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Review

RGS proteins have a signalling complex: Interactions between RGS proteins and GPCRs, effectors, and auxiliary proteins

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Abstract

The intracellular regulator of G protein signalling (RGS) proteins were first identified as GTPase activating proteins (GAPs) for heterotrimeric G proteins, however, it was later found that they can also regulate G protein–effector interactions in other ways that are still not well understood. There is increasing evidence that some of the effects of RGS proteins occur due to their ability to interact with multiprotein signalling complexes. In this review, we will discuss recent evidence that supports the idea that RGS proteins can bind to proteins other than Gα, such as G protein coupled receptors (GPCRs, e.g. muscarinic, dopaminergic, adrenergic, angiotensin, interleukin and opioid receptors) and effectors (e.g. adenylyl cyclase, GIRK channels, PDEγ, PLC-β and Ca^{2+} channels). Furthermore, we will investigate novel RGS binding partners (e.g. GIPC, spinophilin, 14-3-3) that underlie the formation of signalling scaffolds or govern RGS protein availability and/or activity.

Keywords: Regulator of G protein signalling protein (RGS protein); GTPase activating protein (GAP); Heterotrimeric G protein; G protein-coupled receptor (GPCR); Effector protein; Signalling complex; Scaffolding protein

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1. Introduction

Heterotrimeric G proteins convey extracellular signals that activate 7-transmembrane-spanning G protein-coupled receptors (GPCRs) to the inside of cells, communicating this information to effector proteins and thus initiating changes in cell behaviour. GPCRs turn on G proteins by promoting the binding of the activating nucleotide GTP in exchange for GDP on the Gα subunit. In recent years, the intracellular regulator of G protein signalling (RGS) proteins have been discovered to serve additional, mostly negative, modulatory roles in G protein-mediated signal transduction. RGS proteins first were identified as GTPase activating proteins (GAPs) for heterotrimeric G proteins, and later it was found that they also can regulate G protein–effector interactions in other ways that are not currently well understood [1,2]. In mammals, twenty distinct genes for RGS proteins have been identified (numbered RGS1-21, omitting 15), some of which have splice variants, and these are categorized into four subfamilies (R4/B, RZ/A, R7/C, and R12/D). In addition there are a similar number of structurally divergent "RGS-like" proteins, a few of which have GAP activity [3,4].

Early studies on RGS proteins showed that they could act on multiple equivalents of GTP-bound Gαi and/or Gαq proteins to increase the speed with which these G proteins hydrolyze GTP [1–3]. Such findings were originally interpreted to mean that RGS proteins act catalytically to turn off activated, free Gα subunits in vivo, consistent with the widespread assumption that Gα upon GTP binding would dissociate from both the activating GPCR and the Gβγ dimer. In this prevailing paradigm, activated Gα-GTP and/or Gβγ are thought to shuttle between GPCRs and effector proteins [5]; the presumed role of RGS proteins in this context would be to "intercept" activated G proteins. The shuttling dogma has been questioned [5,6], and an alternative school of thought postulates that chemical messages are transduced via signalling complexes that contain GPCRs, G proteins, effectors, and possibly other proteins [5–8]. In addition to GPCRs, G proteins and effectors, signalling complexes can contain a variety of other structural and modulatory protein components [9]. Although the exact compositions and molecular arrangements of most signalling complexes remain to be elucidated, not to mention the mechanisms by which they are formed and disassembled, there is mounting evidence that they can at least transiently interact with RGS proteins.

The GAP effects of RGS proteins are sensitive to a wide variety of factors, for example cations such as sodium, potassium and magnesium [10,11], and phospholipids such as phosphatidylinositol 3,4,5-trisphosphate (PIP_3) [12] and phosphatidylserine [13]. Also, post-translational modifications can have profound influences on both the intracellular localization of RGS proteins and on their interactions with G proteins and other binding partners [2]. In this review, we will examine how the abilities of RGS proteins to modulate G protein-mediated signals are governed by binding to proteins other than Gα, focusing in particular on how RGS proteins may function within signalling complexes, and how auxiliary proteins such as scaffolds can play a major role in determining the availability of RGS proteins to their G protein, receptor, and effector targets.

2. Interactions between RGS proteins and GPCRs

In most cases, effects of individual RGS proteins on Gα GTPase activities can be observed in solution-based assays in the absence of other proteins. Notwithstanding, some findings suggest that RGS function may be suboptimal under such simple conditions and that RGS–Gα interactions may be enhanced in the presence of other cellular constituents. For example, when a G protein is co-reconstituted into phospholipid vesicles together with a GPCR, both the G protein affinity of an RGS protein [14] and the degree to which it accelerates GTPase activity [15] may be increased. It follows that GPCRs may govern RGS–G protein interactions [1–3], which conversely suggests the possibility that RGS proteins may modulate GPCR function. Since GTP turnover is limited by the ability of the receptor to promote nucleotide exchange [16], one would expect to see equal maxima with all RGS proteins if the receptor were acting independently. In contrast, we have observed that RGS4 and RGS17 yield different maximal rates of steady-state, M_2 muscarinic receptor-driven Gαi and Gαo GTPase activity [17], consistent with an effect of RGS proteins on receptor activity.

Evidence points to interactions between GPCRs and members of all four RGS protein subfamilies. Mammalian GPCRs identified as real or putative RGS protein targets include the M_1 [18] and M_2 muscarinic [14,19], D_2 [20–22] and D_3 dopaminergic [23], α_{1A}- [24], β_1- [25] and β_2-adrenergic [19], angiotensin AT_1 [19] interleukin-8 B (CXCR2) [26] and μ opioid receptors [27]. While mechanisms vary among RGS subfamilies, these GPCR interactions tend to involve regions

outside of the conserved RGS domain, and in some cases require auxiliary proteins.

2.1. B/R4 subfamily

Most members of this family (RGS1-5, RGS8, RGS13, RGS16, RGS18, RGS21) are relatively small (20–30 kDa) and simple in structure, containing a conserved RGS domain flanked by short amino and carboxy termini [4]. Most of these isoforms have been implicated in studies showing that the ability of an RGS protein to modulate a G protein signal can vary depending upon the identity of the activating receptor [28–32], suggesting that receptors regulate the affinity of RGS proteins for their G protein targets and/or the efficiency of RGS–G protein interactions. Furthermore, GPCRs can recruit RGS2 and RGS4 to the plasma membrane in living cells in a manner apparently not requiring either receptor or G protein activation [19]. The amino terminal domains of both RGS4 [33] and RGS2 [18] have been implicated in GPCR binding, as the deletion of this region reduces the ability of these RGS proteins to inhibit receptor-mediated signals. Moreover, the region between the RGS domain and the N-terminus of RGS2 has been shown to bind selectively to the third intracellular loop of the M_1 muscarinic acetylcholine receptor [18], as well as that region of the α_{1A} [24] and β_2-adrenergic receptors [34] (Fig. 1A).

2.2. A/RZ subfamily

These proteins (RGS17, RGS19, RGS20) are similar in size to members of the B/R4 subfamily, but they differ in that their amino-terminal regions contain a cysteine string region that is thought to be multiply palmitoylated and thereby promote membrane association [1,35]. RGS19 (also called GAIP) additionally contains a carboxy terminal PDZ binding motif [36]. As discussed elsewhere in this review, the binding of this motif to the PDZ domain-containing scaffolding protein GIPC promotes the interaction of RGS19 with the D_2 dopaminergic receptor, and possibly other PDZ binding motif-containing GPCRs [20], ultimately targeting RGS19 to specific receptor signalling pathways. In addition, recent studies have reported that both RGS17 and RGS20 can be selectively co-immunoprecipitated with the μ-opioid receptor in an agonist-sensitive manner from various mouse brain regions, although the specific binding motifs involved in this interaction were not determined [37,38].

2.3. C/R7 subfamily

Members of this subfamily (RGS6, RGS7, RGS9, RGS11) contain two functional regions that may facilitate interactions with GPCRs. These are the Gγ-like, or GGL domain, which forms a requisite stable complex with the G protein subunit Gβ5

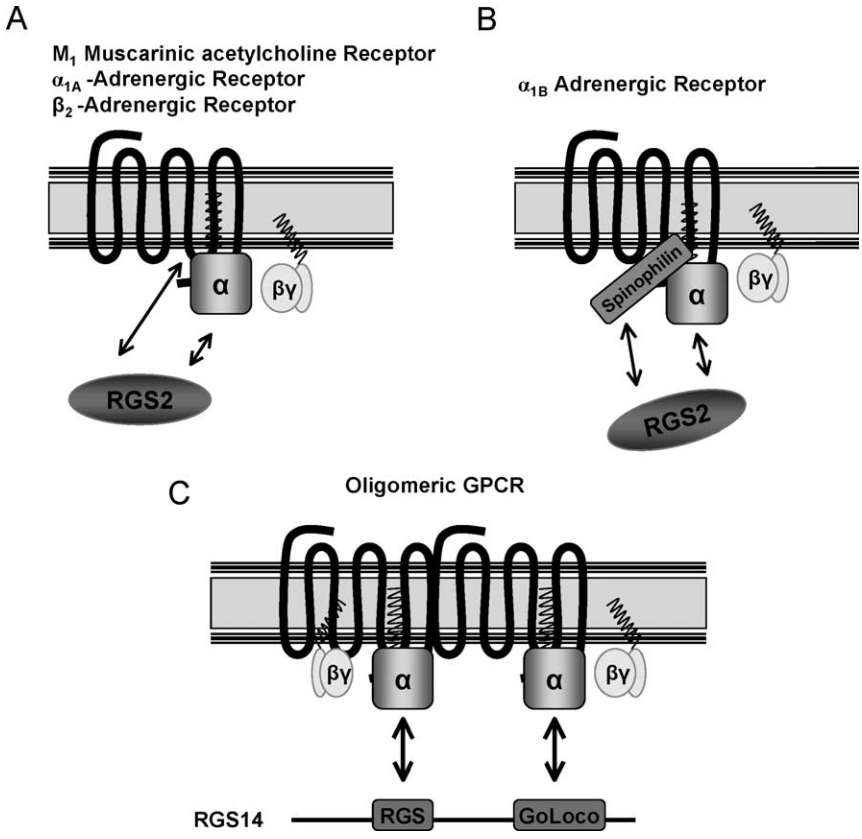

Fig. 1. Interactions between RGS proteins and GPCRs. RGS proteins in some cases bind directly to GPCRs (A), while in other cases binding occurs indirectly via an adaptor or scaffolding protein such as spinophilin (B). RGS12 and RGS14 each have a second G protein binding GoLoco domain in addition to an RGS domain, and it is postulated that both of these domains may bind simultaneously to two linked G proteins within a signalling complex (C). Further details are indicated in the text.

[39] (but which can also bind to other proteins [40,41]), and the DEP (Disheveled/EGL-10/Plextrin homology) domain, which is a module of about 100 amino acids found in a variety of signalling-related proteins and which serves as a binding interface for other proteins [42].

Both Gα and Gβγ subunits of heterotrimeric G proteins bind to receptors, and the Gβγ dimer greatly enhances GPCR-promoted nucleotide exchange on Gα [43]. It has been hypothesized that the GGL domain/Gβ5 complex may be able to substitute for ordinary Gβγ to serve this function [1,44], which would imply direct binding between GPCRs and C/R7 RGS proteins via the GGL domain. Whether this can actually occur is unclear—on one hand, full length RGS9-2/Gβ5 is able to support to a limited degree the M_2 muscarinic receptor-dependent steady-state GTPase activity of Gαo in the absence of Gβγ, but on the other hand, attempts to observe an effect of RGS9-2/Gβ5 on receptor-promoted GDP dissociation per se have been unsuccessful (AM Krumins, personal communication).

C/R7 RGS proteins appear to be targeted to their G proteins via DEP domain-associated syntaxin-like proteins called R7BP (R7 binding protein) [45,46] and R9AP (RGS9 associated protein) [47]. In mammalian retina, R9AP is required for the rapid turnoff of rhodopsin-activated transducin (G_t) signals by RGS9-1 (the splice variant of RGS9 found in retina) [48], and the absence of R9AP leads to the loss of the RGS9-1/Gβ5 complex in vivo [49]. R9AP binds to both RGS9 and RGS11, and R7BP binds to all four C/R7 family members in vitro [45]. The physiological implications of most of these interactions still await investigation although the presence of R7BP was recently shown to be necessary for recruitment of C/R7 family RGS proteins to the plasma membrane in HEK293 cells [46].

Interestingly, the DEP domain of RGS9-2 (the non-retinal splice variant) has been implicated in the selective targeting of this RGS protein to D_2 dopaminergic receptor signalling [21,22]. Also, RGS9-2 was found to co-immunoprecipitate with the μ-opioid receptor in membranes from mouse periaquedictal grey matter [38]. However, it is not clear whether the two proteins bind directly to one another nor has it been established which domain(s) of RGS9-2 might be involved. Thus, in total three distinct GPCRs (i.e., rhodopsin, D_2 dopaminergic and μ opioid) have been identified whose signals are targeted by C/R7 RGS proteins, and the available evidence points to the DEP domain as playing an important, but as yet unspecified, role in these interactions.

2.4. D/R12 subfamily

Two members of this subfamily are large multidomain proteins (RGS12 and RGS14) that contain a second Gα binding region (GoLoco), while the remaining member (RGS10) is similar in size to the B/R4 proteins [1]. RGS12 exists as multiple splice variants, some of which include a PDZ domain [50] capable of binding to the C terminus of interleukin-8 receptor B (CXCR2) [26] and potentially to other GPCRs. Also, RGS14 has been found to co-immunoprecipitate with the μ-opioid receptor in homogenates of mouse periaqueductal grey matter [38]. As discussed in the following section, the fact that RGS12 and RGS14 each have two Gα binding domains suggests the possibility that they could bind simultaneously to two G proteins within a signalling complex containing multiple GPCRs and multiple G proteins (Fig. 1C).

3. Oligomeric GPCRs—relevance to RGS protein function

Over the past decade or so, it has gradually become accepted that many if not most GPCRs exist as dimers or larger oligomers [51,52], although the functional implications of this with respect to signal transduction mechanisms have received relatively little attention. If signalling complexes contain two or more copies of a GPCR, it stands to reason that multiple G protein units might also be present. Consistent with this notion, ligand binding both to receptors [53,54] and to G proteins [55] appears to be cooperative. Recent studies examining native disk membranes from rod outer segments using atomic force microscopy suggest a 4:2 stoichiometry of rhodopsin : transducin [52], however ratios for other combinations of receptor and G protein remain to be determined.

Although biophysical evidence is not available, the behaviour of RGS proteins in steady-state, receptor-driven GTPase assays point to the possibility that RGS proteins interact in a complex manner with multiple G proteins contained within heteromeric signalling complexes, as behaviour tends to be inconsistent with simple mass-action. For example, the concentration-dependence of the GAP activity of RGS2 on M_2 muscarinic receptor-activated Gi2 is characterized by Hill coefficients in excess of 2 [11]. The high Hill coefficient of RGS2 implies its positive homotropic binding to multiple interacting units of G protein. The behaviour of RGS4 in the same system is defined by bell-shaped curves [11], which also occur with M_2-activated Gαo [56]. Similarly RGS16 exhibits a bell-shaped concentration-dependence in assays using an M_1 muscarinic receptor-Gα11 fusion protein [18]. Such bell-shaped curves suggest auto-antagonism at high concentrations of RGS protein, analogous to comparable patterns produced by GPCR agonists that can be described in terms of a homotropic two-state system [57].

As noted above, RGS12 and RGS14 each have two distinct Gα binding domains. In contrast to the RGS domain, the GoLoco domain binds preferentially to inactive Gα in solution and impedes the dissociation of GDP from free Gα [58]. The physiological relevance of this is unclear, as a recent study has shown that a truncated form of RGS14 containing the GoLoco domain but lacking the RGS domain (R14-RBD/GL) does not inhibit as predicted the steady-state GTPase activity of GPCR-activated G proteins [56]. Surprisingly, R14-RBD/GL *increased* the steady-state GAP activity of RGS4, apparently by increasing its affinity [56]. One possible explanation is that the binding of R14-RBD/GL to one G protein within a signalling complex cooperatively increases the affinity for RGS4 of a second G protein within the signalling complex. This in turn suggests that the GoLoco domain of full length RGS14 may serve to promote the binding of the RGS domain and anchor

it to a receptor-G protein multimer, although for technical reasons that possibility could not be tested experimentally [56].

4. Interactions between RGS proteins and effectors

RGS proteins, by virtue of their GAP activities, are able to rapidly deactivate G proteins, thus curtailing GPCR signals. An early study by Hepler and colleagues showed that an RGS protein could also impede signalling via G proteins that are stably activated by a non-hydrolysable GTP analogue [59]. Since then, RGS proteins have been found to bind to a variety of effector proteins, and this clearly contributes to their regulatory effects on signalling. In some cases, RGS proteins act as effector antagonists, binding to either the effector protein or the Gα subunit to prevent an operative physical interaction between the two. However, the RGS–effector protein interaction can also have a positive effect on GPCR signalling, for example by creating a complex between the activated G protein and effector, resulting in fast transduction of the activated Gα signal. In this section, we will discuss the evidence for direct interactions between RGS proteins and a number of effector proteins (Fig. 2), and the physiological implications of these interactions.

4.1. Adenylyl cyclase

Adenylyl cyclase (AC) activity is modulated by two G protein α subunits—Gαs and Gαi, which increase and decrease cAMP accumulation, respectively, and some AC subtypes additionally are regulated by Gβγ [60]. Many RGS proteins are able to inhibit the effects of Gαi on AC activity as a result of their GAP activity [61]. In addition, a number of RGS proteins including RGS2, RGS3, RGS4, RGS10 and RGS13 inhibit Gαs-stimulated AC activation [19,62–68]. This effect is most likely not due to RGS GAP activity since (1) few if any RGS proteins are able to increase the rate of GTP hydrolysis by Gαs [2]; (2) RGS proteins also inhibit forskolin-stimulated activation of AC in the absence of activated Gαs [66]; and (3) the inhibition occurs when the non-hydrolysable GTP analogue GTPγS is substituted for GTP [66,67]. Thus, the inhibition of AC activity by RGS proteins could be due to a direct interaction between the RGS protein and either Gαs or AC, or possibly both. With respect to Gαs, RGS2 co-immunoprecipitates with Gαs in cellular extracts [69], binds to activated Gαs in solution [68], and the two proteins interact in living cells, as determined by bioluminescence resonance energy transfer (BRET; [34]). These observations suggest that the direct binding of RGS2 to Gαs is at least partly responsible for the inhibitory effect of

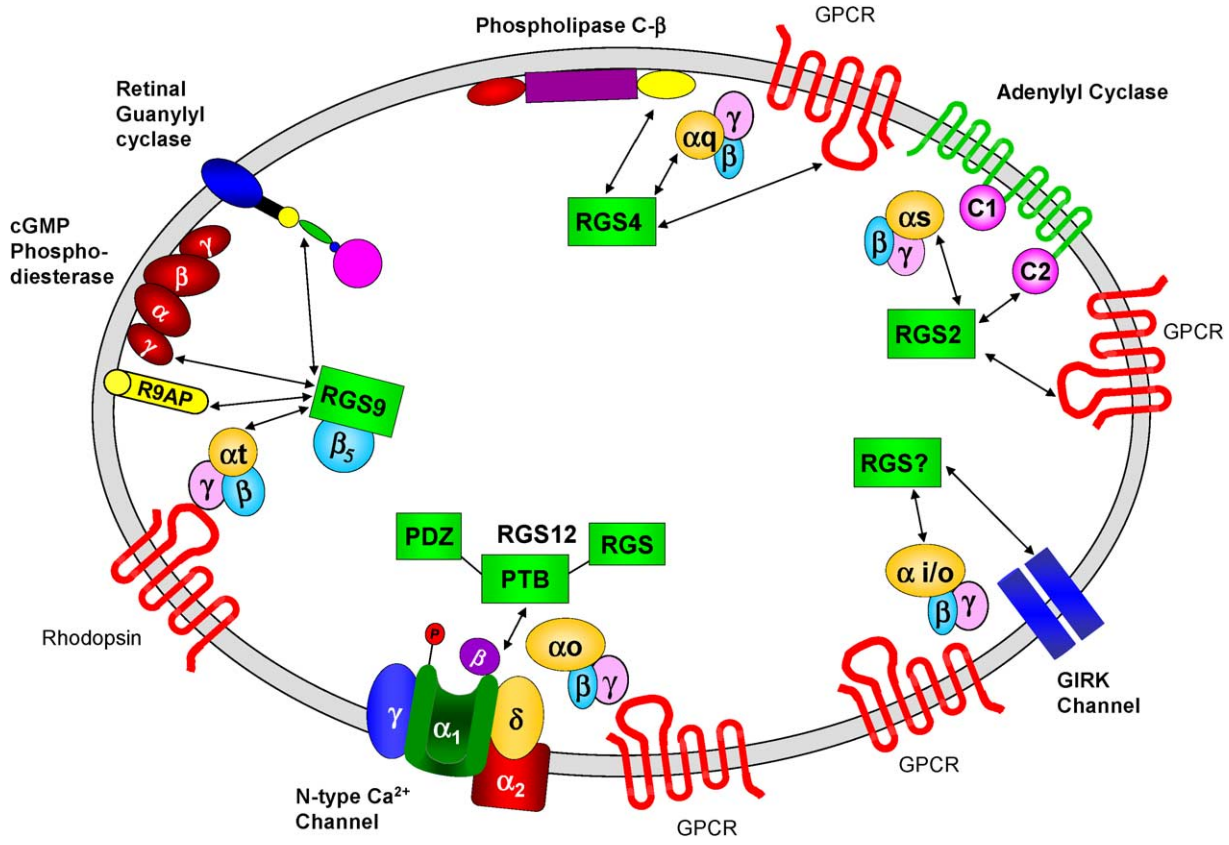

Fig. 2. Interactions between RGS proteins and G protein effectors. RGS proteins have been demonstrated to directly interact with adenylyl cyclase (the second cytosolic (C2) domain), phospholipase C-β (in the C-terminal tail region), cGMP phosphodiesterase (the γ subunit), and retinal guanylyl cyclase, as well as GIRK (K_{ir} 3.1 and K_{ir} 3.4 channel subunits) and N-type Ca^{2+} channels (the phosphorylated tyrosine residue on the α1 channel subunit). RGS proteins also bind to the Gα subunits of the heterotrimeric G proteins which activate these effectors, and in some cases, the third intracellular loop of the GPCRs which respectively couple to these G proteins. See text for a discussion of the functional implications of these interactions.

RGS2 on cAMP accumulation. Alternatively, there is evidence for a direct interaction between RGS2 and AC. RGS2 was found to decrease cAMP production by reconstitution of purified cytoplasmic domains of type V AC in the absence of Gαs [66]. Correspondingly, Dessauer and colleagues demonstrated that RGS2 binds directly to the C1 cytoplasmic domain of type V AC in cell extracts [67]. This study found that while the RGS catalytic domain is redundant in this interaction, the N-terminal 19 amino acids are both necessary and sufficient for RGS2 binding, and for inhibition of type V AC. In our laboratory, we have recently shown that expression of AC recruits RGS2 to the plasma membrane and that RGS2 binds to ACII and ACVI in protein pull-down assays. In addition, we used BRET to show that this interaction can be translated to living cells where RGS2 directly interacts with a number of AC isoforms [34]. Taken together, these data suggest that RGS2 attenuates AC activity by binding to both Gαs and AC, although the exact mechanism remains to be elucidated.

4.2. GIRK channels

G protein-gated inwardly rectifying potassium channels (GIRK) mediate hyperpolarization of excitable cells in response to GPCR activation of pertussis toxin-sensitive G proteins. Gαi/o activation in response to GPCR stimulation in turn activates Gβγ, and K^+ currents are elicited due to contacts between Gβγ and the N- and C-termini of GIRK channels [70]. In recombinant systems, GIRK channel activation (rate of channel opening) and deactivation (rate of channel closing) kinetics are significantly slower than in physiological systems [71]. However, physiological channel kinetics can be restored by the expression of RGS proteins (including RGS1, RGS3, RGS4, RGS5 and RGS8) in Xenopus oocytes, HEK293T and CHO-K1 cells, implicating RGS proteins as crucial in vitro mediators of GIRK channel function [72–77].

The steady-state concentrations of Gα-GTP (and hence activated Gβγ) should be determined by the relative rates of Gα-GTP formation and GTP hydrolysis. Therefore, the RGS-mediated increase in the rate of current deactivation presumably reflects RGS protein GAP activity [78], although it is difficult to rationalize the ability of RGS proteins to increase the onset rate of GIRK activation by this effect. Evidence shows that the increased rates of channel opening and closing are mediated via distinct mechanisms. Herlitze et al. [75] found that the kinetic effects of RGS2, RGS5 and RGS8 are more pronounced on channel deactivation than on activation in Xenopus oocytes, whereas another study, (also using Xenopus oocytes) showed that RGS7 expression accelerates channel activation, but has only weak effects on channel deactivation [73]. Further work using RGS-insensitive Gαo mutants indicated that RGS proteins promote fast activation and deactivation kinetics of α_2 adrenoceptor-mediated GIRK channel activation through distinct mechanisms in rat superior cervical ganglion neurons [79]. In that study, over-expression of RGS8 or RGS10 reversed the loss in the rate of activation, but further slowed the impaired deactivation caused by the mutant G protein. Finally, mutants of RGS4 [80] and RGS2 [75] with reduced affinity for Gα were found to accelerate activation but not deactivation in Xenopus oocytes. Thus it follows that acceleration of deactivation of GIRK channels is dependent on GAP activity subsequent to the binding of RGS to Gα, whereas the acceleration of activation is not.

The distinct effects on GIRK channel behaviour map to distinct domains within the RGS protein. The GTPase-dependent acceleration of channel closing requires an intact RGS domain, but the acceleration of channel opening does not. Jeong and Ikeda showed that the NH_2-terminus (but not the RGS domain) of RGS8 accelerates the activation kinetics of GIRK channels [79], and thus the effect on channel activation arises from a non-core domain of this RGS protein. Overall, these data suggest a mechanism wherein an RGS protein functionally completes a signalling complex that additionally contains a receptor, a heterotrimeric G protein and a GIRK channel. This would limit the diffusion time required for Gβγ channel activation after exchange of GTP for GDP on the Gα subunit, thereby increasing speed of channel opening [3,72,78]. There is some physical evidence to support the existence of such signalling complexes, as Fujita et al. [81] have demonstrated that K_{ir} 3.1 and K_{ir} 3.4 can be co-immunoprecipitated with RGS4.

4.3. cGMP phosphodiesterase and guanylyl cyclase

Vertebrate visual signals are transduced via signalling cascades that are highly organized and tightly regulated to maximize acuity, and RGS proteins play a key role in coordinating this process. The visual GPCR rhodopsin activates the G protein transducin (Gαt), and signals are conveyed via decreases in cGMP, which is produced by guanylyl cyclase and broken down by cGMP phosphodiesterase (PDE). PDE is a heterotetramer consisting of two catalytic subunits (PDEα and PDEβ) and two regulatory subunits (PDEγ). Activated Gαt turns on PDE by binding to the regulatory PDEγ subunits, to disinhibit the catalytic activity of the PDEα and PDEβ subunits [82].

The slow intrinsic GTPase rate of transducin is accelerated by RGS9-1, however several other proteins are necessary to support this activity—these include PDEγ [83], R9AP [47], and the long splice variant of Gβ5 (Gβ5L) [84]. As noted previously, both R9AP and Gβ5 are required to prevent RGS9-1 protein degradation in vivo. In contrast, PDEγ serves as a co-GAP that enhances the ability of RGS9-1 (but not other members of the C/R7 family) to promote Gαt GTPase activity, and both RGS9-1 and the effector PDE, are needed to produce full GAP activity [82].

The mechanism by which PDEγ enhances the GAP activity of RGS9-1 seems to be via an increased affinity of RGS9-1-Gβ5L for Gαt-PDEγ, compared to its affinity for Gαt alone [85,86]. The physiological implication of this is that the RGS protein will only exert its effects when Gαt is bound to its effector (PDEγ). Thus, free activated Gαt is able to interact with PDE to produce high efficiency signalling, and once this has occurred, the signal is rapidly terminated due to the GAP activity of RGS9-1 [82]. Both the catalytic and non-catalytic domains of RGS9-1 are required for its interaction with Gαt-

PDEγ [83,87]. RGS9-1 and PDEγ cooperatively bind to Gαt at distinct residues that are located in close proximity within its switch II region, [85,88], which functionally leads to increased GAP activity of RGS9-1 on activated Gαt.

Intriguingly, there is evidence to suggest that RGS9-1 may limit not only the breakdown but also the formation of cGMP. Yamazaki and co-workers isolated RGS9-1 from rod outer segments as a guanylyl cyclase binding protein [89], and found that this binding, which maps to the amino terminus of the RGS protein [90], can inhibit the production of cGMP in vitro. Although the physiological significance of this remains to be established, it suggests the possibility that visual impairment associated with RGS9-1 deficiencies may not be due entirely to the slow deactivation of Gαt.

4.4. Phospholipase C-β

Phospholipase C-β (PLCβ) is activated by G proteins of the $G\alpha q_{/11}$ subfamily to regulate inositol lipid signalling. PLCβ promotes the GTPase activity of receptor activated Gαq [91] and acts as a GAP on free Gαq [92]. Furthermore, rapid rates of GTP binding and hydrolysis imply that agonist-activated muscarinic M_1 receptor, Gαq and PLCβ exist as a complex in vitro [93], and this paradigm can be generalized to RGS proteins and receptor-activated G proteins [1].

By virtue of their GAP effects on Gαq, many RGS proteins can inhibit PLCβ activity [94]. In addition, a number of RGS proteins, including RGS2, RGS3, RGS4 and RGS10, can block PLCβ activation by GTPγS-activated Gαq, indicating that they have the capacity to inhibit signalling through this effector without deactivating the G protein [59,95–97]. One possible explanation for this might be that RGS proteins directly inhibit the interaction between Gαq and PLCβ by competitively binding to Gαq. Alternatively, Dowal et al. [98] demonstrated that RGS4 can directly interact with both PLCβ and Gαq, and that PLCβ can bind to a complex of RGS4 and Gαq, suggesting that RGS4 may remain anchored to the Gαq, Gβγ, PLCβ signalling complex in order to rapidly shut down the activated Gαq signal. Although the precise RGS4-binding domain on PLCβ has not been established, the site of interaction appears to be neither the membrane-binding face of PLCβ, nor the region of the C-terminal domain that binds to Gαq [98,99].

4.5. Calcium channels

In neurons, voltage-gated calcium channels play essential roles in neurosecretion and other functions. The activities of Ca_v1 (L-type) and Ca_v2 (P/Q, N, and R-type) channels are increased by voltage, but decreased by G protein-mediated signals, which in turn are tempered by RGS proteins.

GPCR activation causes Gβγ to directly inhibit channel opening by binding to specific regions of the α1 subunits of N-, P/Q-, and R-type Ca^{2+} channels [100,101]. Both the on and off rates of current inhibition by activated Gαz, Gαq and Gαi/o proteins are increased by RGS proteins (e.g. RGS2, RGS4, RGS10, RGS12) in recombinant [102–104] and endogenous [104–106] systems. As previously discussed for GIRK channel modulation by RGS proteins, the acceleration of the onset of channel inhibition is unlikely to be due to GAP activity, suggesting that RGS proteins subserve other roles in mediating calcium channel inhibition.

One suggested mechanism for the accelerated rate of channel inhibition by RGS proteins is that they sequestering Gα, which through an unknown process blocks the inhibitory actions of Gα on Gβγ-mediated inhibition of Ca^{2+} channels [107,108]. A second possible explanation is that the RGS proteins act by bind to Gα, thereby making Gβγ available to inhibit calcium currents [102,103,109].

Finally, in the case of the multidomain protein RGS12, it is possible that a direct interaction with the Ca^{2+} channel subunits is responsible for the acceleration of inhibition. This is supported by recent work from Diverse-Pierluissi and colleagues [104] who showed that RGS12 selectively accelerates the rate of voltage-independent GABA-mediated inhibition of the N-type calcium channel. The authors demonstrated that the phosphotyrosine-binding (PTB) domain of RGS12 directly interacts with the SNARE (soluble *N*-ethyl maleimide-sensitive factor attachment protein receptors)-binding region of the α1-subunit of the N-type calcium channel, which is tyrosine-phosphorylated in response to $GABA_B$ receptor stimulation [110]. The SNARE binding region of calcium channels has been shown to bind syntaxin, SNAP-25 and the vesicular SNARE synaptotagmin [111]. The syntaxin-channel interaction is important in the stabilization of the binding of Gβγ subunits [112], suggesting that syntaxin and RGS12 may compete for binding to the N-type calcium channel, which may offer a greater degree of effector regulation.

5. Interactions between RGS proteins and auxiliary proteins

In addition to the primary participants in G protein-mediated signal transduction, i.e., receptors, G proteins and effectors, RGS proteins have been found to interact with a wide variety of other proteins [113] and these associations can influence their subcellular localization, function and stability. Novel binding interactions by both non-RGS and RGS domains have introduced more complexity to our understanding of the potential role of RGS proteins in vivo. For example, it has been observed that the RGS domain, primarily known for its binding to Gα proteins, also interacts with small GTPases, protein kinase A, and components of the Wnt signalling pathway [113]. In this section, we will discuss in detail a number of RGS binding partners. In particular, we will focus on how RGS interactions can lead to the formation of scaffolding complexes, and the role of RGS protein partners in the regulation of RGS activity.

5.1. GIPC

GIPC belongs to a central PDZ (PSD95/DLG/ZO-1) domain-containing group of proteins [114,115]. It was first identified by its ability to bind to the C terminus of RGS19/GAIP (hence its name RGS-*G*AIP *i*nteracting *p*rotein *C*

terminus) through a PDZ-binding motif [36,116,117]. However, since then it has been shown that GIPC-binding partners are not defined by a common, prototypical PDZ-binding motif, but rather GIPC interactions are flexible and for this reason, it is able to associate with a variety of proteins with divergent PDZ-binding motifs [118–120]. Many GIPC binding partners have been identified and these include GPCRs (D_2 and D_3 dopaminergic and β_1 adrenergic, [20,23,25]) and tyrosine kinase receptors (IGF-1 and TGF beta type III, [121,122]).

5.1.1. GIPC and RGS proteins

It has been well-established that GIPC forms a complex with RGS19 (A/RZ family) that is membrane-anchored by its N-terminus and is mostly localized in clathrin-coated vesicles [123]. This localization supports a role for the RGS19-GIPC complex in the regulation of vesicular trafficking and endocytosis [36,116,124–126]. It is possible that the function of GIPC in this complex is simply to promote RGS19-mediated Gαi-GTP hydrolysis, ultimately terminating G protein signalling. However, this seems unlikely since overexpression of both RGS19 and Gαi3 (the preferred substrate of RGS19) produces inhibition of vesicular trafficking [3], suggesting that the mechanism is primarily independent of RGS19's GAP activity. Thus, the real significance of the RGS19–GIPC interaction is probably to create a complex which can further associate with other proteins [119,127].

5.1.2. GIPC function in GPCR signalling

PDZ-containing proteins are known to (1) stabilize large functional complexes; (2) spatially cluster and anchor transmembrane proteins to specific subcellular domains; (3) act as adaptors or (4) scaffolds; (5) regulate trafficking of cytosolic proteins to and from the plasma membrane and (6) interact with the cytoskeleton [128]. Therefore the GPCR–GIPC complex can be viewed as part of a dynamic protein network, necessary for fine-tuning downstream signalling and introducing functional diversity within different cell types, by acting as a bridge between GPCRs and other types of signalling molecules [115]. Evidence for these diverse interactions has been demonstrated by immunoprecipitation, pull-down assays and yeast-two hybrid screens in which GIPC has been found to bind to the cytoplasmic domain of a number of membrane proteins, such as the glucose transporter Glut-1 [129], semaphorin-F and its receptor neuropilin-1 [118,130], syndecan-4 [131], and the nerve growth factor receptor TrkA [119]. These findings imply that GIPC has an important function as a scaffolding protein, capable of spatially clustering and assembling receptors and signalling molecules in a particular cellular domain.

GIPC is able to affect downstream signalling events of GPCRs by interacting directly with certain receptors [20,23,25] and this may help to explain functional differences between receptor subtypes through G protein-independent mechanisms. For example, Hu et al. [25] showed that GIPC specifically interacts with β_1 AR and through this PDZ–protein interaction is able to decrease β_1AR-mediated ERK activation (Fig. 3B). Since GIPC associates with clathrin-containing vesicles and with specialized domains on the plasma membrane, it might be important in the regulation of GPCR internalization, compartmentalization and recycling [36,130], as well as be involved in receptor-subtype specific regulation of vesicular trafficking [23,119]. Hence, GIPC plays a prominent role in processing, trafficking and stabilization of receptors at the cell surface [23].

The RGS19–GIPC complex may be important in clustering transmembrane receptors with signalling molecules. As mentioned previously, it has been shown for the dopamine D_2 receptor that, once stimulated by dopamine agonists, the signal is fine-tuned by the GIPC-dependent protein complex, consisting of D_2 receptor and RGS19, in which the GIPC–receptor interaction actively recruits and clusters RGS19 to the plasma membrane [20] (Fig. 3A). As yet, it is unclear whether this complex involves GIPC dimerization and/or other accessory proteins (i.e. G proteins) and/or post-translational modifications [23,27]. However, it has been suggested that GIPC may accomplish this function by forming homo-oligomers which contain multiple PDZ-binding sites through interactions at its N terminus [23,120,131].

The interaction between GIPC and RGS19 is also able to promote cross-talk between non-G protein and G protein signalling networks [117–119,130]. It has been observed that the RGS19–GIPC complex is likely to associate with the phosphorylated NGF receptor TrkA, where GIPC co-localizes with the receptor in retrograde transport vesicles and inhibits MAP kinase activation by NGF. In this example, GIPC facilitates a link between NGF tyrosine kinase pathways and G proteins [119]. Similarly, Lou et al. [117] presented evidence that megalin, belonging to the LDL (low-density lipoprotein) receptor family, binds to GIPC in clathrin-coated pits in the renal proximal tubule epithelium. Megalin was shown to be concentrated in endocytic compartments of the proximal tubule along with Gαi3, RGS19 and GIPC. Hence, it may be regulated partially by Gαi3, RGS19 and GIPC, suggesting a model in which G protein-mediated signalling modulates megalin's endocytic function and/or trafficking (Fig. 3C).

5.2. C/R7 binding proteins

A number of RGS proteins have been found to interact with novel intracellular proteins to create scaffolding complexes. In particular, the C/R7 RGS family (RGS6, RGS7, RGS9 and RGS11) contains two functional regions, the GGL and DEP domains which as mentioned previously, serve as binding interfaces for other signalling proteins [42,127,132]. As noted, the SNARE-like proteins R9AP and R7BP bind to the DEP domain of C/R7 RGS proteins. R9AP serves a scaffolding function and is required for the localization and function of RGS9-1 in the retina [133]. Likewise, R7BP probably plays a similar role in the nervous system and forms part of a regulatory complex [45,49].

The GGL domains of C/R7 RGS proteins may bind to GPCRs, and also can interact with other proteins to produce effects distinct from their role in G protein-mediated signalling. For instance, recently a Gβ5-independent function of the GGL domain in RGS localization has been observed, in which both

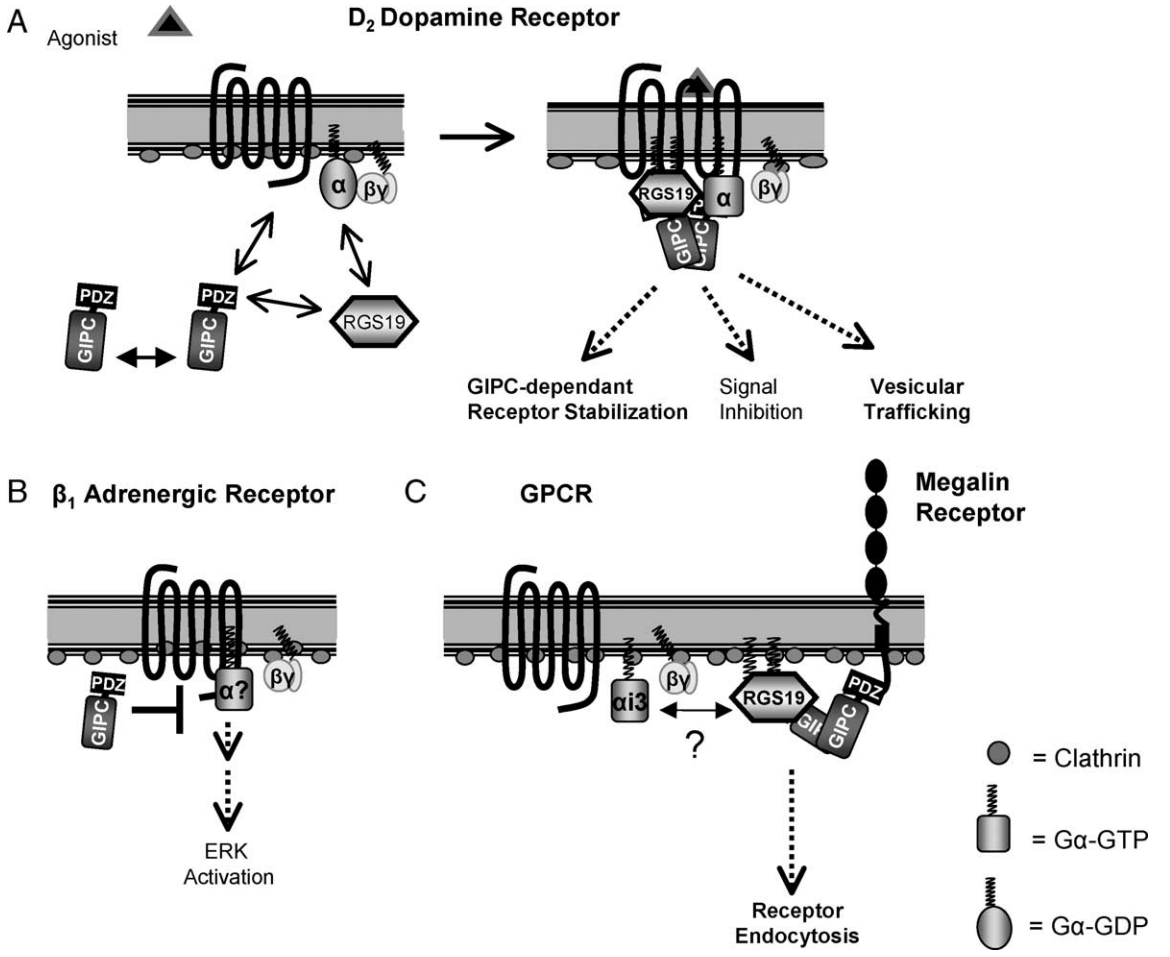

Fig. 3. Role of GIPC in GPCR signal transduction pathways. The diagram summarizes the diverse functions of GIPC that involve its direct interaction with various GPCRs, other types of receptors and RGS19. GIPC seems to be recruited by RGS19 to the plasma membrane following agonist-dependent activation of the D_2 dopamine receptor to form a complex that results in receptor stabilization, receptor trafficking and inhibition of the signal transduction pathway (A). GIPC also blocks ERK activation by the β_1 adrenergic receptor through an unknown mechanism (B) and may promote crosstalk between G protein and non-G protein pathways for example by forming a complex with the megalin receptor and RGS19, although the involvement of the GPCR and the Gαi3 still remains unclear. In this context, the GIPC/RGS19 interaction possibly plays a role in the regulation of megalin receptor endocytosis (C).

the long N-terminal and the GGL domain sequence prevented nuclear/nucleolar accumulation of several distinct transcripts of human RGS6 [132]. Moreover, RGS6 through a motif in the GGL domain that is distinct from the binding site of Gβ5, is able to bind to the transcriptional repressor protein DNA methyltransferase-associated protein 1 (DMAP1) and thus become part of a complex with DNA methyltransferase 1 [41]. In this context, RGS6 seems to decrease DNA methylation through the inhibition of DMAP1's transcriptional repressor activity. These data suggest that RGS proteins can be involved in transcriptional regulation and have a G protein-independent function in the nucleus.

Finally, it was observed that the GGL domain of RGS6 can also interact with SCG10, a neuronal growth-associated protein. Co-expression of both these proteins results in co-localization and a synergistic enhancement of PC12 cell differentiation induced by NGF [40]. Thus, this example emphasizes a role for RGS6 in neuronal differentiation via a G protein-independent mechanism. To complicate matters further, SCG10 also appears to bind to RGS20 (RGSZ1) which has no GGL domain, and this interaction has the opposite effect, resulting in the blockage of microtubule disassembly [134].

5.3. Spinophilin

Interactions between RGS and scaffolding proteins are not limited to the more complex RGS proteins. A recent report demonstrated that spinophilin, a multi-domain protein is able to interact with RGS1, RGS2, RGS4, RGS16 and RGS19 in pull-down assays [135]. Spinophilin also binds to the 3rd intracellular loop of a number of GPCRs including α_{1B}-, α_{2A}-, α_{2B}-, and α_{2C}-adrenergic and D_2 dopaminergic receptors [135]. This suggests a possible scaffolding role, as was demonstrated using Xenopus oocytes, where spinophilin was found to enhance the ability of RGS2 to inhibit Ca^{2+} signalling via the α_{1B} adrenoceptor [135]. Spinophilin also contains a PDZ domain and interacts with protein phosphatase-1 and the cytoskeletal proteins F-actin [136] and doublecortin [137]. Moreover spinophilin binds to the nucleotide exchange factor Tiam-1, where it promotes the activation of p70 S6 kinase by the small

GTPase Rac [138], further implying a role for spinophilin as an organizer of signalling complexes.

5.4. 14-3-3 proteins

14-3-3 proteins are small (27–32 kDa) proteins which, despite having no detectable catalytic or functional domains [139], appear to be regulators of key signalling components. 14-3-3 proteins are involved in many diverse cellular pathways and function primarily as chaperones, adaptors and scaffolds [140–143]. In mammals, there are seven highly conserved isoforms (β, γ, ζ, σ, ε, η and τ,), although their functions appear to be largely similar [139,144–146]. 14-3-3 proteins were initially thought to bind to either of two specific phosphorylated motifs (RSXpSXP and RXY/FXpSSXP) [143,147]. However, it is now recognized that 14-3-3 binding sites vary widely among the many (~200) binding partners that have been identified, with some interactions occurring in a phosphorylation-independent manner [148,149]. The binding partners of 14-3-3 proteins include a number of regulatory proteins and integral components of signal transduction. These consist of several GPCRs ($GABA_B$, [150], α_2-adrenergic [151] and parathyroid hormone receptors [152]), as well as tyrosine kinase receptors [153–155], kinases [156–160], phosphatases [161], apoptosis-related proteins [162,163] and protooncogene products [164]. The manner in which 14-3-3 proteins regulate such a large number of substrates is still under investigation. However, it has been shown that they are able to assemble oligomeric complexes [139], act as phosphoprotein adaptors [140,165–167], affect nuclear localization [168,169], regulate apoptosis [162,170–172] and bind to and sequester proteins in subcellular compartments, preventing further interactions with their targets [162]. Intriguingly, some evidence suggests that 14-3-3 may compete with spinophilin for binding to α_{2A}-adrenergic receptors [173].

5.4.1. 14-3-3 and RGS proteins

Recently, RGS3, RGS7 and RGS8 have been identified as 14-3-3 binding partners [174–176]. RGS protein function seems to be impeded by such binding, as indicated by 14-3-3 dependent decreases in the inhibitory effects of both RGS3 and RGS7 on G protein-mediated signals and a reduced GAP effect of RGS7 on free Gαi1 [174–176]. The primary 14-3-3-binding site of RGS7 and RGS8 is located within the Gα-binding RGS domain at a conserved 'SYP' motif [175]. In addition, RGS3 appears to have a second 14-3-3-binding site that is outside the RGS domain, located near the N-terminus [176]. Phosphorylation of the serine residues within these binding sites in RGS3 and RGS7 increases their affinity for 14-3-3 proteins [174,176]. Conversely, cellular phosphatases appear to increase the pool of active RGS7 [175], implying a mechanism to regulate RGS GAP activity without altering their expression levels.

Interestingly, the 'SYP' motif is present in about half of the RGS proteins, suggesting that other isoforms also may be subject to 14-3-3 regulation. Divergence from this is thought to preclude 14-3-3–RGS interactions, for example the SYR-containing RGS4 was found to lack sensitivity to 14-3-3, but did interact when a proline residue was substituted in for the divergent arginine [175]. In contrast, a yeast 2-hybrid screen in our lab yielded 14-3-3ε as a novel RGS4 binding partner (M Abramow-Newerly, H Ming and P Chidiac, manuscript in preparation), suggesting that 14-3-3–RGS protein binding interactions may be less limited than originally perceived. Similarly a recent study found that RGS9-2, which has an alanine substituted for the conserved proline, also could bind to 14-3-3 [177].

5.5. Ca^{2+}/calmodulin

The calcium sensor calmodulin undergoes a pronounced conformational change in response to the binding of calcium and regulates multiple signalling proteins [178]. Ca^{2+}/calmodulin directly binds to RGS1, RGS2, RGS4, RGS10, RGS16, and RGS19, in a Ca^{2+}-dependent manner [179]. This binding does not seem to affect the GAP activity of the RGS proteins [179], despite the finding that intracellular RGS activity can be increased by binding to Ca^{2+}/calmodulin. For instance in cardiomyocytes, RGS action on GIRK channels was facilitated via an increase in intracellular Ca^{2+} in a Ca^{2+}/calmodulin dependent manner [180]. Ca^{2+}/calmodulin competes with PIP_3 for binding to RGS4 and this is significant since PIP_3 binding has been shown to inhibit GAP activity in a concentration-dependent manner [179]. Therefore it seems likely that calmodulin positively regulates RGS4 activity in cells not by increasing GAP activity per se, but by preventing the inhibition of GAP activity by PIP_3.

Calmodulin and PIP_3 both bind to the C-terminal portion of helix 4 of the RGS domain of RGS4 [181]. This binding site is well-conserved in different RGS proteins, suggesting that reciprocal regulation by PIP_3 and Ca^{2+}/calmodulin may be important for the physiological control of multiple RGS subtypes. The mutually exclusive binding of Ca^{2+}/calmodulin and PIP_3 to RGS proteins implies an elegant mechanism for RGS protein-mediated modification of intracellular Ca^{2+} oscillations in vivo [3,182]. PIP_3 may initially prevent the RGS protein from inhibiting PLCβ activity (described above), allowing an increase in intracellular Ca^{2+} and activation of Ca^{2+}/calmodulin. Ca^{2+}/calmodulin will then compete for the PIP_3 binding site on the RGS protein, thereby promoting the RGS inhibitory effect on PLCβ activation. This in turn will decrease intracellular Ca^{2+}/calmodulin activation, thereby allowing PIP_3 to rebind to the RGS protein. In this way, the dual regulation of RGS activity can cause Ca^{2+} oscillations [179,183].

6. Summary/conclusion

Our understanding of RGS protein function has expanded greatly in recent years. Far from being independent "hit and run" deactivators of G proteins, RGS protein activity is now known to be regulated by a complex web of intracellular factors. RGS proteins are presented to G proteins in the context of their associated receptors and/or effectors, both of which may be

secondary RGS targets. Cells employ a variety of additional mechanisms to facilitate or hinder functional contact between RGS proteins and their target G proteins. RGS protein activity thus can be modulated such that they essentially serve as "dimmer switches" to fine-tune agonist potency and efficacy. For the majority of G protein-mediated signalling cascades, the stoichiometric and temporal aspects of how this is accomplished remain the pursuits of further study.

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