Regulators of G protein signaling (RGS) are GTPase-accelerating proteins (GAPs), which can inhibit heterotrimeric G protein pathways. In this study, we provide experimental and theoretical evidence that high concentrations of receptors (as at a synapse) can lead to saturation of GDP-GTP exchange making GDP hydrolysis rate-limiting. This results in local depletion of inactive heterotrimeric G-GDP, which is reversed by RGS GAP activity. Thus, RGS enhances receptor-mediated G protein activation even as it deactivates the G protein. Evidence supporting this model includes a GTP-dependent enhancement of guanosine 5'-3-O-(thio)triphosphate (GTPγS) binding to Gi by RGS. The RGS domain of RGS4 is sufficient for this, not requiring the NH2- or COOH-terminal extensions. Furthermore, a kinetic model including only the GAP activity of RGS replicates the GTP-dependent enhancement of GTPγS binding observed experimentally. Finally in a Monte Carlo model, this mechanism results in a dramatic “spatial focusing” of active G protein. Near the receptor, G protein activity is maintained even with RGS due to the ability of RGS to reduce depletion of local Go-GDP levels permitting rapid recoupling to receptor and maintained G protein activation near the receptor. In contrast, distant signals are suppressed by the RGS, since Go-GDP is not depleted there. Thus, a novel RGS-mediated “kinetic scaffolding” mechanism is proposed which narrows the spatial range of active G protein around a cluster of receptors limiting the spill-over of G protein signals to more distant effector molecules, thus enhancing the specificity of Gi protein signals.

A critical question in cellular signaling is what determines the specificity of signal transduction processes. There is much recent evidence for the formation of complexes maintained by protein scaffolds to control signaling specificity. This contrasts with a classical model in the G protein signaling field, the collision-coupling model (1), which relies entirely on the structure of receptor-G protein and G protein-effector contact sites to determine signaling specificity. The collision coupling model also suggests that there would be significant spread of G protein signals in a cell upon receptor activation, since all components are freely diffusible. There have been numerous studies indicating that such free transfer of information over long distances may not occur for Gi or Gq mediated signals (2, 3). Thus, similar to the localized signaling by postsynaptic nontropic receptors via protein complex assembly (4), mechanisms to limit the “spread” of G protein signaling appear necessary.

G protein-coupled receptors (GPCRs) activate cellular signals by inducing nucleotide exchange on the G protein α subunit, while inactivation occurs upon GTP hydrolysis by the intrinsic Go GAP (5). Regulator of G protein signaling (RGS) proteins are a recently discovered family of proteins which act as GAP-activating proteins (GAPs) for Gi subunits (6–9). The GAP activity of RGS proteins generally reduces steady state levels of GTP-bound Go subunits and inhibits the activity of G proteins (6, 10). However, some studies of receptor-stimulated signaling show that RGS proteins can speed the kinetics of responses without compromising steady state signaling strength (11–13). The mechanism and significance of this paradoxical result is not understood.

The maintained signaling in the face of RGS-enhanced GTPase activity suggests that the RGS proteins somehow increase the efficiency of G protein activation. One possible mechanism for this could be “physical scaffolding” in which the RGS protein binds to both receptor and G protein and stabilizes a complex between them. This could involve the diverse amino- and carboxyl-terminal domains of the RGS proteins such as GGL, DEP, DH/PH, and PDZ domains (6, 10, 14, 15). Indeed, RGS12 does bind to the carboxyl terminus of the IL8 receptor through a PDZ domain (16). Alternatively, Ross and co-workers (17) have suggested that the GAP activity of phospholipase C-β1, which is both a Gi GAP and its effector, serves to enhance muscarinic receptor-Gq coupling (6, 17). In that model, the GAP activity causes rapid hydrolysis of GTP so that the Go-GTP does not have time to completely dissociate from receptor, which is then able to rapidly catalyze the next round of GDP/GTP exchange.

In this report, we propose that RGS proteins, via their ability to accelerate GTP hydrolysis, reduce depletion of local Go-GDP levels to permit rapid recoupling to receptor and maintained G protein activation near the receptor with decreased activity farther away. This narrows the spatial range of active G protein...
tein around a cluster of receptors by a “kinetic scaffolding” rather than by a physical scaffolding mechanism. While local signaling to a nearby effector is not significantly reduced in the presence of RGS, both the kinetics and spatial focus of signaling are sharpened, thus limiting the spill-over of G protein signals to more distant effector molecules.

**EXPERIMENTAL PROCEDURES**

**Materials—Guanosine 5’-3-O-[^35S]thiophosphosphate ([^35S]GTPγS, 1250 Ci/mmol) and[^35P]GTP (30 Ci/mmol) were from PerkinElmer Life Sciences. His10RGS2 in PET-1Bb was from Dr. John Hepler (Emory University). His10-tagged RGS4–(58–517) in pQE60 was from Dr. Thomas Wilkie (University of Texas Southwestern Medical Center).**

**Cell Culture and Membrane Preparation—**The TAG-Li CHO cell line with stable expression of an HA-epitope tagged porcine α1A-AR adrenoceptor (α1A-AR-CHO, 10–20 pmol/mg) was cultured and cell membranes prepared as described (18).

**Purification of RGS Proteins—**GST fusion proteins containing rat RGS4, RGS7 (aa 305–453), RGS8 were prepared as described (19). His6-RGS2 was expressed in BL21/D3 and purified as described (20) yielding >90% purity. His6-RGS4-box (aa 58–177) was expressed in JM109 and purified under denaturing conditions as described (19).

**Kinetic Scaffolding of G Protein Signals**

![Fig. 1. RGS4 enhanced receptor-stimulated[^32P]GTPase activity.](image)

A, steady state GTPase activity CHO membranes expressing high levels of the α1A-AR was measured at 30 °C for 10 min in buffer containing 1 μM GDP and 100 nM [γ-[^32P]GTP as described under “Experimental Procedures.” The effect of RGS4 was determined in the absence (open circles) or presence (filled circles) of the α1 agonist UK 14,304 (10 μM). Data were fit to sigmoidal dose-response curves using GraphPad Prism 3. B, the dependence of UK 14,304-stimulated[^32P]GTPase activity on the type of RGS was also determined. One μM GST-RGS4, GST-RGS8, His6-RGS2, or GST-RGS7 (box domain, aa 305–453) were added with boiled GST-RGS4 or GST alone as controls. Data shown are mean ± S.E. of three experiments. Statistical significance was determined using one-way ANOVA with Dunnett's post test. ***, p < 0.001; *, p < 0.05.

Table 1: Parameters used in simulating the RG model

| Step | Function | Forward ratea | Backward ratea |
|------|----------|---------------|---------------|
| 1    | Agonist binding to receptor | 10^6 | 1 |
| 2    | Receptor-G protein coupling | 10^6 | 1 |
| 3    | Stimulated GDP release | 5 | 10^6 |
| 4    | GTP binding to DRG | 10^6 | 0.1 |
| 5    | Dissociation of DRGGTP complex | 2 | 10^6 |
| 6    | GTP hydrolysis without RGS | 0.02 | 0 |
| 7    | Binding of RGS to GTP | 2 × 10^6 | 2 |
| 8    | GTP hydrolysis with RGS | 30 | 0 |
| 9    | Release of RGS from GGDP | 100 | 10^3 |
| 10   | GTPγS binding to DRG | 10^6 | 0 |
| 11   | Dissociation of DRGGTPγS | 2 | 0 |

a Units are s⁻¹ for first order and s⁻¹ s⁻¹ for second order reactions.

The reaction rates for each step of the RG model shown in Fig. 3A are indicated. Parameters were derived from literature estimates (19, 22, 26, 29, 32).

The effect of RGS4 was determined in the absence (open circles) or presence (filled circles) of the α1 agonist UK 14,304 (10 μM). Data were fit to sigmoidal dose-response curves using GraphPad Prism 3. B, the dependence of UK 14,304-stimulated[^32P]GTPase activity on the type of RGS was also determined. One μM GST-RGS4, GST-RGS8, His6-RGS2, or GST-RGS7 (box domain, aa 305–453) were added with boiled GST-RGS4 or GST alone as controls. Data shown are mean ± S.E. of three experiments. Statistical significance was determined using one-way ANOVA with Dunnett's post test. ***, p < 0.001; *, p < 0.05.

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[^35S]GTPγS Binding—[^35S]GTPγS binding was determined in 100 μl of reaction mixture containing 50 mM Tris (pH 7.6), 5 mM MgCl₂, 1 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol, 1 μM GDP, 400 nM GTP₂, unless otherwise indicated. Reactions also contained 4 μg of CHO cell membrane and 0.2 nM[^35S]GTPγS with or without the full α1 agonist UK 14,304 (10 μM). The reaction was started at 30 °C by adding[^35S]GTPγS and was stopped at 10 min with ice-cold washing buffer (20 mM Tris, 25 mM MgCl₂, 100 mM NaCl (pH 7.7)) using a Brandel cell harvester. For the kinetic study in CHO membranes (Fig. 4), 50 nM[^35S]GTPγS was used with 1 μM GTP, but no GDP was added. The reactions were initiated at 25 °C in reverse order 10–60 s prior to simultaneous filtration on a Brandel harvester.

[^32P]GTPase Assay—Steady state[^32P]GTPase activity was measured in a reaction mixture (100 μl) containing 4 μg of membranes, 0.2 mM ATP, 0.2 mM MgCl₂, 1 μM GDP, 50 units/ml creatine phosphokinase, 5 mM phosphocreatine, 20 mM NaCl, 2 mM MgCl₂, 0.2 mM EDTA, 10 mM Tris/HCl, 1 mM dithiothreitol, and 0.1 μM [γ-[^32P]GTP (pH 7.6). Reactions were started by addition of[^32P]GTP containing mixture to the incubation mixture in the presence or absence of RGS proteins and UK 14,304 (10 μM) and incubated at 30 °C for 10 min. Reactions were then terminated by adding ice-cold charcoal slurry as described previously (19). Release of[^32P]IP, was linear with time up to 15 min.

**Model Simulations—**A chemical kinetic model of receptor/G protein/RGS interactions was simulated using the RK4 method in the chemical reaction module of Berkeley Madonna (Version 8.0.2 for Windows, Kagi Shareware, Berkeley, CA). The model (see Fig. 3A) used a standard collision-coupling mechanism (22) with the addition of RGS serving only as a GAP for GTP-bound Gα proteins (G-GTP). No stable R-G-RGS complex was included. Rate parameters listed in Table 1 and initial reactant concentrations were designed to closely approximate the con-
ditions used in our assays. Receptor concentrations were estimated by [3H]yohimbine binding (~20 pmol/mg of protein or ~0.8 nM final), and G protein concentrations were similar (1 nM). Other model parameters were derived from measured literature values in the indicated references (Table I).

Monte Carlo simulations (23) were used to model the spatial effect of RGS proteins on the distribution of active and inactive G proteins. Simulations were run on a 600 × 600 × 600 triangular lattice (2.5 nm per grid step) with three distinct diffusible species: receptors, inactive G proteins, and active G proteins. Receptors and G proteins had a diameter of two lattice spacings and could interact with adjacent particles separated by one or fewer lattice spacings. In each time step, receptors could activate one adjacent inactive G protein if available, otherwise the receptor caused no reactions. Active G proteins were allowed to revert to inactive G proteins with a probability proportional to the GTP hydrolysis rate, $k_{on}$. Inactive G proteins were passive. All species in the simulation were assumed to have the same diffusion rate. The simulation was started with 600 inactive G proteins and 1 receptor and allowed to equilibrate for 5 million iterations. Over the next 5 million iterations, data sets were gathered to determine the radial distribution from the receptor of inactive and active G proteins. Diffusion coefficients were $10^{-10}$, $10^{-9}$, and $10^{-8}$ cm²/s, and $k_{on}$ values were 0.02, 0.2, 2, 20, and 200 s⁻¹.

Data Analysis—Data were analyzed with non-linear curve fitting equations using GraphPad Prism 3 (San Diego, CA). Data are reported as mean ± S.E. Statistical comparisons were conducted using one-way ANOVA.

RESULTS AND DISCUSSION

RGS Effects on $\alpha_{2a}$-Receptor-coupled G Proteins in CHO Cell Membranes—To study RGS function in the presence of receptor we examined CHO cell membranes expressing high levels (10 pmol/mg) of $\alpha_{2a}$ adrenergic receptor ($\alpha_{2a}$AR). Since RGS does not affect steady state GTPase activity of purified G proteins because GDP release is rate-limiting, any effect on GTPase in these membranes should be attributable to receptor-stimulated G protein. Surprisingly, the $\alpha_{2a}$AR membranes without added RGS showed only a modest agonist-induced increase in GTPase activity (from 2.3 to 3.3 pmol/mg/min) but RGS4 further increased the $\alpha_{2a}$AR-stimulated GTPase activity 3.8 ± 0.1-fold (Fig. 1A) with an EC₅₀ for RGS4 of 0.46 ± 0.06 μM (n = 3). As expected, the basal (or non-agonist stimulated) GTPase activity was only marginally increased by RGS4 (1.2-fold). Also, membranes expressing lower amounts of receptor gave proportionally smaller increases in GTPase activity (data not shown). Thus the RGS-mediated enhancement of GTPase is receptor-dependent and requires that the rate of receptor-stimulated GDP release exceed that of the unstimulated GTP hydrolysis, making hydrolysis rate-limiting in the G protein cycle. This raises the interesting possibility that at these receptor densities, the rate of receptor activation of G protein may become limited by depletion of the G-GDP receptor substrate in the absence of RGS activity.

The RGS specificity of the GTPase stimulation was RGS4 > RGS8 > RGS2 (Fig. 1B). This is expected, since $G_{i3}$ and $G_{i5}$ subunits are activated by the $\alpha_{2a}$AR in CHO cells (24). RGS4 and RGS8 are good GAPs for $G_{i3}$ and $G_{i5}$, while RGS2 and RGS7 are specific for $G_{i3}$ and $G_{i6}$ (19), respectively, which are either not activated by $\alpha_{2a}$AR ($G_{i3}$) or not expressed in CHO (Ga). This ability of RGS4 and RGS8 to increase steady state GTPase of receptor-stimulated G proteins is similar to recent data from Milligan and co-workers (25) using receptor-Go fusion proteins.

With our receptor/G protein/RGS system established we wanted to test possible mechanisms leading to the unexplained effect of RGS proteins to increase on- and off-rates of channel kinetics without significantly decreasing the steady state signal amplitude (11, 12). If RGS could enhance G protein activation as well as deactivation, that might account for those results.

Therefore, we tested the ability of RGS4 to enhance receptor-stimulated [35S]GTPγS binding. As expected, the full $\alpha_{2a}$AR agonist UK 14,304 stimulated [35S]GTPγS binding (4.1 ± 0.3-fold) (Fig. 2A), indicating efficient receptor-G protein coupling in the absence of RGS. Consistent with the possibility that RGS could enhance activation, RGS4 caused a small (~50%) increase in UK 14,304-stimulated [35S]GTPγS binding with an EC₅₀ of 0.4 μM (Fig. 2A). In attempting to optimize the magnitude of the RGS-stimulated [35S]GTPγS binding we tested the ability of GDP and GTP to enhance the effect of RGS4. Unlike receptor-stimulated GTPγS binding for which the -fold increase is enhanced by GDP, the RGS effect was strongly increased only upon addition of GTP (Fig. 2, B and C, insets). The small increase (~30%) in GTPγS binding in the absence of added nucleotide probably results from endogenous GTP present in

### Fig. 2. RGS4-stimulated [35S]GTPγS binding: nucleotide dependence.

#### A

- **GDP**
- **GTP**

**Panel A** shows the effect of GDP, GTP, and GDP/GTP on [35S]GTPγS binding in the absence (open circles) and presence (solid circles) of 1 μM GDP. Data plotted are receptor-stimulated [35S]GTPγS binding calculated by subtracting binding with 10 μM GDP from binding with 10 μM GDP and 1 μM GDP, to the binding with 10 μM GDP (which represented 15%–20% of the total binding). Insets show the RGS-stimulated -fold increase in [35S]GTPγS binding at 0, 1, and 10 μM GDP added GDP and 0, 10, and 100 μM added GDP, to permit a comparison at similar degrees of inhibition with each nucleotide. Data show mean ± S.E. values from three experiments each conducted in triplicate. Curves are non-linear least squares fits to a sigmoid function.

#### B

**Panel B** shows the effect of GDP and GTP on [35S]GTPγS binding in the absence (open circles) and presence (solid circles) of 1 μM GDP. Data plotted are receptor-stimulated [35S]GTPγS binding calculated by subtracting binding with 10 μM GDP from binding with 10 μM GDP and 1 μM GDP, to the binding with 10 μM GDP (which represented 15%–20% of the total binding). Insets show the RGS-stimulated -fold increase in [35S]GTPγS binding at 0, 1, and 10 μM GDP added GDP and 0, 10, and 100 μM added GDP, to permit a comparison at similar degrees of inhibition with each nucleotide. Data show mean ± S.E. values from three experiments each conducted in triplicate. Curves are non-linear least squares fits to a sigmoid function.

#### C

**Panel C** shows the effect of GDP and GTP on [35S]GTPγS binding in the absence (open circles) and presence (solid circles) of 1 μM GDP. Data plotted are receptor-stimulated [35S]GTPγS binding calculated by subtracting binding with 10 μM GDP from binding with 10 μM GDP and 1 μM GDP, to the binding with 10 μM GDP (which represented 15%–20% of the total binding). Insets show the RGS-stimulated -fold increase in [35S]GTPγS binding at 0, 1, and 10 μM GDP added GDP and 0, 10, and 100 μM added GDP, to permit a comparison at similar degrees of inhibition with each nucleotide. Data show mean ± S.E. values from three experiments each conducted in triplicate. Curves are non-linear least squares fits to a sigmoid function.

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3 All RGS proteins were prepared and used as GST fusion proteins unless indicated otherwise.
the membrane preparations. Furthermore, the micromolar GTP concentrations used in the subsequent studies are appropriate, since cellular GTP levels are in the high micromolar range.

With added GTP present, RGS4 stimulated GTP\textsubscript{S} binding 3.0-fold with an EC\textsubscript{50} of 1.0 \textmu M (Fig. 3A). There was also a small increase in GTP\textsubscript{S} binding in the absence of UK 14,304 (EC\textsubscript{50} of 1.3 \textmu M and a maximum effect of 2.3-fold), possibly due to constitutive receptor activity or GTP-bound G\alpha subunits. To ensure that the effect of RGS4 was specific, we also tested the protein buffer (phosphate-buffered saline), boiled RGS4, and GST alone (Fig. 3B), which had no effect.

Also, thrombin-cleaved purified RGS4 protein\textsuperscript{4} gave effects similar to GST-RGS4 (data not shown). We examined the specificity of the different RGS proteins and found that it was identical to that for GTPase stimulation (compare Figs. 1B and 3B).

Physical Scaffold Mechanism?—One mechanism by which RGS could stimulate GTP\textsubscript{S} binding is by enhancing receptor-G protein coupling. The simplest scheme would be for RGS to act as a GAP of GTP-bound G\alpha subunit. Parameters for the individual steps are shown in Table I. Components include: D, drug or agonist; R, receptor; G, G protein. Reversible reactions are indicated as double-headed arrows. Molecular complexes are represented by concatenated names of individual components. Initial reactant concentrations (M) were: R, 8 \times 10\textsuperscript{-10}; D, 10\textsuperscript{-5}; GDP, 10\textsuperscript{-6}. B, the concentrations of G\alpha complexes (DRG, open circles; GGTP, filled circles; and GGDP, open squares) were simulated at steady state (10 min) using Berkeley Madonna (Version 8.02). For this simulation, the initial GTP was 400 nM with the indicated concentrations of RGS. C, simulated GTPase activity was determined at 10 min with an initial GTP concentration of 0.1 \textmu M and varied RGS concentrations. P, release is plotted with values converted to units relevant to our experimental measurements (4 \mu g of membrane protein in a reaction volume of 100 \mu l with receptor and G protein concentrations at values in Table I).

D, GTP\textsubscript{S} binding. To simulate GTP\textsubscript{S} binding an extra step was added to the model in which GTP\textsubscript{S} binds irreversibly to DRG (steps 10 and 11 in Table I). The initial reactant concentrations were GTP\textsubscript{S}, 0.2 nM and GTP, 4 nM (open circles) or 400 nM (filled circles). The GTP\textsubscript{S} bound after a 10-min simulation was transformed into fmol/mg as in C.

\textsuperscript{4}The thrombin-cleaved RGS4 construct has an amino-terminal extension of GSPGIRL and was >90% pure by Coomassie staining on SDS-PAGE.
to bind directly to both proteins (R and G) forming a physical scaffold perhaps enhancing receptor-G protein pre-coupling (26, 27). Indeed, the amino-terminal amphipathic sequence of RGS4 confers receptor specificity in regulation of G<sub>i</sub> signaling, and it has been suggested that it may directly interact with receptors (28). Thus that region would be a logical candidate to engage in the formation of a receptor/RGS/Gi protein complex. To determine whether the amino-terminal sequence of RGS4 was necessary or sufficient for enhancing receptor-stimulated GTP<sub>S</sub> binding to Gi, we prepared the catalytic domain fragment of RGS4 (RGS4box, aa 58–177, His<sub>6</sub>-tagged) and an amino-terminal synthetic peptide (1–51), which has previously been shown to enhance RGS regulation of G<sub>i</sub> in cells (28). The catalytic domain alone (i.e. RGS4box) stimulated GTP<sub>S</sub> binding to Gi in a manner identical to that of full-length RGS4 (Fig. 3, C and D). In addition, the amino-terminal fragment alone had no effect (Fig. 3, C and D). Furthermore the peptide did not potentiate or inhibit the effects of the RGSbox construct (data not shown). These results rule out a physical scaffolding model that depends on the amphipathic amino-terminal sequence of RGS4. While we cannot rule out a physical scaffold mechanism mediated by the RGS domain itself, these observations taken together prompted us to consider other mechanisms.

**Kinetic Scaffolding Mechanism**—Since the RGS specificity and concentration dependence in enhancing GTPase and [35S]GTP<sub>S</sub> binding were strikingly similar and only the RGS GAP domain was required, we reasoned that these two phenomena might depend only on the GAP activity. Furthermore, the dependence of RGS-stimulated GTP<sub>S</sub> binding on GTP versus GDP in the reaction mixture suggested a mechanism involving GTP hydrolysis. If a strong receptor stimulus caused sufficient accumulation of activated Gα-GTP to deplete the receptor substrate (i.e. heterotrimeric G-GDP) in the vicinity of receptor, then the GAP activity of the RGS could: restore local G-GDP substrate levels, permit receptor to stimulate more GDP release, produce more “empty” DRG state, and permit more GTP<sub>S</sub> binding per unit time. Such a mechanism has been proposed for muscarinic receptors and G<sub>i</sub> by Ross and co-workers (6, 17). In that case the effector, phospholipase C-β, serves as the GAP to maintain a complex of receptor/G protein/effecter, but RGS4 can also enhance the rate of GTP binding and GTPase in that system (29).

To determine whether G-GDP depletion could account for our results, we constructed a kinetic model (Fig. 4) to examine RGS effects on receptor-G protein interactions and GTP hydrolysis. Simulating the presence of GTP (0.4 μM) in the face of a strong receptor stimulus, the steady state levels of G-GTP calculated by the model actually exceed those of G-GDP (Fig. 4B). This indicates that GDP release driven by the receptor can exceed the basal rate of GTP hydrolysis by the G protein. As RGS is added and the GTPase rate increases, the ratio of G-GDP/G-GTP increases. These results show that G-GDP substrate depletion is feasible with this set of reasonable kinetic parameters for the G protein cycle. In addition to enhancing the G-GDP to G-GTP ratio, RGS also increased the amount of...
nucleotide-free DRG (albeit still at low levels). We then asked whether this model could also replicate our experimental findings with steady state GTPase and GTP\(\gamma\)S binding. Fig. 4C shows that agonist-simulated GTP hydrolysis increased from 12 to 20 pmol/mg/min, and RGS was also able to increase GTP\(\gamma\)S binding from 105 to 241 fmol/mg (Fig. 4D). Interestingly, this effect was dependent on the GTP concentration, since modeling with a GTP concentration of 4 nM (i.e. the amount present endogenously in the membranes) showed a marginal effect on GTP\(\gamma\)S binding (527–539 fmol/mg). Thus the kinetic model predicts that the ability of RGS to increase GTP\(\gamma\)S binding should only be evident when GTP is included in the assay (Fig. 4D), which is consistent with our data. Thus, the structural data (Fig. 3D) and model predictions (Figs. 2C and 4D) are all consistent with the kinetic model in which enhanced GTP\(\gamma\)S binding is caused simply by the accelerated GTP hydrolysis in the face of a strong receptor stimulus.

To ensure that the effect of RGS to enhance \([^{35}\text{S}]\text{GTP}\)\(\gamma\)S binding was not dependent on the long incubation times and very low GTP\(\gamma\)S concentration used in these experiments, we also tested the effect of RGS4 on binding of 50 nM \([^{35}\text{S}]\text{GTP}\)\(\gamma\)S (Fig. 5). In the presence of agonist, RGS4, and 1 \(\mu\)M GTP, \([^{35}\text{S}]\text{GTP}\)\(\gamma\)S binding occurred very fast (\(t_{1/2} = 20\) s) and a substantial fraction of the G protein pool was occupied (2 pmol/mg, about 20% of total G\(\text{P}\), present in CHO cell membranes (24)). Under these conditions, the RGS stimulation of agonist-induced \([^{35}\text{S}]\text{GTP}\)\(\gamma\)S binding was even more striking.

Spatial Implications of Kinetic Scaffolding—The chemical kinetic model, just described, assumes that reactions are occurring in a homogenous three-dimensional system with free mixing of all components. Since receptor-mediated G protein activation in cells occurs in a two-dimensional membrane which may have diffusion limitations, we used a Monte Carlo model similar to that developed by Mahama and Linderman (23) to examine spatial effects of the kinetic scaffolding mechanism. Simulations were run over a range of diffusion coefficients (10.\(10^{-10}\)–10.\(8\) cm\(^2\) s\(^{-1}\)) (30, 31) and GTP hydrolysis rates (from the intrinsic G\(\text{a}\) GTPase rate of 0.02 s\(^{-1}\) to 200 s\(^{-1}\)).

Fig. 6 illustrates the results of these Monte Carlo simulations. With the lowest rate of GTP hydrolysis (\(k_{\text{hyd}} 0.02\) s\(^{-1}\)) and \(D = 10.\(10^{-8}\) cm\(^2\) s\(^{-1}\)), a single receptor can activate the entire pool of 600 G proteins over the simulation area of 1 \(\mu\)m\(^2\) leading to profound and extensive G-GDP depletion (total G protein density is constant so G-GDP is almost fully depleted throughout the membrane system). As the GRS concentration and the rate of GTP hydrolysis increase, the envelope of active G protein (and depleted G-GDP) narrows. One can define the range of activity by the radius at which a given concentration of active G\(\text{a}\) is reached. Fig. 6B illustrates, for different diffusion coefficients and GTPase rates, the distance from receptor at which the active G\(\text{a}\) level is 200 \(\mu\)m\(^{-2}\) or G protein is 40% active. The zone of active G\(\text{a}\) can range from less than 20 nm to over 450 nm depending on the GRS activity (\(i.e.\ k_{\text{hyd}}\)) and the diffusion coefficient. At any given diffusion coefficient, RGS dramatically narrows the range of G protein activation around a single receptor or a cluster of receptors. Interestingly, the amount of active G protein immediately adjacent to the receptor (\(i.e.\ within about 10–20\) nm) is not reduced significantly even at very high GRS concentrations, so an effector in close proximity would not show an RGS-dependent decrease in activity. Such an effect could explain the ability of RGS to speed the kinetics of G protein-coupled inwardly rectifying K\(^+\) channel responses without altering steady state activity (11, 12).

In ongoing work, we show that three effector responses produced by the \(\mu\) opioid receptor in a C6 glioma cell line are differentially sensitive to the influence of RGS proteins. Using an RGS-insensitive G\(\text{a}\), (19) it is shown that inhibition of adenyl cyclase and activation of ERK are greatly enhanced in the absence of RGS effect while the increase in intracellular calcium is not. Thus different effectors may be differentially modulated by RGS action. Those results are consistent with the RGS-mediated kinetic scaffolding model proposed here (for example if the Ca\(^{2+}\) response effectors were more closely associated with \(\mu\) opioid receptor and G\(\text{a}\), than those for adenylyl cyclase and ERK responses). Clearly other models may also account for the differential RGS effects, but kinetic scaffolding is one possible mechanism.

Functional Roles of RGS—RGS proteins play numerous roles in G protein signaling. They reduce G protein signals via their GAP activity and/or by competing for G protein binding to effectors (6, 10). This inhibition of G protein signaling may be regulated either by changes in RGS expression (7) or perhaps by post-translational modifications (34). RGS proteins are required for the fast kinetics of turnoff during ion channel regulation by G proteins (12, 35). RGS can participate in many other protein-protein interactions via amino- and carboxy-terminal extensions from the RGS domain (14). These likely serve to coordinate signaling between heterotrimeric G proteins and low molecular weight Ras superfamily G proteins (36, 37) and between pairs of heterotrimeric G proteins (38, 39). They can also cause signal-dependent translocation of other types of regulatory molecules to the site of active G\(\text{a}\) subunits (40) and may play a role in nuclear processes (33).

To this long list of established or hypothesized functions of RGS proteins, we present the concept of kinetic scaffolding and its contribution to spatial focusing of G protein signals. This may occur around a single receptor but could also play an important role in localizing signals around small clusters of receptors in dendrites or synaptic areas of neuronal cell bodies. Localization of ionotropic receptors in these regions is quite exquisite (4). Thus the ability of RGS to narrow the spatial range of signal output from G protein-coupled receptors to the 10–100 nm scale could permit a similar fine localization of signaling via G protein systems.

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