Channels 4:6, 1-6; November/December 2010; © 2010 Landes Bioscience

Calcium channel regulation by RGK proteins

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Key words: RGK protein, calcium channel, β subunit, Rem, Rem2, Rad, Gem

The RGK family of proteins, small GTPases of the Ras superfamily, are known to regulate calcium currents. It is commonly thought that this is due to an interaction with the $Ca_V\beta$ subunit, however, the mechanism of this inhibition is unclear. There have been conflicting reports of whether RGK proteins can affect channel trafficking or whether they reduce calcium currents by interacting with channels at the membrane. In the last year, several studies have emerged which explore the intricacies of RGK protein interaction with the channel itself and the importance of the $Ca_V\beta$ subunit for this interaction, in addition to providing some tantalizing suggestions for the mechanism by which RGK proteins reduce or eliminate calcium currents. In this review, we present an overview of these recent advances and suggest a model that may synthesize these latest works.

Introduction

In the last 10 years, small GTPases of the RGK protein family have emerged as potent regulators of HVA calcium channels. The RGK family (Rad, Gem, Kir) consists of four related proteins within the Ras GTPase superfamily: Rad, Gem/Kir, Rem and Rem2. While structurally related, they are subject to differential tissue expression and transcriptional regulation. Rad is expressed in muscle and is upregulated in response to insulin, as well as in diabetic muscle.[1,2] Gem is found in immune cells, endothelial cells, and some neuronal cells, and is upregulated upon an immune challenge, e.g., with mitogens or cytokines, or following treatment with carbachol in the case of neuroblastoma cells.[3-7] Less work has been done on regulation of Rem and Rem2. They are known to be expressed in heart muscle (Rem) and kidney, brain and endothelial cells (Rem2) and, similarly to other family members, appear to show transcriptional regulation.[8-13]

Because RGK proteins have differential tissue expression, each one also exerts differential effects in its own unique milieu. Being expressed in brain, for example Rem2 is important for early synapse development,[11] promotes endothelial cell sprouting[13] and enhances survival of embryonic stem cells by regulating the p53 and cyclin D1 pathways.[16] In addition to each protein's unique effects, there are functions that all RGK proteins have in common: alterations in cytoskeletal architecture, possibly by interaction with the Rho-kinase pathway and, pertinent to this article, a reduction in calcium currents (reviewed in ref. 14 and 15). All RGK proteins have been shown to bind directly to the $Ca_V\beta$ subunit,[10,17-19] an interaction which has been assumed to underlie the effect of RGK proteins on calcium currents. Recent studies, however, have challenged this assumption. Here, we provide an overview of the latest state of knowledge pertaining to regulation of voltage-gated calcium channels by RGK proteins.

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Submitted: 09/23/10; Accepted: 09/23/10
Previously published online:
www.landesbioscience.com/journals/channels/article/12865
DOI: 10.4161/chan.4.6.12865

Structural Features and Protein Interactions of RGK Proteins

RGK proteins are comprised of a central Ras-homology domain flanked by long unique N- and C-termini (**Fig. 1A and B**). Homology between the family members varies from 7% in the N-terminal extension to 40% in the C-terminal extension to 52% in the central Ras domain.[15] Typical Ras GTPase protein domains contain five conserved GTP-binding motifs (G1-G5), which can exist in two conformational states, an active GTP-binding and an inactive GDP-binding state. Critical substitutions in these five motifs of RGK proteins suggest that, although they bind guanine nucleotides, they may not behave as canonical GTPases (reviewed in ref. 14). Measured rates of GTP hydrolysis of RGK proteins range from zero (Gem[20]) to low (Rem and Rem2[9]), to almost 30-fold higher than H-Ras (Gem[21]). Thus there is little consensus in the field as to whether or not RGK proteins act as GTPases—a discrepancy that may be due to different experimental conditions used.

RGK proteins bind calmodulin (CaM) in a calcium dependent manner via a conserved binding site in their C-termini.[22-25] A single point mutation Leu→Gly reduces or abolishes this binding.[18,22,24,26] This mutation seems to increase the localization of most RGK proteins in the cell nucleus, although overexpression of the molecular chaperone protein 14-3-3 appears to clear them back to the cytoplasm.[24,26,27] Thus, in addition to the $Ca_V\beta$ subunit, CaM is a common binding partner between RGK proteins and calcium channels.

RGK Proteins Reduce or Eliminate Calcium Currents

All RGK proteins negatively regulate calcium currents, but the mechanism(s) by which this happens are still being elucidated. This phenomenon has been shown using both exogenously expressed and endogenous channels, including L-type channels,[10,17-19,25,27-35] N-types,[36-38] and P/Q types,[39] but not T-types.[17,36,40] Since all RGK proteins bind the $Ca_V\beta$ subunit, it was assumed that this interaction was necessary for ablation of calcium currents, especially in

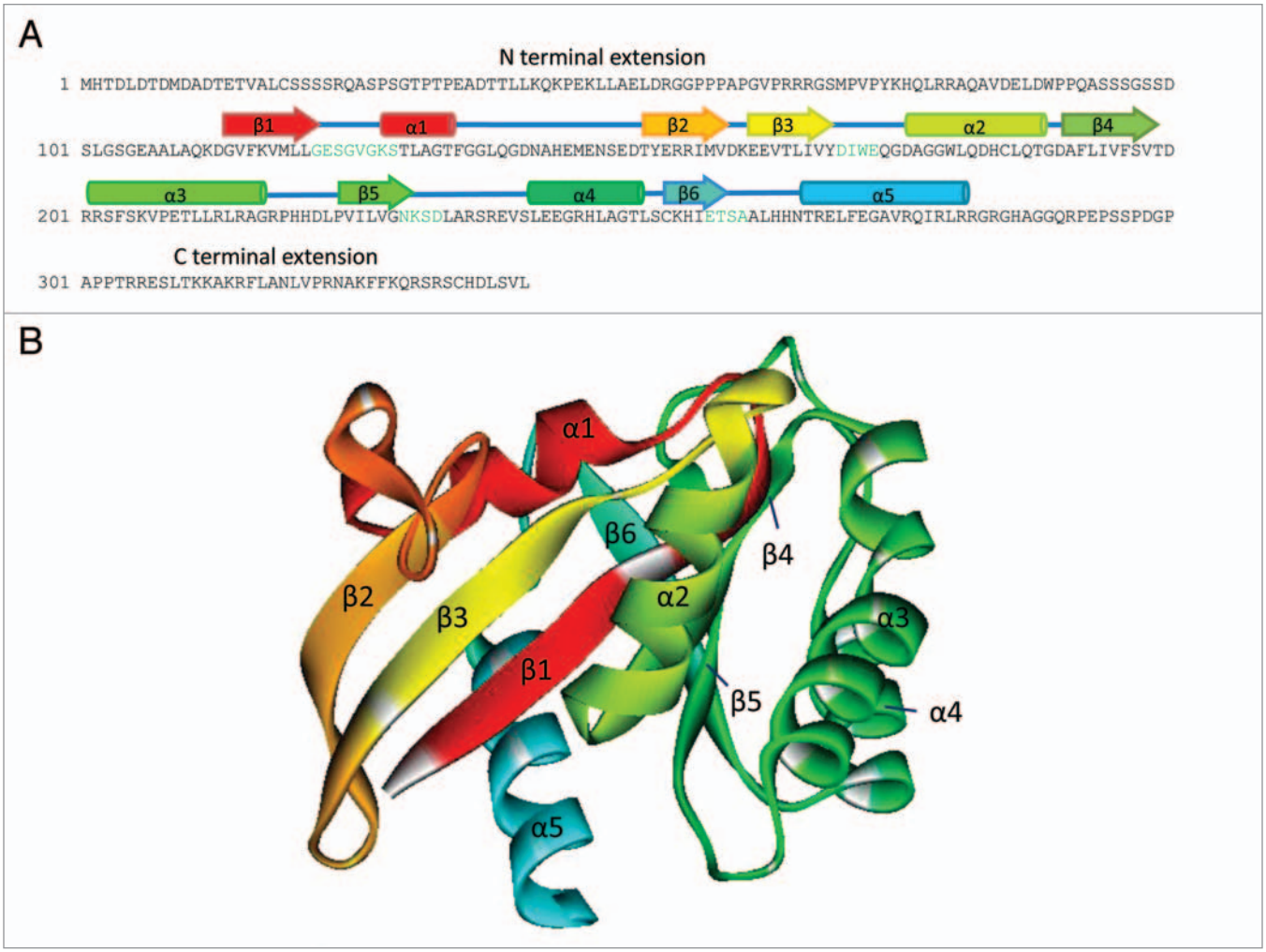

Figure 1. RGK protein structure. (A) RGK protein structure consists of long N- and C-termini flanking a central Ras-homology domain. The C-terminal 31 residues comprise the membrane-binding region, including a calmodulin binding site. The crystal structure of the Ras domain of Rem2 (PDB entry 3CBQ) is shown in (B). Helices and strands are marked as in (A).

light of the fact that T-type channels, which do not associate with accessory subunits, are unaffected by RGK proteins. Indeed, most reports have demonstrated RGK-$Ca_V\beta$ interaction to be required for modulation of calcium current density (reviewed in ref. 14). Lately, however, studies have emerged that shed new light on the mechanisms of Ca_V regulation by RGK proteins and suggest that complex molecular dynamics are at work between channels, $Ca_V\beta$ subunits and RGK proteins.

Two general hypotheses have been proposed to explain the mechanism of the effects of RGK proteins on calcium currents: one, that RGK proteins inhibit channel surface expression; and alternatively, that they inhibit the function of channels already inserted into the membrane.

RGK Proteins may Exert Multiple Simultaneous Effects on Surface Channels

The reduction in surface expression hypothesis proposes that an RGK protein disrupts $Ca_V\alpha_1$-$Ca_V\beta$ subunit association, thus retaining newly synthesized channels in the ER and reducing the numbers of functional channels in the cell membrane.[18,26,27,41] Using an extracellular epitope to probe for surface expression of calcium channels, these studies showed a dramatic reduction of fluorescent signal when channels and their subunits were coexpressed with RGK proteins. An alternate hypothesis proposes that RGK proteins inhibit channel function in situ, and has been tested using a variety of methods. By labeling N-type channels with a fluorescent variant of ω-conotoxin, Chen[36] showed no change in channel cell surface expression upon coexpression of Rem2. Similarly, Finlin[10,19] found no reduction in membrane expression of channels in the presence of Rem or Rem2 when using surface biotinylation assays.

A recent paper from the Colecraft group[32] has demonstrated by flow cytometry a reduction in surface expression of $Ca_V1.2$ by Rem in HEK cells (~60%) with a concomitant abolition of calcium currents. Rather than Rem sequestering the $Ca_V\beta$ subunit, however, this reduction seems to occur by enhancing

the dynamin-dependent endocytosis pathway. Interestingly, restoring $Ca_V1.2$ surface expression by coexpressing a dominant negative dynamin mutant was not sufficient to restore $Ca_V1.2$ currents, suggesting that Rem has at least one other mode of action to inhibit calcium currents. Additionally, they found that Rem could reduce the open probability of the channel, by immobilizing voltage sensors as well as by another mechanism that leaves voltage sensors free. Lately, another study from this group found that expressing Rem in cardiac myoctes leads to decreased channel open probability.[35] Thus, the modes of action by which RGK proteins exert their effects on calcium currents may be more complex than previously realized, and may involve a combined effect on cell surface expression levels and alterations in channel function.

The Role of $Ca_V\beta$ Subunits in RGK Protein Regulation of Calcium Channels

RGK protein association with the $Ca_V\beta$ subunit has been a subject of recent intense investigation (reviewed in ref. 14), since it could potentially present a target for therapeutic regulation of calcium currents. The region of the $Ca_V\beta_{2a}$ subunit required to bind Rem was identified to be a 130 amino acid stretch within the GK domain, a region which also contains the major interaction site between the $Ca_V\beta$ subunit and the channel, a helix in the I-II linker termed the alpha interaction domain (AID). Truncated GK domains that lost the AID binding retained Rem binding, however, showing there are discrete residues responsible for each. The GK domain is sufficient for RGK-mediated calcium current inhibition in Xenopus oocytes,[39,42,43] and it can also simultaneously bind both the AID region and Rem, suggesting that competing for the $Ca_V\beta$ subunit is not a mechanism by which RGK proteins work;[19] these conclusions also hold for Rem2.[44] Although one study found that an AID peptide could inhibit the interaction between Gem and $Ca_V\beta$,[45] most other studies support the separation of RGK-$Ca_V\beta$ binding and $Ca_V\beta$-AID interaction.[30,41,44]

How essential is the RGK protein-$Ca_V\beta$ subunit interaction to achieve the functional effect of reducing calcium currents, and what other factors are necessary? $Ca_V1.2$ currents from channels expressed in HEK cells in the absence of $Ca_V\beta$ subunits are reduced 64% by Rem,[33] but the same channels expressed in Xenopus oocytes in the absence of $Ca_V\beta$ are minimally affected by Gem or Rem2.[18,30] A fragment of $Ca_V\beta_{2a}$ that interacts with Rem but not the AID region can partially rescue calcium currents by competition when coexpressed with Rem and $Ca_V1.2$,[19] showing that RGK-β subunit binding is important for the effect. But $Ca_V\beta$ subunit binding alone can also not exclusively account for the effect. Indeed, Rem and Rem2 must contain the membrane-targeting sequence in the C-terminus as well as bind the $Ca_V\beta$ subunit to reduce calcium currents.[38,44] Thus, the necessity of involving the $Ca_V\beta$ subunit in RGK modulation of calcium currents is variable, certainly between RGK proteins and possibly between expression systems as well.

Direct RGK-channel Interaction

To add further complexity to RGK regulation of calcium channels, Pang et al.[29] recently reported that Rem (as well as Rad and Rem2) can bind the C-terminus of $Ca_V1.2$ channels directly, independently of any interaction with the $Ca_V\beta$ subunit. To achieve both $Ca_V1.2$ C-terminus binding and calcium channel knockdown, Rem had to be targeted to the cell membrane. Calcium-bound calmodulin inhibited this interaction and also partially rescued Rem-mediated calcium current inhibition, but not through Ca-CaM interaction with the Rem C-terminus. This suggests that Rem competes with Ca-CaM for the C-terminus of the channel, perhaps near the IQ motif. In these experiments, the $Ca_V\beta$ subunit was included in all electrophysiological recordings of channels with or without RGK proteins, and as a result, it is not possible to determine from this study if the RGK-channel C-terminus interaction is sufficient for RGK calcium current knockdown, or if the $Ca_V\beta$ subunit must be present to achieve it. Thus, this study left open the question: what role, if any, does the $Ca_V\beta$ subunit play a role in the channel-RGK protein association?

This question was recently addressed in a new report which examined Gem regulation of $Ca_V2.1$ channels.[42] After demonstrating a direct biochemical interaction between Gem and the channel, the authors showed that the $Ca_V\beta$ subunit is required for Gem inhibition of channels in situ. This was done by using inside-out membrane patches from Xenopus oocytes expressing exogenous $Ca_V2.1$ and a β subunit that could be washed off due to point mutations in the AID-binding region that resulted in a weakened $Ca_V\beta$-channel interaction. Interestingly, though, disrupting Gem-$Ca_V\beta$ interaction with point mutations did not rescue calcium currents, suggesting that although $Ca_V\beta$ is required to be present, a direct binding between Gem and $Ca_V\beta$ is not essential for Gem to reduce calcium current. To explain this, the authors proposed a model whereby the $Ca_V\beta$ subunit serves to prime the channel to receive the inhibitory effects of Gem. Even more interestingly, by exchanging loops between P/Q-type channels and T-type channels, they showed that the region comprised of the S1-S3 transmembrane segments of domain II conferred Gem sensitivity (not necessarily Gem binding, which was not tested). This was surprising, because sites of channel interaction with modulatory proteins are typically located in the linkers between domains or the N- or C-termini. Therefore, this raises the question as to whether this may reflect a potential mechanism of RGK protein lowering the open probability of the channel (perhaps by immobilizing the voltage sensor) as found by Colecraft's group.

Further Complexity: Rem2 Multimerization

To add yet another dimension of complexity to calcium channel regulation by RGK-proteins, we found that Rem2 can form dimers and multimers when expressed in HEK cells. When coexpressing two full-length epitope-tagged Rem2 proteins, we discovered that HA-Rem2 can pull down myc-Rem2 (**Fig. 2A**), and that this interaction was independent of CaM binding (**Fig. 2B**). Additionally, the short C-terminal fragment comprising Rem2

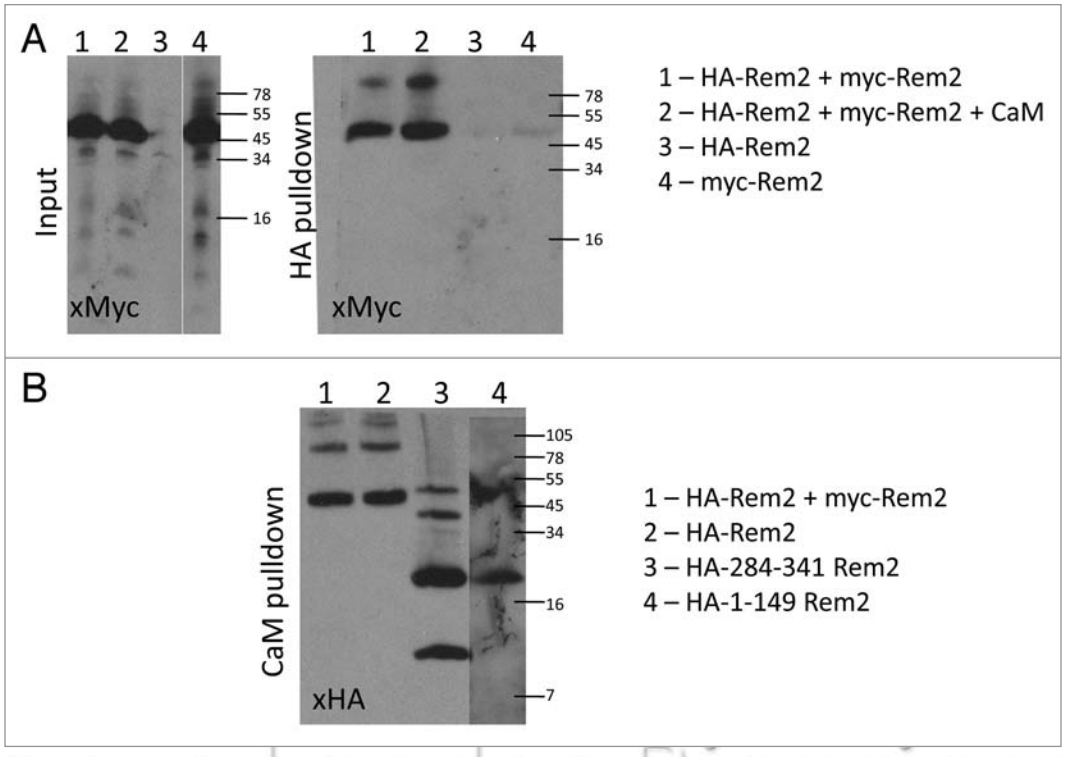

Figure 2. Rem2 forms multimers under non-reducing conditions. HA-Rem2 and myc-Rem2 were coexpressed in HEK cells, and cells were lysed 2 days after transfection in buffer containing 2 mM Ca. Cleared lysates were incubated with either HA-agarose beads or calmodulin-sepharose beads, then washed 3x and eluted with non-reducing sample buffer (lacking DTT or β-mercaptoethanol). Lysates and eluates were separated by SDS-PAGE on layered 10%/16% tricine gels, then transferred to nitrocellulose membranes and probed with anti-myc antibody (Roche). Results are representative of at least three different experiments. (A) Pulldown assay showing an interaction between myc-Rem2 and HA-Rem2. Left, cleared cell lysates probed with anti-myc antibody. Approximate molecular weights are given (kD), and contents of each lane are listed at right. Right, pulldown assay from HA beads. The lowest band in each lane represents the monomeric Rem2. (B) Pulldown assay using calmodulin sepharose beads. Contents of each lane are listed at right, and approximate molecular weights are given (kD). Again, the lowest band in each lane represents the monomeric form of that protein.

residues 284–341 expressed alone in HEK cells shows multimers on a non-reducing gel, but the N-terminal fragment composed of residues 1–149 shows a monomer under the same conditions (**Fig. 2B**), suggesting that the Rem2 domain involved in self-association is in the C-terminal fragment. Whether multimeric Rem2 is a normal cellular form, or if multimers are formed in response to a physiological event such as neuronal stimulation, is unknown. Since Rem2 seems to use its C-terminus to form multimers, it is possible that other RGK proteins may also form higher-order complexes, as the C-terminus is ~40% conserved within the RGK family. The possibility of multimeric RGK protein complexes must be taken into consideration when one interprets experiments involving RGK mutant constructs, especially when expressed in a system that contains endogenous RGK proteins.

Model for RGK Protein Reduction of Calcium Currents

There has been a recent emergence of several new insights into how RGK proteins interact with the calcium channel machinery to regulate currents. Reports from independent groups have demonstrated a direct binding between RGK proteins and the calcium channel (summarized in **Fig. 3A**), but numerous questions remain unanswered. For example, what role does the $Ca_V\beta$ subunit play in RGK protein-mediated calcium current inhibition? Is it an essential part of an RGK protein-$Ca_V\beta$ subunit-channel complex as reported in the early literature? Does it serve a chaperoning function to bring the RGK protein and channel close enough together to interact directly? Is it dispensable? There may be no clear-cut unifying answers that apply equally to all RGK subtypes and all types of voltage gated calcium channels or their ancillary subunits. Nonetheless, when compiling information from recent studies, a model emerges (**Fig. 3B**) in which the $Ca_V\beta$ subunit may serve to orient or chaperone the RGK protein to the channel (aided perhaps by the membrane-association domain of RGK proteins). Once at the channel, RGK proteins may then bind the proximal C-terminus of the channel (potentially competing with calmodulin) while the $Ca_V\beta$ subunit binds to the domain I-II linker. The RGK protein may then be in position to exert an effect on the voltage sensors, thus reducing the functionality of the channel. Additionally, it may confer some allosteric interference with normal channel function by holding the C-terminus close to the membrane. Third, multimerization of RGK proteins can provide additional points of regulation of a given channel. If one RGK protein can bind the C-terminus of the channel and be linked to another RGK protein, this leaves open the possibility of additional interactions with other parts of the channel and/or the $Ca_V\beta$ subunit, or even an unknown third-party regulatory protein.

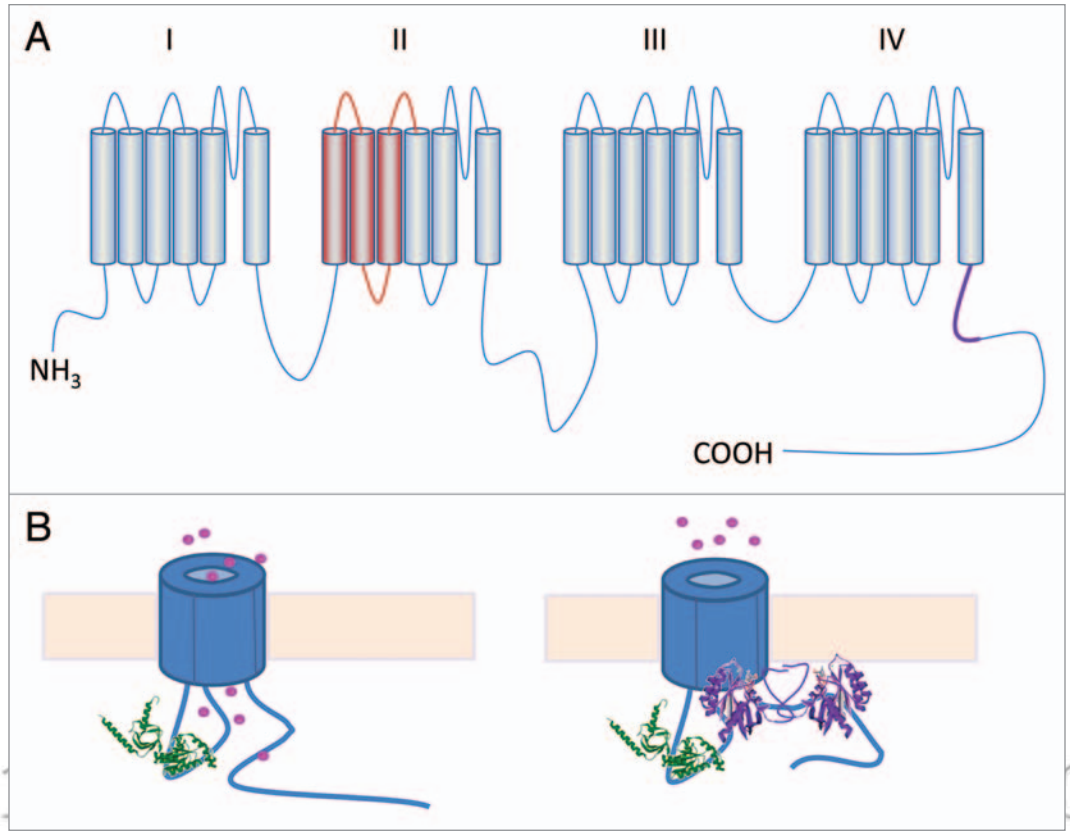

Figure 3. Model of RGK protein modulation of calcium channels. (A) The structure of a calcium channel is shown. The red region (domain II S1-S3) confers sensitivity to the current-reducing effects of the RGK protein Gem. The purple region (proximal C-terminus) indicates an area of direct binding to Rem. (B) Proposed model of RGK regulation of calcium channels. Left, a calcium channel unaffected by RGK proteins contains the $Ca_V\beta$ subunit bound to the I-II linker and passes calcium in response to depolarization. Right, the calcium channel bound to an RGK protein, which is itself associated with the plasma membrane, does not pass calcium, possibly due to immobilization of voltage sensors, allosteric interference from the channel C-terminus, or by displacing calmodulin from the IQ motif region. Multimerization of RGK proteins can also provide more potential points of RGK regulation of a single channel.

This model accounts for only a part of the known effects of RGK proteins on channels, thus additional work will be needed to address issues such as how RGK proteins affect voltage sensors, how they enhance channel endocytosis, and how the relationship between the RGK protein and the $Ca_V\beta$ subunit leads to decreased channel open probability. Only then will we truly understand the precise mechanism of action of these multifunctional proteins.

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