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Regulators of G protein signaling (RGS proteins): Novel central nervous system drug targets

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Abstract: Many drugs of abuse signal through receptors that couple to G proteins (GPCRs), so the factors that control GPCR signaling are likely to be important to the understanding of drug abuse. Contributions by the recently identified protein family, regulators of G protein signaling (RGS) to the control of GPCR function are just beginning to be understood. RGS proteins can accelerate the deactivation of G proteins by 1000-fold and in cell systems they profoundly inhibit signaling by many receptors, including mu-opioid receptors. Coupled with the known dynamic regulation of RGS protein expression and function, they are of obvious interest in understanding tolerance and dependence mechanisms. Furthermore, drugs that could inhibit their activity could be useful in preventing the development of or in treating drug dependence.

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Cell–cell communication is fundamental to brain function and the G-protein-coupled receptor (GPCR) superfamily (1) is one of most abundant and diverse protein families in the central nervous system (CNS). In the human genome there are 616 GPCRs, excluding olfactory receptors, with only the ribosomal proteins being more numerous (2). The guanine nucleotide binding proteins (G proteins), which act downstream of the GPCRs, perform crucial functions in the regulation of neural processes. They are critical signaling elements for drugs of abuse, such as opioids, and dopamine-modulating drugs, such as cocaine and amphetamine. A tremendous amount of structural and mechanistic information is known about GPCR signaling (3). The G protein consists of alpha and beta-gamma subunits, which undergo interlocking kinetic cycles of nucleotide binding,

hydrolysis, and release interacting with subunit association and dissociation (4). Thus, G-protein function is intrinsically kinetic. The recent identification of the novel family of regulators of G protein signaling, or RGS proteins (5–7), provided emphatic confirmation of the role of kinetics in G-protein function.

RGS proteins were identified genetically in 1996 by their ability to reduce signaling by G proteins in yeast (*Saccharomyces cervesiae*) (8) and *Caenorhabditis elegans* (9). The main mechanism of RGS-mediated inhibition of G-protein signaling is through RGS binding to the Gα subunit and acting as a GTPase accelerating protein (GAP) to rapidly deactivate Gα. RGS proteins may also competitively inhibit Gα binding to effectors such as phospholipase C (10). There are at least 30 members of the RGS protein family (11). The majority of RGS proteins interact with either G_i or G_q family G proteins and influence cAMP, Ca^{2+}, MAP kinase, or ion channel signaling, however, RGS proteins that bind Gα12/13 (12,13) and Gαs (14) have also recently been identified. There is strong evidence implicating RGS proteins in the subsecond kinetics of G_i- and G_o-mediated ion channel activation and deactivation in neurons (15,16). More recently, the conserved RGS protein domain has been found to serve as a multifunctional protein adapter which can recruit many effectors or regulators to the vicinity of activated G proteins (for reviews see Refs 6,7,17). Notable examples include P115rhoGEF (12), the A kinase anchoring protein D-AKAP2 (18) and GRK2 (19).

One other intriguing aspect of RGS proteins is that they undergo rather profound regulation of expression by signal transduction events. This makes them uniquely well-suited to play a role in the cellular changes that underlie tolerance and/or dependence that are the hallmarks of drugs of abuse. Indeed, Burchett *et al.* (20) demonstrated amphetamine-induced upregulation of RGS2 and -3 with chronic administration, whereas RGS5 was upregulated only by acute amphetamine. Similarly, stimulation of both mu and delta opioid receptors induced RGS4 upregulation in PC12 cells expressing those receptors (21).

There is a wealth of information about the effects of RGS proteins on G proteins *in vitro* and in overexpression studies in transfected mammalian cells in which different RGS proteins modulate signaling (6,7,22). In general, expression of RGS proteins that target G_i, G_o, and G_q reduces signaling by those G proteins. The specificity of different RGS proteins *in vitro* is fairly well established though it appears that specificity in intact tissues or in differentiated cell types may be greater than predicted by the RGS-Gα specificity alone (11). In particular, there is evidence for receptor-specific effects of RGS proteins in pancreatic acinar cells (23) and in vascular smooth muscle (24). Such specificity has not been established in CNS systems but it is likely that similar observations will be made in neurons. More recently, evidence of differential RGS effects on opioid signaling to different effectors has been found (M.J. Clark *et al.*, manuscript submitted).

Approaches to Studying the Function of Endogenous RGS Proteins

A major limitation of current knowledge is that *the physiological functions of RGS proteins remain very poorly defined*. Only two RGS knockouts have been reported, RGS9 in the eye and RGS2 (25,26). The RGS9 knockout shows dramatically slowed visual potentials (25) and the RGS2 knockout has subtle behavioral and immunologic phenotypes (26). The phenotype of the RGS9 knockout with respect to CNS function, behavior, and drug abuse has not yet been reported and will be of significant interest. One difficulty in unraveling the function of RGS proteins has been the many subtypes of both G proteins and RGS proteins which may render some functions redundant. This limits an understanding of their function *in vivo* using standard antisense or knockout strategies.

To determine the contribution of RGS proteins as a group to biological responses mediated by G_o and G_i, we have taken advantage of a point mutation in the Gα subunit that abrogates the RGS–Gα protein interaction (27). This glycine to serine mutation in the switch 1 region of the yeast Gα subunit renders it insensitive to inhibition by the yeast RGS (28). We characterized the mammalian homologs (G184S in α_o and G183S in $G\alpha_{i1}$) and found that they prevent the GAP activity of RGS4 and RGS7 and block RGS4 binding to aluminum fluoride-activated Gα subunits. The mutation does not affect other functions of the Gα subunit (29) such as: the intrinsic GTPase activity of the G protein or its coupling to βγ subunits, receptors, or effectors (adenylyl cyclase). Thus, the only known effect of the G_o G184S and $G\alpha_{i1}$ G183S mutation is to prevent RGS action on G. Based on this mechanism, one would expect that both the inhibitory GAP activity and potential effector functions of any RGS at that Gα subunit would be blocked by this mutation.

Recent publications from the Ikeda and Lambert laboratories (30,31) demonstrate profound changes in response kinetics and sensitivity in neurons expressing Gα subunits with these RGS-insensitive mutations. Furthermore, studies

with mu opioid receptors show that the G184S mutant of $G\alpha_o$ dramatically enhances morphine-induced inhibition of adenylyl cyclase in C6 glioma cells (M.J. Clark *et al*., manuscript submitted). A 5-fold increase in maximal adenylyl cyclase inhibition and an 8-fold reduction in EC_{50} was seen. This shows that endogenous RGS proteins in C6 glioma cells have a profound effect on opioid signaling, and elimination of the RGS action (such as by an RGS inhibitor drug) could dramatically potentiate opioid responses. This could lead to enhanced analgesic responses without leading to tolerance and dependence. This is possible because opioid tolerance depends in part on the GRK/β-arrestin-mediated receptor desensitization which occurs at very high receptor occupancy. If an RGS inhibitor could permit opioid analgesia to be produced by low agonist doses, which only occupy a small fraction of receptors, tolerance, and possibly dependence, could be avoided. This scenario of combining an agonist drug with an agent enhancing its function is similar to the use of the aromatic amino acid decarboxylase inhibitor carbidopa with L-Dopa to permit lower doses to be used and to direct signaling to the tissue of interest (i.e. CNS vs. peripheral tissues). The combination of an RGS inhibitor with an opioid agonist would permit lower doses of opioid to be used, which might reduce tolerance and may also enhance signaling in selected brain regions if the RGS proteins important in those regions could be targeted by an inhibitor. Two preliminary reports have described RGS inhibitor peptides with micromolar potency (32,33). Further developments in this area are eagerly awaited.

Studies with the RGS-insensitive Gα subunits should provide a broad-brush view of RGS actions at a particular Gα subunit. A more targeted approach to deciding which RGS protein is mediating a particular function is also required. Ribozymes are unique RNA enzymes that can recognize and cleave other RNA molecules in a sequence-specific fashion (34). They were initially used to inhibit gene expression in HIV virus (35) and cancer (36). Compared with the antisense oligos, ribozymes offer significant advantages: (i) they operate as site-specific ribonucleases, resulting in catalytical cleavage of the target mRNAs; (ii); they are more stringent than antisense oligos in binding mRNA targets; (iii) controls for ribozyme activity can be made by substituting nucleotides in the catalytic core to produce an inactive ribozyme (37). Among the several types of ribozymes, the hammerhead ribozymes are the most extensively characterized with enhanced catalytic turnover and stability (38). They cleave 3′ to a 'GUC' sequence motif in the target mRNA (38). Wang *et al*. (24) recently demonstrated that chemically synthesized ribozymes targeted against specific RGS proteins can be used to 'knock-down' their expression in vascular smooth muscle cells. Interestingly, the RGS3-ribozyme selectively enhanced M3 muscarinic responses, whereas the RGS5-ribozyme selectively enhanced angiotensin II responses. Using the related antisense oligodeoxynucleotide approach, Garzon *et al.* (39) showed that an RGS9 antisense construct injected i.c.v. produced an enhanced opioid analgesic effect with morphine and beta-endorphin, whereas an RGS2 antisense reduced the opioid effects. In contrast, Potenza *et al*. (40) found that overexpression of RGS2 inhibited opioid responses in a melanophore response system – an effect opposite to that predicted by the Garzon results. Thus, additional study is clearly needed to better define the role of RGS proteins, in general, and RGS2, -4, and -9 in particular, in both the acute actions and long-term regulation of opioid signaling. Similar approaches should also be relevant in studies of other drugs of abuse.

Tools Needed to Advance the Study of RGS Protein Physiology

A better understanding of the *in vivo* physiology and pharmacology of the RGS protein family is important for a full understanding of drug abuse. A number of limitations have slowed the pace of research in the RGS field. First, studies of the expression of RGS protein have been largely limited to assessments at the mRNA level. RGS proteins are often expressed at low levels and many antibodies are not specific enough to detect them in Western blots of complex tissues. Thus, good antibodies to identify the location and regulation of RGS at the protein level are important. Second, experimental approaches to selectively block RGS protein function (knockouts, ribozymes, RNAi, chemical inhibitors) need to be developed to dissect the roles of specific RGS proteins. The availability of specific chemical inhibitors is also the first step toward the development of RGS inhibitor drugs. Third, is the need for good assays to permit rapid screens for potent RGS inhibitors. The 'gold standard' assays for RGS function are complex single-turnover [^{32}P]GTPase assays that are not amenable to high-throughput approaches. Improved and simplified assays for RGS function are needed. Finally, creative approaches to dissect the roles and interactions among RGS and non-RGS domains will be essential to fully understand the biological roles of RGS proteins. This article has focused on the GAP activity of the RGS domains but the RGS domains are also scaffolds to permit assembly and

regulation of the other diverse signal transduction modules found in RGS proteins.

Future Prospects and Key Questions

As noted above, the potential for RGS proteins as drug targets is substantial but completely unexploited. To develop this potential fully many questions need to be answered. What are the distribution, regulation, and role of the more than 30 endogenous RGS proteins in receptor signaling? What determines the specificity of RGS proteins for different receptor responses and what are the functions of the N- and C-terminal non-RGS modules? What is the role of RGS proteins in drug tolerance and dependence? Can RGS inhibitors prevent or reverse tolerance and/or dependence (perhaps by permitting the use of lower doses of drugs, e.g. opiates)? The RGS protein family thus provides fertile ground for additional study in drug abuse.

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