Regulators of G-protein Signaling Form a Quaternary Complex with the Agonist, Receptor, and G-protein

A NOVEL EXPLANATION FOR THE ACCELERATION OF SIGNALING ACTIVATION KINETICS

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Regulators of G-protein signaling (RGS) proteins modulate signaling through heterotrimeric G-proteins. They act to enhance the intrinsic GTPase activity of the Gα subunit but paradoxically have also been shown to enhance receptor-stimulated activation. To study this paradox, we used a G-protein gated K⁺ channel to report the dynamics of the G-protein cycle and fluorescence resonance energy transfer techniques with cyan and yellow fluorescent protein-tagged proteins to report physical interaction. Our data show that the acceleration of the activation kinetics is dissociated from deactivation kinetics and dependent on receptor and RGS type, G-protein isoform, and RGS expression levels. By using fluorescently tagged proteins, fluorescence resonance energy transfer microscopy showed a stable physical interaction between the G-protein ε subunit and RGS (RGS8 and RGS7) that is independent of the functional state of the G-protein. RGS8 does not directly interact with G-protein-coupled receptors. Our data show participation of the RGS in the ternary complex between agonist-receptor and G-protein to form a “quaternary complex.” Thus we propose a novel model for the action of RGS proteins in the G-protein cycle in which the RGS protein appears to enhance the “kinetic efficacy” of the ternary complex, by direct association with the G-protein α subunit.

The G-protein cycle is initiated by binding of an agonist to its target seven-helical G-protein-coupled receptor (GPCR), which associates with a heterotrimeric G-protein on the cytoplasmic side of the cell membrane. Once assembled, this “ternary complex” promotes GDP release and stimulates GTP binding on the G-protein ε subunit and dissociation of the G-protein subunits Gα-GTP and Gβγ. Both subunits can activate downstream signaling molecules, including enzymes and ion channels (1, 2). Regulators of G-protein signaling (RGS) proteins modulate signaling through heterotrimeric G-proteins. Cloning studies have identified a large RGS gene family, each endowed with a conserved RGS domain of 120–130 amino acids that is flanked by N and C termini of varying lengths (3–7). By itself, the RGS domain is capable of interacting with G-protein α subunits to accelerate the GTP hydrolysis rate of the Gα subunit, thereby promoting termination of the G-protein signal (3–7). Based on primary sequence similarities, mammalian RGS proteins have been grouped into five subfamilies (7). In this study we focus largely on RGS8, belonging to the R4 subfamily of RGS proteins that are generally considered prototypical in that they appear to have little function other than to act as GTPase-activating proteins (GAPs) on Gαo and Gα11 G-protein α subunits. However, we also examine RGS7, which belongs to the R7 family, and G4IP, which belongs to the RZ family. RGS7 is particularly interesting because it contains a number of protein-protein interaction domains in the N terminus, but it is of particular relevance for our study because it has a substantially attenuated GAP activity compared with other RGSs (8).

The discovery of the RGS protein family had important ramifications for the study of the G-protein gated K⁺ channel (GIRK) that was first identified in atrial myocytes and activated by acetylcholine acting at muscarinic M2 receptors (9). Activation of GIRK is responsible, in part, for slowing of the heart rate in response to vagal stimulation (10, 11). Analogous GIRK currents are present in neurons and neuroendocrine cells (12). The native channel is a heterotetrameric complex composed of the inwardly rectifying K⁺ (Kir) channel subunits, Kir3.1–3.4 (12). Activation of native and cloned G-protein gated K⁺ channels has been shown to involve a direct, membrane-delimited interaction with the Gβγ subunit (13, 14). A number of studies have sought to explain why these channels, when expressed in Xenopus laevis oocytes, deactivate more slowly than the native atrial current upon termination of the receptor stimulus. Identification of the RGS family has resolved this discrepancy. For example, expression of RGS4 or RGS8 in X. laevis oocytes accelerates GIRK deactivation kinetics such that the measured time constants are more consistent with those occurring after stimulation of native channels in atrial cells (15, 16).

A paradox arose, however, because RGS protein expression not only enhanced channel deactivation but also accelerated GIRK activation (15, 16). Furthermore, RGS expression did not attenuate the overall signal, an effect that would be predicted if the only function of the RGS were to act as a GAP (16, 17). This interesting phenomenon has resulted in a number of new proposals for the mechanism of action of RGS proteins and has important consequences for our understanding of the G-protein cycle. RGS proteins may function as scaffolds to help pre-assembly signaling complexes of receptor with G-protein, for...
example (17–19). Alternatively, RGS proteins may promote entry into the G-protein cycle by physically aiding the dissociation of Go-GTP and Gβγ subunits or by encouraging the formation of fast cycling signaling complexes, acting according to a “kinetic scaffolding” mechanism (7, 20, 21). Previously, we have presented data showing the importance of the ternary complex in determining the selectivity and kinetics of GIRK activation (22–24). Briefly stated, this hypothesis ascribes a unique conformation for the complex formed between various combinations of agonist, GPCR, and G-protein, which dictates the speed and efficiency of G-protein activation. This hypothesis is consistent with many of the recent models used to explain G-protein activation (22–24). Briefly stated, this hypothesis ascribes a unique conformation for the complex formed between various combinations of agonist, GPCR, and G-protein, which dictates the speed and efficiency of G-protein activation.

**MATERIALS AND METHODS**

**Molecular Biology, Cell Culture, and Transfection**—The human clones of RGS8, RGS7, and Gαi3 were obtained from the Guthrie cDNA Resource Centre. A PCR-based approach was employed to clone RGS8, RGS7, and Gαi3 in-frame into pEYFP-N1 (Clontech) by using HindIII and BamHI (RGS8 and Gαi3) and Xhol and BamHI (RGS7) as cloning sites. Fluorescently tagged receptors (D2-CFP, A1-CFP, a2A-CFP, and M4-YFP) were constructed by using a similar general approach in the pECFP-N1 and pEYFP-N1 vectors using Kpnl and HindIII as the cloning sites. Inducible expression of RGS8-YFP was achieved using the TREx system (Invitrogen). RGS8-YFP was excised from pEYFP-N1 and ligated into pCDNA5/TTO and pCDNA6/TR (both Invitrogen) with a HindIII/Ncol restriction digest. The methods for cell culture and transient transfection and the techniques for establishing stable cell lines with HEK293 cells have been described previously (22, 27). “Quadripluripotent” inducible stable cell lines were established after the transfaction of RGS8-YFP in pcDNA5/TTO and pCDNA6/TR (both Invitrogen) into the HKIR3.1/3.2/M4 cell line and subsequent selection with 727 g/ml hygromycin, and 5 μg/ml blasticidin. In this stable cell line, designated as HKIR3.1/3.2/M4/RSYYPT, gene expression was conditional upon the addition of the antibiotic tetracycline (Sigma). Treatment of cells with tetracycline (0.01–10 μg/ml) for 24–36 h resulted in graded expression of the fluorescently tagged protein, Gαi-CFP, and Gαi-CFP were constructed in an analogous fashion to Gαi3-CFP (28). Gα2-CFP was the kind gift from Dr. S. Ikeda. All other plasmids were used as described previously. A CFP-YFP dimer was constructed by cloning an in-frame fragment of enhanced YFP (Clontech) into enhanced CFP-N1 in the Xhol-BamHI sites of the polyclin

**Electrophysiology**—Whole-cell membrane currents were recorded with an Axopatch 200B amplifier, and data were acquired with a Digidata 1200B interface (both Axon Instruments) and analyzed with pClamp software (version 6.0; Axon Instruments). Cell capacitance was 15 picoFarads, and series resistance (20–40 MΩ) was at least 75% uncompensated using the amplifier circuitry. After an equilibration period of ~5 min, cells were voltage-clamped at ~60 mV; records were digitized at 100 Hz, and agonist-induced currents were measured at this potential. Rapid drug application and removal were achieved as described previously (24, 29, 30). Agonist was applied for 20 s. Upon agonist application, the current was activated with an initial lag and then a subsequent rise to peak current amplitude (time-to-peak). Activation kinetics are described as lag + time-to-peak. On removal of the agonist, the current deactivated back to base-line levels. Deactivation was generally well described by a single exponential function, \( I(t) = A \exp(-t/\tau) + C \), where \( A \) is the current amplitude at the start of the fit; \( t \) is time; \( \tau \) is the deactivation time constant, and \( C \) is the steady state asymptote. Curve fitting was performed by using Clampfit software (pClamp version 6.0). After removal of the agonist dopamine from HKIR3.1/3.2/M4 cells for 15–30 s, the deactivation phase was repositioned as described by the “sum of two exponentials” equation: \( I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + C \). After removal of the agonist adenosine from HKIR3.1/3.2/M4, the deactivation phase was not adequately fit by a single exponential function. Therefore, the measure “time to half-current decay” was used to describe the rate of deactivation. This is defined as the time taken from removal of agonist for the agonist-induced current to deactivate to half its peak value.

**Fluorescence Microscopy**—Cells for imaging were subcultured onto 25-mm glass coverslips or 35-mm culture dishes with integral number of 0 glass coverslip bottoms (Matttek) and, if transiently transfected, were allowed 24–48 h to express the protein(s) of interest.

**Confocal Microscopy**—Prior to imaging, a coverslip was placed into a water-tight cell imaging chamber at room temperature, and cells were overlaid with HEPEM-buffered Opti-MEM without phenol red (Invitrogen). HEK293 cells were imaged by using a Bio-Rad Radiance 2100 confocal microscope using a 60× Nikon Plan Apo objective (1.40 NA). CFP was excited with a 457-nm laser line, and images were obtained using a 514-nm band-pass filter. YFP was excited with a 543-nm laser line, and emission was measured between 530 and 570 nm. The FRET imaging conditions were obtained with excitation by using a 470-nm laser line, and emission was measured between 530 and 570 nm. Multiple images were acquired sequentially. Intensities in the CFP, YFP, and FRET set of imaging conditions were determined from immunodominated-regions of interest drawn by hand at high magnification using the LaserPix software. The background-subtracted intensities were analyzed to determine FRET ratios using three-cube protocols (see below). 16-Bit images were obtained with identical laser powers, photomultiplier gain, and pinhole size and were optimized to examine cells with moderate expression of both constructs. Care was taken to avoid saturating images.

**CCD-based Fluorescence Microscopy**—We also used a standard fluorescence microscope (Nikon TE2000 60× Plan Apo objective 1.40 NA), equipped with a back-illuminated digital CCD camera (Roper Scientific MicroMax 1024 EB) and high speed CCD detector control, to measure FRET ratios and changes in these ratios with perfusion. Samples were excited by using a mercury lamp with an excitation filter wheel, and emission filters were selected through an emission filter wheel (Sutter Instruments). Lambda-1020 emission filter sets were used (excitation and emission): YFP (500 ± 10 nm; 535 ± 15 nm), CFP (430 ± 12.5 nm; 465 ± 20 nm), and FRET (430 ± 12.5 nm; 535 ± 15 nm). 16-Bit images were acquired and analyzed with the Universal Imaging Corp. metamorph software (version 6.1). Background values employed were regions containing no cells in the viewing field. 3-Cube parameters and FRET ratios were calculated as above.

It is worth noting that in both microscopy systems the S.E. of the FRET ratio was less than 5% of the mean, and this compares very favorably with other methods (31). Given the different imaging conditions in the two setups, the relationship between FRET efficiency and FRET ratio will be different as it depends on the ratio of the CFP and YFP molar extinction coefficients at the respective excitation wavelength (32). Practically, exciting CFP at the suboptimal 457 nm on the laser-scan confocal microscope leads to a smaller FRET ratio given a particular FRET efficiency.

**FRET Ratio and Other Calculations**—Colocalization ratios were determined using the LaserPix software. The FRET ratio (FR) measures the fractional increase in YFP emission because of FRET and was calculated according to Equation 1 (32),

\[
FR = \left( \frac{S_{\text{FRET}}(DA) - R_{\text{DA}} \times S_{\text{YFP}}(DA)}{R_{\text{YFP}} \times S_{\text{YFP}}(DA) - R_{\text{DA}} \times S_{\text{YFP}}(DA)} \right) = \left( \frac{R_{\text{YFP}} - R_{\text{DA}}}{R_{\text{YFP}} - R_{\text{DA}}} \right) \tag{1}
\]

where \( S_{\text{YFP}}(DA) \) is the intensity measurement of YFP (500 nm; 535 nm) in the presence of YFP alone, and \( S_{\text{FRET}}(DA) \) is the intensity measurement of YFP in the presence of CFP and YFP alone.

**Data Analysis**—Membrane currents were measured at −60 mV, and agonist application was performed for 15–30 s, which was the average number of cells recorded. Data were analyzed for statistical significance using either Student’s t test or one-way analysis of variance with Dunnett’s post-test where appropriate; * indicates p < 0.05, ** indicates p < 0.01, and *** indicates p < 0.001. Time measurements were reciprocated prior to statistical analysis.

**Materials and Drugs**—Solutions for electrophysiology were as follows (concentrations in mM): pipette solution, 107 KCl, 1.2 MgCl₂, 107 NaCl, 1.2 CaCl₂, 10 HEPES, 2 Na₂SO₄, 1 MgSO₄, 0.5 NaH₂PO₄, and 0.24 NaHCO₃. Extracellular solution contained 5 C6-HCl, 2 Glu, 0.5 MgCl₂, 2 CaCl₂, 15 NaCl, 100 KCl, 10 HEPES, 2 Na₂SO₄, 1 MgSO₄, 0.5 NaH₂PO₄, and 0.24 NaHCO₃ buffered to pH 7.4 with 10 HEPES.
CaCl$_2$, 10 EGTA, 5 HEPES, 2 MgATP, 0.3 Na$_2$GTP (KOH to pH 7.4, ~140 mM total K$^+$; bath solution, 140 K$_2$Cl, 1.2 MgCl$_2$, 5 HEPES (pH 7.4). Cell culture materials were from Invitrogen. The following drugs were made up as concentrated stock solutions and kept at ~20 °C: 5′-N-ethylcarboxamidoadenosine; adenosine (9-d-ribofuranosyladenine); baclofen hydrochloride; carbachol (carbamylcholine chloride); dopamine hydrochloride; (-)-norepinephrine hydroxide (all from Sigma).

**RESULTS**

As in our previous studies (22, 23), we used a HEK293 stable cell line that expresses the GIRK channel subunits Kir3.1 and Kir3.2A (HKIR3.1/3.2) and dual channel plus GPCR stable lines (adenosine A1, HKIR3.1/3.2/A1; adrenergic α2A, HKIR3.1/3.2/α2; dopamine D2S, HKIR3.1/3.2/D2; muscarinic M4, HKIR3.1/3.2/M4; and the heterodimeric GABA-B1b + GABA-B2, HKIR3.1/3.2/GGB). Cells were studied with the whole-cell configuration of the patch clamp technique, and drugs were applied using a rapid agonist application system.

Initial transfection studies were carried out to confirm that RGS8-YFP and RGS7-YFP cDNAs could express functional proteins that mediated effects similar to the untagged protein. Fig. 1A illustrates the effects of expression of RGS8, RGS8-YFP, and RGS7-YFP in a cell line expressing the heterodimeric GABA-B1b/2 receptor. Application of a saturating dose of the appropriate agonist, in this case 100 μM baclofen, rapidly activated an inward K$^+$ current. Activation was characterized by an initial delay (lag) and then a subsequent rise to a peak value (time-to-peak, “tp”). After expression of either RGS8 or RGS8-YFP, there was enhancement of both GIRK activation (Fig. 1B (i)) and deactivation kinetics (Fig. 1B (ii)) by both constructs. Thus RGS8-YFP is functional with effects equivalent to that of the untagged protein; there was no significant difference between RGS8 and RGS8-YFP in the acceleration of activation and deactivation. Saitoh et al. (57) have reported that expression of RGS7 profoundly accelerated activation kinetics but had a much weaker effect on deactivation kinetics. We were able to reproduce this observation, and we also observed that RGS7-YFP had a similar effect (Fig. 1B). Thus, RGS7-YFP is functional with effects equivalent to that of the untagged protein; there were no significant differences between RGS7 and RGS7-YFP in the acceleration of activation and in the failure to accelerate deactivation kinetics (Fig. 1B, (i) and (ii)). We did not find it necessary to coexpress G$\beta\gamma$ with RGS7 to observe pronounced functional effects and presumably RGS7 complexes with endogenously expressed G$\beta\gamma$ (33, 34). It is also known that G$\beta\gamma$ containing G$\beta\gamma$ complexes can inhibit GIRK channels (35), and overexpression of this protein would potentially complicate our electrophysiological analysis.

We have found previously that with certain agonist/GPCR combinations, the deactivation rate is not determined by the rate of GTP hydrolysis by the G-protein but by processes occurring at the level of the receptor, particularly the agonist-ubinding rate (30). For example, for the synthetic agonists 5′-N-ethylcarboxamidoadenosine at the A1 receptor or quinpirole at the D2S receptor, the deactivation rate was slow and was not altered by overexpression of RGS8 (30). However, with the agonists adenosine and dopamine at their respective GPCRs, the rate of current deactivation was faster and strongly enhanced by RGS8-YFP expression (Fig. 2). We then questioned whether the kinetic effects of RGS8 on activation might be selective for different GPCRs. We examined the effects of RGS8-YFP expression on GIRK channel kinetics in cells stably expressing different GPCRs, in response to fast application and removal of agonists. We have previously used radioligand binding to show equivalent levels of receptor expression in the HKIR3.1/3.2/A1, HKIR3.1/3.2/α2A, and HKIR3.1/3.2/D2 cell lines (24).

When RGS8-YFP was expressed in HKIR3.1/3.2/M4, HKIR3.1/3.2/D2, and HKIR3.1/3.2/GGB cells, both current activation and deactivation were significantly accelerated upon...
the application and removal of an appropriate agonist (Figs. 1A and 2). In contrast, in the HKIR3.1/3.2/A1 and HKIR3.1/3.2/α2 cell lines, only deactivation kinetics were accelerated by RGS8-YFP (Fig. 2). In the HKIR3.1/3.2/A1 cell line, basal currents were significantly reduced after RGS8-YFP expression (control, 125 ± 26 pA/pF; +RGS8-YFP, 69 ± 6 pA/pF; p < 0.05, t test), which is consistent with a GAP function. Thus it appears that the effects of RGS8-YFP on the kinetics of current activation are dependent on the GPCR studied. For those GPCRs that exhibit relatively slow GIRK activation, RGS8-YFP can accelerate the kinetics, whereas for those that are intrinsically fast (A1 and α2A), the expression of RGS8-YFP has no effect. Finally, to ensure these observations were not because of YFP tagging the C terminus of RGS8, untagged RGS8 (1 g) was expressed in the HKIR3.1/3.2/α2A cell line, and the kinetics were analyzed. Activation was similarly unchanged (α2A control, lag + ttp (s), 1.13 ± 0.05; +RGS8, 1.42 ± 0.25, n = 12; not significant), and deactivation was significantly enhanced (α2A control, OFF (s), 5.05 ± 1.00; +RGS8, 2.39 ± 0.60s, n = 12, p < 0.01). Therefore, activation kinetics appeared to approach a limiting value.

There are some additional points to note. First, with dopamine as an agonist, deactivation was significantly accelerated by RGS8-YFP overexpression, but on closer kinetic analysis, two distinct deactivation time constants were revealed, a fast and slow component (Fig. 2A). Second, it was necessary to use the measurement time to half-current decay (see “Materials and Methods”) in the A1 recordings due to difficulty in fitting single exponential curves to the current deactivation phase as noted previously (30). Finally, we have shown previously that untagged RGS8 overexpression did not significantly accelerate activation kinetics in the M4 line (30); however, this was based on fewer observations, and there is more intrinsic biological variability in this line as evidenced by the error bars (Fig. 2B).

It is known that different Gαi/coupled GPCRs interact preferentially with certain Gαi/0 isoforms and that RGS proteins may show selectivity for different G-proteins. Therefore, we addressed the question of whether RGS8 was acting selectively on certain Gαi/0 isoforms.

Mechanism of RGS Action

Fig. 2. Receptor specificity of channel activation by RGS. A, gallery of representative current traces recorded at −60 mV from stable cell lines as indicated. Control recordings are shown in the top panel, and recordings from cells transfected with RGS8-YFP (1 μg of cDNA) are shown in the lower panel. B, summary of the activation kinetics (i) and deactivation kinetics (ii) for each of the cell lines studied. Numbers are shown in parentheses. RGS8-YFP expression (+RGS8 in figure) had no effect on the rate of channel activation via the A1 and α2A receptors but enhanced activation via the D2, GABA-B1b/2, and M4 receptors. In each cell line there was a significant enhancement of deactivation kinetics. The bar charts show analysis to maximal concentrations of full agonists as follows: A1, 1 μM adenosine; α2A, 3 μM noradrenaline; D2, 10 μM dopamine; GABA-B1b/2, 100 μM baclofen; M4, 100 μM carbachol. Inset shows an expanded view of the activation phase. The arrow indicates the onset of agonist application.
Mechanism of RGS Action

FIG. 3. Receptor selectivity is not because of preferential regulation of different Gαi/o G-protein α isoforms by RGS8-YFP. A, equivalent regulation of Gαs and Gαi/o G-protein isoforms by RGS8-YFP. Transfections of PTx-insensitive Gα subunits (500 ng cDNA) plus either 500 ng of pEYFP-N1 vector (control) or 500 ng RGS8-YFP (+ RGS8-YFP) were made into HKIR3.1/3.2/GBB cells. Cells were PTx-treated for at least 16 h, and recordings were made 24–72 h after transfection. The upper panel shows representative control traces from cells expressing Gαs (C-G) (left) and Gαi/o (C-G) (right). The lower panel shows recordings from cells additionally expressing RGS8-YFP. The solid bars indicate applications of 100 μM baclofen. B, summary of activation (lag + ttp) and deactivation (t1/2) data. Current activation and deactivation kinetics through both G-protein isoforms was significantly enhanced by RGS8-YFP expression. C and D, the HKIR3.1/3.2/A1 cell line was transiently transfected with 500 ng of Gαs (C-G) plus 500 ng of RGS8-YFP or 500 ng of pEYFP-N1. As before, cells were PTx-treated (100 ng/ml >16 h) and recorded from 24 to 72 h post-transfection. Recordings of current traces are shown from cells transfected as indicated. The horizontal bar depicts a 20-s application of 1 μM adenosine. Activation and deactivation kinetics are summarized in D. Activation through the A1 receptor, constrained to signal through Gαi/o (C-G) subunits, is not enhanced by RGS8-YFP expression, whereas deactivation, measured using the time to half-current-decay parameter, is significantly accelerated by RGS8-YFP.

We next investigated how varying the concentration of RGS8-YFP affected the kinetics of current responses. We used a “Tet-On” system and developed a monoclonal cell line on the background of the HKIR3.1/3.2/M4 cell line, additionally expressing RGS8-YFP in an inducible vector. In the absence of tetracycline (Tet), there was little basal RGS8-YFP expression. Concentrations of Tet were selected that yielded intermediate (0.3 μM) and saturating (10 μM) levels of RGS8-YFP expression (Fig. 4A). We observed a graded acceleration of deactivation kinetics with increasing Tet concentrations (Fig. 4B). In contrast, the change in activation kinetics was smaller and did not reach statistical significance. Thus it seems that at lower expression levels there is a graded acceleration of deactivation kinetics before activation kinetics are enhanced.

We have shown previously that HEK293 cells possess endogenous RGS proteins, and we found that endogenous RGS proteins solely modulate deactivation kinetics via the GABA-B receptor, without affecting activation kinetics (30). We further investigated the role of endogenous RGS proteins in the HKIR3.1/3.2/D2 line by expressing a PTx-resistant G-protein with a point mutation, G184S (designated as RGSiGoA), rendering it resistant to the actions of RGS proteins (39). Fig. 5 shows that constraining signaling through the RGS-resistant G-protein substantially slowed deactivation and also slowed transfectad mutant G-protein. We have shown that these mutant G-proteins can substitute for native G-proteins, and we have used this approach to dissect out the interaction of different GPCRs with distinct Gi/o isoforms (23, 24). When initially characterized, RGS8 was found to coimmunoprecipitate with Gαs and Gαi/o subunits (16). Therefore, we asked if RGS8 showed substrate specificity for specific G-protein α isoforms. In Fig. 3, PTx-resistant forms of either Gαs or Gαi/o were expressed in the HKIR3.1/3.2/GBB cell line. The activation and deactivation kinetics of currents mediated by either Gαs or Gαi/o were significantly enhanced by coexpression of RGS8-YFP (Fig. 3B). As noted previously (24), the GABA-B1b/2 receptor activates currents significantly faster via the Gi/o G-protein isoforms than through the Gi subset, leading it resistant to the actions of RGS proteins (39).
channel activation. The effect on activation (less than a 3-fold change) was not as substantial as that on deactivation (greater than 10-fold change).

To investigate the trafficking and the interaction of components in this signaling cascade, we used laser-scanning confocal microscopy to examine the subcellular distribution of RGS8-YFP. Another RGS protein from a separate family, namely GAIP (RGS19), was also YFP-tagged and imaged to test the generality of our observations. We expressed RGS8-YFP and GAIP-YFP in D2S receptor-expressing cells (HKIR3.1/3.2/D2) with and without cotransfection of the Gαi subunit. Alone, RGS8-YFP was located in the cytosol and nucleus and GAIP-YFP in the cytosol (Fig. 6A). Cotransfection of Gαi resulted in a dramatic redistribution of RGS8-YFP to the membrane (Fig. 6A). This effect was quantified by colocalization studies with CFP-tagged G-protein α subunits. We have shown previously (28) such constructs to be functional and able to participate in receptor-mediated coupling. However, more subtle differences between untagged and tagged G-protein cannot be excluded. In control experiments, RGS8-YFP was coexpressed with mem-CFP (CFP targeted to the membrane by a dual palmitoylation sequence). Our findings were that coexpression of Gαi-CFP trafficked RGS8-YFP to the plasma membrane (Fig. 6B, upper two panels), whereas Gα-CFP (Fig. 6B, lower panel) and mem-CFP did not (colocalization data are given in Fig. 6D). Coexpression of GAIP-YFP with Gαi-CFP or Gα-CFP resulted in a similar translocation to the plasma membrane; again this did not occur with mem-CFP (Fig. 6, C and D). RGS7-YFP also showed similar membrane translocation with Gαi-CFP (see below). Finally, the potential for RGS8-YFP to translocate to the plasma membrane after activation of a Gαi-coupled receptor was examined. RGS8-YFP was expressed in HKIR3.1/3.2/D2 cells, and no translocation was observed on application of dopamine (n = 7, Fig. 6E).

To investigate the potential for protein-protein interaction between RGS8 and Gαi in living cells, we performed FRET measurements between RGS8-YFP and Gαi-CFP. By using laser-scanning confocal microscopy, we examined whether there was basal FRET between these two proteins transiently expressed in HEK293 cells. We collected images corresponding to CFP, FRET, and YFP wavelengths in cells expressing RGS8-YFP and Gαi-CFP alone (Fig. 7A, top two panels) or the combination of the two proteins (Fig. 7A, bottom panel). It can be seen from the images (Fig. 7A, top two panels) that there is

**FIG. 4. Varying the concentration of RGS8-YFP.** The HKIR3.1/3.2/MA/RGS8-YFP cell line was established using a four-pronged antibiotic selection strategy (see “Materials and Methods”). This monoclonal isolate was selected for low basal expression of RGS8-YFP and dose-dependent induction of RGS8-YFP expression on treatment with tetracycline (Tet, 24 h prior to imaging or recording). A, the top panel shows laser-scanning confocal images of fields of cells induced with Tet as indicated. Corresponding bright field images of the same field of cells are shown in the lower panel. Very low basal expression of RGS8-YFP (0 Tet) was detected in this cell line. Moderate induction of RGS8-YFP at 0.3 and 1.0 μg/ml Tet shows some membrane localization of the fluorescent protein. At high [Tet] (10 μg/ml), some RGS8-YFP appears to express more prominently in the cell nucleus. B, activation (i) kinetics and deactivation (ii) are summarized here. No significant difference in activation kinetics across treatment groups was detected, although there is a clear trend toward faster activation in the 10 μg/ml Tet group. Deactivation kinetics were significantly faster in HKIR3.1/3.2/M4/RGS8-YFP cells treated with 0.3 Tet and 10 μg/ml Tet.

**FIG. 5. Effect of endogenous RGS proteins.** A, examples of current traces recorded from HKIR3.1/3.2/D2 cells transiently transfected with either Gαi (upper left panel) or RGSIGαi (lower left panel). Dopamine (10 μM) was applied for 20 s as indicated. B, current densities are shown in the bar chart. C, (i), channel activation kinetics are summarized, and (ii), deactivation kinetics on shown. Both are significantly slower in RGSiGoαi-expressing cells.
only modest bleed through of signal into the FRET wavelength. Parameters obtained from such images were used to correct for bleed through using the three-cube method. Fig. 7B (upper panels) shows application of the three-cube method as follows: the “net FRET” image clearly demonstrates that cotransfection of G\textsubscript{\alpha}2\textsubscript{YFP} and RGS8-YFP led to a significant net FRET signal delimited to the plasma membrane. A further confirmation of FRET between G\textsubscript{\alpha}2\textsubscript{YFP} and RGS8 comes from examining acceptor photobleaching on cells cotransfected with G\textsubscript{\alpha}2\textsubscript{YFP} and RGS8-YFP (Fig. 7B, lower panels). Under conditions where FRET occurs, photobleaching the acceptor leads to an increase in donor fluorescence intensity. Our experiments show that intensity of G\textsubscript{\alpha}2\textsubscript{YFP} increases by 11 ± 2% (n = 11) if RGS8-YFP is bleached (RGS8-YFP intensity decreases by 75 ± 4%, n = 11). In addition, under these microscopy conditions, the CFP signal bleaches by ~10% after obtaining each image, and thus the true increase is probably larger. We performed a similar analysis on RGS7-YFP. Coexpression of G\textsubscript{\alpha}2\textsubscript{YFP} CFP with RGS7-YFP led to a significant net FRET signal delimited to the membrane (Fig. 7C).

We also performed experiments with a mutant RGS8-YFP, RGS8(N122H)-YFP. When introduced into RGS4, this mutation had no effect on deactivation kinetics but preserved acceleration of GIRK activation (40) thereby converting RGS4 to act in a kinetic manner equivalent to RGS7. In the HKIR3.1/3.2/GGB cell line, we found that expression of RGS8(N122H)-YFP did not significantly accelerate deactivation, whereas activation was enhanced (deactivated \( \tau \) is 1.8 ± 0.26 s, not significant; \( \text{lag} + \text{tp} = 1.07 ± 0.11 \text{ s} (n = 13) p < 0.05 \)). The RGS8(N122H)-YFP protein was more diffusely distributed in the cytoplasm on coexpression with G\textsubscript{\alpha}2\textsubscript{YFP}, and there was still significant but reduced FRET (FRET ratio = 1.31 ± 0.10, n = 15, p < 0.01).

We next examined whether there was FRET signal between relevant GPCRs and RGS8-YFP indicative of interaction. We first tested the functionality of GPCRs (D2S, A1, a2A, and M4) fused with fluorescent proteins. After transient expression in the HKIR3.1/3.2 cell line, D2-CFP (Fig. 8A), a2A-CFP (not shown), A1-CFP (not shown), and M4-CFP (Fig. 8A) were all able to activate the channel in the presence of the appropriate agonist. Cotransfection of D2-CFP (Fig. 8B), A1-CFP (Fig. 8B), and a2A-CFP (not shown) with RGS8-YFP led to poor delivery of RGS8-YFP to the plasma membrane, and there was no net FRET signal (Fig. 8B).

It is possible to quantify the FRET signal by calculating three-cube FRET ratios from regions of interest defined on the relevant cellular compartment. We first performed control experiments to verify that three-cube methods provide sensitive and non-artifactual detection of FRET. Cells coexpressing CFP and YFP showed no FRET (FRET ratio ~1; Fig. 9). Coexpression of two membrane-localized GPCRs D2-CFP and M4-CFP, which are not thought to form heterodimers (41), also did not show FRET (FRET ratio ~1; top panel of Fig. 8B and Fig. 9).

Cells expressing the CFP-YFP tandem dimer showed a FRET ratio of ~2.5, a positive control that calibrated the dynamic range of the system. Coexpression of RGS8-YFP and G\textsubscript{\alpha}2\textsubscript{YFP} with and without overexpression of G\textsubscript{\beta}1 and G\textsubscript{\gamma}2 and RGS7-YFP.
YFP and Go<sub>A-CFP</sub> gave a FRET ratio of 1.7 to 1.8. Measurement of the FRET ratio after cotransfection of the CFP-tagged GPCRs with RGS8-YFP revealed the value was not different from 1 (lower panels of Figs. 8B and 9).

To test further the validity of these FRET measurements, it was necessary to exclude the possibility that FRET had arisen from random collision of fluorescent species in the membrane (42–44). This phenomenon is known to happen in regions of high fluorophore intensity. Therefore, we plotted the FRET ratio against donor and acceptor intensity per pixel, which is equivalent to the concentration (Fig. 10, A and B). Three-cube measurements show a tendency for increased FRET ratios at higher donor concentration probably because of random collision (44). This trend was present in our data, although at similar intensity levels, the FRET ratio was significantly higher for RGS8-YFP and Go<sub>A-CFP</sub> compared with the two non-interacting membrane proteins, D2-CFP and M4-YFP, expressed at comparable levels but below that for the tandem CFP-YFP dimer (Fig. 10A). The dependence of FRET between RGS8-YFP and Go<sub>A-CFP</sub> on the donor concentration is also likely to reflect complex formation occurring at higher levels of expression. Edidin and co-workers (42, 43) have shown that FRET occurring from stable interaction should result in a FRET ratio that is independent of the acceptor intensity at a fixed donor:acceptor ratio. Indeed there was only a weak correlation between FRET ratio and acceptor intensity for the RGS8-YFP and Go<sub>A-CFP</sub> interaction (Fig. 10B).

The Go<sub>A-CFP</sub> construct contains a mutation to render it resistant to the action of PTx (28). We examined whether RGS8-YFP was able to functionally interact with Go<sub>A-CFP</sub>; overexpression of RGS8-YFP in PTx-treated cells coexpressing Go<sub>A-CFP</sub> led to an acceleration of deactivation and also under these conditions activation kinetics via the Go<sub>2A</sub> receptor (Fig. 11A). Therefore, there is functional coupling between the two fluorescently tagged proteins. To examine whether the FRET signal is dependent on the functional state of the G-protein, we
used a CCD-based microscopy system by which we could more easily perfuse the cell under investigation. As conditions for the excitation of CFP are better optimized on the CCD-based system (see "Materials and Methods"), the dynamic range of the FRET ratio for a given FRET efficiency is greater. For example, the CFP-YFP dimer yielded a FRET ratio of 5.25 ± 0.2 (n = 12), whereas coexpressed CFP/YFP gave a FRET ratio close to 1, and RGSS8-YFP/G,αA-CFP had an FR significantly greater than 1 (Fig. 11B). To encourage the formation of the heterotrimer, we first coexpressed RGSS8-YFP with Gβ1 and Gγ2. The FRET signal between RGSS8-YFP/G,αA-CFP was marginally reduced but was still substantial (Fig. 11B). Equivalent experiments on the confocal microscope yielded a similar result (Fig. 9). Second, we examined for FRET between RGSS8-YFP and Gγ2-CFP (cotransfected with G,αA and Gβ1 for efficient membrane localization). Once again we observed a significant FRET ratio suggesting close proximity of RGSS8-YFP to the assembled heterotrimer (Fig. 11B). The next step was to look at the possible modulation of the FRET signal between G,αA-CFP and RGSS8-YFP by agonist activation of the GPCR. In the D2S and α2A cell lines, FRET ratios were unchanged by activation of the GPCR with the relevant agonist (Fig. 11C).
DISCUSSION

There are two major hypotheses to account for enhancement of GIRK channel activation by RGS proteins: "physical scaffolding" of the GPCR, G-protein, and possibly channel by the RGS protein, or a kinetic mechanism. The physical scaffolding hypothesis has the potential to account for receptor-selective effects and kinetic effects on GIRK channel activation by bringing certain components of this signaling system into closer proximity. In pancreatic acinar cells, RGS4 was found to preferentially inhibit Ca\(^{2+}\) signaling initiated at the M3 receptor compared with the cholecystokinin receptor (45). A scaffolding function for RGS4 has also been proposed in the preassembly of a signaling complex of the M2 receptor, heterotrimeric G-protein and GIRK channel, and the \(\alpha2A\) adrenergic receptor, RGS8 and GIRK channel (15, 18, 19). More recently, it has been proposed that these effects can be explained solely by the GAP activity of RGS proteins: a process termed kinetic scaffolding (7, 20, 21). In this formulation, in an area of high receptor density with a saturating dose of agonist, receptor to G-protein signaling could potentially be attenuated by the rapid depletion of G-protein heterotrimers. By increasing G-protein turnover, the GAP activity of RGS proteins should prevent a rate-limiting depletion of heterotrimeric G-protein. Currently, the only evidence in support of this hypothesis is biochemical: Zhong et al. (21) demonstrated increased binding of GTP\(\gamma\)S to membranes in the presence of RGS4, the \(\alpha2A\) receptor, and agonist.

In our studies there is dissociation between the effects of RGS proteins on activation and deactivation kinetics. The data obtained with the GAP-defective RGS7 is particularly telling; RGS7 is ineffective at enhancing GIRK deactivation, which is consistent with the biochemical data (8), but we found RGS7 to be as effective as RGS8 on activation. Another example is that low concentrations of RGS8-YFP enhance deactivation without having an effect on activation kinetics. Therefore, the effects of RGSs on GIRK activation cannot be explained solely by a kinetic scaffolding mechanism as one would expect a parallel increase in activation and deactivation kinetics. However, we cannot completely exclude a kinetic component from the mechanism of action of RGS proteins. For example, an N-terminally deleted RGS8 construct was found to exert equivalent effects to the full-length protein.\(^2\)

Our data support a novel model that contains many elements of the physical scaffolding hypothesis. We propose that RGS8 and RGS7 can form a stable complex with the G-protein \(\alpha\) subunit at the plasma membrane that is independent of the state of the G-protein. To support this claim, we have performed a series of experiments using FRET microscopy between fluorescently labeled signaling components. Our data indicate that the RGS binds the heterotrimeric G-protein in addition to Go-GTP and transition complex. Furthermore, data obtained in living cells show an interaction that is not competed away by \(\beta\gamma\) overexpression and occurs in the presence and absence of receptor activation. In addition, FRET can occur between the RGS and Go. Given our current methodology, it would be difficult to detect a subtle change in FRET ratio indicative of conformational rearrangement, and this cannot be excluded. In agreement with the findings of other investigators (46), the G-protein \(\alpha\) subunit appears to recruit the RGS (RGS8, RGS7, and GAIP) to the plasma membrane. Recent studies (47) indicate that some activity in the G-protein cycle may be necessary to promote membrane attachment through RGS7 palmitoylation. It is worth emphasizing that the fluorescent Go subunits used here tend to preferentially adopt an inactive conformation (28), and no further increase in translocation or in the FRET signal was observed upon G-protein activation. FRET is purely a distance-dependent phenomenon, and it is possible that intermediary proteins may be involved in supporting the interaction between the RGS and Go subunit.

Our theory goes against the grain of conventional biochemical thinking that RGS proteins only associate with the active Go subunit, with the highest affinity for the transition state of the G-protein and little or no affinity for Go-GDP (6). In addition, from the crystal structure it is clear that the binding sites for RGS and \(\beta\gamma\) overlap on Go in its hydrolytic transition state (48). However, there are some recent indirect indications that other RGS proteins may be able to interact more weakly with other states of the G-protein (49, 50). Therefore, it is plausible that the interaction we observe between RGS8 and

\(^2\)A. Benians and A. Tinker, unpublished observations.
the heterotrimeric G-protein may be weaker in affinity and may occur over different domains of both proteins. Other investigators (50) have detected an ability of the M2 receptor to traffic RGS2 and RGS4 to the plasma membrane. We were unable to observe a direct interaction between three different GPCRs (H2A-CFP, D2-CFP, and A1-CFP) and RGS8-YFP. Thus, at least for RGS8, scaffolding with the receptor appears to only occur by virtue of interaction with the G-protein.

The second important point to our model is the role that the RGS may have in shaping the behavior of the ternary complex. Although acceleration of deactivation kinetics (using an appropriate agonist) was an invariable feature of RGS8 overexpression, enhancement of activation kinetics only occurred with a subset of receptors. We have shown previously that the particular combination of agonist/receptor/G-protein can have a profound influence on the channel activation kinetics (24). Activation kinetics with a saturating dose of agonist at the D2S, GABA-B1b/2, and M4 receptors was accelerated by RGS8 whereas that via A1 and a2A receptors was not. Closer inspection of the data reveals that activation via A1 and a2A is intrinsically fast. Our interpretation is that activation kinetics essentially reach a limiting value that may or may not require RGS, depending on the receptor. Hence, we propose that the intrinsically rapid kinetics seen with the A1 and a2A receptors reflect an efficient ternary complex of agonist, receptor, and G-protein. Essentially the kinetic efficacy cannot be improved upon. In the case of the D2, M4, and GABA-B receptors, in which the ternary complex displays less kinetic efficacy, RGS8-YFP strengthens it. Our data show that with all five receptors the RGS participates by direct protein-protein interaction in the ternary complex with the G-protein to form a quaternary complex; it is just the functional consequences that differ. We have shown previously that endogenous RGSs may significantly influence receptor-mediated GIRK channel activation and deactivation kinetics (30), and this unavoidable complication should be borne in mind in the interpretation of receptor-selective effects. Such factors may explain inconsistencies between different expression systems.

This complex formation, accounting for the acceleration of activation kinetics, may only occur at higher RGS8 and G-protein expression levels as both the functional effects (Fig. 4) and FRET ratio (Fig. 10A) are dependent on concentration. That the level of expression should influence signaling is important. There is evidence that RGS concentrations may be dynamically regulated by intracellular signaling pathways (51), in a use-dependent manner by persistent activation of a
GPCR or in disease states, for example (52). How this relates to signaling in neurons and cardiac cells, in which these signaling components may be clustered in specialized microdomains at high concentration, is intriguing. Furthermore, whether all RGSs behave in such a fashion is an important topic for future investigation.

In our view, it is the ternary complex that matters and not just the receptor. In particular, we have examined the role of the G-protein a subunit. At one level, RGS8 receptor selectivity does not reflect G-protein coupling profiles (23) as the pattern of receptor selectivity is unaffected when signaling is constrained through a single G-protein isoform (as shown in Fig. 3). However, the data are more subtle than this. In Fig. 1A, we make the observation that when the o2A receptor is constrained to signal through Goa, the activation kinetics are slower and now become modulated by RGS-YFP. In addition, the GABA1b/2 receptor activates current more slowly via Gi and RGS8-YFP accelerates this, but the absolute rates of activation differ. These observations all support the idea that it is the conformation of the signaling complex that dictates the rate of activation, and this is uniquely dependent on the nature of all of the agonist, receptor, G-protein, and associated RGS. These ideas are coherent with much of the recent signaling literature (25, 26). It is also interesting that for the o2A and A1 receptors, with which we see intrinsically fast activation, are those for which receptor-G-protein “prechanging” has been described (53–56). The kinetic consequences of this should be to intrinsically accelerate the activation process. A further feature is that it opens the possibility that different RGSs may have differential effects on activation and deactivation kinetics. For example, it has been shown here (Fig. 1) and previously (57) that RGS7 is particularly effective at accelerating activation kinetics.

In summary, our data reveal that the RGS can enter into the ternary complex to form a quaternary complex, thus accounting for the behavior of accelerated activation kinetics. Our data contribute to an emerging picture in which channel, G-protein, GPCR, and now RGS may all be scaffolded into a macromolecular signaling complex (22–24, 28–61). Furthermore, they illustrate the central role of the Gα subunit in organizing the specificity and dynamics of signaling to the G-protein-gated inwardly rectifying K+ channel.