2 showing considerable identity, while CaV3.3 has a much smaller loop (204 vs. 372 a.a.). This study also revealed important differences in the surface expression of wild-type CaV3 channels, as only 13% of wild-type CaV3.2 channels were expressed at the surface, while 25% and 34% of CaV3.1 and CaV3.3 channels were expressed at the surface, respectively. It is interesting to speculate that trafficking of CaV3.2 channels is regulated by extrinsic factors, and underlies the increase in T-currents observed in several disease states, such as temporal lobe epilepsy and diabetic neuropathy.

**Figure 1.** Conservation of the gating brake. (A) Alignment of the three human CaV3 amino acid sequences to that determined for the freshwater pond snail, *Lymnaea stagnalis*. The location of helix-turn-helix of the gating brake is shown above, and secondary structure prediction by the SOPMA algorithm. Bottom line shows consensus sequence for residues that are identical in all four sequences. Amino acids are colored based on their physical properties to emphasize conservative amino acid changes. (B) Model of the CaV3.2 I–II loop gating brake with residues conserved in Lymnaea displayed in Corey-Pauling-Koltun (CPK) space filling model. Model was originally reported by Arias et al. and modified according to the Lymnaea sequence reported by Senatore and Spafford. The model is viewed from the side, with the plasma membrane on top and helix to the left. Note how conserved residues face away from the hydrophobic core, suggesting they form conserved protein-protein contacts. Substitutions in the core are conservative—hydrophobic for hydrophobic and the putative salt bridge is conserved. (C) Second view of the model is from the bottom looking up at the membrane. Note how the conserved “arginine fingers” jut out perpendicular to the plane of the gating brake. These residues are comprised of five Arg residues on one face and 2 on the other.
circular dichroism spectrum indicated that the proximal I–II loop contains a high α-helical content, essentially matching the predicted value.

Four types of mutations were used to test the helix-loop-helix model. First, we used deletion analysis to find the distal end of the gating brake. Second, we tested for the existence of α-helical regions by replacing 6 consecutive amino acids with prolines and glycines to disrupt the helix, and as a control we replaced the same residues with alanine to conserve the helix. Third, we tested for the existence of the loop by either converting it to an α-helix, or by deleting it entirely, effectively fusing helix 1 to helix 2. Finally, we also tested whether addition of alanines to the middle of helix 1 would alter the pitch of the helix, and thereby alter the orientation of the helix-loop-helix with respect to the channel. Overall, the results were consistent with the helix-loop-helix model, as disruption of any of this structure recapitulated the same phenotype: channels whose voltage dependence of activation and inactivation were shifted 10–15 mV and accelerated kinetics. Surprisingly, the poly-alanine mutations also affected function. This finding suggests that the mutated residues play an additional role other than forming an α-helix, possibly forming points of contact with other parts of the gating machinery.

3-D Model of the Brake

To interpret our findings, we generated three dimensional models of the gating brake and examined how the mutations affected its structure. Specifically, we constructed de novo models obtained using the Quanta program and molecular dynamics simulations using HyperChem 7.1. The predicted structure supported the hypothesis that the gating brake forms a helix-loop-helix structure. The models provided insights into the structural modifications caused by the mutations. For example, the poly-alanine mutations disrupted function greater than predicted if this region were a simple helix, and the model shows the loss of a salt bridge between E429 and both K470 and R473. The model also predicted the end of the gating brake, coinciding perfectly with the experimental results. Our studies of the loop region were complicated by the presence of two putative hair-pin turns, since only one turn would be required to orient helix 2 in an antiparallel manner with respect to helix 1. Indeed, replacement of either turn region with six alanines produced channels with only modest effects on gating. In contrast, mutation of both turn regions or deletion of the entire loop region (DC1), produced dramatic shifts in gating. The modeling studies agree well with the experimental findings: replacement of either turn was insufficient to disrupt the helix-loop-helix structure due to compensation by the remaining turn. Modeling of DC1 indicates that the loop was effectively removed, and that helix 1 was fused to helix 2. The gating phenotype of this mutant is similar to the D2 mutant (which lacks helix 2); therefore, we infer that function of the gating brake was totally disrupted. The model also predicts that the epilepsy mutation, C456S, is at a critical location in the brake, forming the start of helix 2, and projecting towards helix 1.

Proposed Mechanism of Action of the Gating Brake

Taken together, our studies establish that the first 62 amino acids immediately following the IS6 segment plays the role of a gating brake in all three CaV3 channels. We called it a gating brake since it appears to stabilize channels in the closed state, and disruption of its function leads to channels that open at normal membrane potentials. Similarly, destabilization of the closed state was proposed to explain how mutations in hydrophobic residues in the S4 voltage sensor of K+ channels shifted gating to more negative voltages. Deletion of the gating brake also led to faster channel opening and increased IP, as predicted by this hypothesis.

Previous measurements of T-channel gating currents indicate that 80% of the channels open after only 20% of total charge movement. This suggests that T-channels open after minimal movement of their voltage sensors and modeling studies suggest this might explain why T-channel kinetics are so voltage-dependent. We propose two mechanisms by which the brake region stabilizes the closed state. These models were developed by superimposing the gating brake on the crystal structure of the K+1.2-K+2.1 paddle chimera channel. In one version, the gating brake is oriented towards the S6 segments of other repeats (Fig. 2A). Voltage-gated channels are now accepted to have two gates, one at the pore loop and the second at the intracellular crossing of the S6 bundles. We suggest that the T-channel S6 segments form an internal gating ring similar to that determined for K+ channels, and that the gating brake acts on this intracellular gate. The model shows how the gating brake could reach both II6 and IIIIS6. Support for an S6 gating ring comes from studies showing that mutations in S6 segments slow open channel inactivation as observed in HVA channels. A second scenario is that the gating brake is oriented in the opposite direction (X-Y plane), facing the IS1-S4 voltage-sensing paddle (Fig. 2B). In this manner it could interact directly with the S4-S5 linker, which plays a major role in coupling S4 voltage-sensor movement to opening of the lower S6 gate. The model also predicts that in either orientation the gating brake would lie over the IS1-S4 gating paddle. A limitation of these models is that the orientation of the gating brake in the Z-axis is unknown. Therefore we make the assumption that the orientation of the S6 segment is the same as observed in the K+ crystal structure, which projects horizontally with respect to the membrane. It is tempting to speculate that the conserved positively charged residues at the end of helix 2 (Fig. 1) are important for this interaction. Interestingly, deletion of the gating brake in Ca,3.3 induces a large -35 mV shift in the gating current-voltage relationship (Karmazinova M and Lacinova L, unpublished observations). At first glance it seems unlikely the gating brake could shift the entire G(V) curve by interaction with a single S4 paddle, yet closer examination of voltage-gated K+ channel structures reveals an interdigitation between subunits, resulting in an extended hot-spot where the three repeats converge. In this manner, the gating brake could interact with not only repeat I machinery, but also interact with repeats II and IV, and thereby affect movements in 3 of the 4 repeats (Fig. 2C and D). In addition, an allosteric model with coupling of the S4 voltage sensors could account for this large shift in the G(V) curve.
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In conclusion, the I–II loops of T-channels play critical roles in the trafficking and gating of these channels. This loop contains an intracellular gating brake that is essential to their ability open after small depolarizations of the membrane. Finally, the ability of sequence variants of the I–II loop to alter gating and trafficking provides insights into how these variations increase seizure susceptibility.

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