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Regulation of voltage-dependent calcium channels by RGK proteins

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Abstract

RGK proteins belong to the Ras superfamily of monomeric G-proteins, and currently include four members– Rad, Rem, Rem2, and Gem/Kir. RGK proteins are broadly expressed, and are the most potent known intracellular inhibitors of high-voltage-activated Ca^{2+} (Ca_V1 and Ca_V2) channels. Here, we review and discuss the evidence in the literature regarding the functional mechanisms, structural determinants, physiological role, and potential practical applications of RGK-mediated inhibition of Ca_V1/Ca_V2 channels.

1. INTRODUCTION

RGK (Rad, Rem, Rem2, Gem/Kir) proteins are a four-member subfamily of the Ras superfamily of monomeric G-proteins. Rad was first discovered as a protein over-expressed in skeletal muscle of type II diabetic humans [1]; Gem as a mitogen-induced gene in human T cells [2]; Rem was originally cloned using a degenerate PCR strategy based on similarity to Rad and Gem [3]; and Rem2 was cloned from a rat brain cDNA library [4]. Functionally, individual RGKs have been linked to diverse functions in different cell types and tissues including (but not limited to): promotion of cell shape remodeling via regulation of cytoskeletal dynamics (Gem) [5-9]; induction of apoptosis in cardiac myocytes (Rad) [10]; regulation of synapse development and dendritic morphology (Rem2) [11, 12]; control of neuronal proliferation and apoptosis during embryogenesis, and survival of human embryonic stem cells (Rem2) [13, 14].

All RGK proteins powerfully inhibit high-voltage-activated Ca^{2+} (Ca_V1/Ca_V2) channels [15-17]. In this review, we discuss the experimental evidence that underlies current understanding of the mechanisms and structural determinants underlying RGK regulation of Ca_V1/Ca_V2 channels, its potential physiological role, and the state of efforts to exploit this channel regulation for practical applications.

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2. BASIC STRUCTURE-FUNCTION OF RGK PROTEINS

Similar to other Ras superfamily proteins, RGKs contain a guanine nucleotide binding domain (G-domain) [18-23]. In comparison to Ras, RGK proteins have relatively large N– and C–termini extensions, and non-conservative substitutions in the G-domain of residues critical for GTP binding and hydrolysis [1-4] (Fig. 1). Within the RGK family, the N-termini extensions have variable lengths and display low sequence conservation. The functional significance of the N-terminus extensions in RGKs is unknown. The C-terminus extensions consist of a distal conserved region (~40 residues) separated from the G-domain by a relatively short (12 – 22 residues) variable linker sequence. The C-termini of RGK proteins lack the CAAX (C= cysteine, A= aliphatic, X= any amino acid) prenylation motif that is common to many Ras superfamily proteins, and directs their anchoring to membranes [18]. Nevertheless, RGKs target to the plasma membrane using basic and hydrophobic residues in their C-terminus extensions [24, 25]. The membrane-targeting region in the C-termini of RGK proteins overlaps with a calmodulin (CaM) binding domain (CBD) that mediates RGK interactions with Ca^{2+}-CaM [26-29] (Fig. 1A). 14-3-3 proteins bind as dimers to all four RGKs, and this requires phosphorylation of two distinct serines in the N- and C-termini extensions, respectively [6, 26-28, 30] (Fig.1A). The functional significance of CaM and 14-3-3 binding to RGKs is unknown, though it has been suggested that these interactions may regulate the subcellular localization or stability of RGK proteins [6, 27, 28].

The G-domains of all RGKs have been functionally demonstrated to be *bona fide* guanine nucleotide binding proteins, although there may be quantitative differences within the family [1-4, 22, 23]. For example, Rad displays higher affinity ($K_d \approx 100$ nM) for guanine nucleotides compared to Gem ($K_d \approx 1 – 20$ μM) [23]. Moreover, Gem displays a 5- to 10-fold higher affinity for GDP vs GTP, whereas Rad displays no such preference [23]. Crystal structures of RGK proteins confirm that their G-domains adopt a fold comprised of a six-stranded β-sheet surrounded by five α-helices [20-22], similar to that found in other Ras superfamily members [18, 19] (Fig. 1B). In Ras superfamily proteins, five loops within the G-domain (G1 – G5) are highly conserved and form the guanine nucleotide-binding site (Fig. 1). The G1-box (or P-loop) with consensus sequence GXXXXGKS/T is conserved in RGKs, and similar to Ras, engages in interactions that co-ordinate the α/β phosphates and Mg^{2+} [20-22]. Similarly, G4 (NKXD) and G5 (ETSA) motifs are conserved in RGKs and participate in recognition of the guanine base. In Ras, G2 (XTX) and G3 (DXAG) motifs reside within regions referred to as Switch I and Switch II, respectively. In Ras-GDP, Switch I and Switch II are disordered, suggesting a high flexibility of these regions. The Thr^{35} and Gly^{60} residues in Ras G2 and G3, respectively, act as sensors for the γ phosphate of GTP. Hence, in Ras, GTP binding leads to a conformational change in which switches I and II are stabilized [31]. Since RGKs do not have the equivalent of Thr^{35} in switch I and show non-conservative substitutions in G3 (DXWE instead of DXAG), it has long been suggested that they may not display the canonical GTP-regulated switch mechanism evident in most Ras superfamily G-proteins [31]. Indeed, crystal structures of Rad and Rem2 bound to a non-hydrolyzable GTP analog showed little structural changes from the GDP-bound forms, with no evidence of a GTP-mediated stabilization of switch I and II [23]. A caveat that must be mentioned is that all RGK protein crystal structures to date are of truncated proteins in

which the N- and C-termini extensions have been removed (Fig. 1B). In some other G-proteins such as Ran and Arf, the canonical switch mechanism is modified by additional conformation changes in C- and N-termini extensions, respectively [31]. To this point, a crystal structure of GDP-bound Gem that includes the initial part of the C-terminus extension has been reported [22]. In this structure, the proximal C-terminus extension forms a helix that contacts with an interswitch region between the β2 and β3 strands of the G-domain, as well as the α5 helix. Moreover, in functional assays, the presence of the N- and C-termini extensions in Gem increases its GTPase activity by 20-fold [22].

The rate of intrinsic GTP hydrolysis has been measured for Rad [23, 32] and Gem [22, 23]. Purified Rad has a low rate of intrinsic GTPase activity which can be dramatically increased by cytosolic fractions derived from different tissues [32]. Sub-fractionation of liver cytosol led to the identification of nucleoside diphosphate kinase (nm23 or NDPK) as a GTPase activating protein (GAP) for Rad and Gem (but not Rem) [33]. In an unusual twist, nm23 also acted as a functional guanine nucleotide exchange factor (GEF) for Gem due to its diphosphate kinase catalyzing the transfer of phosphate from ATP to a bound GDP in Gem [33]. To date, nm23 is the only known GAP (and GEF) for any RGK protein.

3. BASIC STRUCTURE-FUNCTION OF Ca_V CHANNELS

Ca^{2+} influx through voltage-dependent Ca^{2+} (Ca_V) channels plays a critical role in various biological functions including muscle contraction, synaptic transmission, hormone secretion, and gene expression [34, 35]. Ca_V channels are divided into two main families based on their threshold for activation: low-voltage-activated (LVA) Ca^{2+} channels and high-voltage-activated (HVA) Ca^{2+} channels. There are three types of LVA Ca^{2+} channels (T-type: $Ca_V3.1$-3.3) [36], and seven types of HVA Ca^{2+} channels (L-type: $Ca_V1.1$–1.4; P/Q-type: $Ca_V2.1$; N-type: $Ca_V2.2$; R-type: $Ca_V2.3$) [34, 37].

HVA Ca_V channels are hetero-multimeric proteins comprised of pore-forming α_1 subunits and auxiliary β, $\alpha_2\delta$, and sometimes γ subunits. So far, seven genes encoding HVA α_1 subunits ($Ca_V1.1$ [α_{1S}]; $Ca_V1.2$ [α_{1C}]; $Ca_V1.3$ [α_{1D}]; $Ca_V1.4$ [α_{1F}]; $Ca_V2.1$ [α_{1A}]; $Ca_V2.2$ [α_{1B}]; $Ca_V2.3$ [α_{1E}]) [37], four genes encoding $Ca_V\beta$ ($Ca_V\beta$1-4), and four genes encoding $\alpha_2\delta$ ($\alpha_2\delta$1-4) have been identified [34]. Pore-forming α_1 subunits are all comprised of four homologous domains (I–IV) each with six transmembrane segments (S1–S6). $Ca_V\beta$s are required for efficient targeting of α_1 subunits to the plasma membrane [38-42], enhancing channel open probability (P_o) [41], normalizing the voltage-dependence of channel activation [43-46], and modulating inactivation [43, 47-50]. $Ca_V\beta$s bind with high affinity to a conserved 18-residue sequence (the α interaction domain, or AID) located in the loop connecting domains I and II (I-II loop) of α_1 subunits [51-53].

4. RGK INHIBITION OF VOLTAGE-DEPENDENT CALCIUM CHANNELS

4.1. Discovery and basic properties

The separate fields of RGK proteins and Ca_V channels intersected when a yeast two hybrid screen of insulin-secreting MIN6 cells identified Gem/Kir as a $Ca_V\beta_3$-binding protein [15]. Co-expression of recombinant $Ca_V1.3$ or $Ca_V1.2$ channels with Gem in *Xenopus* oocytes

resulted in a complete and constitutive inhibition of both channel types [15]. Subsequently, it was found that the ability to inhibit $Ca_V1.2$ channels applied to all RGK proteins [16, 54]. Since these seminal studies, RGK proteins have been shown to indiscriminately and potently inhibit all high-voltage-activated channels tested including, $Ca_V1.1$ [55], $Ca_V1.2$ [17, 26, 28, 54, 56-60], $Ca_V1.3$ [15], $Ca_V2.1$ [15, 61, 62], and $Ca_V2.2$ [15, 56, 63]. By contrast, low-voltage-activated T-type ($Ca_V3.1$ – $Ca_V3.3$) channels are unaffected by RGK proteins [16, 63]. RGK inhibition of Ca_V1 and Ca_V2 channels takes place in all cell types studied to date, including heterologous expression systems (e.g. HEK cells, *Xenopus* oocytes), cell lines containing endogenous Ca_V channels (e.g. PC12 cells, MIN6 cells), and primary cells (heart, skeletal muscle and neurons).

4.2. RGKs use multiple mechanisms to inhibit Ca_V channels

The question of how RGKs inhibit Ca_V1 and Ca_V2 channels has been intensely studied by several groups. The whole-cell calcium current (I_{Ca}) is related to microscopic channel properties by the relation: $I_{Ca} = N \times F_A \times i \times P_o$, where N is the total number of channels in the surface membrane, F_A is the fraction of activatable channels, i is the unitary current amplitude, and P_o is the single-channel open probability. In principle, RGKs could inhibit I_{Ca} by reducing any one of the four parameters or a combination of them. To address whether RGKs reduce N, Beguin and colleagues used a $Ca_V1.2$ pore-forming α_{1C} subunit harboring a hemagluttinin (HA) epitope tag in an extracellular loop. Combining immunofluorescence and confocal microscopy, they found that all four RGKs prevent surface expression of HA-tagged $Ca_V1.2$ channels reconstituted in either PC12 or HEK 293 cells [15, 26, 27]. By contrast, using a surface biotinylation/Western blot detection approach, Finlin et al, found that Rem2 inhibited I_{Ca} in mouse insulinoma MIN6 cells without reducing the number of endogenous $Ca_V1.2$ channels at the membrane [54]. Similarly, Ikeda and colleagues found that Rem2 inhibits $Ca_V2.2$ channels stably expressed in tsA201 cells without decreasing channel surface density as determined by radio-labeled ω-conotoxin GVIA binding assays [63]. These two latter reports suggested that Rem2 inhibited $Ca_V1.2$ and $Ca_V2.2$ channels directly at the cell surface, although the exact mechanisms were not investigated.

By combining optical detection of surface epitope-tagged α_{1C} subunits with quantum dot and high throughput flow cytometry measurements, our group discovered that Rem partially decreases (by 60%) the surface density of recombinant $Ca_V1.2$ channels reconstituted in HEK 293 cells [57]. This decrease was completely prevented by co-expressing dominant negative dynamin, suggesting that Rem increased the rate of dynamin-dependent $Ca_V1.2$ endocytosis, rather than interfered with forward trafficking of the channel [57] (Fig. 2, *mechanism I*). Interestingly, even when Rem-induced decrease in channel surface density was completely reversed with dominant negative dynamin, the inhibition of I_{Ca} was not rescued, suggesting that Rem could also block the activity of surface channels. We distinguished two separate mechanisms Rem used to block $Ca_V1.2$ channels at the cell surface. First, Rem could decrease I_{Ca} by diminishing channel P_o without accompanying reductions in voltage sensor movement (Fig. 2, *mechanism II*). Targeting the Rem G-domain to the membrane either by the Rem C-terminus or a generic membrane targeting module is sufficient to reconstitute *mechanism II* [57]. Second, we discovered that Rem reduced

maximal gating charge (Q_{max}) of $Ca_V1.2$ channels in a manner that was not accounted for by a decrease in *N*. This result suggested that Rem partially immobilizes $Ca_V1.2$ channel voltage sensors (Fig. 2, *mechanism III*). On the assumption that all four voltage sensors are required to move for the channel to open, the decreased Q_{max} suggests that Rem reduces the fraction of activatable (F_A) $Ca_V1.2$ channels on the cell surface. Overall, this study established that within the same experimental system Rem utilized at least three separable mechanisms to inhibit recombinant $Ca_V1.2$ channels [57, 64].

Several groups have reported that over-expressing distinct RGKs in cardiac myocytes or skeletal myotubes dramatically decreases endogenous $I_{Ca,L}$ [55, 58-60]. In three of these studies the impact of RGKs on Q_{max} was also measured. Murata et al [60] found that over-expressing Gem in adult guinea pig ventricular myocytes profoundly inhibited $Ca_V1.2$ channel Q_{max} (70% reduction). By contrast, over-expressing Rem in guinea pig ventricular myocytes [59] or skeletal myotubes [55] yielded smaller reductions in $Ca_V1.2$ (33% reduction) and $Ca_V1.1$ (44% reduction) Q_{max}, respectively. RGK mediated decrease in $Ca_V1.1$/$Ca_V1.2$ channel Q_{max} could result from a decrease in the surface density of channels or an ability of RGKs to partially immobilize channel voltage sensors. The impact of RGKs on $I_{Ca,L}$ in cardiac myocytes has often been interpreted to reflect a decrease in the surface density of $Ca_V1.2$ channels [58, 60, 65]. However, we discovered that acute treatment of control and Rem-over-expressing myocytes with the $Ca_V1.2$ channel agonist, BAY K 8644, eliminated the inhibitory effect of Rem and resulted in $I_{Ca,L}$ of comparable amplitude between the two conditions [59]. This result not only demonstrated that Rem-inhibited $I_{Ca,L}$ can be rescued pharmacologically, but also indicated that the majority of $Ca_V1.2$ channels must be present at the cell surface in cardiac myocytes over-expressing Rem. This contrasts with the finding that Rem significantly reduces the surface density of recombinant $Ca_V1.2$ channels reconstituted in PC12 or HEK 293 cells [17, 57], suggesting that although RGKs can inhibit $Ca_V1.2$ channels using multiple distinct mechanisms, only particular subsets of these may be available and used in different cellular contexts.

5. STRUCTURAL DETERMINANTS ON RGKs IMPORTANT FOR I_{Ca} INHIBITION

5.1. Role of the RGK C-terminus

Much work has focused on defining the important structural elements on RGKs that mediate their potent inhibition of Ca_V1/Ca_V2 channels. Deleting the N-terminus extension from different RGKs does not abolish their ability to inhibit I_{Ca} [61-63], suggesting that this non-conserved feature is not critical for this effect. By contrast, several investigators have demonstrated that deleting the distal C-terminus of individual RGKs generates truncated versions (e.g. Rem_{265}, Rad_{276}, Gem_{264}) that do not block I_{Ca} [16, 56, 57, 59, 62, 63, 66]. Hence, the distal C-terminus of RGKs is clearly critical for the mechanism of Ca_V channel block. Nevertheless, the precise manner in which the C-termini of RGKs participate in I_{Ca} inhibition is not completely clear, though the available evidence suggests multiple mechanisms may be in play. Ambiguities arise in part due to the overlapping roles of RGK C-termini– as membrane-targeting modules, as CaM binding sites, and as bearers of nuclear localization signals (NLS) (Fig. 1A).

5.2. Is RGK C-terminus sufficient to inhibit I_{Ca}?

Several investigators have sought to determine whether the C-terminus of different RGK proteins is sufficient to inhibit I_{Ca} with mixed results. Ikeda and colleagues found that over-expressing the C-terminus of Rem2 in SCG neurons did not block endogenous $Ca_V2.2$ channels, whereas full-length Rem2 strongly inhibited I_{Ca} [63]. Similarly, expression of the Rem distal C-terminus (final 32 residues) did not inhibit recombinant $Ca_V1.2$ channels in tsA201 cells [66]. By contrast, Leyris et al found that the final 75 residues of Gem, which includes the entire C-terminus extension, was sufficient to fully inhibit recombinant $Ca_V2.1$ channels reconstituted in *Xenopus* oocytes [67]. Recently, a 12-amino acid peptide containing residues K265–K276 in the Gem C-terminus was shown to be sufficient to acutely inhibit $Ca_V2.1$ channels expressed in *Xenopus* oocytes, albeit at high concentrations [62]. In this study, the Gem K265–K276 peptide was directly applied to $Ca_V2.1$ in the inside-out patch configuration, explicitly demonstrating that the inhibitory effect was achieved at the level of surface channels. Remarkably, a scrambled Gem K265–K276 peptide, which contained the same amino acid residues as the original but in different positions, also effectively inhibited $Ca_V2.1$ in inside-out patches [62], suggesting that the amino acid content of this region rather than its sequence was important for channel inhibition. The region corresponding to Gem residues K265-K276 is quite well conserved amongst RGK proteins (Fig. 1A). Therefore, the inability of Rem2 and Rem C-termini to inhibit $Ca_V2.2$ channels in SCG neurons [63] and $Ca_V1.2$ in tsA201 cells [66], respectively, may indicate that the sufficiency of this region to block I_{Ca}, as observed with Gem K265–K276 inhibition of $Ca_V2.1$ channels [62, 67] may not be a general property. Alternatively, one possible explanation for the discrepancies could be that the effective concentration of the expressed C-terminal fragment was different in these studies-higher in oocyte studies and lower in cell lines. Further work is needed to clarify this issue, as well as the mechanism K265-K276 peptide uses to decrease $Ca_V2.1$.

5.3 Role of RGK membrane targeting

In many cell types, RGK proteins autonomously target to the inner leaflet of the plasma membrane via their C-termini interacting with membrane phosphatidylinositol lipids [24, 25, 56, 63, 66]. Deleting the distal C-terminus of RGKs eliminates both their ability to inhibit I_{Ca} and their membrane targeting [56, 63, 66]. The potential importance of RGK membrane targeting in the mechanism of I_{Ca} inhibition has been investigated by several groups. Replacing the C-termini of either Rem2 [63] or Rem [66] with the tail of K-Ras4B which consists of a polybasic region and a CAAX prenylation motif, restored both membrane targeting and inhibition of $Ca_V2.2$ and $Ca_V1.2$ channels, respectively. For Rem2, the polarity of the membrane targeting domain appeared to be important, as attaching the first 10 residues from $G\alpha_{i1}$, which contains myristoylation and palmitoylation motifs, to the N-terminus of truncated Rem2 (Rem2ΔC) restored membrane targeting but not inhibition of $Ca_V2.2$ channels [63]. By contrast, attaching a palmitoylated peptide to the N-terminus of truncated Rem (Rem_{265}) restored both membrane targeting and inhibition of $Ca_V2.2$ channels stably expressed in tsA201 cells [56]. Overall, the available data suggest that one way the RGK C-terminus participates in I_{Ca} inhibition depends on its ability to target the RGK G-domain to the plasma membrane. We exploited this feature to create a small-

molecule-inducible Ca_V1/Ca_V2 channel inhibitor by fusing the C1 domain from protein kinase Cγ to either the N- or C-terminus of YFP-Rem_{265} [17, 56, 57]. The resulting constructs, $C1_{PKC\gamma}$-YFP-Rem_{265} or YFP-Rem_{265}-$C1_{PKC\gamma}$, are cytosolic when expressed in HEK 293 cells, but are rapidly recruited to the membrane upon exposure to phorbol-12,13-dibutyrate (PdBu) [56, 57]. Inhibition of $Ca_V1.2$ and $Ca_V2.2$ channels occurs concomitantly with the dynamic translocation of $C1_{PKC\gamma}$-YFP-Rem_{265} or YFP-Rem_{265}-$C1_{PKC\gamma}$ to the plasma membrane [17, 56, 57]. These molecules were termed genetically encoded molecules for inducibly inactivating Ca_V channels (GEMIICCs) [56]. GEMIICCs acutely inhibited I_{Ca} without affecting Q_{max}, suggesting they lowered current solely by decreasing channel P_o [56, 57] (Fig. 2, *mechanism II*). Beyond providing insights into the mechanism by which membrane targeting of RGKs results in I_{Ca} inhibition, GEMIICCs provide a proof-of-concept that new functionalities (in this case inducible inhibition) can be engineered into RGKs.

5.4. Role of CaM binding and nuclear localization of RGKs

In addition to mediating targeting to the membrane, the C-terminus of RGKs binds CaM and possesses nuclear localization signals (Fig. 1A). A useful mutation widely used in the field converts a hydrophobic residue in the RGK C-terminus (corresponding to Gem^{W269} Rem^{L271} Rad^{L281} $Rem2^{L317}$) to glycine (Fig. 1A). The effects of this mutation are complex since it diminishes CaM binding [26-28], prevents membrane targeting [57], and dramatically increases nuclear localization of RGKs [26-28, 57]. Hence, the functional consequences of this mutation in RGKs need to be interpreted carefully. Gem^{W269G} consistently shows a diminished ability to inhibit I_{Ca} compared to wild-type Gem. This effect has been demonstrated for Gem^{W269G} inhibition of $Ca_V1.2$ channels in PC12 cells [15, 28] and cardiac myocytes [60], and $Ca_V2.2$ channels in SCG neurons [6]. Similarly, Rad^{L281G} displayed a diminished ability to inhibit $Ca_V1.2$ channels in PC12 cells, whereas Rem^{L271G} retained full inhibitory activity in the same system [26]. The precise reason for the functional differences between Gem^{W269G}/Rad^{L281G} and Rem^{L271G} is not clear. Because these mutations markedly increase the nuclear localization of the respective RGK protein, it was suggested that nuclear sequestration of $Ca_V\beta$ by Rad^{L281G} and Rem^{L271G} could represent a mechanism to regulate surface $Ca_V1.2$ channels [26]. An ambiguity with these experiments is that though the mutant RGKs are enriched in the nucleus, a significant portion remains in the cytosol. Hence, in the case of Rem^{L271G} it was unclear whether inhibition of $Ca_V1.2$ channels was achieved via the nuclear or cytosolic pools of the mutant protein. We examined this question by examining the effect of Rem^{L271G} on $Ca_V1.2$ channels reconstituted in HEK 293 cells [57]. YFP-Rem^{L271G} in HEK cells was present in both the nucleus and cytosol and caused a partial inhibition of $I_{Ca,L}$ when compared to wild type Rem. Attaching a nuclear localization signal (NLS) to YFP-Rem^{L271G} localized it exclusively to the nucleus. Interestingly, NLS-YFP-Rem^{L271G} was completely inert with respect to $I_{Ca,L}$ inhibition. Conversely, attaching a nuclear export signal (NES) to YFP-Rem^{L271G} targeted it exclusively to the cytosol, and NES-YFP-Rem^{L271G} completely blocked $I_{Ca,L}$, explicitly demonstrating that the cytosolic pool is the active component for channel inhibition. These results support three consequential conclusions. First, the ineffectiveness of Gem^{W269G} and Rad^{L281G} to inhibit I_{Ca} may be because their nuclear localization reduces their active concentration in the cytosol. This interpretation is consistent

with the finding that RGK inhibition of I_{Ca} is dose-dependent [68]. Second, that CaM binding is not necessary for RGK inhibition of I_{Ca}. Third, that membrane targeting of RGKs is not an absolute requirement for I_{Ca} inhibition, since the non-membrane-targeted NES-YFP-Rem^{L271G} is an effective blocker of $Ca_V1.2$ channels.

5.5 Role of nucleotide binding and hydrolysis in RGK inhibition of I_{Ca}

A canonical feature of Ras superfamily G-proteins is that they function as guanine nucleotide-regulated molecular switches, cycling between inactive GDP-bound and active GTP-bound conformations [18]. There is tremendous ambiguity as to whether and how this basic defining property plays a role in RGK regulation of Ca_V1/Ca_V2 channels. All RGKs have been demonstrated to be *bona fide* guanine nucleotide binding proteins though there may be quantitative differences among them with respect to relative affinities for GDP and GTP, and the intrinsic rate of GTP hydrolysis. Comparing crystal structures of GTP- and GDP-bound Rad and Rem2 suggest RGKs do not undergo the classical switch mechanism observed in Ras, which involves a GTP-mediated stabilization of otherwise disordered Switch I and Switch II regions [23, 31]. An important approach used to investigate the potential role of GTP binding and hydrolysis of RGKs is to introduce point mutations that decrease their affinities for guanine nucleotides. In Ras, a S17N mutation locks the protein in a GDP-bound state [69]. Ras^{S17N} has a dominant negative effect on Ras signaling in cells because it has a higher affinity for, and sequesters, Ras-GEFs [69]. The residue corresponding to Ras S17 is conserved in all RGKs (Rad^{S105}, Gem^{S89}, Rem^{T94}, Rem2^{S129}) (Fig. 1A). Similar to the S17N mutation in Ras, GTP binding is abolished in Rad^{S105N} and Gem^{S89N}, although the affinity to GDP is also reduced [23, 32].

Over a series of papers, Beguin and colleagues demonstrated using pull-down assays that Rad^{S105N}, Gem^{S89N}, Rem^{T94N}, and Rem2^{S129N} displayed decreased binding to $Ca_V\beta$ subunits compared to their wild-type counterparts [26-28]. This was interpreted as indicating that GTP binding was necessary for RGKs to bind $Ca_V\beta$s. However, the impact of these mutations on the ability of the distinct RGKs to inhibit I_{Ca} was not evaluated in these studies. A different conclusion regarding the role of nucleotide binding in RGKs was reached in a study where the Rem2/$Ca_V\beta_{2a}$ interaction was unaffected when Rem2 was loaded with either GDP or GTP [24].

A number of studies have focused on evaluating the functional impact of the nucleotide binding state of RGKs on their ability to inhibit I_{Ca}, with mixed results Chen et al found that Rem2^{S129N} down-regulated I_{Ca} to the same extent as wild type Rem2 in sympathetic neurons [63]. However, the same group also found that Gem^{S89N} lost the ability to inhibit I_{Ca} in sympathetic neurons [6]. This discrepancy hints at a fundamental difference between these two RGKs with respect to the role of nucleotide binding in I_{Ca} inhibition in neurons. Rad^{S105N} has been reported to have no effect on recombinant $Ca_V1.2$ channels reconstituted in HEK 293 cells, but to exert a dominant negative effect in cardiac myocytes, increasing endogenous $I_{Ca,L}$ [70]. By contrast, we found that Rem^{T94N} potently inhibited $Ca_V1.2$ channels in HEK 293 cells, but with an interesting difference from wild-type Rem. Whereas wild-type Rem significantly decreased gating currents, Rem^{T94N} blocked I_{Ca} without impacting Q_{max} [57]. This result suggested that the ability of Rem to inhibit voltage sensor

movement is selectively dependent on GTP binding (Fig. 2, *mechanism III*). Surprisingly, over-expressing Rem^{T94N} in heart cells had no impact on endogenous $I_{Ca,L}$, in contrast to wild-type Rem which markedly decreased $I_{Ca,L}$ [59]. Given that Rem^{T94N} does inhibit $Ca_V1.2$ channels in HEK 293 cells, this result suggests the existence of a cardiac specific mechanism that inactivates the ability of GDP-bound Rem to inhibit $I_{Ca,L}$ in heart. A caveat of all the functional studies described here is they rely on mutations predicted to lock RGK proteins in a GDP-bound state. It is possible that these mutations may also have some unanticipated effects that could confound interpretation of results. To circumvent this problem, Chen et al attempted to reverse Rem2 inhibition of I_{Ca} in sympathetic neurons by dialyzing in GDP-β-S via the patch pipette [63]. They observed no reversal of I_{Ca} inhibition over 20 minutes of GDP-β-S dialysis, suggesting that GTP binding may not be necessary for Rem2 inhibition of I_{Ca} in sympathetic neurons.

6. STRUCTURAL DETERMINANTS ON Ca_V1/Ca_V2 CHANNELS IMPORTANT FOR RGK INHIBITION

6.1. Role of auxiliary β subunits in RGK regulation of Ca_V channels

All RGK proteins bind $Ca_V\beta$ subunits [15-17, 26, 56, 71-73]. The affinity of RGK/$Ca_V\beta$ association is about an order of magnitude lower than the interaction of $Ca_V\beta$ with the AID present in the I-II loop of Ca_V1/Ca_V2 pore-forming α_1 subunits [56]. The relatively low affinity of the RGK/$Ca_V\beta$ interaction may explain the lack of a high resolution crystal structure for this complex. Beguin et al. conducted an extensive mutagenesis screen of $Ca_V\beta$s and RGKs to identify mutations that interrupted their mutual interaction without disrupting their global tertiary structures [72]. Mutations on $Ca_V\beta$ that eliminated interaction with RGKs clustered at a hotspot region that was distinct from the α-binding pocket that binds the AID [74-76] (Fig. 3A). Based on results from this mutagenesis screening and the known structures of RGKs and $Ca_V\beta$s, the authors generated a homology model in which Gem was docked to the identified hotspot on $Ca_V\beta$ (Fig. 3B).

It was initially suggested that RGKs bind to $Ca_V\beta$s and prevent their interaction with α_1 subunits, thereby compromising their chaperone function and severely limiting channel trafficking to the membrane [15, 26, 77]. However, subsequent work has shown that RGKs do not disrupt the α_1-β interaction, and there is consensus that RGK inhibition of I_{Ca} involves a ternary α_1/β/RGK complex [24, 54, 56, 57, 63]. Within this ternary complex framework, there are two possible ways in which $Ca_V\beta$ could play a role in RGK inhibition of I_{Ca}. In the first scenario, a direct RGK/$Ca_V\beta$ interaction is not necessary for I_{Ca} inhibition. However, $Ca_V\beta$ could play a role by promoting a permissive conformation of the channel complex that is necessary for functional interaction with RGKs. Alternatively, a direct RGK/$Ca_V\beta$ interaction could be obligatory for the mechanism of RGK inhibition of I_{Ca}. Evidence has been provided for both scenarios, and there are indications that there may be specificity for different RGK/Ca_V channel combinations as discussed below. An important tool that made these advances possible was the discovery of specific mutations in $Ca_V\beta$s that selectively eliminated binding to RGKs without compromising functional regulation of Ca_V α_1 subunit trafficking and gating [17, 61, 72] (Fig. 3C).

Jian Yang's group used a mutated $Ca_V\beta$ that no longer binds RGKs to demonstrate that direct interaction with $Ca_V\beta$ was not necessary for Gem to acutely inhibit $Ca_V2.1$ channels in *Xenopus* oocytes [61]. Nevertheless, they further showed that β binding to α_{1A} was necessary for Gem to downregulate $Ca_V2.1$. This latter effect was demonstrated using a β with weakened affinity for α_{1A}, which could be readily washed off in inside-out patches. With the β washed off, Gem no longer inhibited the channel, though it could still bind α_{1A}. On the basis of these results, they proposed a model where the presence of β exposes an inhibitory site on the channel complex that is then engaged by Gem to block $Ca_V2.1$ [53, 61]. The location of this putative inhibitory site on the $Ca_V2.1$ channel complex that binds Gem is currently unknown.

We investigated whether the RGK/β interaction has any role in the mechanism of I_{Ca} inhibition, or merely represents an unrelated epiphenomenon, by examining Rem inhibition of $Ca_V1.2$ channels in HEK 293 cells [17]. We found that $Ca_V1.2$ channels containing a β_{2a} mutant that selectively loses binding to RGK proteins (Fig. 3C), are less potently inhibited (74% inhibition) by Rem than channels containing wild-type β_{2a} (96% inhibition), suggesting the prevalence of both β-binding dependent and independent modes of inhibition [17] (Fig. 3, E-H). We further found that two mechanistic signatures of Rem inhibition of $Ca_V1.2$ channels (decreased N and P_o), but not a third (reduced Q_{max}), depend on Rem binding to $Ca_V\beta$. Surprisingly, we discovered a functional dichotomy amongst the RGKs– while Rem and Rad used both β-binding-dependent and independent mechanisms to inhibit $Ca_V1.2$, Gem and Rem2 solely utilized a β-binding-dependent method to do so [17] (Fig. 4). These findings may explain why $Ca_V1.2$ channels expressed in the absence of β subunits are partially blocked by Rem in HEK 293 cells [78], but minimally affected by Gem or Rem2 in *Xenopus* oocytes [15, 68, 79]. Finally, we found that Rem inhibition of $Ca_V2.2$ channels was completely dependent on the Rem/$Ca_V\beta$ interaction, in contrast with $Ca_V1.2$ [17]. Therefore, the mechanisms of RGK inhibition of Ca_V1/Ca_V2 channels are customized at the levels of both the RGK and channel type. Overall, these data suggest a dualistic view for RGK inhibition of Ca_V channels. First, all RGKs can inhibit all Ca_V1/Ca_V2 channels via mechanisms that depend on direct RGK/$Ca_V\beta$ interactions. Because $Ca_V\beta$s are necessary for the formation of all functionally mature Ca_V channels, this could explain the promiscuity of RGKs in blocking all Ca_V1/Ca_V2 channel types. Second, distinct RGKs may initiate β-binding-independent channel inhibition through selective interaction with specific Ca_V1/Ca_V2 channel pore-forming α_1 subunits [17].

6.2. Direct interactions of RGKs with Ca_V α_1 subunits

A logical explanation for the observation that specific RGKs can inhibit $Ca_V2.1$ and $Ca_V1.2$ channels without binding to β subunits is that particular RGKs directly interact with individual Ca_V1/Ca_V2 α_1 subunits. Consistent with this idea, Gem co-immunoprecipitates with $Ca_V2.1$ α_{1A} in the absence of $Ca_V\beta$ [61]. Nevertheless, the exact Gem binding sites on α_{1A} that underlie channel inhibition are unknown. By exchanging corresponding fragments between P/Q- (α_{1A}) and T-type channels ($Ca_V3.1$ α_{1G}), Fan et al. showed that the region comprised of the S1-S3 transmembrane segments of domain II conferred Gem sensitivity [61]. However, since Gem is an intracellular protein, it is most likely that IIS1-IIS3 may be involved in the transduction of the effect, rather than being a binding site for Gem [61, 79].

There have also been efforts to identify direct RGK binding sites on $Ca_V1.2$ α_{1C} subunit. Pang et al. [80] reported that Rem (as well as Rad and Rem2) directly binds to proximal and distal regions C-terminus of α_{1C}. The proximal α_{1C} C-terminus contains structural elements, including a CaM binding domain and an EF hand motif, that are essential for Ca^{2+}-dependent regulation (inactivation and facilitation) of $Ca_V1.2$ [81-86]. Ca^{2+}-CaM was found to block the Rem/α_{1C} C-terminus interaction *in vitro*. This interplay was not due to Ca^{2+}-CaM interaction with the Rem C-terminus, suggesting that Rem directly competes with Ca^{2+}-CaM for α_{1C} C-terminus [80]. Furthermore, co-overexpression of CaM was found to partially relieve Rem inhibition of $Ca_V1.2$ channels in tsA201 cells. The authors concluded that direct binding to the proximal α_{1C} C-terminus is important for Rem-mediated regulation of CDI and $I_{Ca,L}$ blockade in $Ca_V1.2$ [80]. Results from our group support a different conclusion. Based on the idea that the putative Rem binding site was most likely localized within an intracellular region of the channel, we conducted an unbiased screen for potential Rem interaction sites on α_{1C} (N- and C-termini, I-II, II-III, and III-IV loops) using three independent approaches (fluorescence resonance energy transfer, co-localization analyses, and co-immunoprecipitation assays) [17]. We found that Rem interacts solely with the α_{1C} N-terminus in the region immediately upstream of transmembrane segment I in domain I (IS1). Moreover, over-expressing α_{1C} N-terminus completely rescued Rem-mediated β-binding-independent inhibition of $Ca_V1.2$ channels, suggesting that Rem/α_{1C} N-terminus interaction underlies this mode of regulation. Neither Rem2 nor Gem bound α_{1C} N-terminus, providing an explanation for why they exhibit only a β-binding-dependent mechanism of $Ca_V1.2$ inhibition. Although the distal N-terminus shows homology among distinct Ca_V1/Ca_V2 α_1-subunits (60% identical residues or conservative substitutions), Rem does not bind $Ca_V2.2$ α_{1B} N-terminus [17]. This explains why Rem inhibits $Ca_V2.2$ channels solely through a β-binding-dependent mechanism.

7. CROSSTALK OF RGK AND PROTEIN KINASE SIGNALING ON $Ca_V1.2$ CHANNELS

In heart, up-regulation of $Ca_V1.2$ channels by protein kinase A (PKA) is an important physiological modulation that contributes to sympathetic regulation of the heartbeat, a critical component of the flight-or-fight mechanism [87, 88]. Two reports have documented an interesting cross talk between RGKs and PKA signaling at the level of $Ca_V1.2$ channels in heart. Specifically, in primary cardiac myocytes over-expressing either Rad [58] or Rem [59], the remaining $I_{Ca,L}$ was completely insensitive to PKA modulation. By contrast, the $Ca_V1.2$ channel agonist BAY K 8644 robustly and acutely rescued $I_{Ca,L}$ in myocytes over-expressing Rem [59]. The mechanism by which RGKs prevent PKA-mediated modulation of $Ca_V1.2$ channels in heart is unknown.

A recent study found that α_1-adrenergic receptor stimulation could attenuate Rem inhibition of $Ca_V1.2$ channels in both HEK 293 cells and cardiac myocytes [65]. The mechanism was found to involve activation of protein kinase D1 (PKD1) and subsequent phosphorylation of Rem at residue Ser18. The authors propose that this leads to sequestration of Rem by 14-3-3 proteins and permits $Ca_V1.2$ channels trapped intracellularly to traffick to the cell surface [65].

8. PRACTICAL APPLICATIONS OF RGK INHIBITION OF Ca_V CHANNELS

Inhibition of Ca_V1/Ca_V2 channels is an important or potential therapy for many cardiovascular and neurological diseases including: hypertension, cardiac arrhythmias, neuropathic pain, Alzheimer's disease, and Parkinson's disease [89-92]. Under certain circumstances, intracellular genetically encoded Ca_V channel inhibitors may have advantages over traditional small-molecule blockers [93]. For example, localized expression of RGKs may permit a more restricted inhibition of Ca_V channels, permitting a targeted therapeutic result while minimizing unwanted off-target effects [93]. In a proof of concept demonstration of this principle, focal gene delivery of Gem to the atrioventricular (AV) node slowed AV nodal conduction and reduced the heart rate in a porcine atrial fibrillation model [60].

The potential practical applications of RGKs may be widely expanded if new functionalities such as inducibility and selectivity could be engineered into them. To this end, we have developed engineered derivatives of Rem which are inert but can be acutely activated by small molecules to inhibit I_{Ca} [56]. Recently, a caveolae-targeted Rem has been developed that selectively inhibits caveolae-localized Cav1.2 channels in heart cells [94]. This molecule selectively inhibits hypertrophic Ca^{2+} signaling pathways in cardiac myocytes without impairing contractility.

9. PHYSIOLOGICAL SIGNIFICANCE OF RGK INHIBITION OF I_{Ca}

In contrast to the steady progress in evaluating the mechanisms by which RGKs inhibit Ca_V1/Ca_V2 channels, the physiological role of this channel regulation remains somewhat mysterious. Many different excitable cell types co-express RGKs and specific Ca_V1/Ca_V2 channel isoforms. For example, Rad and Rem are expressed in cardiac and skeletal muscle [3, 16, 95], Rem2 is expressed in neurons and pancreatic β-cells [4, 11, 54, 96], and Gem is present in mitogen-activated T cells and pancreatic β cells [2, 15, 97]. This leads to the expectation that RGK inhibition of Ca_V channels plays a physiological role in these cells. However, this is difficult to prove for two main reasons.

First, RGKs have been shown to interact with many other signaling molecules besides Ca_V channel subunits. Therefore, it is difficult to specifically assign any (patho)physiological consequences that arise from knockout of individual RGKs to their effect on Ca_V channels. Knockout mice for Rad [95], Gem [97], and Rem [98] have been generated. Rad knockout mice display an increased susceptibility to transverse aortic constriction (TAC) induced hypertrophy, and down-regulation of Rad is correlated with development of heart failure in humans [95]. $I_{Ca,L}$ has not been measured in these mice so it is unknown whether this contributes to the observed phenotype. However, deciphering the potential role of $I_{Ca,L}$ is further complicated by the fact that Rad functionally interacts with CaMKII [29, 95] and Rho kinase [99, 100], two signaling molecules well known to be involved in the development of pathological cardiac hypertrophy [101, 102]. Gem knockout mice displayed glucose intolerance, impaired insulin secretion in response to high glucose, and abnormal Ca^{2+} handling in pancreatic β-cells [97]. I_{Ca} in β-cells was not measured, precluding any inferences about a possible role of Ca_V channel dysregulation in these abnormalities. Rem

knockout mice exhibit a moderately increased I_{Ca} in cardiac myocytes, without showing any overt cardiac phenotype [98]. Interpretation of the results may be confounded by compensatory mechanisms as well as the fact that other RGKs present in heart may provide a redundant pathway for I_{Ca} inhibition. To date, the most direct evidence that an RGK may mediate constitutive inhibition of I_{Ca} in an excitable cell comes from a study which demonstrated that knockdown of Rad with shRNA resulted in an increase in $I_{Ca,L}$ in cultured cardiac myocytes [58]. Similarly RNAi knockdown of Rem2 in neurons demonstrated a role in synaptic development and dendritic morphology [11, 12], and reduced the frequency of miniature excitatory postsynaptic currents [96]. However, the Rem2 knockdown in hippocampal neurons had no impact on I_{Ca}, suggesting that other effectors underlie the observed functional effects.

A second reason for the difficulty in evaluating the physiological role of RGK inhibition of Ca_V channels in different systems has to do with the likely constitutive, background nature of this regulation. Development of approaches that can selectively and acutely relieve RGK inhibition of Ca_V channels would appear to be necessary for progress in determining the physiological role of this form of Ca_V1/Ca_V2 channel regulation.

10. CONCLUSION

All RGKs powerfully and promiscuously inhibit all Ca_V1 and Ca_V2 channels. Surprisingly, this seemingly homogenous and simple phenomenon is underlain by a rich variety of mechanisms and structural determinants [57, 68]. The mechanisms and structural determinants of RGK inhibition of Ca_V channels appear to be customized based on the RGK type, Ca_V1/Ca_V2 channel isoform, and cellular context. This may have contributed to apparently contradictory reports in the field. Further work is needed to define the precise mechanisms that different RGKs use to inhibit distinct Ca_V1/Ca_V2 channel isoforms in specific cell types. Nailing down the physiological role and importance of RGK inhibition of Ca_V channels has proven difficult due to the constitutive nature of this inhibition, the fact that RGKs functionally interact with other important signaling molecules, and the existence of compensatory mechanisms and redundant pathways in knockout mice. New tools that permit acute relief of RGK inhibition of Ca_V channels are needed to probe the physiological significance of this phenomenon. Finally, RGKs and engineered derivatives have potential utility as therapeutics and useful molecular tools. Exploring these dimensions of the RGK/Ca_V channel functional interaction is an exciting area for future research.

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Highlights

- RGK proteins inhibit voltage-dependent Ca_V1 and Ca_V2 channels.
- RGKs use multiple mechanisms and determinants to inhibit Ca_V1/Ca_V2 channels.
- The mechanisms of channel inhibition are customized for different RGK/Ca_V channel types.
- New functionalities can be engineered into RGKs for practical applications.

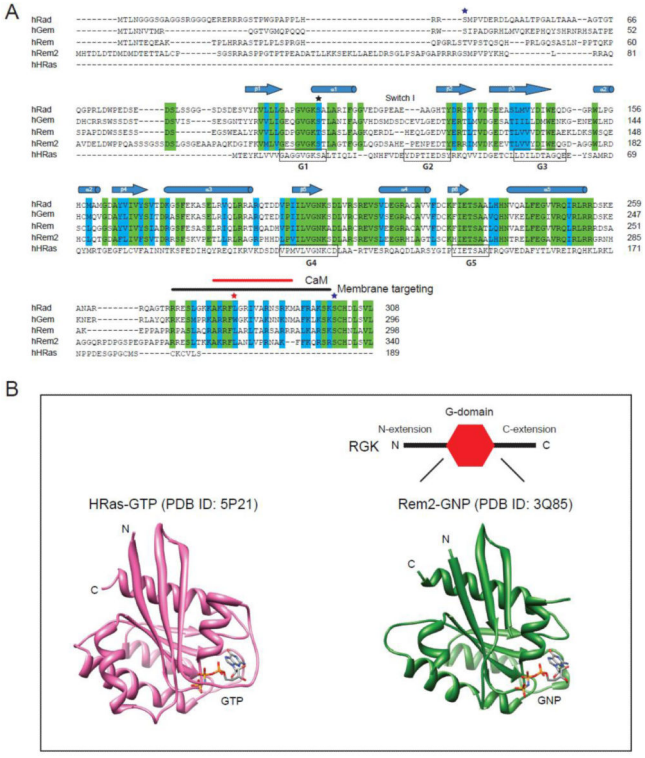

Figure 1. Structural features of RGK proteins

(A) Sequence alignment of human RGK proteins and H-Ras. Identical residues in RGKs are shaded green, and similar residues shaded in cyan. ★ residues homologous to Ras^{S17}; ★ hydrophobic residues important for CaM binding and membrane targeting; ★serine residues important for 14-3-3 binding. (B) Crystal structures of GTP-bound H-Ras (PDB ID: 5P21) and GNP-bound Rem2 G-domain (PDB ID: 3Q85).

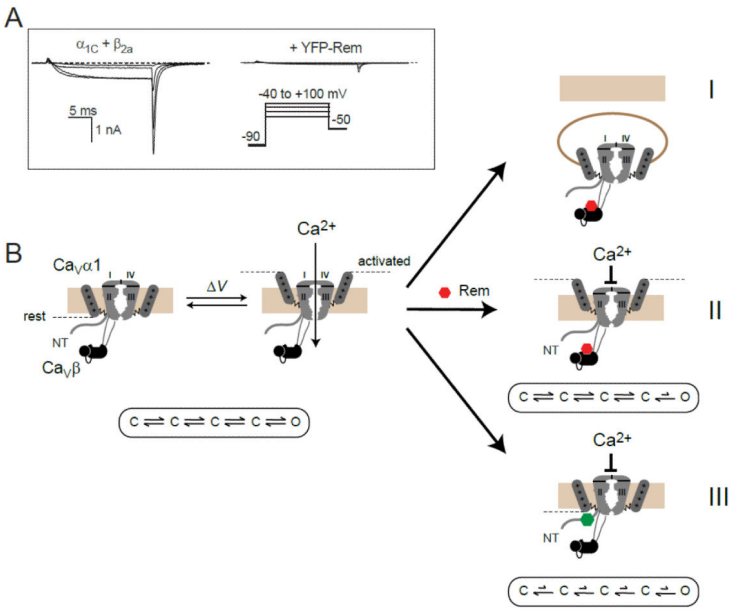

Figure 2. Rem inhibits $Ca_V1.2$ channels using multiple mechanisms

(A) *Left*, exemplar whole-cell currents from HEK 293 cells expressing $Ca_V1.2$ $\alpha_{1C} + \beta_{2a}$. *Right*, representative $\alpha_{1C} + \beta_{2a}$ channel currents in the presence of Rem. (B) Rem inhibits I_{Ca} using three independent mechanisms: by reducing channel surface density via enhanced dynamin-dependent endocytosis (mechanism I); by reducing channel P_o independently of channel voltage sensor movement (mechanism II); by partially immobilizing channel voltage sensors as reported by a decrease in maximal gating charge (Q_{max}; *mechanism III*). Mechanisms I and II persist with Rem^{T94N}, suggesting they do not require GTP to be bound to the Rem nucleotide binding domain (represented as a red hexagon). By contrast, mechanism III is selectively lost with Rem^{T94N} suggesting a requirement for GTP binding to Rem (represented as a green hexagon). Figure modified from [57] and [17].

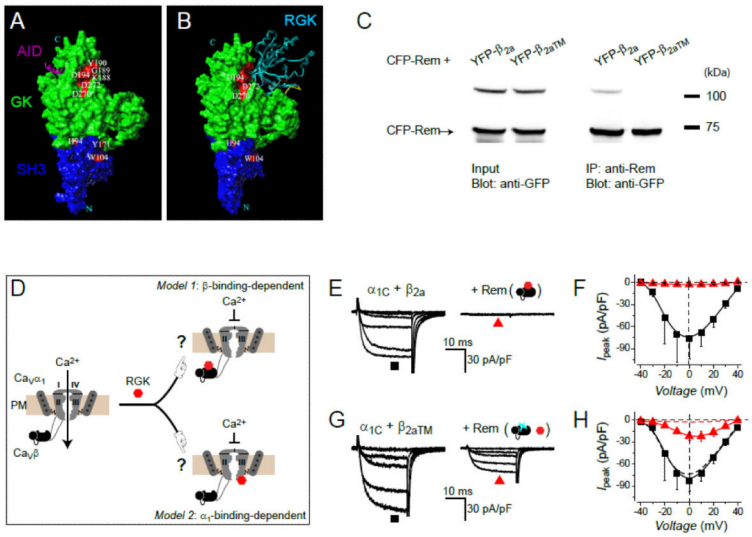

Figure 3. Role of $Ca_V\beta$-binding in Rem inhibition of $Ca_V1.2$ channels

(A) Mutations on $Ca_V\beta$ that disrupt binding to RGK proteins aggregate at a hotspot that is separate from the α_1-binding pocket that binds AID. Modified from [72]. (B) Computational model showing docking of Gem to the $Ca_V\beta$ RGK-binding hotspot. Modified from [72]. (C) Co-immunoprecipitation assay demonstrating that a mutated β_{2a}, β_{2aTM}, no longer interacts with Rem. (D) Cartoon showing two possible modes of RGK inhibition of $Ca_V1.2$ channels–β-binding-dependent and α_1-binding-dependent. (E) Exemplar currents from HEK 293 cells expressing α_{1C} + β_{2a} in the absence (*left*) and presence (*right*) of Rem. (F) Population current-voltage (*I-V*) relationships for α_{1C} + β_{2a} in the absence (■) and presence (▲) of Rem. (G, H) Data for α_{1C} + β_{2aTM} ±Rem. Same format as E and F. Figure reproduced from [17].

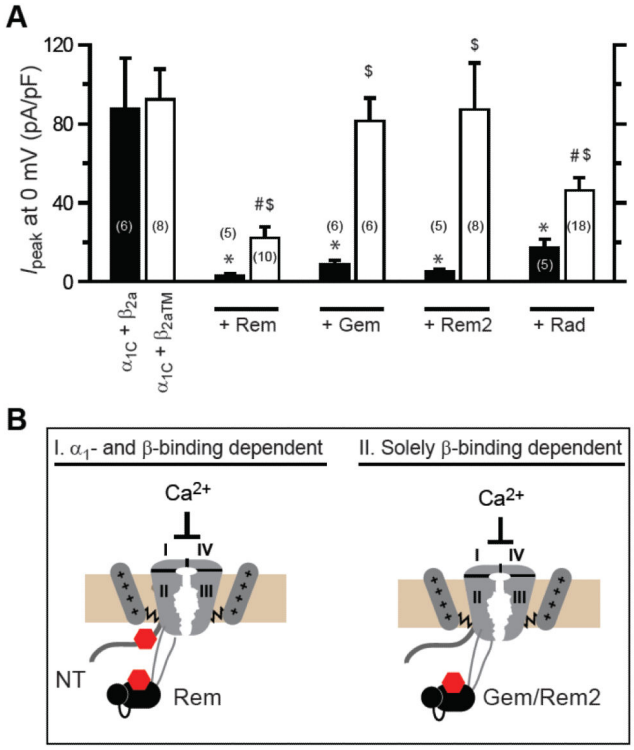

Figure 4. Distinct RGKs differentially use β-binding-dependent and β-binding-independent mechanisms to inhibit $Ca_V1.2$ channels

(A) Impact of distinct RGKs on wild-type (α_{1C}+β_{2a}) and mutant (α_{1C}+β_{2aTM}) $Ca_V1.2$ channels. *, #, $ $P < 0.05$ when compared to α_{1C}+β_{2a}, α_{1C}+ β_{2aTM}, or α_{1C}+β_{2a}+RGK, respectively, using two-tailed unpaired Student's *t* test. (B) Cartoon showing dichotomy in determinants used by distinct RGKs to inhibit $Ca_V1.2$ channels. Rem can inhibit $Ca_V1.2$ by both β-binding-dependent and α_1-binding-dependent mechanisms. Gem and Rem2 inhibit $Ca_V1.2$ solely using a β-binding-dependent mechanism. The stoichiometry of Rem binding to the $Ca_V1.2$ complex has not been investigated. One possibility is that two Rem proteins can simultaneously associate with a single $Ca_V1.2$ channel. Alternatively, it is also possible that within a single $Ca_V1.2$ channel complex, the interaction of Rem with α_{1C} N-terminus or β subunit is mutually exclusive, thus ensuring a 1:1 binding stoichiometry. Figure reproduced from [17].