Journal of Medicinal Chemistry

PERSPECTIVE

pubs.acs.org/jmc

Regulators of G Protein Signaling (RGS) Proteins as Drug Targets: Modulating G-Protein-Coupled Receptor (GPCR) Signal Transduction

Miniperspective

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INTRODUCTION

Regulator of G protein signaling (RGS) proteins are emerging as important negative modulators of G-protein-coupled receptor (GPCR) signaling. Multiple lines of evidence ranging from biochemical characterizations to genetic studies using engineered mice are converging to demonstrate an important role of RGS proteins in the physiology of many organ systems and therefore as potential drug targets in pathologies including CNS diseases,[1−3] cardiovascular disease,[4−6] and diabetes.[7] The discovery of these roles together with the knowledge that RGS proteins function to modulate GPCR-mediated signal transduction provides an opportunity to target GPCR signaling pathways in a unique way. Agonist or antagonist drugs acting at GPCRs represent well over 50% of all drugs currently on the market. Targeting the protein−protein interactions that govern intracellular signaling processes downstream of GPCRs is challenging[8] but expands the number of potential targets and exploits the discrete roles that specific interactions may play within cellular signaling cascades.

GPCRs couple to heterotrimeric G proteins that consist of a Gα subunit and a $\beta\gamma$ heterodimer. In the resting state, the Gα of the heterotrimer G protein binds the guanine nucleotide GDP. Activation of a GPCR by agonist causes the exchange of GDP for GTP on the Gα subunit of the heterotrimeric G protein and separation from the $\beta\gamma$ heterodimer.[9] Both the Gα and $\beta\gamma$ subunits interact with downstream effector proteins. Hydrolysis of the bound GTP by the inherent GTPase activity of the Gα subunit provides inactive Gα-GDP that reassociates with $\beta\gamma$ heterodimer, thus terminating the signaling of both Gα-GTP and $\beta\gamma$ (Figure 1). RGS proteins, by acting as GTPase accelerating proteins (GAPs), increase this rate of hydrolysis and so serve to terminate signaling more rapidly. Gα proteins can be divided into several families. The Gαi family of proteins that inhibit adenylate cyclase and the Gαq family that activates the enzyme phospholipase C leading to the release of calcium from intracellular stores bind RGS proteins and are susceptible to their GAP activity. However, the GTPase activity of Gαs that activates adenylate cyclase already hydrolyzes GTP rapidly and is insensitive to GAP activity of RGS proteins. The associated $\beta\gamma$ subunits also interact with a variety of intracellular effectors including inwardly rectifying potassium channels, calcium channels, phospholipase C, and the mitogen-activated protein kinase pathway. Since $\beta\gamma$ signaling is terminated by reassociation with Gα-GDP subunits, these pathways are also negatively regulated by RGS proteins.

GTPase accelerating activity is a hallmark of RGS proteins and is functionally conserved within a RGS homology (RH) domain ("RGS box") comprising approximately 120 amino acids and containing the structural determinants necessary for interaction with Gα subunits. However, RGS proteins number more than 30 members, defined by the presence of the canonical RH domain, and have been divided into several families, based upon structural similarity and the presence of accessory domains outside the RH domain (Table 1). In addition to acting as GAPs for Gαi, Gαo, and Gαq, some RGS family members have more specialized roles. For example, RGS9-1 is a retinal specific protein that acts as a GAP for the function-specific G protein transducin (Gα_t) in retina and is necessary to ensure that the visual system rapidly responds to new incoming signals. The proteins p115-RhoGEF, PDZ-RhoGEF, and leukemia associated RhoGEF contain domains that function as guanine nucleotide exchange factors (GEFs) but also have RH domains that interact with the Gα_{12} family of G proteins to link GPCRs to Rho-mediated signaling pathways for the control of cellular processes such as cell growth, proliferation, and differentiation.[10] The RH domains of the G protein receptor kinases (GRK) 2 and 3 bind Gαq and reduce the activation of phospholipase C, inhibiting formation of inositol triphosphate (IP_3) and diacyglycerol and limiting increases in intracellular Ca^{2+} and activation of protein kinase C.[11]

RGS PROTEIN PHYSIOLOGY

Studies using cellular models of RGS protein function and biochemical experiments using purified RGS proteins revealed the GAP activity of RGS proteins in vitro.[12,13] However, it is a greater challenge to study RGS protein action in vivo, even in a model system. A significant problem to overcome is the large number of RGS proteins, many of which have nonselective GAP activity and therefore redundant functions. Several RGS proteins have been targeted in knockout strategies, with the most characterized being mice lacking RGS2,[6,14,15] RGS4,[16] and RGS9.[17] RGS2 knockout mice have a profound hypertensive phenotype,[6] some neurobehavioral effects including anxiety-like behavior[14] and altered NO-mediated vasodilatation responses,[15] whereas RGS9 knockout mice exhibit markedly enhanced sensitivity to drugs of abuse due to the loss of the striatal specific RGS9-2 variant.[18] An RGS4 knockout mouse exhibits only subtle sensorimotor deficits,[16] although a

Received: December 10, 2010
Published: September 14, 2011

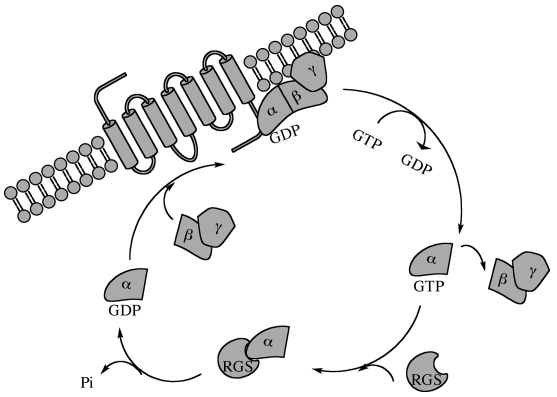

Figure 1. G protein cycle. After activation, GTP bound to the Gα subunit is hydrolyzed to GDP by the intrinsic GTPase of Gα. This allows for recombination of the Gα and G$\beta\gamma$ subunits and termination of signaling. RGS proteins bind to the activated Gα-GTP subunit to accelerate the hydrolysis of bound GTP (GAP activity) and thereby act as negative modulators by increasing the rate of inactivation of Gα-GTP and G$\beta\gamma$.

Table 1. RGS Protein Families

family	member	accessory domain[a]
	Classical RGS Proteins	
RZ	17, 19, 20[b]	N-terminal cysteine string
R4	1, 2, 3,[c] 4, 5, 8, 13, 16, 18, 21	N-terminal ampipathic helix
R7	6, 7, 9, 11	GGL, DEP, DHEX
R12	10, 12, 14	GoLoco, RBD, PDZ
	Proteins Containing RGS Homology (RH) Domains	
RA	Axin, Conduction	GSK, CAT, PP2A, DIX
RL	p63RhoGEF, p115-RhoGEF, GRK2, GRK3	DH, PH Ser/Thr kinase, PH

[a] CAT = β-catenin binding site. DEP = Disheveled, EGL-10, Pleckstrin homology domain. DH = Dbl-homology (RhoGEF) domain. DHEX, DEP helical extension domain. GEF, guanine-nucleotide-exchange factor. GGL= G protein γ-like domain. GoLoco = $G\alpha_{i/o}$-GDP binding motif. GRK = G-protein receptor kinase. GSK = glycogen synthase kinase 3β-binding domain. PDZ = PSD95/Dlg/Z0-1 domain. PH = Pleckstrin homology domain. PP2A = protein phosphatase 2A homology region. RBD = Rap1/2-binding domain. DIX = dimerization domain. [b] RGS 17 and 20 are also known as RGSZ2 and RGSZ1, respectively. RGS20 is also known as GAIP (Gα-interacting protein). [c] RGS3 has splice variants, some of which include a PDZ domain.

second strain of mice was seen to show altered behaviors following chronic morphine,[19] and an inducible RGS4 knockout mouse allowed identification of effects of RGS4 on certain aspects of morphine pharmacology.[19] Mice null for several other RGS proteins are commercially available (Mutant Mouse Resource Center, www.mmrrc.org, supported by NCRR-NIH), and as additional genetically modified mice are studied, the near future should see the revelation of even more roles of RGS proteins in physiology.

Although genetically manipulated mice with targeted knockouts of various RGS proteins have been engineered, a caveat, as with any knockout model, is the concern of compensatory expression

Figure 2. Structure of RGS4 (green) bound to Gαi1 (maroon)[22] from the RCSB (Research Collaboratory for Structural Bioinformatics) PDB database (www.pdb.org) generated using PyMOL (www.pymol.org).

of other RGS transcripts. Because of the redundancy of G protein interactions for many RGS proteins, the functional consequences of compensatory expression could obscure interesting phenotypes, as may be the case with the RGS4 knockout mouse.[16] In order to address the *overall* role of RGS proteins in physiological processes, our laboratory has utilized a "knock-in" approach to neutralize the effects of all RGS protein GAP activity.[20] This process involves knock-in of an RGS-insensitive mutation (G184S) into the *GNAI2* ($G\alpha i_2$) allele. The mutation of glycine to serine, discovered through a yeast genetic screen, disrupts RGS binding to Gα.[21] G184 is located in the critical switch I region of the G protein where the Gα interacts with RGS,[22] and studies indicate that the steric effect of the hydroxymethyl side chain of the serine or disruption of the local conformation of the switch I region prevents RGS from binding Gα (Figure 2).[21] A mouse model with the G184S mutation knocked-in to the *GNAI2* allele ($G\alpha i2^{GSGS}$) exhibits a pleiotropic phenotype with several interesting alterations, such as short bones, low body weight, altered adipose tissue distribution, and splenomegaly.[20] However, the heterozygote ($G\alpha i2^{GS/+}$) is much less affected and yet is resistant to diet-induced obesity[7] and shows antidepressant-like activity due to increased serotonin signaling via 5HT1A receptors.[3]

BENEFITS OF TARGETING RGS PROTEINS

Since RGS protein function is at the initial steps of signal transduction immediately after receptor activation, one questions the benefit of targeting an RGS protein over the receptor itself. On the other hand, there are several reasons why targeting the RGS may prove feasible and also advantageous from a therapeutic standpoint. Many RGS proteins have discrete expression profiles, particularly in the central nervous system, that could provide for a selective target. This is most clearly exemplified by

RGS9-2 which is discretely expressed in dopaminergic regions such as the basal ganglia and nucleus accumbens[23] where it has a similar expression to other striatal specific proteins.[24−26] However, even for RGS proteins that are more widely expressed, selectivity can be impacted by the cognate GPCR itself, since there is evidence that GPCRs recruit specific RGS proteins to modulate signaling. For example, it has been shown in a heterologous cell system that RGS2 is recruited to the plasma membrane by adrenergic β2 receptors and $AT1_A$ receptors, whereas RGS4 is recruited by muscarinic M2 receptors.[27] Additionally, several studies have identified receptor-specific effects of RGS protein action. Thus, RGS3 negatively modulates ERK (extracellular signal-regulated kinase) activation by muscarinic M3 receptors but RGS5 modulates $AT1_A$ receptor-mediated activation of ERK.[28] RGS3, but not RGS1, RGS2, or RGS4, suppresses gonadotropin-releasing hormone-induced IP_3 responses.[29] RGS4 selectively inhibits muscarinic but not cholecystokinin-mediated calcium signaling.[30] Moreover, we have shown RGS4 to act as a GAP at μ- but not δ-opioid receptors in cells expressing endogenous RGS proteins.[31] As a consequence, it should be possible to exploit additional selectivity occurring as a result of structural determinants that may be specific to particular Gα-RGS pairs.[32]

It is feasible that inhibitors of RGS proteins selectively expressed in particular tissues and/or specific for GPCR-Gα pairs could modulate the beneficial effects of GPCR agonist drugs, thus allowing lower doses to be used therapeutically, leading to fewer side effects while also enhancing specificity. The potential widening of the therapeutic window would introduce additional safety for existing drugs, as well as possibly allowing for drugs previously abandoned for having too narrow a therapeutic window to be used in conjunction with an RGS inhibitor. For example, genetic knockout of all RGS activity at Gαi2 provides a mouse with antidepressant-like phenotype behavior and promotes the beneficial antidepressant actions of selective serotonin reuptake inhibitors (SSRIs) by enhancing signaling of the $5HT_{1A}$ receptor; there is no alteration at other serotonin receptors and no effects on other antidepressant drugs.[3] SSRIs exert their antidepressant action by increasing serotonin levels. This increased serotonin acts at all 5HT receptor subtypes. It is likely then that by promotion of only the beneficial $5HT_{1A}$-mediated antidepressant effects, the therapeutic window of these drugs could be increased. Another example would be opioid partial agonists. Such compounds have a lower incidence of side effects (e.g., respiratory depression, constipation) but also a lower therapeutic analgesic efficacy than more robust agonists such as morphine. It is possible, given a variety of brain circuits are involved in the diverse activities of opioids, that different RGS proteins are involved in the antinociceptive responses to opiates compared to the unwanted actions. Accordingly, inhibition of those RGS proteins that are associated only with the antinociceptive pathways would selectively improve analgesic efficacy. In relation to this, it has recently been demonstrated that knockout of RGS9-2 has opposite effects on morphine-mediated spinal versus supraspinal antinociception[33] and even promotes the antinociceptive action of some opioids while inhibiting others,[34] suggesting that parsing out the selectivity of RGS action is feasible.

An alternative potential benefit of compounds that affect RGS protein function is to alleviate conditions that may have an RGS component to their etiology. This is highlighted by RGS4 and RGS9. RGS4 mRNA is up-regulated in the dorsal horn of the spinal cord in response to nerve injury[35] but, together with RGS3, is down-regulated in small diameter primary sensory neurons.[36] These changes may contribute to the etiology of neuropathic pain and to the lack of effectiveness of opioids in these pain states. An association between the RGS4 gene and schizophrenia has been reported in several studies, including one that showed decreases in RGS4 mRNA and protein product in the cortices of schizophrenic patients[37] and another in a rat model of schizophrenia.[38]

The general function of RGS4 makes it an intriguing target, even if the linkage data to schizophrenia are not fully established. On the other hand, the idea of RGS proteins as a target for schizophrenia is far from simple because of the highly complex nature of this disease and the number of receptors with which current antiopsychotic drugs interact.[39] Dopamine D_2 receptor antagonism has long been the basis for the treatment of schizophrenia. Such drugs improve the positive symptoms of the disease (e.g., delusions, hallucinations, disordered thoughts) but not the negative (e.g., lack of motivation, anhedonia) or cognitive defects and have extrapyramidal side effects. A drug that acts to increase RGS (e.g., RGS4) protein activity and so reduce D_2 receptor signaling will presumably have similar effects. Alternatively, atypical antipsychotic agents have $5HT_{2A}$ antagonist actions that help to alleviate negative and cognitive symptoms and $5HT_{1A}$ agonists may also be useful in controlling cognition;[40] thus, pharmacological manipulation of RGS protein activity at these receptors (to decrease activity at $5HT_{2A}$ receptors and increase activity $5HT_{1A}$ receptors) may be helpful. However, in addition to these bidirection effects on serotonin signaling, specificity of the RGS protein target is vital because actions at muscarinic receptors, adrenergic α2 receptors, and histamine H1 receptors could promote side effects similar to those of currently available antipsychotic agents.

The splice variant RGS9-2 represents another potentially important target.[1] This RGS protein has been identified as being overexpressed in the striatum of Parkinson's disease patients[41] and in rats after dopamine depletion.[42] Dopamine D_2 receptors appear to couple specifically to Gαo,[43] and RGS9-2 is a specific GAP for Gαo-mediated signaling. A study by Gold and colleagues[44] using a non-human primate model of Parkinson's disease demonstrated that overexpression of RGS9-2 in the striatum reduced the degree of L-DOPA-induced dyskinesia compared to control animals, without affecting the antiparkinsonian effects of the drug, although with the D_2/D_3 agonist ropinirole, both responses were reduced. These results further support the idea that interventions in receptor subtype specific signaling events might be used to limit the undesired effects of a drug.

Whereas these cases are representative of the benefits that might come from modulating RGS activity, additional targets will no doubt emerge as new physiological roles for RGS proteins are discovered. For example, both RGS9-2 and RGS4 have been implicated as possible targets for the treatment of addiction to opiates, cocaine, and amphetamine,[2] and as mentioned earlier, RGS2 knockout mice exhibit a hypertensive phenotype[6] and so increasing RGS2 activity might provide an approach for the management of hypertension.

■ STRATEGIES FOR DEVELOPMENT OF RGS LIGANDS

RGS proteins as a family present a variety of intriguing structural properties that could be exploited with small molecules or peptides to modify their function. Interest in modulating RGS activity has led several groups on discovery pathways in search of RGS ligands. Targeting the RGS-Gα interaction surface in the RH domain of the RGS proteins (the so-called A site)[45] is

an obvious and direct way of modifying RGS function and has been the subject of several approaches as described below. However, there are other potential "drugable" sites on RGS molecules. The R4 family of RGS proteins consists mainly of the RGS domain and an amphipathic helix and so lacks any of the accessory domains found in other RGS families (Table 1 and Figure 3). On the other hand, RGS4 does contain a modulating domain within the RH domain but distal from the Gα interaction (A) site termed the "B" site. The phospholipid phosphatidylinositol 3,4,5-trisphosphate (PIP_3) can bind to this site and inhibit GAP activity, a phenomenon that is blocked by the binding of calmodulin.[46] The binding of Ca^{2+}/calmodulin to the same B site relieves the inhibition by phospholipids, thereby forming a regulator pair.[46] Exploiting the B site could provide an avenue for the rational design of small molecules that modulate the function of even the most structurally simple RGS proteins of the R4 family. In spite of this, compounds designed to directly act at this site have not yet been reported.

An alternative approach to developing molecules that alter the GAP activity of RGS proteins is to increase or decrease RGS protein expression levels and therefore activity. This can potentially be achieved with compounds that modify the metabolism of RGS proteins or their targeting to correct cellular sites. Several of the smaller RGS proteins (RGS2, RGS4, and RGS5) are unstable and metabolized by N-end rule degradation.[47,48] Hence, levels of RGS4 can be markedly increased by inhibition of proteasomal degradation with such compounds as MG132 (*N*-(benzyloxycarbonyl)leucinylleucinylleucinal) and lactacystin; this results in increased negative modulation of GPCR signaling.[49] The larger RGS proteins exhibit a greater degree of structural diversity outside the RGS domain with several protein-binding sites necessary for proper targeting and stability, providing alternative ideas for drug design (Figure 3). The R7 family is hallmarked by the presence of G protein γ-like (GGL) domain that binds the G protein β5 subunit (Gβ5) and a DEP (Dishevelled, Egl-10, and Pleckstrin) domain with a helical extension domain that binds an R7 binding protein (R7BP) or, in the retina and specifically for RGS9-1, binds RGS9 anchor protein (R9AP).[50] Binding of the Gβ5 subunit promotes protein stability,[51,52] and binding of R7BP (or R9AP) promotes membrane association and also protects against protein degredation.[53,54] In the absence of these two binding partners RGS7 family members' expression levels, localization, and therefore function are severely attenuated, highlighting the critical nature of these protein−protein interactions. Targeting such protein−protein binding domains to stabilize or inhibit the interaction of R7 family members with their protein partners provides another potential approach to pharmacological manipulation of RGS protein levels.

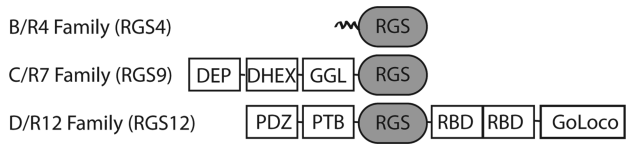

Figure 3. Domain arrangement of three RGS protein families. The R4 family, to which RGS4 belongs, is the simplest with the RH domain and an N-terminal amphipathic helix. The R7 family, to which RGS9 belongs, contains a DEP (Dishevelled/EGL-10/Pleckstrin) domain with a helical extension domain (DHEX) that bind R7BP (R7 binding protein) or, for RGS9 specifically in the retina R9AP (RGS9 anchor protein), that direct cellular localization and increase stability, a GGL or G protein γ-like domain that binds Gβ subunits, as well as the conserved RH domain. RGS12 is a member of the R12 family that possesses a PDZ domain, a phosphotyrosine binding domain (PTB), and dual Ras binding domains (RBDs). The GoLoco domain binds GDP-bound Gα subunits, acting as a guanine nucleotide dissociation inhibitor or GDI.

To date, the common approach toward modulating RGS protein function has focused on disrupting the protein−protein interaction between the A site on RGS proteins and their Gα subunit partners, mainly using high-throughput screening methods to identify lead compounds. Such methods include the synthesis of peptide libraries,[55] yeast-based screening methods for RGS4 and RGSZ1 (RGS20) inhibitors,[56] and flow cytometry protein interaction assays (FCPIA) and/or time-resolved fluorescent resonance energy transfer assays (TR-FRET) focused on RGS4 inhibitors.[57,59] These efforts have resulted in the identification of both peptide and non-peptide compounds that disrupt RGS activity at Gα proteins.

Peptides. The first steps toward the identification of inhibitors were pioneered using rational design of peptides modeled on the switch 1 region of Gαi, one of the three key regions on inhibitory Gα subunits that interact with RGS proteins (Figure 2).[22] These peptides mimic a sequence in the switch region (Table 2) to prevent RGS-Gα interaction.[60] A constrained cyclic octapeptide YJ34 (Table 2) was found to inhibit the GAP activity of RGS4 with a potency of 26 μM.[61] When the Gly at position 5 was mutated to Ser to mimic the RGS-sensitive mutation in Gαi1, the resulting peptide was inactive indicating that YJ34 does indeed bind as designed. Development of the structure−activity relationship of peptides has resulted in compounds with varying potencies to inhibit RGS/Gα interactions.[62] In furthering the identification of peptide inhibitors, Roof et al[55] used a one-bead one-compound library approach to screen 2.5 million peptide sequences based on the essential features of YJ34 for interaction with an Alexa Fluor 532 labeled N-terminal truncated form of RGS4. Two peptides were identified, one (peptide 2, Table 2) that directly inhibited binding of RGS4 to Gαi1 and appeared selective for RGS4 being inactive against RGS7, RGS16, and RGS19[55] and one (peptide 5, Table 2) that acted by cysteine modification and was less selective.[63] None of the compounds, however, showed any marked improvement in potency over YJ34, although they did require the disulfide bridge for activity. Using RGS4 as bait in a yeast two-hybrid system to screen a

Table 2. Peptide Inhibitors of RGS GAP Activity

peptide	sequence	reference
Gαi switch region	-Val-Lys-Thr-Thr-Gly-Ile-Val-Glu	
YJ34	Ac- Val-Lys-c[Cys-Thr-Gly-Ile-Cys]-Glu-NH_2	60
compound **2**	Gly-Thr-c[Cys-Phe-Gly-Thr-Cys]-Trp-NH_2	55
compound **5**	Tyr-Trp-c[Cys-Lys-Gly-Leu-Cys]-Lys-NH_2	63
compound P17	Val-Arg-His-Val-Ala-Val-Glu-Val-Gly-Gly-Val-Val-Val-Val-Val-Gly	64

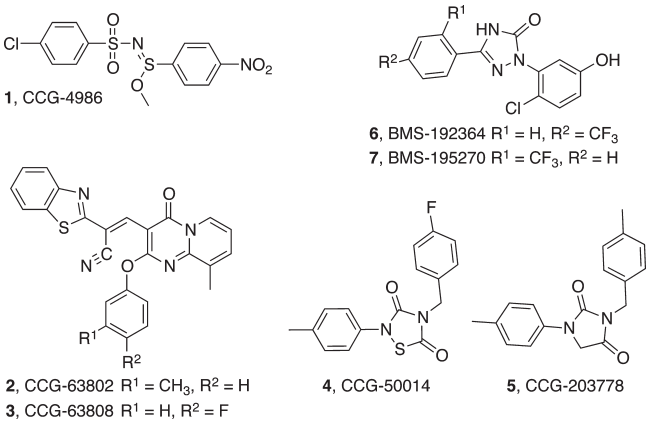

Figure 4. Small molecules that affect RGS protein function.

random peptide library, Wang and colleagues[64] identified a unique peptide sequence (P17, Table 2) that blocked the interaction between RGS4 and Gαi1, with specificity over RGS7, and functionally inhibited RGS4 action. The N-terminal Arg was seen to be essential for activity as was the carboxy Val-Gly.

Because of their instability and poor membrane penetration, including access to CNS targets, peptides are unlikely to lead to marketable drugs. However, advances are being made to progress peptidic molecules to drugs. Such methods include development of glycopeptide analogues[65] and conjugation to cell penetrating peptides as delivery vehicles.[66] Nonetheless the major role for these peptide discovery efforts is to provide information on the structural and conformational requirements of the protein−protein interaction site and thereby pave the way for the design of small molecule peptidomimetics.

Small Molecules. A yeast-based RGS4 screen utilizing the endogenous pheromone-responsive GPCR signal transduction pathway has been employed to detect modulators of the pathway, and an RGSZ1 screen using yeast two-hybrid technology has been used to detect compounds that disrupted the RGSZ1/Gα interaction.[56] These yeast-based screens identified a number of compounds with mid to low micromolar potency, some of which exhibited RGS selectivity in follow-up assays. Although structural information on the compounds was not published, these screens represent a unique methodology for high throughput yeast-based screening for RGS inhibitors.

Efforts by our group to identify small molecule RGS4 inhibitors, using FCPIA to determine the interaction between a bead-immobilized RGS protein and a fluorescently labeled Gαo binding partner, resulted in the discovery of methyl *N*-[(4-chlorophenyl)-sulfonyl]-4-nitrobenzenesulfinimidoate, **1** (CCG-4986, Figure 4). Compounds that inhibited the RGS/Gα interactions were further validated through GTPase accelerating (GAP) assays. **1** exhibited some specificity for RGS4. Additional studies indicated that the compound functions through an irreversible mechanism by covalently modifying RGS4 at cysteine residues,[58,67] and indeed, activity is lost in the presence of free thiols. More recent work[59] using both FCPIA and TR-FRET assays has identified two related pyrido[1,2-*a*]pyrimidine derivates, **2** (CCG-63802) and **3** (CCG-63808, Figure 4), that bind to RGS4 to prevent association with Gαo and are 10-fold selective for RGS4 over other related RGS proteins. Both **2** and **3** require three of the four cysteines in the amino acid sequence of RGS4 for full activity, although the action of the compounds is reversed by washing, which suggests that a proposed Michael reaction between the vinyl cyanide and sensitive cysteines on the protein must be reversible. Since two of these cysteines are in the allosteric B site that binds PIP_3, it is postulated that the compounds may, at least partially, act as allosteric modulators. The 1,2,4-thiadiazolidine-3,5-dione (**4**, CCG-50014; Figure 4) has also been revealed as an inhibitor of RGS4.[68] The compound is an improvement on the earlier molecules in that it has about 100-fold selectivity for RGS4 over the other R4 family members RGS8 and RGS16, highlighting the fact that it is possible to exploit structural differences even in the RH homology domain of these proteins.[32] Moreover, **4** inhibits RGS4 activity in a cell based system, indicating both membrane permeability and stability in a cellular environment. Unfortunately, the action of this compound also involves irreversible binding to cysteine residues in the RGS molecule, since it is inactive against the cysteineless mutant protein and replacement of the thiadiazolidine-3,5-dione ring with an imidazolidine-2,4-dione ring as in **5** (Figure 4) destroys activity. In silico docking studies suggest that **4** also binds to the B site but at some distance from the sensitive cysteine residues. Moreover, RGS4 is not particularly sensitive to nonspecific cysteine alkylating agents. Consequently the authors postulate that **4** binds reversibly at first, and this causes a conformational change in RGS4 that brings the thiadiazolidine-dione ring closer to the sensitive SH groups.

As with the peptides, these small molecules that target sulfhydryl groups are useful as tools to probe roles for RGS proteins in physiology and also may help inform on the design of reversible molecules. However, the irreversible mechanism of action probably precludes further development of these particular compounds. On the other hand, the proton pump inhibitor omeprazole works, following conversion to a sulfenamide, by covalent cysteine modification of H^+/K^+-ATPase.[69]

Fitzgerald and colleagues have discovered other structures that affect G protein signaling and RGS function.[70] Studies that focused on the development of compounds to treat urinary incontinence identified a series of 1,3-diaryl-1,2,4-(4*H*)-triazol-5-ones that caused relaxation of rat isolated bladder smooth muscle strips by an unknown mechanism. Further investigation of two of these compounds, **6** (BMS-192364) and **7** (BMS-195270, Figure 4), through the use of the nematode *Caenorhabditis elegans*, yeast genetics, cell culture experiments, and biochemistry showed that the compounds function to limit Gαq signaling by an action involving RGS proteins downstream of muscarinic GPCRs and so reduce muscle contraction. The data were most consistent with a mechanism whereby **6** and **7** interact with Gαq/RGS protein complexes and lead to increased binding affinity between the two proteins such that Gαq cannot reassociate with the Gβγ subunits and cannot recycle to renew heterotrimeric G protein substrate for the GPCR. Mechanistically therefore the compounds lead to a "dead-end" Gαq/RGS complex. Analogues with a methyl ether in place of the phenolic OH of the aryl ring in the 1-position were inactive. The discovery of this class of compounds is very promising in that it provides evidence that targeting downstream (i.e., postreceptor) signaling can be effective for treating conditions that may be triggered by inappropriate receptor activation, as is the case in urinary incontinence. This example, while not the first example of a compound targeting and stabilizing a protein complex, showcases how the G protein/RGS complex dynamic association can be modulated for possible therapeutic benefit.

The peptides and small molecules that have so far been identified as modulating the function of either the RGS protein or the RGS/Gα complex are but initial steps toward developing

RGS proteins as a drug target. These moieties represent the beginning stages of establishing RGS pharmacology.

CONCLUSIONS AND OUTLOOK

Targeting RGS proteins presents a unique and challenging paradigm to modulate the intracellular signaling cascades initiated by an activated GPCR. The point of control can be as direct as physically blocking the RGS/Gα protein−protein interaction or targeting an allosteric domain or indirect by altering the expression levels or localization of an RGS protein within a cell. Whereas some RGS proteins are expressed almost ubiquitously, others, such as RGS9-2, have discrete expression patterns that can provide for selectivity that can be increased by the specificity of targeting receptor−G protein pairings. Thus, targeting RGS proteins with small molecule modulators or inhibitors could provide specific control or treatment of pathophysiological states, such as Parkinson's disease, schizophrenia, addiction, urinary incontinence, and hypertension.

As with many attempts to target protein−protein interactions, the development of RGS ligands is still at an early stage, with compounds that interfere with these interactions being discovered only comparatively recently. As discovery efforts expand and we learn more about the pharmacology of compounds that alter RGS protein function, the next stages of developing structure−activity relationships and pharmacophore modeling will help guide us toward an understanding of how RGS function can be modulated. These early discovery efforts will serve as a step for the development of novel therapeutic agents.

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ACKNOWLEDGMENT

D.L.R.'s research is supported by the American Cancer Society (Institutional Research Grant IRG-77-004-31 administered through the Holden Comprehensive Cancer Center at the University of Iowa) and University of Iowa College of Pharmacy startup funds. J.R.T.'s research on RGS proteins is supported by National Institutes of Health Grant DA04087.

ABBREVIATIONS USED

ERK, extracellular signal-regulated kinase; FCPIA, flow cytometry protein interaction assay; GPCR, G-protein-coupled receptor; GAP, GTPase accelerating activity; Gβ5, G protein β5 subunit; GEF, guanine nucleotide exchange factor; GRK, G protein receptor kinase; IP_3, inositol trisphosphate; PIP_3, phosphatidylinositol 3,4,5-trisphosphate; R7BP, RGS7 binding protein; R9AP, RGS9 anchor protein; RH, RGS homology; RGS, regulator of G protein signaling; TR-FRET, time-resolved fluorescent resonance energy transfer

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