Published in final edited form as:
Cell Signal. 2008 February ; 20(2): 292–300.

The RGK family of GTP-binding Proteins: Regulators of Voltage-dependent Calcium Channels and Cytoskeleton Remodeling

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Abstract

RGK proteins constitute a novel subfamily of small Ras-related proteins that function as potent inhibitors of voltage-dependent (VDCC) Ca^{2+} channels and regulators of actin cytoskeletal dynamics. Within the larger Ras superfamily, RGK proteins have distinct regulatory and structural characteristics, including nonconservative amino acid substitutions within regions known to participate in nucleotide binding and hydrolysis and a C-terminal extension that contains conserved regulatory sites which control both subcellular localization and function. RGK GTPases interact with the VDCC β-subunit ($Ca_V\beta$) and inhibit Rho/Rho kinase signaling to regulate VDCC activity and the cytoskeleton respectively. Binding of both calmodulin and 14-3-3 to RGK proteins, and regulation by phosphorylation controls cellular trafficking and the downstream signaling of RGK proteins, suggesting that a complex interplay between interacting protein factors and trafficking contribute to their regulation.

1. Introduction

The Ras superfamily of low-molecular-mass GTP-binding proteins is composed of a diverse group of over 170 structurally related proteins that have been grouped into the broad Ras, Rab, Rho, Arf, and Ran families [1]. Functioning in unison with their affiliated regulatory and effector protein networks, Ras-related GTPases serve as central control elements in signal transduction cascades that contribute to almost every aspect of cellular physiology. The members of each family can be further subdivided into evolutionarily conserved subfamilies reflecting additional levels of structural, biochemical, and functional conservation [1]. Despite these family differences, all Ras-related GTPases contain five highly conserved domains (G1–G5) and function as guanine nucleotide-dependent molecular switches, alternating between an active GTP-bound and an inactive GDP-bound conformational state [2]. Guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) [2] influence the relative proportions of molecules in the active and inactive conformation.

The RGK subfamily of small GTP-binding proteins is comprised of four members, Rem (also known as Rem1 or Ges), Rem2, Rad, and Gem (mouse homolog also referred to as Kir) that exhibit conserved structural features that distinguish them from the other Ras proteins [3–9] (see Fig. 1). These include several nonconservative substitutions within regions of the Ras core known to be involved in both GTP/GDP binding and hydrolysis, a conserved C-terminal extension that functions to direct membrane association but also serves as a critical regulatory domain, and a large N-terminal domain that is not conserved within the family. RGK proteins

also differ from one another and other Ras-related proteins within their putative effector (G2) domains, suggesting that they may interact with distinct regulatory and effector proteins. While classical GEFs and GAPs remain to be identified for the RGK GTPases (see section 2), additional methods of regulating these proteins have been identified (see sections 4–6). Members of the RGK family are expressed in a tissue-specific manner and subject to both transcriptional and posttranscriptional control mechanisms that regulate protein levels in response to a diversity of environmental stimuli (see section 4). RGK proteins have recently been shown to control the activity of voltage-dependent Ca^{2+} channels by interacting with their accessory β-subunits (see section 6), as well as regulating the Rho/Rho kinase signaling cascade to modulate actin cytoskeletal remodeling (see section 5). Here we discuss the structural and functional properties of the RGK proteins and examine the roles of phosphorylation, regulatory protein binding, and lipid interactions as mechanisms for controlling RGK family function.

2. RGK proteins are guanine nucleotide-binding proteins

RGK proteins contain a number of non-conserved amino acids at positions important for GTP/GDP binding and hydrolysis [3–9]. For example, RGK GTPases contain substitutions within the G1 motif that is involved in phosphate binding. The residue equivalent to Gly^{12} in Ras, mutation of which results in the constitutive activation of both Ras and Rho family GTPases, is altered in all RGK proteins (to Glu in Gem, Ser in Rem, and Pro in Rad and Rem2), and the highly conserved threonine residue within G1 (Thr^{35} in Ras) is lacking. There are also major substitutions within the G2 and G3 domains, which function to sense GTP binding and promote conformational change within Ras family proteins during their GTP/GDP activation cycle [3–8]. All RGK proteins share a conserved DXWEX G3 motif which diverges significantly from the DTAGQ motif found in other Ras family GTPases [10]. In particular, the glutamine residue (Gln^{61} in H-Ras) plays a critical role in GTP hydrolysis, and is conserved in Rad and Rem2 but is replaced by alanine in Rem and asparagine in Gem. These alterations to the RGK primary structure initially raised questions about the mechanism of GTP hydrolysis. A final striking difference is the sequence divergence within the putative G2 effector domain among the four RGK proteins. This domain is highly conserved within Ras GTPase subfamilies, since it constitutes the primary effector protein docking site. This suggests that individual RGK proteins either associate with distinct cellular effectors (see Table 1), or perhaps that the RGK effector domain involves residues beyond the canonical switch I and switch II regions (see section 3). Despite these structural alterations, biochemical studies have revealed that all RGK proteins display micromolar affinity for GDP and GTP, and exhibit intrinsic GTPase activity [3–9,11–13].

Because RGK proteins are capable of undergoing a classical GTP/GDP cycle, it is predicted that they will be regulated by the actions of both GEF and GAP proteins. While these proteins have not yet been identified, the *in vitro* GTP/GDP state of Rad has been found to be modified by the tumor suppressor nm23, which can either phosphorylate GDP or dephosphoryate GTP while the nucleotide is bound to Rad [14]. It remains to be determined whether nm23 functions as an *in vivo* regulator of Rad, or regulates the actions of other RGK proteins. In addition to nm23, calmodulin was proposed to modulate GTP binding to Gem [15], although this result has recently been challenged [8]. Indeed, it remains unclear whether the *in vivo* nucleotide status of RGK proteins is regulated in response to extracellular stimuli, or if GTP-binding is required for RGK downstream signaling. Thus, the identification of RGK regulatory factors remains an important goal for the field. Isolation of these GEF/GAP proteins may assist in the identification of the physiological stimuli that control RGK G-protein activation.

3. RGK Structure

The recent crystal structures of GDP-bound Rad and Gem demonstrate that the RGK proteins adopt a canonical GTP-binding domain (G-domain) fold similar to those found in other Ras family members [7–9]. However, because of the amino acid substitutions discussed above, fewer contacts with the bound guanine nucleotide are observed [7–9] and the nucleotide is not covered by the G2 (switch I) domain [7,8]. Interestingly, a portion of the extended Gem C-terminus makes contacts with the core G-domain, reminiscent of that seen with the N-termini of GDP-bound Arf proteins [8,16] and the C-terminus of Ran [17]. This combination of structural alterations likely results in the lower GTP/GDP affinity found for RGK proteins when compared to Ras [4,8]. De Gunzburg and colleagues also note that the GTPase activity of Gem is reduced by removal of the N-terminus, or following deletion of both the N- and C-terminal extensions from the conserved GTPase core [8]. Together with the structural studies, these biochemical data suggest that conformational changes involving rearrangement of the N- and C-termini might contribute importantly to the RGK GTPase cycle, and indicate that the molecular mechanism of RGK GTPase activity may be quite different from that of other small GTP-binding proteins [8]. Additional crystal structures, particularly of full length RGK proteins in the GTP-bound structural state, will be needed to further clarify this potentially novel GTPase cycle.

The crystal structures combined with *in vitro* binding studies have recently been used to model both Gem-$Ca_V\beta$ [7,18] and the Gem-Rho kinase interactions [7]. Interestingly, residues in distinct regions of Gem were found to disrupt $Ca_V\beta$ association when subjected to site-directed mutagenesis [17,18]. These include residues within the G2 and G3 regions that inhibited $Ca_V\beta$ but not Gem-Rho kinase mediated neurite binding outgrowth [7], as well as residues in the G4–G5 region of Gem required for $Ca_V\beta$ binding [18]. Intriguingly, these data suggest that multiple contact sites contribute to these interactions, and appear to involve residues outside of the classical G2 effector loop. Thus, the picture of how RGK proteins interact with their binding partners has begun to be clarified, although resolution of the details of protein association await co-crystalization studies.

4. Regulation of RGK Activity

The majority of Ras-related GTP-binding proteins function as molecular switches regulated primarily through nucleotide exchange facilitated by regulatory proteins, including the actions of selective GEFs and GAPs [19]. Since it remains unclear whether RGK protein function is controlled via a classical GTP/GDP cycle (see section 2), a critical question becomes how RGK protein function is regulated. While definitive answers await additional study, it appears that both transcriptional and translational control is used to regulate RGK protein levels. Furthermore, the C-terminus has emerged as a key regulatory domain being subject to phosphorylation, as well as protein-protein and protein-lipid interactions, to control both the subcellular localization and activity of all RGK GTPases.

4.1 Transcriptional Regulation

Among the most unique features of RGK proteins is their ability to be transcriptionally regulated [3,5,6,11,20–37] (Table 2). Indeed, Gem/Kir was discovered as a gene upregulated in human T cells after stimulation with mitogens [5] or in BCR-Abl-transformed B cells [11]. Subsequent studies have found Gem to be induced following cytokine stimulation [36], in response to muscarinic receptor agonists [26], by glucose in pancreatic β-cell lines [28], during acute inflammation [37], and in the brains of tau-deficient mice (see below) [29]. Finally, Gem/Kir expression has been correlated, via ribozyme-mediated knockdown, to cell invasiveness [38], a key feature of metastatic cancer.

The other RGK subfamily members also demonstrate tissue and molecule-specific transcriptional regulation [3,4,6,20,21,24,25,27,30–35]. Rem is expressed predominantly in cardiac muscle, but also at more modest levels in lung, kidney, and skeletal muscle [3]; Rem2 is highly expressed in the brain and kidney, but also in neuroendocrine tissues [4]; Rad is found in abundance in cardiac and skeletal muscle [6]; while Gem/Kir is found in a diverse set of tissues, including myeloid cells, kidney, liver, and lung [5]. Rad was initially identified as a gene overexpressed in the skeletal muscle of non-insulin dependent diabetics [6], though subsequent studies in Pima Indians and the Zucker diabetic rat model imply that this effect is not universal [39]. Regulation may result from the hyperinsulinemia associated with the disease [40] as insulin has been shown to increase Rad mRNA levels in muscle [25]. In addition, Rad expression is upregulated in both developing and post-amputation de-differentiating muscle cells in the newt [33]. A similar regulation occurs in developing and regenerating muscle [20,24], as well as in denervated or injured muscle [20,27], and in vascular smooth muscle cells following balloon injury [21]. While the majority of Rad transcriptional regulation has been reported in muscle, other intriguing, but unexplored observations have emerged from other cell types. For instance, Rad is upregulated in human peripheral blood mononuclear cells after acute heat shock [34] and in human placenta post-hypoxia [22,31]. Rad expression is also correlated with the grade, size, and metastatic potential of breast cancer tumors [35].

Less is known concerning the regulation of Rem and Rem2. Rem2 expression is induced by glucose treatment in MIN6 cells, a pancreatic β-islet cell line [23], a change that suppresses insulin secretion. Similarly, Gem/Kir expression is induced in the same cell line under elevated glucose, or following insulin and KCl treatment [28]. Rem2 is upregulated in developing neurons and siRNA-mediated Rem2 silencing profoundly inhibits the development of glutamatergic and GABAergic synapses [30]. Since Rem2 is the only RGK protein abundantly expressed in neuronal tissues [4], Rem2 signaling appears crucial for synapse development and maturation. Finally, Rem expression is increased in cardiomyocytes following treatment with isoproterenol, a β-adrenergic receptor agonist [32], but decreased in cardiac muscle upon injection of mice with lipopolysaccharide [3]. While these results are intriguing, the physiological relevance of RGK transcriptional regulation is still unclear. However, it should be noted that RGK signaling may control a previously unappreciated negative feedback cascade operating to protect both pancreatic β-cells (Rem2) and cardiomyocytes (Rem) from uncontrolled Ca^{2+} signaling in the presence of persistent hyperglycemia or chronic β-adrenergic stimulation [23,32]. Since disruption of intracellular Ca^{2+} homeostasis readily induces cellular dysfunction, this putative pathway may play a significant protective role. Overall, RGK GTPases are found to be upregulated in both developmental and disease processes and also during adaptive responses to extracellular stimuli.

4.2 Post-Translational Modification

Phosphorylation is the major post-translational modification observed for RGKs to date, and appears to regulate both their interactions with multiple binding partners and intracellular trafficking (Table 1). Rad is an *in vitro* substrate for several kinases, including PKA, PKC, CaMKII, and casein kinase II [12], which phosphorylate several distinct serine residues within the protein. 14-3-3 proteins interact specifically with RGK proteins phosphorylated on N- and C- terminal serines [41–45] and 14-3-3 binding appears to modulate the subcellular localization of Rad, Rem, Rem2, and Gem/Kir proteins [41,43,44,46,47]. Phosphorylation of Rad by PKC or casein kinase II, on the other hand, reduces binding to CaM [12]. PKCζ-mediated phosphorylation of Ser261 in Gem [42] has been suggested to play a role in cytoskeletal rearrangements ([42]; see below) although it does not appear to contribute to either 14-3-3 or calmodulin binding [42]. On the other hand, these same residues do not contribute to Gem-mediated inhibition of Ca^{2+} channels [42], indicating that the two known functions of Gem, and possibly other RGKs, are independently regulated.

4.3 Subcellular Localization

RGK proteins do not contain canonical lipid modification motifs [48], though there is enrichment of these proteins at the plasma membrane, and individual proteins have been shown to be localized to the cytosol, nucleus, and with both the actin and microtubule networks [4, 5,18,41,43,44,46–54]. The carboxyl terminus is well conserved (Fig. 1) and has been shown to play a critical role in the function of all RGK GTPases [49,55], regulating subcellular distribution [4,5,53], and directing protein-protein interactions [45,53,55], to control both Ca^{2+} channel activity [49,53,55] and cytoskeletal reorganization [41–44,46,47,52]. This region contains a cysteine residue within a highly conserved domain termed the C-7 motif (Fig. 1 and [56]), however, despite the potential to serve as a site of lipidation, there is no evidence that the cysteine residue is modified, and deletion of the C-7 domain does not alter RGK function [8,48,53,55]. The question of how RGK GTPases localize to the plasma membrane was, at least partially, answered by the recent work of Heo and colleagues illustrating the importance of phosphatidylinositol phosphate (PIP) lipids in the membrane recruitment of RGKs, as well as other Ras-related GTPases containing polybasic tracts in their C-termini [57] (Figs. 1 and 3). Additional studies will be necessary to determine whether PIP lipid binding is required for RGK activity, although the recent analysis of Rem and Rem2 C-terminal truncation mutants suggests a strong correlation between PIP-lipid interaction, plasma membrane localization, and Rem/Rem2-dependent Ca^{2+} channel regulation ([53] and unpublished data). In addition to plasma membrane targeting through the C-terminus, three specific nuclear localization signals are well conserved in all RGK proteins [46,47]. Recent studies suggest that nuclear sequestration may play a role in regulating RGK GTPase function, and appears to be controlled in part through interactions with both calmodulin and 14-3-3 proteins (see sections 5 and 6) [41–44,46,47].

5. RGK regulation of cytoskeletal dynamics: modulation of Rho-dependent signaling

RGK proteins have been found to promote cell shape remodeling through the regulation of the actin [21,26,41–44,58–61], and perhaps microtubule, cytoskeletons [29,51]. The identification of the microtubule- associated protein, kinesin-like protein (KIF9), as a Gem-binding partner suggests that microtubule localization may facilitate Gem-dependent activation of downstream effector proteins [51]. Furthermore, a second microtubule-associated protein, tau, has been shown to abolish Gem-induced cell elongation [29]. Although data indicating a direct interaction between Gem and tau is lacking, Gem mRNA is significantly upregulated in the brains of tau-deficient mice, suggesting that Gem/tau signaling may play a role in neuronal function [29]. More importantly, overexpression of Gem or Rad has been shown to antagonize Rho kinase (ROK) induced neurite retraction and cause morphological differentiation in neuroblastoma cells [26,42,50], stress fiber disassembly and focal adhesion dissolution in fibroblasts and epithelial cells [42,50], while expression of a putative dominant-negative Rad mutant inhibits these processes in vascular smooth muscle cells [21]. ROK is a central effector for Rho GTPases [62–65] and the direct interaction of Gem with Rho kinase-β [50] inhibits ROKβ-mediated phosphorylation of both the myosin light chain (MLC) and myosin-binding subunit (MBS) of myosin light chain phosphatase [50]. In a similar fashion, Rad has been shown to associate with the ROK-α isoform [50] (Fig. 2).

While the precise mechanism is unknown, 14-3-3 binding contributes to RGK-mediated cytoskeleton reorganization [41–44]. Phosphorylation of a pair of conserved serines located in the N- and C-terminus of Gem generates a bi-dentate 14-3-3 binding site [42], and similar 14-3-3 binding sites are conserved in all RGK proteins [41,43,44] (Fig. 1). Since 14-3-3 binding stabilizes the overall Gem protein [42], it has been suggested that the Gem-14-3-3 complex regulates cytoskeletal remodeling [41,43,44]. However, the regulatory role of 14-3-3 binding

appears to depend on both the individual RGK protein and specific cell type under study [41, 43,44]. Phosphorylation of the Gem C-terminus, and other RGK proteins, may also regulate subcellular distribution [41,44,46,47], with 14-3-3 binding modulating nuclear localization in heterologous expression systems [41,43,44,47]. Therefore, nucleocytoplasmic shuttling of RGK proteins may represent a distinct mechanism for controlling RGK-dependent cell shape remodeling, and this process appears to be dynamically regulated by calmodulin and 14-3-3 binding [41,43,44,46,47,61]. Whether these regulatory processes are correlated with RGK-mediated Rho/ROK-cytoskeleton reorganization remains to be clarified. Finally, phosphorylation of these residues is dispensable for Gem-mediated inhibition of voltage-gated Ca^{2+} channel activity (see section 6), indicating that Ca^{2+} channel blockade and skeleton organization are controlled by distinct regulatory cascades [42].

In addition to directly inhibiting ROK activity, recent yeast-two hybrid studies have identified a second Gem-mediated cytoskeletal regulatory cascade [60] which involves both the novel Gem-interacting protein Gmip [66] and the membrane cytoskeletal linker protein Ezrin [60] (Fig. 2). GTP-bound Gem binds to active Ezrin at the plasma membrane-cytoskeleton interface, and this complex appears to be required for both the recruitment and activation of Gmip, which functions as a RhoGAP [66]. The recruitment of Gmip to the cell surface leads to the localized inactivation of Rho signaling [60]. Although the exact mechanism underlying Gem-Ezrin-Gmip signaling awaits further study, it might act synergistically with the ROK-inhibition pathway described above. In this case, Gem would antagonize Rho GTPase signaling at two levels, both down-regulating overall Rho signaling through recruitment of Gmip and specifically inhibiting ROK function via direct interaction. This would be similar to the ability of the Rnd3/RhoE GTPase to both inhibit ROK [67] and act to recruit and activate p190Rho-GAP [68]. Finally, it is important to note that both Rem and Rem2 have been reported to induce cytoskeletal reorganization [41,43,47,58]. However, it remains unclear whether Rem or Rem2 can activate Gmip and neither protein associates with ROK [50], suggesting that an additional regulatory mechanism may remain to be defined. Thus, while RGK GTPases regulate both cell morphology and migration, questions remain concerning both the molecular mechanism(s) and cellular stimuli that control these pathways.

6. RGK inhibition of voltage-dependent Ca^{2+} channels

Voltage-dependent Ca^{2+} channels (VDCCs) transduce electrical activity into increased intracellular Ca^{2+} that mediates a diverse array of essential cellular processes, including hormone secretion, neurotransmitter release, and excitation-contraction coupling in muscle systems [69]. These channels are multiprotein complexes consisting of the pore-forming $Ca_V\alpha_1$ subunit and a variety of auxiliary proteins, including important contributions from accessory $Ca_V\beta$ subunits [69]. While the pore forming $Ca_V\alpha_1$-subunit determines the ion selectivity and single channel conductance of the mature channel, co-expressed $Ca_V\beta$ facilitates cell surface trafficking of the α_1 subunit, increases Ca^{2+} current amplitude, and alters channel gating properties [69].

Recently, all members of the RGK family have been identified as potent inhibitors of voltage-dependent Ca^{2+} channel currents [23,52,55] (Fig. 3). Ectopic expression of RGK proteins in variety of heterologous and endogenous cell models consistently demonstrates an almost complete inhibition of VDCC current, including L- [23,52–55,70–73], P/Q- [52], and N- [49, 52], but importantly not T-type channels which do not require accessory β-subunits for ionic current expression [55,69]. Recent studies in *Xenopus* oocytes demonstrate that this may be a dose-dependent effect [72], and recent work indicates that RGK-mediated $Ca_V\beta$ binding is essential for VDCC regulation [71]. Currently, at least two mechanisms appear to contribute to RGK-mediated Ca^{2+} channel inhibition (Fig. 3). The seminal work of Beguin and colleagues provided the first evidence in support of a model in which Gem binding to cytosolic $Ca_V\beta$-

subunits would disrupt $Ca_V\beta$-$Ca_V\alpha_1$ association [52] (Fig. 3A). RGK-mediated $Ca_V\beta$ sequestration would result in the retention of newly synthesized $Ca_V\alpha_1$ subunits in an intracellular compartment [52], resulting in a chronic reduction in surface expressed Ca^{2+} channels [74]. Subsequent studies have supported this observation [18,44,75], and extended it to demonstrate inhibition of epitope-tagged $Ca_V\alpha_1$ trafficking by co-expressed Rad, Rem and Rem2, as detected by immunofluorescence microscopy [18,41,43]. The finding that RGK protein localization is controlled in part through their association with calmodulin (CaM) and 14-3-3 proteins [41,43,44,46,47], prompted Beguin and colleagues to extend the sequestration regulatory model. Although the CaM-binding site does not appear to directly contribute to *in vitro* $Ca_V\beta$ binding, mutation of this domain results in nuclear localization of the Gem mutant, and is required for Gem-mediated VDCC inhibition [42,44,52]. Indeed, Rem and Rad mutants deficient for CaM binding have been shown to translocate to the nucleus along with their associated $Ca_V\beta$ binding partners, suggesting that RGK:$Ca_V\beta$ sequestration within the nucleus might provide a second mode for regulating α_1 trafficking [41,43,44,46,47].

Exogenous Gem expression in cardiomyocytes results in reduced gating currents, suggesting a loss of functional channel complexes at the plasma membrane consistent with inhibition of channel trafficking [76]. However, the ability of RGK expression to alter Ca^{2+} channel trafficking to date has only been directly demonstrated for L-type channels in heterologous expression systems [18,41,43,44,52]. Indeed, Chen et al. [49] reported that Rem2 does not affect the surface expression of endogenous N-type Ca^{2+} channels in primary neurons, while Finlin et al. [23,71] have reported similar finding for the regulation of endogenous L-type Ca^{2+} channels by both Rem and Rem2 in HIT-T15 pancreatic β cells at a time when each RGK protein generates an almost complete block of Ca^{2+} channel currents. Furthermore, cyclohexamide-mediated blockade of new Ca^{2+} channel synthesis does not result in the inhibition of endogenous Ca^{2+} currents seen following RGK expression in neurons [49], suggesting that the majority of RGK-mediated channel regulation in these cells is acute and not due to turnover of preformed channel complexes. Thus, while there is evidence to support a role for RGK proteins in VDCC trafficking, additional studies are needed to characterize the Ca^{2+} channel subtypes that are subject to this mode of regulation, and whether additional cellular co-factors are required.

A key feature of the sequestration model is the notion that RGK proteins inhibit $Ca_V\alpha$:$Ca_V\beta$ association [52,75]. All $Ca_V\beta$ subunits share a conserved domain structure with three variable regions separated by conserved SH3-like and GK-like domains [77]. The $Ca_V\alpha$ interaction site is located within the GK domain of all $Ca_V\beta$ subunits and supports high affinity (low nanomolar) association with a conserved domain (termed the AID) of the α-subunits [77–79]. As first shown with Rem [71], and subsequently with the remainder of the RGK GTPases [18], deletion mapping studies have located the RGK binding domain to a region within the larger GK domain of $Ca_V\beta$. In these same studies, $Ca_V\beta$ mutants were identified that had lost the ability to bind $Ca_V\alpha$ but retained Rem binding, demonstrating that the AID and Rem association sites on $Ca_V\alpha_1$ are structurally distinct [71]. Furthermore, both *in vitro* [71] and *in vivo* [18] binding studies indicate that RGK association does not inhibit the association of $Ca_V\beta$ with AID. Instead, $Ca_V\beta$ plays a scaffolding role, simultaneously associating with both RGK and $Ca_{V\,1}$ [18,71]. Together these studies suggest that formation of a RGK-$Ca_V\beta$-$Ca_V\alpha_1$ regulatory complex acutely produces a nonconducting channel species, without the need to disrupt $Ca_V\alpha_1$-$Ca_V\beta$ association or alter $Ca_V\alpha_1$ expression at the plasma membrane (Fig. 3B). In addition, it remains a formal possibility that a similar RGK-$Ca_V\beta$-$Ca_V\alpha_1$ regulatory complex might be found on the endoplasmic reticulum and function to inhibit $Ca_V\alpha_1$ trafficking to the plasma membrane, serving to unify the apparent contradictions in the two regulatory models (Fig. 3A).

An important question raised by these studies is the nature of the regulatory mechanisms that act on RGK proteins to modulate their VDCC-inhibitory signaling. *In vitro* $Ca_V\beta$ binding appears to require GTP-bound Gem [52], but channel regulation has been reported to be nucleotide-independent for Rem2 [49]. In addition, Rem-mediated VDCC regulation is modulated by cellular kinase pathways, although it remains to be determined whether this is true for other RGK proteins [54]. Moreover, recent studies indicate that RGK regulation involves the conserved C-terminus (Figs. 1 and 3). Deletion of the Rem or Rem2 C-termini inhibits plasma membrane localization and eliminates Ca^{2+} channel regulation, but does not disrupt $Ca_V\beta$ binding [49,53], indicating that RGK proteins do not inhibit channel function solely through association with β-subunits. Instead, plasma membrane localization is critical to RGK-mediated Ca^{2+} channel regulation, since anchoring C-terminal Rem [53] or Rem2 truncation mutants [49] to the plasma membrane is sufficient to restore Ca^{2+} channel inhibition. As discussed above (see Section 4), the RGK C-terminus would appear to provide an excellent regulatory domain, being subject to both complex patterns of phosphorylation [12,41–45], serving as the docking site for various binding factors [15,41–45,80] (Table 1), and as a phosphatidylinositol lipid (PIP) binding domain [53,57] (Fig. 1). Indeed, calmodulin binding to Gem and Rad has been reported to be required for VDCC blockade [41,42,44]. In addition, Rem2/PIP lipid association may be subject to regulation, since PIP lipid interaction is inhibited by association with 14-3-3 proteins (unpublished data). Importantly, this is a reversible process, regulated by the phosphorylation of a pair of serine residues in Rem2 [43], and loss of 14-3-3 binding restores PIP association (unpublished data). Thus, the regulation of cellular PI lipid kinases/phosphatases as well as modulation of RGK/14-3-3 binding may play important roles in regulating localization, which in turn would provide a physiological mechanism for controlling RGK-mediated VDCC inhibition (Fig. 3).

Regardless of the mechanism of RGK-dependent Ca^{2+} channel inhibition, recent studies suggest that RGK overexpression may prove useful as a treatment for some forms of heart disease. Murata et al. have shown that Gem overexpression in the AV node both slows conduction and provides protection during atrial fibrillation [76]. Although this study provides a compelling summation of the effects of RGK overexpression in the heart, there is a notable absence of studies designed to explore the function of native RGK proteins expressed at endogenous levels in their respective tissues. To that end, a recent study by Yada and colleagues in which they attempted to ablate the function of endogenous RGK proteins by overexpression of a mutation within the GTPase core of Rad (Rad^{S105N}), is notable [73]. Although biochemical studies are lacking for Rad^{S105N}, it is postulated that the mutant will function in a dominant-negative manner, inhibiting the activity of both endogenous Rad, and perhaps all RGK GTPases by sequestering putative RGK GEFs [81]. Indeed, mutant expression in cardiomyocytes increases both current density and action potential duration, suggesting that Rad^{S105N} may interfere with the normal functioning of wild-type RGK proteins and thus modulate Ca^{2+} channel activity. Cardiomyocytes from transgenic mutant mice overexpressing Rad^{S105N} display a longer QT interval and an increased incidence of arrhythmia under both resting and stimulated conditions [73]. To date, this study is the most compelling evidence to suggest that endogenous RGK proteins function in the regulation of Ca^{2+} channels. Overexpression of Rad^{S105N} has also been found to inhibit neointimal development following injury by inhibiting the migration of vascular smooth muscle cells [21]. However, both studies rely upon Rad^{S105N} overexpression, and it will be important to demonstrate that genetic knockout of an RGK protein(s) results in a similar phenotype before the physiological role of individual RGK proteins can be established. Indeed, transgenic mice overexpressing Rad specifically in skeletal muscle have been generated, and shown to develop more severe diet-induced insulin resistance and glucose intolerance, and exhibit lower plasma triglyceride levels than wild-type littermates [82]. Whether these effects result from dysfunctional regulation of Ca^{2+} channel activity, cytoskeletal reorganization, or a novel function of Rad signaling, remains to be determined.

7. Conclusions

RGK proteins have emerged as critical regulators of VDCC function and cytoskeletal reorganization. These effects appear to be mediated by distinct effector pathways, involving both blockade of the Rho/ROK signaling cascade, and inhibition of voltage-gated Ca^{2+} channel current expression mediated by association with $Ca_V\beta$ subunits and the plasma membrane. Despite this recent progress, much remains to be learned concerning the molecular mechanisms that govern these diverse actions. Insights to date have been generated largely through overexpression, and must be balanced in future by studies investigating the actions of RGK proteins at physiological concentrations, as well as the cellular effect of genetic ablation of individual family members. Despite the wealth of data concerning the roles of phosphorylation, calmodulin, 14-3-3, and PIP-lipid binding as potential means toward RGK protein regulation, almost nothing is known concerning the environmental signals that control RGK protein function, and whether the subcellular distribution or binding partners of endogenous RGK proteins are dynamically controlled. Recent three-dimensional structures of RGK proteins have provided insight into potential mechanisms for controlling GTP hydrolysis and will assist in the identification of protein interaction domains. Understanding how RGK GTPases are regulated, together with the isolation of additional RGK binding partners, are crucial steps toward defining the physiological functions of this novel Ras GTPase subfamily.

Acknowledgements

We apologize to the scientists whose work was not cited due to space constraints. Research of the authors was supported by Public Health Service Grants HL072936 (to D.A.A.), a National Center for Research Resources grant P20 RR20171 (to D.A.A.), an American Diabetes Junior Faculty Award 7-05-JF-16 to (B.S.F.), and an NIH Interdisciplinary Cardiovascular Training Grant T32 HL072743 (to R.N.C.).

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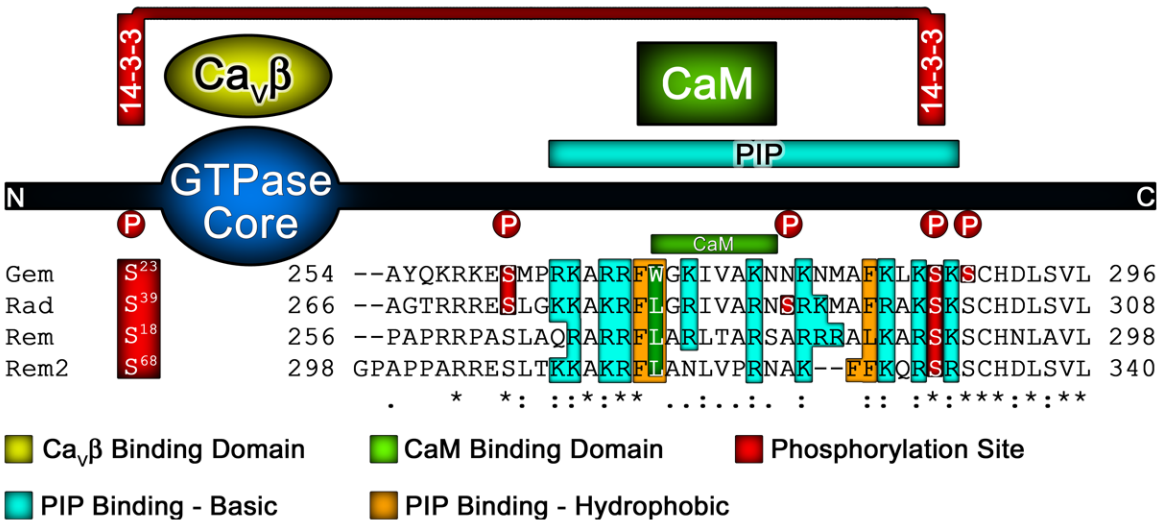

Figure 1. RGK proteins interact with a variety of factors to regulate their localization and functions

Human RGK proteins were aligned using ClustalW and columns are scored as "*" (residues identical among aligned sequences), ":" (conserved substitutions among aligned sequences), or "." (semi-conserved substitutions among aligned sequences). The positions of $Ca_V\beta$ accessory channel subunits (through the GTPase core), calmodulin (CaM), and PIP lipid binding are indicated. Residues highlighted in green are known to reduce or eliminate CaM binding, while those highlighted in teal (basic residues) and orange (hydrophobic residues) are proposed to be involved in PIP lipid binding. Residues highlighted in red are phosphorylation sites that have been directly confirmed through experimentation with the exception of S18/S290 in mouse Rem (and corresponding residues in other RGK proteins) that are thought to be phosphorylated based on their roles in 14-3-3 association.

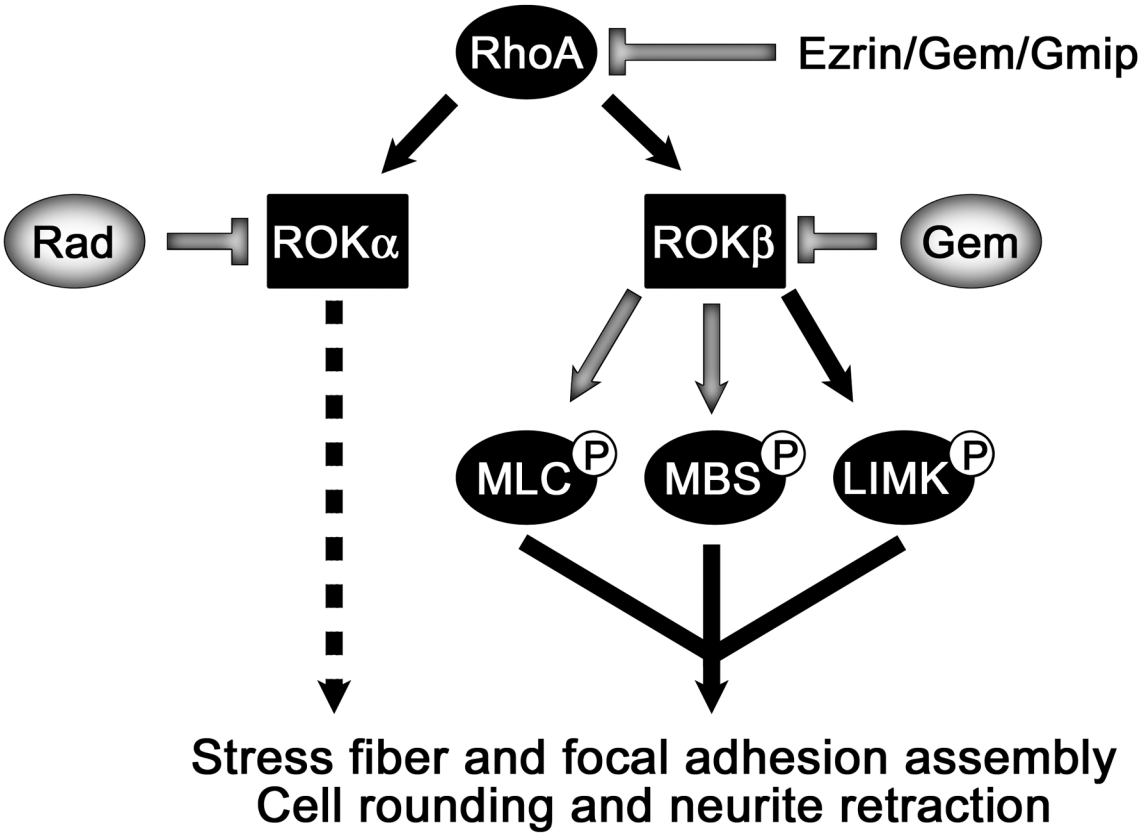

Figure 2. Proposed mechanisms for Gem/Rad mediated cytoskeleton reorganization

Gem/Rad has been proposed to antagonize Rho GTPase signaling at two levels. First, Gem has been shown to interact with ROKβ, which inhibits (grey arrows) the phosphorylation of myosin light chain (MLC) and myosin binding subunit (MBS) of the myosin phosphatases, but not (black arrow) LIM kinase. Rad interacts with ROKα and induces similar cellular effects, likely through a similar mechanism. Moreover, GTP-bound Gem has also been shown to interact with Ezrin at the plasma membrane-cytoskeleton interface and recruit the Gmip RhoGAP to down-regulate RhoA signaling.

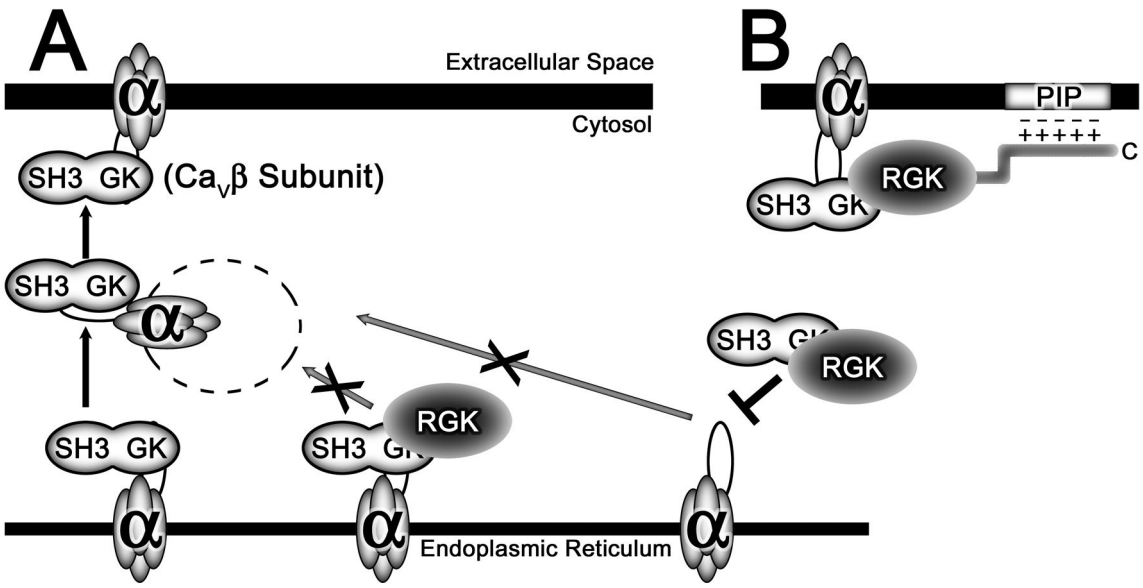

Figure 3. Potential mechanisms used by RGK proteins inhibit the function of voltage-dependent Ca^{2+} channels

A) Chronic regulation of VDCC function by altered trafficking. Left, newly-synthesized Ca_Vαsubunits are retained in the endoplasmic reticulum (ER) until association with a Ca_Vβ subunit promotes trafficking to the plasma membrane. Center, Ca_Vβ subunits have been found to simultaneously associate with both Ca_Vαand RGK proteins. This scaffolded complex may serve to inhibit channel trafficking. Right, RGK proteins have also been demonstrated to bind and sequester Ca_Vβ subunits. In this model, the RGK/Ca_Vβ complex never associates with newly-synthesized Ca_Vα subunits which remain trapped in the ER. B) Acute regulation of VDCC at the plasma membrane by RGK proteins. Acute regulation is proposed to involve two independent molecular events, association of RGK proteins with Ca_Vβ/Ca_Vα, and membrane anchoring of the conserved RGK C-terminus. Membrane association of the C-terminus involves the interaction of the positively charged RGK polybasic region with negatively charged inositol 1,4,5-bisphosphate and –trisphosphate lipids (PIP lipids).

Table 1

RGK Interacting Molecules

Accessory Molecule	Rem	Rem2	Rad	Gem/Kir	Effect	References
Ca_v β (1,2,3,4)	+	+	+	+	Inhibition of VDCC	[7,18,23,41,43,44,46,47,49,52,53,55,71]
Calmodulin	+	+	+	+	Subcellular localization; VDCC modulation	[12,15,41–44,46,47,53,80]
14-3-3 proteins	+	+	+	+	Subcellular localization	[41–47]
importins (β, α_3 α_4 α_5	+	−	+	+	Nuclear import	[46,47]
Rho kinase α	−	−	+	−	Regulation of actin organization	[21,50]
Rho kinase β	−	−	−	+	Regulation of actin organization	[7,26,50]
KIF9	N.D.	N.D.	−	+	Facilitates RGK interaction with microtubules	[51]
Gmip	N.D.	N.D.	N.D.	+	Inhibition of Rho GTPase activity	[60,66]
Ezrin	N.D.	N.D.	N.D.	+	Inhibition of Rho GTPase activity	[60]
β-Tropomyosin	N.D.	N.D.	+	N.D.	N.D.	[83]
NM23	N.D.	N.D.	+	−	GTP/GDP (de) phosphorylation	[14]
CaMKII	N.D.	N.D.	+	N.D.	RGK phosphorylation	[80]
PIP lipids	+	+	+	+	Membrane localization	[53,57] unpublished data

Abbreviations: + = demonstrated interaction, − = demonstrated lack of interaction, **CaMKII** = calmodulin-dependent kinase II, **N.D,** = not determined, **PIP** = phnosphatidylinositol phosphate, **VDCC** = voltage-dependent Ca^{2+} channel

Table 2

RGK Transcriptional Regulation

RGK Protein	Cell/Tissue Type	Regulator	Direction	References
Rem	cardiac muscle	lipopolysaccharide	down	[3]
	mouse heart	isoproterenol	up	[32]
Rem2	MIN6	glucose	up	[23]
	developing mouse neurons	KCl	up	[30]
Rad	diabetic muscle	N.D.	up	[6]
	muscle	insulin	up	[25]
	developing and dedifferentiating muscle	N.D.	up	[33]
	developing and regenerating muscle	MyoD/Myf-5/MEF2	up	[24]
	injured skeletal muscle	N.D.	up	[20]
	denervated mouse skeletal muscle	N.D.	up	[27]
	VSMCs after balloon injury	PDGF/TF-α	up	[21]
	peripheral blood mononuclear cells	heat shock	up	[34]
	placenta	hypoxia	up	[22,31]
	breast cancer specimens and cell lines	N.D.	up	[35]
Gem/Kir	MIN6	glucose/KCl	up	[28]
	T-cells	mitogens	up	[5]
	B-cells	BCR/ABL v-Abl	up	[11]
	endothelial cells	cytokines	up	[36]
	endothelial cells	NLF1/NLF2	up	[37]
	neuroblastoma cells	carbachol	up	[26]
	primary neurons from tau-deficient mice	N.D.	up	[29]

Abbreviations: N.D. = not determined, **PDGF** = platelet-derived growth factor, **TNF** = tumor necrosis factor, **VSMCs** = vascular smooth muscle cells