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# Endogenous Regulators of G Protein Signaling Differentially Modulate Full and Partial $\mu$-Opioid Agonists at Adenylyl Cyclase as Predicted by a Collision Coupling Model

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## ABSTRACT

Regulator of G protein signaling (RGS) proteins accelerate the endogenous GTPase activity of $G\alpha_{i/o}$ proteins to increase the rate of deactivation of active G$\alpha$-GTP and G$\beta\gamma$ signaling molecules. Previous studies have suggested that RGS proteins are more effective on less efficiently coupled systems such as with partial agonist responses. To determine the role of endogenous RGS proteins in functional responses to $\mu$-opioid agonists of different intrinsic efficacy, $G\alpha_{i/o}$ subunits with a mutation at the pertussis toxin (PTX)-sensitive cysteine (C351I) and with or without a mutation at the RGS binding site (G184S) were stably expressed in C6 glioma cells expressing a $\mu$-opioid receptor. Cells were treated overnight with PTX to inactivate endogenous G proteins. Maximal inhibition of forskolin-stimulated adenylyl cyclase by the low-efficacy partial agonists buprenorphine and nalbuphine was increased in cells expressing RGS-insensitive $G\alpha_o^{CIGS}$, $G\alpha_{i2}^{CIGS}$, or $G\alpha_{i3}^{CIGS}$ compared with their $G\alpha^{CI}$ counterparts, but the RGS-insensitive mutation had little or no effect on the maximal inhibition by the higher efficacy agonists DAMGO and morphine. The potency of all the agonists to inhibit forskolin-stimulated adenylyl cyclase was increased in cells expressing RGS-insensitive $G\alpha_o^{CIGS}$, $G\alpha_{i2}^{CIGS}$, or $G\alpha_{i3}^{CIGS}$, regardless of efficacy. These data are comparable with predictions based on a collision coupling model. In this model, the rate of G protein inactivation, which is modulated by RGS proteins, and the rate of G protein activation, which is affected by agonist intrinsic efficacy, determine the maximal agonist response and potency at adenylyl cyclase under steady state conditions.

Regulator of G protein signaling (RGS) proteins negatively modulate signaling by receptors coupled to the $G_{i/o}$ and $G_q$ family of G proteins by accelerating the endogenous GTPase activity of activated GTP-bound G$\alpha$ subunits. This speeds the return to the inactive GDP-bound G$\alpha$ with subsequent recoupling to, and inactivation of, the $\beta\gamma$ subunits. As a result of the GTPase accelerating protein (GAP) activity on $G\alpha_{i/o}$, RGS proteins reduce maximal agonist inhibition of adenylyl cyclase (Huang et al., 1997; Clark et al., 2003; Ghavami et al., 2004), maximal agonist stimulation of mitogen-activated protein kinase and Akt phosphorylation (Clark et al., 2003; Huang et al., 2006) and increase deactivation and activation kinetics of GIRK currents (Doupnik et al., 1997). RGS proteins also decrease agonist potency for inhibition of adenylyl cyclase (Clark et al., 2003), stimulation of mitogen-activated protein kinase phosphorylation (Clark et al., 2003), and inhibition of calcium channels (Jeong and Ikeda, 2000). Although the above studies mostly have used full agonists, there is evidence that RGS proteins have a greater effect on signaling mediated by partial agonists with reduced intrinsic efficacy, which activate receptors and cognate signaling pathways less efficiently (Clark et al., 2003).

The effect of G protein cycle kinetics on agonist potency and maximal response at adenylyl cyclase has been formulated for $G\alpha_s$ by Stickle and Barber (1992) and Whaley et al. (1994) based on the collision coupling model (Tolkovsky and Levitzki, 1978), which included parameters for the G protein cycle of GDP-GTP exchange and GTP hydrolysis. According to this model, both the fractional activation of adenylyl cyclase and the agonist potency at steady state are dependent on the rate constant for G protein activation, the rate constant for inactivation, and the number of receptors. The activation rate constant is dependent on the collision frequency between receptor and G protein (a function of diffu-

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**ABBREVIATIONS:** RGS, regulator of G protein signaling; GAP, GTPase accelerating protein; PTX, pertussis toxin; [$^{35}$S]GTP$\gamma$S, 5′-*O*-(3-[$^{35}$S]thiotriphosphate); IBMX, 3-isobutyl-1-methylxanthine; C6$\mu$, C6 cells stably expressing the $\mu$-opioid receptor; HEK, human embryonic kidney; HEK293$\mu$, HEK293 cells stably expressing the $\mu$-opioid receptor; DAMGO, [D-Ala$^2$,*N*-Me-Phe$^4$,Gly$^5$-ol]enkephalin; GIRK channels, G protein-gated inwardly rectifying $K^+$ channels; CI, RGS-sensitive clone; CIGS, RGS-insensitive clone.

sivity and the numbers of molecules), receptor occupancy, and agonist intrinsic efficacy (Whaley et al., 1994; Krumins et al., 1997). The inactivation rate constant is dependent on the rate of GTP hydrolysis, which is modulated by RGS proteins, thus providing a simple model for predicting the effects of RGS proteins on agonist responses.

In this study, we tested the hypothesis that endogenously expressed RGS proteins affect maximal agonist response and potency in a manner consistent with the collision coupling model and that this explains the differential sensitivity of full and partial agonists to RGS action. Moreover, because different $G\alpha_{i/o}$ subtypes may have different rate constants for activation and inactivation by agonist-occupied receptors and RGS proteins may differentially affect the $G\alpha$ subtypes because of their different GAP activities (Posner et al., 1999; Posner et al., 1999; Cavalli et al., 2000; Welsby et al., 2002; Hooks et al., 2003), we also test the hypothesis that the endogenous RGS proteins may be more effective at $G\alpha_o$ than the $G\alpha_i$ subtypes.

By expressing $G\alpha_{i/o}$ subunits with a mutation at the pertussis toxin (PTX)-sensitive cysteine (C351I) in C6 glioma cells expressing the μ-opioid receptor, we are able to study the action of the exogenously expressed $G\alpha$ subtype on inhibition of adenylyl cyclase after treatment with PTX to uncouple the endogenous $G\alpha$ subunits. There is evidence for the expression of certain RGS proteins in C6 cells (Snow et al., 2002), but a full description is not available. Therefore, to examine the effects of the GAP activity of the total complement of endogenous RGS proteins present in C6 cells, we expressed the PTX-insensitive $G\alpha$ subunits with or without an additional mutation at the RGS binding site (G184S). This prevents binding between $G\alpha$ and the RGS box of all RGS proteins without affecting the endogenous GTPase activity of the $G\alpha$. This eliminates RGS-stimulated GTPase-activity (Clark et al., 2003; Huang et al., 2006), resulting in an accumulation of active $G\alpha$-GTP and $G\beta\gamma$ signaling molecules, which is observed as an increase in agonist potency and/or an increase in the maximal agonist effect (Clark et al., 2003).

Our results demonstrate that, as predicted by the collision coupling model, endogenous RGS proteins have a greater effect on the maximal effect of partial μ-opioid agonists than full agonists at inhibition of adenylyl cyclase but similarly reduce agonist potency for both full and partial agonists.

## Materials and Methods

**Materials.** [$^3$H]Diprenorphine and [$^{35}$S]GTPγS were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). Tissue culture medium, fetal bovine serum, trypsin, LipofectAMINE Plus reagent, LipofectAMINE 2000, G-418 (Geneticin), and Zeocin were from Invitrogen (Carlsbad, CA). Trizma, GDP, forskolin, 3-isobutyl-1-methylxantihine (IBMX), DAMGO, and other biochemicals were from Sigma Chemical Co (St. Louis, MO). Morphine, buprenorphine, nalbuphine, and naloxone were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI).

**RGS-Insensitive Mutation and FLAG-Epitope Tagging.** Human $G\alpha$ containing a cysteine-to-isoleucine mutation at the PTX-sensitive Cys ($G\alpha_{oA}^{C351I}$, $G\alpha_{i2}^{C352I}$, $G\alpha_{i3}^{C351I}$) in the pcDNA3.1+ vector were purchased from the Missouri University of Science and Technology cDNA Resource Center (http://www.cdna.org). For insertion into the Zeocin-resistant vector, pcDNAzeo$^-$, the restriction enzymes Nhe I and Xba I (Promega, Madison, WI) were used to remove $G\alpha_{oA}^{C351I}$ or $G\alpha_{i3}^{C351I}$ from the original vector and Pme I (New England Biolabs, Ipswich, MA) was used for $G\alpha_{i2}^{C352I}$, followed by ligation with calf intestinal alkaline phosphatase-treated pcDNAzeo$^-$ vector. A QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) was used to replace the glycine at the RGS binding site ($G\alpha_oA$, $G\alpha_{i2}$: G184S; $G\alpha_{i3}$: G183S) to a serine using the following primers: 5′-GGGTCAAAACCACTTCCATCGTAGAAAC-3′ for $G\alpha_{oA}^{C351I}$, 5′-GTAAAGACCACGTCGATCGTGGAG-3′ for $G\alpha_{i2}^{C352I}$, and 5′-CGAGAGTGAAGACCACATCCATTGTAGAAAC-3′ for $G\alpha_{i3}^{C351I}$ (underlining indicates mutation sites).

A FLAG tag was added to the N terminus of PTX-insensitive $G\alpha$ in the Zeocin-resistant vector using the QuikChange site-directed mutagenesis kit with the following primers: for $G\alpha_{oA}^{C351I}$, 5′-CCCTTGATCGGTACCACCATGGACTACAAAGACGATGACGACAAG-ATGGGATGTACTCTG-3′; for $G\alpha_{i2}^{C352I}$, 5′-CCCTTGATCGGTACCACCATGGACTACAAAGACGATGACGACAAG-ATGGGCT GCACCGTG-3′; and for $G\alpha_{i3}^{C351I}$ 5′-CCCTTGATCGGTACCACCATGGACTACAAAGACGATGACGACAAG-ATGGGCTGCACGTTG-3′.

**Cell Culture and Expression of PTX-Insensitive Gα.** Human μ-opioid receptor in the pcDNA3.1 vector (http://www.cdna.org) was stably transfected into HEK293 cells using LipofectAMINE 2000 and selected using 0.8 mg/ml G-418. C6 cells stably expressing the μ-opioid receptor (C6μ; Emmerson et al., 1996) or HEK293 cells stably expressing the μ-opioid receptor (HEK293μ) were stably transfected with PTX-insensitive $G\alpha$ using LipofectAMINE Plus and selected using 0.4 mg/ml Zeocin for C6μ or 0.2 mg/ml Zeocin for HEK293μ. Selected clones were maintained in DMEM containing 10% fetal bovine serum, 0.4 mg/ml (HEK293μ) or 0.25 mg/ml (C6μ) G-418, and 0.2 mg/ml (HEK293μ) or 0.4 mg/ml (C6μ) Zeocin.

**Membrane Preparation.** Cells were treated overnight with 100 ng/ml PTX before harvesting and preparation of plasma membranes as described previously (Clark et al., 2003).

**Western Blots.** Membrane proteins (5 μg of HEK293μ-FLAG$G\alpha^{CI}$; 60 μg of C6μ$G\alpha^{CI/CIGS}$) were separated by 12% SDS-polyacrylamide gel electrophoresis (Protogel; National Diagnostics, Inc., Atlanta, GA) and transferred to a nitrocellulose membrane (45 μm; Pierce, Rockford, IL). Membranes with C6μ $G\alpha^{CI/CIGS}$ were blocked overnight with 5% dry milk and probed with 1:200 anti-$G\alpha_o$ (Santa Cruz Biotechnology, Santa Cruz, CA) or 1:6000 anti-$G\alpha_{i2}$ (Calbiochem, San Diego, CA), followed by 1:15,000 goat anti-rabbit IgG-HRP in 5% dry milk (Santa Cruz Biotechnology). Membranes with HEK293μ-FLAG$G\alpha^{CI}$ were blocked overnight with 5% dry milk and probed with 1:2000 anti-M2 FLAG and 1:1000 anti-tubulin (Sigma Chemical Co., St. Louis, MO) in 5% dry milk followed with 1:12,000 goat anti-mouse IgG-HRP in 5% dry milk (Santa Cruz Biotechnology). Bands were visualized using Super Signal West Pico enhanced chemiluminescence (Pierce) and quantified using a KODAK Image Station (Carestream Health, Rochester, NY). We were unable to find a satisfactory antibody to a region of $G\alpha_{i3}$ without the PTX- or RGS-insensitive mutations.

**Receptor Binding Assay.** Membranes (5–20 μg) were incubated with 2 to 3 nM [$^3$H]diprenorphine with or without 50 μM naloxone (to determine nonspecific binding) in 50 mM Tris-HCl, pH 7.4, as described previously (Clark et al., 2003), to determine receptor number.

**[$^{35}$S]GTPγS Binding Assay.** Membranes (5–20 μg) were incubated for 60 min with 0.1 nM [$^{35}$S]GTPγS, 30 μM GDP, 100 mM NaCl, 5 mM $MgCl_2$,1 mM dithiothreitol, 20 mM Tris-HCl, pH 7.4, and 0.01 to 10 μM DAMGO or distilled water and filtered as described previously (Clark et al., 2003) to determine bound [$^{35}$S]GTPγS.

**cAMP Accumulation Assay.** Cells were plated to ~80% confluence 2 days before the assay and treated with 100 ng/ml PTX. To start the assay, medium was replaced with DMEM containing 5 μM forskolin, 1 mM IBMX, and 0.001 to 10 μM DAMGO, morphine, nalbuphine, buprenorphine, or distilled water. After 10 min at 37°C, the reaction was stopped by replacing the medium with ice-cold 3% perchloric acid. After at least 30 min at 4°C, 0.4 ml was removed from each sample, neutralized with 0.08 ml of 2.5 M $KHCO_3$, vortexed, and centrifuged at 15,000*g* for 1 min. A 20-μl aliquot was taken from

the supernatant of each sample, and accumulated cAMP was quantified using a [$^3$H]cAMP assay kit according to manufacturer's instructions (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). Inhibition was determined as the percentage decrease of forskolin-stimulated cAMP accumulation in the absence of opioid agonist.

**Data Analysis.** Concentration-effect data from [$^{35}$S]GTPγS binding and cAMP accumulation assays were fitted to sigmoidal concentration-effect curves using Prism 4.0 (to determine $EC_{50}$ values and maximal effects) (GraphPad Software, San Diego, CA). Data are presented as means ± S.E.M. from at least three separate experiments and are compared using two-tailed Student's $t$ test.

## Results

**Collision Coupling Model Predictions of Maximal Agonist Response and Potency with and without RGS Proteins.** The prediction of the collision coupling model for the maximum agonist response at an effector, $f$, is

$$f = \frac{k_a R_{TOT} f_{active}}{k_{hydrol} + k_a R_{TOT} f_{active}} \quad (1)$$

where $k_a$ is the rate constant for activation, $R_{TOT}$ is the total number of receptors, $f_{active}$ is the fraction of bound receptors in the active form (a measure of agonist intrinsic efficacy), and $k_{hydrol}$ is the rate constant for hydrolysis of Gα-GTP (Kinzer-Ursem et al., 2006; see Appendix for details). For simplicity of notation, we define an overall activation rate constant $k_{act}$ as:

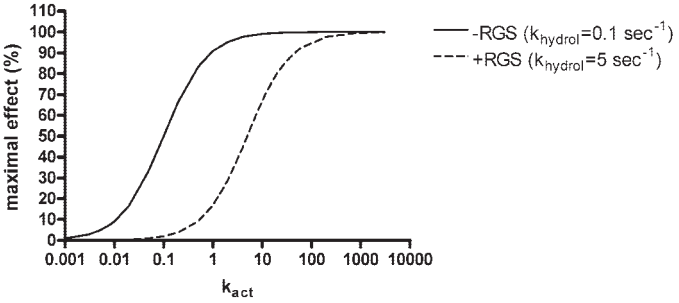



**Fig. 1.** Predicted effect of RGS proteins on the relationship between the overall activation rate constant ($k_{act}$) and agonist inhibition of adenylyl cyclase. The maximal effect (%) at adenylyl cyclase was plotted for a range of values of $k_{act}$ (eqs. 1 and 2) with the rate constant for inactivation by hydrolysis ($k_{hydrol}$) as 0.1 $s^{-1}$ in the absence of RGS proteins and 5 $s^{-1}$ in the presence of RGS proteins.

$$k_{act} = k_a R_{TOT} f_{active} \quad (2)$$

Thus, the activation level of the effector (maximal response) is determined by the overall activation rate constant $k_{act}$ and the hydrolysis rate constant $k_{hydrol}$. To predict the effect of RGS proteins on the maximum agonist response, we used the estimated values for rate constants of hydrolysis ($k_{hydrol}$) of 0.1 $s^{-1}$ in the absence of RGS proteins and 5 $s^{-1}$ in the presence of RGS proteins (Shea et al., 2000). The value of the rate constant for activation, $k_a$, can be estimated from Monte Carlo simulations of receptor and G protein diffusion to be on the order of $10^{-5}$ to $10^{-3}$ (number per cell)$^{-1}$s$^{-1}$ (Mahama and Linderman, 1994; Shea et al., 1997) and the units of $R_{TOT}$ are (number per cell). Values for maximal response were calculated for a range of values for the overall activation rate constant ($k_{act}$) (Fig. 1). When $k_{hydrol}$ is small relative to $k_{act}$, then agonist response approaches 100% and when $k_{hydrol}$ is larger relative to $k_{act}$, then agonist effect approaches zero. Thus, the model predicts that RGS proteins will reduce agonist response most effectively when $k_{hydrol}$ is similar to $k_{act}$. Partial agonists have previously been shown to have slower activation rates than full agonists due to the activation of fewer receptors (Traynor et al., 2002). Therefore, the model predicts that RGS proteins will have less effect on the maximal effect of full agonists than partial agonists.

The prediction of the collision coupling model for potency is

$$EC_{50} = \frac{k_{hydrol} K_D}{k_{hydrol} + k_a R_{TOT} f_{active}} \quad (3)$$

(see Appendix for details). The effect of RGS proteins on agonist potency is predicted using the estimated values for rates of inactivation ($k_{hydrol}$) of 0.1 $s^{-1}$ in the absence of RGS proteins and 5 $s^{-1}$ in the presence of RGS proteins. Values for potency ($EC_{50}$) were calculated for a $K_d$ of 250 nM with a range of values for the overall activation rate constant ($k_{act}$) (Fig. 2). This model predicts that RGS proteins will reduce potency (increase $EC_{50}$) across a wide range of $k_{act}$ values, but when $k_{act}$ is small relative to $k_{hydrol}$, which would occur for example with a very-low-efficacy agonist, then $EC_{50}$ approaches the $K_d$ and RGS proteins will have diminished effect on agonist potency. Therefore, the potency of full agonists could be expected to be decreased by RGS proteins similarly to or more than partial agonists, depending on the rate of activation.

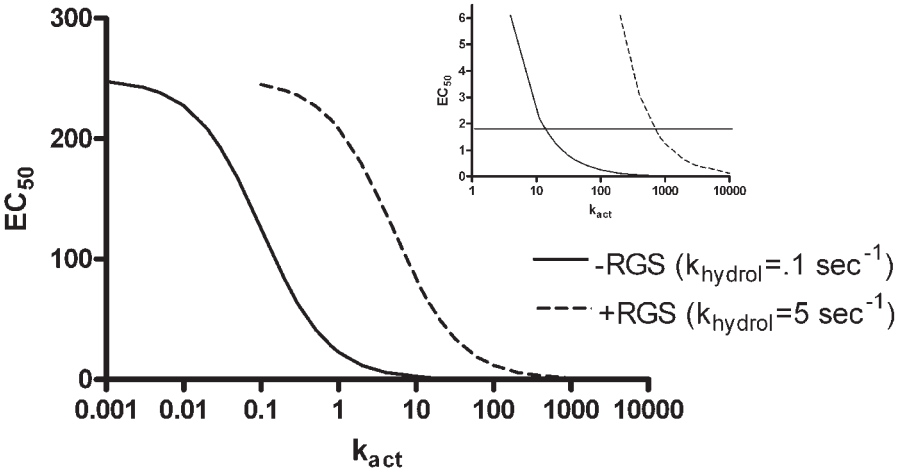



**Fig. 2.** Predicted effect of RGS proteins on the relationship between the overall activation rate constant ($k_{act}$) and agonist potency at adenylyl cyclase. Agonist potency was plotted for a range of rate constants of G protein activation (eqs. 2 and 3) with the agonist $K_d$ set at 250 nM and the rate constant for inactivation by hydrolysis ($k_{hydrol}$) as 0.1 $s^{-1}$ in the absence of RGS proteins and 5 $s^{-1}$ in the presence of RGS proteins.

To test this model, we have studied RGS modulation of μ-opioid inhibition of adenylyl cyclase. Because we do not know which RGS proteins are involved in opioid inhibition of adenylyl cyclase and there are no inhibitors of RGS proteins available, we have made use of RGS-insensitive $G\alpha_{i/o}$ proteins to prevent all RGS protein activity. To avoid a potential confound due to effects of receptor and G protein levels, we have compared sets of clones expressing similar levels of receptor and similar levels of PTX-insensitive Gα with or without RGS sensitivity.

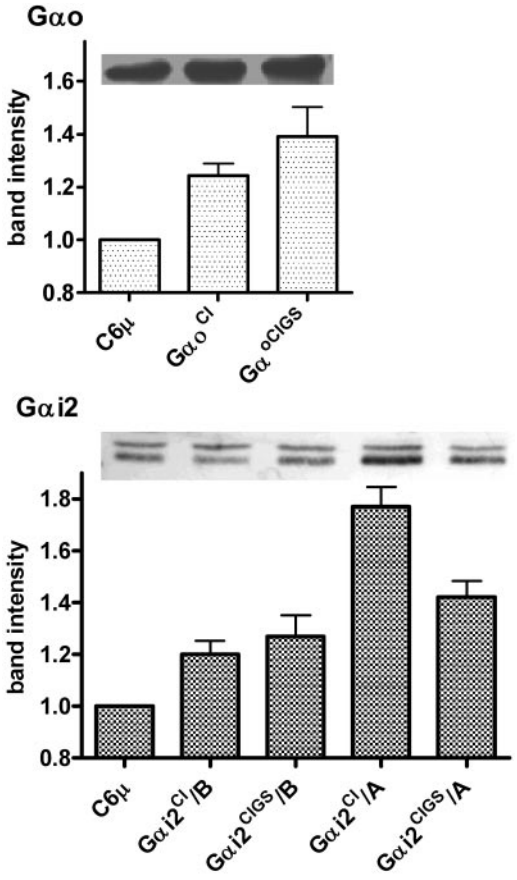



**Fig. 3.** Expression levels of PTX-insensitive Gα in C6μ cells. Membranes were prepared from untransfected C6μ cells or C6μ cells stably expressing PTX-insensitive $G\alpha_{i2}{}^{CI}$ or $G\alpha_{o}{}^{CI}$ with or without the RGS-insensitive mutation (GS) and subjected to Western blot analysis as described under *Materials and Methods*. Shown is the mean intensity of each band(s) relative to the intensity of the band(s) for the untransfected cells performed on the same blot ($n = 3$). Representative blots for $G\alpha_{i2}$ and $G\alpha_{o}$ are shown. Note that C6 cells endogenously express mainly $G\alpha_{i2}$ (Charpentier et al., 1993).

**Gα Expression Levels and [$^{35}$S]GTPγS Binding.** Treatment of C6μ cells with 100 ng/ml PTX overnight has been shown to inactivate the endogenous $G\alpha_{i/o}$ (Clark et al., 2003, 2006) so that only the activity of the exogenously expressed PTX-insensitive $G\alpha_{i/o}$ may be examined. In this study, all cells were treated overnight with PTX to uncouple endogenous $G\alpha_{i/o}$ from the receptors. C6 glioma cells stably expressing the μ-opioid receptor (C6μ) and either PTX-insensitive $G\alpha^{CI}$, or PTX- and RGS-insensitive $G\alpha^{CIGS}$ were selected for similar Gα expression levels (Fig. 3) and receptor expression levels (Table 1), so that the rate of G protein activation will be affected only by agonist intrinsic efficacy and concentration. Levels of expression of different $G\alpha_{i/o}$ mutants within a $G\alpha_{i/o}$ subtype and identified by the same antibody can be compared across clones, but comparisons cannot be made across $G\alpha_{i/o}$ subtypes. In addition, C6 cells express mainly $G\alpha_{i2}$ and little $G\alpha_{o}$ (Charpentier et al., 1993), so the antibody recognizes both the endogenous $G\alpha_{i2}$ and the transfected $G\alpha_{i2}$, making quantification by Western blot difficult. Therefore, we also compared maximal levels of DAMGO-stimulated [$^{35}$S]GTPγS binding, which correlates with the level of functional Gα expression (Clark et al., 2006). DAMGO stimulated a large maximal increase in [$^{35}$S]GTPγS binding in membranes from cells expressing the mutant $G\alpha_{i2}$ or $G\alpha_{o}$ proteins confirming a significant level of expression, although slightly lower than that seen in wild-type cells (Table 1). It is noteworthy that DAMGO-stimulated [$^{35}$S]GTPγS binding was the same in both RGS-sensitive and -insensitive clones matched for $G\alpha_{i2}$ or $G\alpha_{o}$ levels supporting the Western blot data. $G\alpha_{i3}{}^{CI}$ or $G\alpha_{i3}{}^{CIGS}$ did not express well in the C6μ cells, but the set of matched clones with similar receptor levels gave low maximal levels of DAMGO-stimulated [$^{35}$S]GTPγS binding (Table 1). There is the possibility that factors other than Gα expression and receptor levels could differ between the clones, most pertinently the level and type of RGS proteins. This is difficult to assess because of the large number of known RGS proteins. However, our method compares clones expressing the same Gα subtype that is

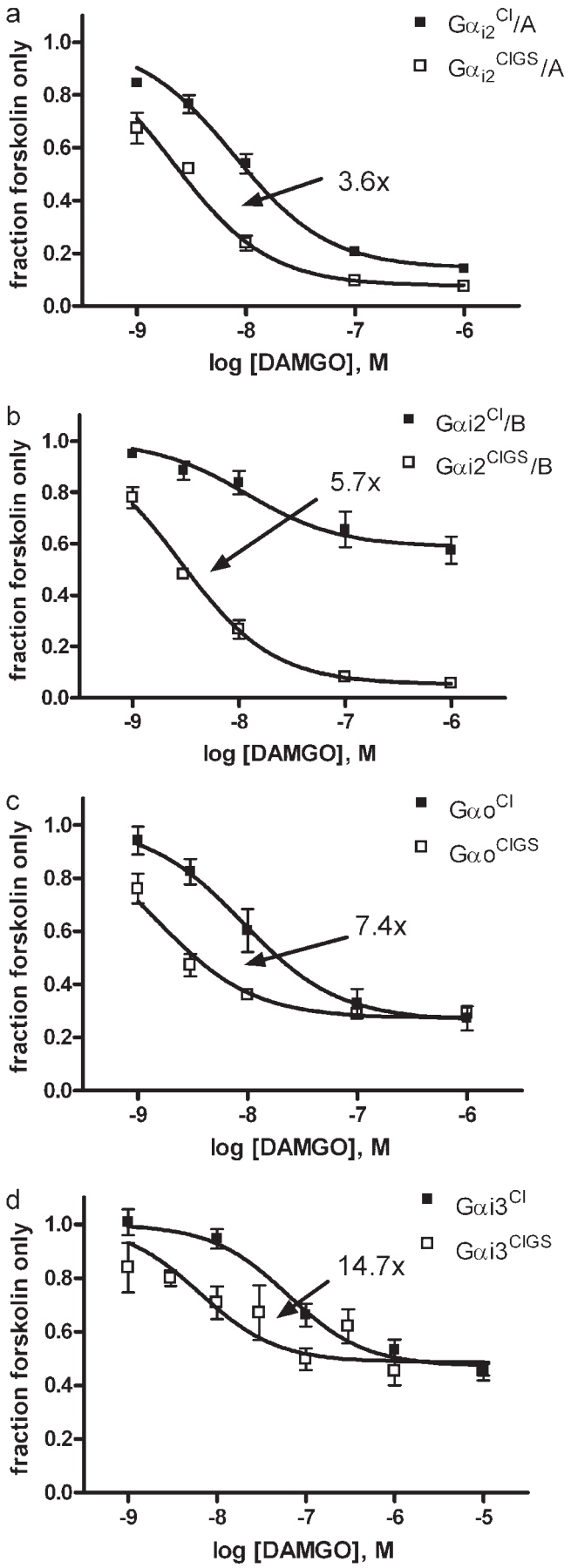
TABLE 1

Receptor number and [$^{35}$S]GTPγS binding in cells expressing RGS-sensitive and -insensitive Gα proteins

RGS-sensitive (CI) and RGS-insensitive (CIGS) clones were matched for Gα expression levels and receptor number. [$^{3}$H]diprenorphine and [$^{35}$S]GTPγS binding in C6μ cells stably expressing PTX-insensitive Gα with or without the RGS-insensitive mutation were measured as described under *Materials and Methods* and given as the mean ± S.E.M. from at least three separate experiments.

| Cells | Receptor Number | DAMGO-Stimulated [$^{35}$S]GTPγS Binding | | Basal [$^{35}$S]GTPγS |
|---|---|---|---|---|
| | | Maximal Stimulation | $EC_{50}$ | |
| | *pmol/mg of protein* | *%* | *nM* | *fmol bound/mg protein* |
| C6μ[a] | 3.2 ± 0.2 | 485 ± 76[b] | 51 ± 12 | 33 ± 5[b] |
| $G\alpha_{i2}{}^{CI}$/A | 5.8 ± 0.3 | 350 ± 100 | 40 ± 10 | 18 ± 2 |
| $G\alpha_{i2}{}^{CIGS}$/A | 6.6 ± 0.4 | 380 ± 80 | 53 ± 6 | 17 ± 1 |
| $G\alpha_{i2}{}^{CI}$/B | 5.9 ± 0.5 | 230 ± 40 | 53 ± 10 | 18 ± 2 |
| $G\alpha_{i2}{}^{CIGS}$/B | 4.8 ± 1.1 | 210 ± 30 | 64 ± 19 | 22 ± 3 |
| $G\alpha_{o}{}^{CI}$ | 4.4 ± 0.3 | 210 ± 10 | 46 ± 15 | 25 ± 1 |
| $G\alpha_{o}{}^{CIGS}$ | 3.7 ± 0.6 | 190 ± 10 | 40 ± 19 | 22 ± 4 |
| $G\alpha_{i3}{}^{CI}$ | 6.2 ± 1.6 | 62 ± 7 | 7.5 ± 2.4 | 19 ± 2 |
| $G\alpha_{i3}{}^{CIGS}$ | 4.5 ± 1.4 | 32 ± 6* | 9.1 ± 2.4 | 18 ± 2 |

* $P < 0.05$ compared to RGS-sensitive $G\alpha_{i3}{}^{CI}$ clone.
[a] Taken from Clark et al. (2006).
[b] In the presence of PTX, DAMGO stimulation of [$^{35}$S]GTPγS binding is completely abolished (0.8 ± 2.7%), and the basal binding is reduced to 20 ± 2 fmol/mg of protein.




either PTX-insensitive or RGS- and PTX-insensitive; thus, we compare a clone with a full complement of functional RGS proteins with a clone in which the GAP activity of all of these proteins has been negated by the RGS-insensitive mutation in the appropriate Gα protein.

The potency of DAMGO to stimulate [$^{35}$S]GTPγS binding was unaffected by the Gα expression level or the RGS-insensitive mutation in any of the Gα subtypes tested (Table 1). Although not matched for Gα expression across clones, the potency of DAMGO to stimulate [$^{35}$S]GTPγS binding in the $G\alpha_{i3}^{CI}$ expressing cells was higher than in the $G\alpha_{o}^{CI}$ and $G\alpha_{i2}^{CI}$ expressing cells (Table 1). This has been demonstrated previously (Clark et al., 2006).

**μ-Opioid Agonist Inhibition of Forskolin-Stimulated cAMP Accumulation.** Maximal inhibition of 5 μM forskolin-stimulated cAMP accumulation by the full agonist, DAMGO, was significantly higher with the RGS-insensitive mutation in the cells expressing either matched pair of $G\alpha_{i2}^{CI}$ and $G\alpha_{i2}^{CIGS}$ (Fig. 4, Table 2). The potency of DAMGO to inhibit forskolin-stimulated cAMP accumulation was also increased (Fig. 4). The potency and efficacy of DAMGO to stimulate [$^{35}$S]GTPγS binding in the matched $G\alpha_{i2}^{CI}$ and $G\alpha_{i2}^{CIGS}$ clones were the same (Table 1), which confirms an effect of endogenous RGS proteins in decreasing the concentration of the active Gα-GTP. In contrast, in cells expressing $G\alpha_{o}^{CI}$ or $G\alpha_{o}^{CIGS}$, the maximal inhibition of forskolin-stimulated cAMP accumulation by DAMGO was not affected by the RGS-insensitive mutation (Fig. 4c). The potency of DAMGO, however, was increased by the RGS-insensitive mutation in $G\alpha_{o}^{CI}$-expressing cells (Fig. 4c). Likewise, the RGS-insensitive mutation increased DAMGO potency in cells expressing $G\alpha_{i3}^{CI}$ without affecting maximal inhibition of forskolin-stimulated cAMP accumulation (Fig. 4d). The increase in DAMGO potency with or without an increase in maximal response with the RGS-insensitive mutation agrees with the predictions based on the collision coupling model under conditions of a fast $k_{act}$. Although the cells were matched within Gα subtypes, there was a difference in the level of forskolin-stimulated cAMP between the matched RGS-sensitive and -insensitive clones, which was especially noticeable for clones

**Fig. 4.** DAMGO inhibition of forskolin-stimulated adenylyl cyclase. C6μ cells stably expressing PTX-insensitive Gα were plated onto 24-well plates and treated with 100 ng/ml PTX for 2 days before assay. The media was replaced with serum-free DMEM containing 5 μM forskolin, 1 mM IBMX, and 0 to 10 μM DAMGO to start the assay, which was allowed to proceed for 10 min at 37°C before termination by replacing the assay media with 3% perchloric acid. Accumulation of cAMP was measured as described under *Materials and Methods* in C6μ cells expressing PTX-insensitive ($G\alpha^{CI}$, closed symbols) or PTX- and RGS-insensitive ($G\alpha^{CIGS}$, open symbols). a, $G\alpha_{i2}$ (clone A) at higher levels; b, $G\alpha_{i2}$ (clone B) at lower levels; c, $G\alpha_{o}$; d, $G\alpha_{i3}$. The accumulated cAMP level in the presence of DAMGO was normalized as a fraction of the cAMP level in the absence of DAMGO for that assay (mean ± S.E.M; $n = 3$ in duplicate). Arrows and accompanying values indicate the degree of shift in the DAMGO concentration effect curves measured as the $EC_{50}$ values. Forskolin-stimulated cAMP for the RGS-sensitive and -insensitive clones, respectively, were (picomoles of cAMP per microgram of protein): 2.2 ± 0.3 and 2.6 ± 0.4 for $G\alpha_{i2}^{CI/CIGS}$/A; 1.0 ± 0.1 and 3.8 ± 0.1 for $G\alpha_{i2}^{CI/CIGS}$/B; 1.7 ± 0.2 and 3.2 ± 0.5 for $G\alpha_{o}^{CI/CIGS}$; or 1.0 ± 0.2 and 1.1 ± 0.1 for $G\alpha_{i3}^{CI/CIGS}$). Alternative $G\alpha_{o}$ clones matched for their forskolin stimulated level of cAMP ($G\alpha_{o}^{CI}$ 0.9 ± 0.08 pmol cAMP/μg of protein; $G\alpha_{o}^{CIGS}$ 1.2 ± 0.1 pmol cAMP/μg of protein) gave similar maximal inhibition by DAMGO ($G\alpha_{o}^{CI}$, 40.2%; $G\alpha_{o}^{CIGS}$, 37 ± 4%), but DAMGO was 8.4 times more potent in the $G\alpha_{o}^{CIGS}$-expressing clones ($EC_{50}$ values: $G\alpha_{o}^{CI}$, 9.2 ± 4.0 nM; $G\alpha_{o}^{CIGS}$, 1.1 ± 0.5 nM). Note that the clones matched for cAMP were not matched for receptor number and G protein expression.

expressing lower levels of the mutant $G\alpha_{i2}$ proteins (Fig. 4b) and clones expressing the mutant $G\alpha_o$ (Fig. 4c). This may relate to an increased adenylyl cyclase sensitization in the presence of the more efficient $G\alpha$ signaling (Clark and Traynor, 2006). Consequently, we repeated the experiment in cells expressing mutant $G\alpha_{i2}$ cells in the presence of fetal bovine serum in the assay medium to prevent expression of sensitization (Clark and Traynor, 2006). Under these conditions, the level of forskolin-stimulated cAMP accumulation in the $G\alpha_{i2}^{CI/B}$ and $G\alpha_{i2}^{CIGS/B}$ clones was similar (1.38 ± 0.11 and 0.98 ± 0.15 pmol/mg of protein, respectively), but the maximal degree of inhibition by DAMGO (34 ± 8 and 85 ± 2%, respectively) and potencies (14 ± 9 and 1.4 ± 0.3 nM, respectively) were unchanged compared with conditions in which the level of forskolin-stimulated cAMP was widely different (Table 2). In confirmation of this, $G\alpha_o^{CI}$ and $G\alpha_o^{CIGS}$ clones that gave a similar level of forskolin-stimulated cAMP gave similar DAMGO-mediated degree of inhibition but retained the shift in potency (see legend to Fig. 4c). In addition, the maximal DAMGO inhibition was not dependent on the forskolin concentration, for example, with the $G\alpha_{i2}^{CI/B}$ clone, maximal DAMGO inhibition was similar (31 ± 9%) using 30 μM forskolin as with 5 μM forskolin (37%; Table 2).

To further examine the model experimentally, relative agonist response was determined for partial agonists providing lower $k_{act}$ values as a percentage of the maximal DAMGO inhibition in the same assay. The relative efficacy of morphine, which behaved as a full agonist in these experiments, was not significantly affected by the RGS-insensitive mutation in cells expressing $G\alpha_o^{CI}$, $G\alpha_{i2}^{CI}$, or $G\alpha_{i3}^{CI}$ (Fig. 5). On the other hand, the relative response of the partial agonists, buprenorphine and nalbuphine, was increased by 1.3- and 1.9-fold, respectively, in the RGS-insensitive mutation in the $G\alpha_{i2}^{CI}$-expressing cells, 1.3- and 2.6-fold in the RGS-insensitive $G\alpha_o^{CI}$-expressing cells, and 2.3- and 6.7-fold in the $G\alpha_{i3}^{CI}$-expressing cells (Fig. 5). The potencies of morphine and buprenorphine were also increased by the RGS-insensitive mutation in each of the $G\alpha$ subtypes (Table 2). These increased potencies and maximal responses with the RGS-insensitive mutation agree with the predictions of the collision coupling model under conditions of slower $k_{act}$.

The potency of the partial agonist with the lowest intrinsic efficacy, nalbuphine, was decreased rather than increased by the RGS-insensitive mutation. It may be speculated that when the $k_{act}$ becomes so slow, steady-state conditions are not reached, so the determined $EC_{50}$ is high. To test this, we repeated the cAMP accumulation assay with nalbuphine using an incubation of 30 min rather than 10 min to allow steady state to be reached. This reversed the shift in potency of nalbuphine to give the expected increase with the RGS-insensitive mutation (Table 2), although under these longer assay conditions, nalbuphine was less potent. As with the shorter period, maximal inhibition by nalbuphine compared with DAMGO was higher with the RGS-insensitive $G\alpha_o^{CIGS}$ (92 ± 5% of DAMGO) than with the RGS-sensitive $G\alpha_o^{CI}$ (68 ± 5% of DAMGO). However, over the longer assay period, the maximal inhibition by DAMGO was lower with the RGS-insensitive $G\alpha_o^{CIGS}$ (69 ± 3% inhibition) than with the RGS-sensitive $G\alpha_o^{CI}$ (85 ± 5% inhibition).

**Calculations of Overall Activation Rate Constants.** The RGS-insensitive $G\alpha$ subunits do not bind RGS proteins, so the rate constant of inactivation ($k_{hydrol}$) for these $G\alpha$ subunits will be the same as if there were no RGS proteins present. Using this assumption, we used the collision coupling model to calculate the overall activation rate constants ($k_{act}$) for the RGS-insensitive clones using the experimental $EC_{50}$ values for DAMGO inhibition of adenylyl cyclase (Table 2), a rate constant of inactivation of 0.1 $s^{-1}$ ($k_{hydrol}$), and $K_i$ values measured in the presence of NaCl and GDP (from Emmerson et al., 1996) for the $K_d$ values. These values are presented in Table 3. As predicted by the model, the calculated $k_{act}$ for DAMGO was slower with decreased receptor number in the clones expressing the same $G\alpha$ subtype. When matched for receptor number, the $k_{act}$ for DAMGO was almost 2-fold faster with $G\alpha_o^{CIGS}$ than with $G\alpha_{i2}^{CIGS}$. In agreement with the collision coupling concept of lower agonist intrinsic efficacy reducing the number of active receptors, and therefore productive "collisions," the overall activation rate constants ($k_{act}$) for the partial agonist, morphine, were

TABLE 2

Inhibition of forskolin-stimulated cAMP accumulation in cells expressing RGS-sensitive or RGS-insensitive Gα proteins

μ-Opioid agonist inhibition of forskolin-stimulated cAMP accumulation was measured for 10 or 30 min in C6μ cells stably expressing PTX-insensitive Gα with (CIGS) or without (CI) the RGS-insensitive mutation as described under *Materials and Methods* and given as mean ± S.E.M. from at least three separate experiments.

| Cells | DAMGO Maximal Inhibition | $EC_{50}$ | | | | |
|---|---|---|---|---|---|---|
| | | DAMGO | Morphine | Buprenorphine | Nalbuphine | Nalbuphine[a] |
| | % | *nM* | *nM* | *nM* | *nM* | *nM* |
| $G\alpha_{i2}^{CI}$/A | 83 ± 2 | 7.9 ± 2.3 | 23 ± 4 | 10 ± 4 | 6.1 ± 2.6 | |
| $G\alpha_{i2}^{CIGS}$/A | 92 ± 1** | 2.2 ± 0.7 | 6.9 ± 3.5* | 3.3 ± 1.8 | 18.7 ± 2.0* | |
| CI/CIGS | | 3.6× | 3.3× | 3.0× | −3.1× | |
| $G\alpha_{i2}^{CI}$/B | 37 ± 6 | 17 ± 7 | 65 ± 12 | 4.2 ± 1.8 | N.A. | |
| $G\alpha_{i2}^{CIGS}$/B | 95 ± 1*** | 3.1 ± 0.5 | 10 ± 4* | 1.9 ± 0.4 | N.A. | |
| CI/CIGS | | 5.5× | 6.5× | 2.2× | | |
| $G\alpha_o^{CI}$ | 72 ± 4 | 13 ± 5 | 15 ± 2 | 9.0 ± 2.2 | 3.8 ± 1.0 | 30 ± 7 |
| $G\alpha_o^{CIGS}$ | 71 ± 3 | 1.7 ± 0.6 | 5.6 ± 2.7* | 3.9 ± 1.7 | 11.8 ± 4.5 | 3.4 ± 1.3* |
| CI/CIGS | | 7.6× | 2.7× | 2.3× | −3.1× | 8.8× |
| $G\alpha_{i3}^{CI}$ | 49 ± 5 | 56 ± 11 | 55 ± 15 | N.A. | N.A. | |
| $G\alpha_{i3}^{CIGS}$ | 49 ± 5 | 3.8 ± 1.9** | 24 ± 7 | N.A. | N.A. | |
| CI/CIGS | | 14.7× | 2.3× | | | |

N.A., not available due to low levels of inhibition.
[a] 30 minute incubation.
*$P < 0.05$ compared to RGS-sensitive clones.
** $P < 0.01$ compared to RGS-sensitive clones.
***$P < 0.001$ compared to RGS-sensitive clones.

at least 7 times slower than for DAMGO at each of the RGS-insensitive clones. However, $k_{act}$ values could not be calculated for nalbuphine or buprenorphine because the $EC_{50}$ values were higher than the $K_i$ value measured in the presence of NaCl and GDP (Emmerson et al., 1996).

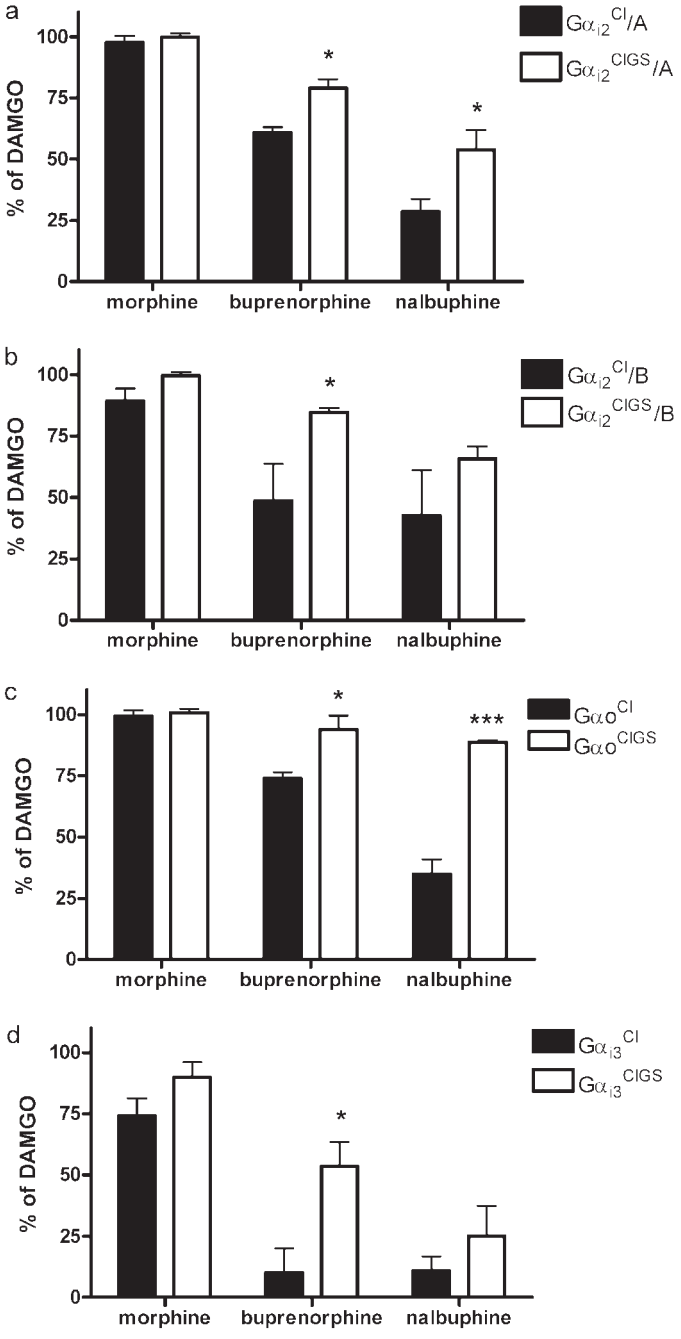



**Fig. 5.** Relative $\mu$-opioid agonist inhibition of forskolin-stimulated adenylyl cyclase. C6$\mu$ cells stably expressing PTX-insensitive G$\alpha$ were plated onto 24-well plates and treated with 100 ng/ml PTX for 2 days before assay. cAMP accumulation was determined as described in legend to Fig. 4 in the presence of 0 to 10 $\mu$M DAMGO, morphine, buprenorphine, or nalbuphine. Maximal percentage of inhibition of forskolin-stimulated cAMP accumulation by each agonist was determined from concentration-response curves and normalized as a percentage of the maximal level of DAMGO inhibition in cells expressing PTX-insensitive $G\alpha^{CI}$ (filled bars) or PTX- and RGS-insensitive $G\alpha^{CIGS}$ (open bars). a, $G\alpha_{i2}$ (clone A) at higher levels; b, $G\alpha_{i2}$ (clone B) at lower levels; c, $G\alpha_o$; d, $G\alpha_{i3}$. Shown are the combined data from three assays carried out in duplicate. *, $p < 0.05$; ***, $p < 0.001$ compared with RGS-sensitive clone.

**[$^{35}$S]GTP$\gamma$S Binding and cAMP Accumulation Using FLAG-Tagged $G\alpha_o{}^{CI}$, $G\alpha_{i2}{}^{CI}$, and $G\alpha_{i3}{}^{CI}$.** To determine whether the faster rates of activation calculated for the $G\alpha_o{}^{CIGS}$ cells were due to higher levels of expression, G$\alpha$ subunits were FLAG-tagged at the N terminus so that expression levels of different G$\alpha$ subtypes could be directly compared by Western blot using the same anti-FLAG antibody. FLAG-$G\alpha_{i2}{}^{CI}$, FLAG-$G\alpha_o{}^{CI}$, or FLAG-$G\alpha_{i3}{}^{CI}$ were stably expressed in HEK293 cells stably expressing the $\mu$-opioid receptor, and clones expressing similar levels of receptor and FLAG-G$\alpha$ were selected (Fig. 6, a and b). None of the FLAG-$G\alpha_{i3}{}^{CI}$ clones expressed higher levels. Maximal stimulation of [$^{35}$S]GTP$\gamma$S binding by DAMGO after overnight treatment with PTX was 3-fold higher in the FLAG-$G\alpha_o{}^{CI}$-expressing clone than in the FLAG-$G\alpha_{i2}{}^{CI}$ or FLAG-$G\alpha_{i3}{}^{CI}$ clones (Fig. 6c). This suggests that the $G\alpha_o{}^{CIGS}$ cells used to calculate the rates of activation (Table 3) actually had lower levels of PTX-insensitive G$\alpha$ than the $G\alpha_{i2}{}^{CIGS}$ clones because they had similar levels of stimulation of [$^{35}$S]GTP$\gamma$S binding by DAMGO.

The degree of inhibition of forskolin-stimulated cAMP accumulation by DAMGO after overnight treatment with PTX was similar in the FLAG-$G\alpha_o{}^{CI}$, FLAG-$G\alpha_{i2}{}^{CI}$, and FLAG-$G\alpha_{i3}{}^{CI}$ clones (Fig. 6d). These results demonstrate that higher levels of [$^{35}$S]GTP$\gamma$S binding or faster rate constants of activation do not correlate with higher levels of inhibition of adenylyl cyclase.

**Effect of Different G Protein Levels.** The simple collision coupling model used here assumes that the G proteins are closely associated with adenylyl cyclase and that agonist potency is independent of G protein and adenylyl cyclase concentrations as shown in turkey erythrocyte membranes (Tolkovsky et al., 1982). In this study, we used clones matched for receptor and G$\alpha$ protein levels to avoid the possible confound of transient G protein and adenylyl cyclase interactions. However, to determine whether G$\alpha$ and adenylyl cyclase are indeed precoupled, agonist potency at inhibition of forskolin-stimulated cAMP accumulation was compared in clones expressing different levels of FLAG-$G\alpha_o{}^{CI}$ but similar levels of $\mu$-opioid receptor (Table 4). DAMGO potency to inhibit cAMP accumulation was decreased at lower levels of FLAG-$G\alpha_o{}^{CI}$ expression, whereas potency to stimulate [$^{35}$S]GTP$\gamma$S binding was not affected, suggesting that G$\alpha$ and adenylyl cyclase are not precoupled in these cells.

TABLE 3

Rates of activation calculated for cells expressing RGS-insensitive G$\alpha$ proteins

$K_{act}$ was calculated from the $EC_{50}$ for inhibition of forskolin-stimulated cAMP accumulation (equation 11). $K_i$ values measured in the presence of NaCl and GDP were used for $K_d$ (DAMGO $K_i$= 279 nM; morphine $K_i$ = 132 nM; from Emmerson et al., 1996) and $k_{hydrol}$ = 0.1 $s^{-1}$ for absence of RGS proteins (from Shea et al., 2000).

| Cells | $\mu$-Opioid Receptors | [$^{35}$S]GTP$\gamma$S Maximal DAMGO Stimulation | $k_{act}$ | |
|---|---|---|---|---|
| | | | DAMGO | Morphine |
| | *pmol/mg protein* | % | $s^{-1}$ | |
| $G\alpha_{i2}{}^{CIGS}$/A | 6.6 ± 0.4 | 380 ± 80 | 13 | 1.8 |
| $G\alpha_{i2}{}^{CIGS}$/B | 4.8 ± 1.1 | 210 ± 30 | 8.9 | 1.2 |
| $G\alpha_o{}^{CIGS}$/B | 6.4 ± 0.4 | 65 ± 20 | 25 | 1.5 |
| $G\alpha_o{}^{CIGS}$A | 3.6 ± 0.6 | 190 ± 10 | 16 | 2.3 |
| $G\alpha_{i3}{}^{CIGS}$/A | 4.5 ± 1.4 | 32 ± 6 | 7.2 | 0.45 |
| $G\alpha_{i3}{}^{CIGS}$/B | 0.08 ± 0.07 | 6 ± 3 | 0.8 | N.A. |

N.A., not available.

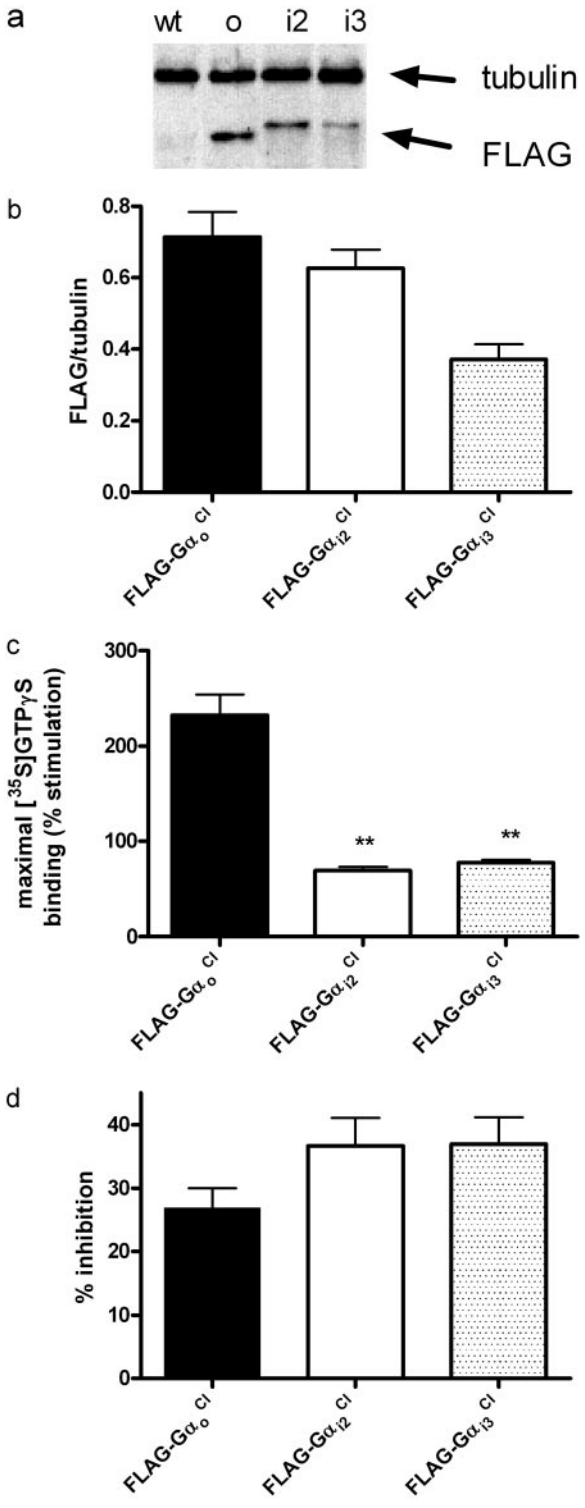



**Fig. 6.** Comparison of FLAG-G$\alpha_{i2}^{CI}$-, FLAG-G$\alpha_{o}^{CI}$-, or FLAG-G$\alpha_{i3}^{CI}$-expressing HEK293$\mu$ cells. PTX-insensitive G$\alpha^{CI}$ was FLAG-tagged and stably expressed in HEK293$\mu$ cells. Cells matched for receptor number (femtomoles per milligram of protein: FLAG-G$\alpha_{i2}^{CI}$, 860 ± 80; FLAG-G$\alpha_{o}^{CI}$, 850 ± 50; FLAG-G$\alpha_{i3}^{CI}$, 950 ± 100) were treated with 100 ng/ml PTX overnight. a and b, Western blot analysis with anti-M2 FLAG antibody and anti-tubulin as described under *Materials and Methods*. a, representative blot; b, combined data from at least three blots normalized to tubulin loading control. c, maximal stimulation of 0.1 nM [$^{35}$S]GTP$\gamma$S binding by DAMGO in presence of 30 $\mu$M GDP for 60 min at 25°C as described under *Materials and Methods*. The $EC_{50}$ of DAMGO to stimulate [$^{35}$S]GTP$\gamma$S binding was lower at FLAG-G$\alpha_{o}^{CI}$ (68 ± 5 nM) and FLAG-G$\alpha_{i3}^{CI}$ (72 ± 24 nM) than at FLAG-G$\alpha_{i2}^{CI}$ (169 ± 14 nM). Basal levels of [$^{35}$S]GTP$\gamma$S binding were similar for FLAG-G$\alpha_{o}^{CI}$ (13 ± 2 fmol/mg), FLAG-G$\alpha_{i2}^{CI}$ (11 ± 2 fmol/mg), or FLAG-G$\alpha_{i3}^{CI}$ (9 ± 2 fmol/mg). d, maximal inhibition of forskolin-stimulated cAMP accumulation by 10 $\mu$M DAMGO measured as described in legend to Fig. 4. The $EC_{50}$ of DAMGO to inhibit cAMP accumulation was 42 ± 22 nM at FLAG-G$\alpha_{o}^{CI}$, 23 ± 5 nM at FLAG-G$\alpha_{i2}^{CI}$, and 95 ± 35 nM at FLAG-G$\alpha_{i3}^{CI}$. The levels of forskolin-stimulated cAMP accumulation in the absence of $\mu$-opioid agonist (picomoles per milligram of protein) were similar for FLAG-G$\alpha_{o}^{CI}$ (1.0 ± 0.1), FLAG-G$\alpha_{i2}^{CI}$ (2.2 ± 0.7), or FLAG-G$\alpha_{i3}^{CI}$ (1.4 ± 0.3). **, $p < 0.01$ compared with FLAG-G$\alpha_{o}^{CI}$. A second set of clones expressing similar levels of FLAG-tagged G$\alpha_{o}^{CI}$ and G$\alpha_{i2}^{CI}$ showed similar results with DAMGO stimulation of [$^{35}$S]GTP$\gamma$S ($EC_{50}$ and maximal stimulation: 69 ± 9 nM and 215 ± 27% for G$\alpha_{o}^{CI}$; 157 ± 21 nM and 104 ± 10% for G$\alpha_{i2}^{CI}$) and inhibition of cAMP accumulation ($EC_{50}$ and maximal inhibition: 29 ± 11 nM and 35 ± 3% for G$\alpha_{o}^{CI}$; 68 ± 31 nM and 42 ± 5% for G$\alpha_{i2}^{CI}$).

**Comparison of Calculated and Experimentally Determined $EC_{50}$ Values.** Using the calculated $k_{act}$ values for the RGS-insensitive clones and the $k_{hydrol}$ of 5 $s^{-1}$ for the presence of RGS proteins, the expected decrease in potency for the RGS sensitive clones is predicted to range from 29- to 38-fold for DAMGO. This is higher than the experimental range of a 4- to 15-fold decrease in DAMGO potency across all of the RGS-sensitive clones compared with their matched RGS-insensitive clone (Table 2). The predicted potency shift for morphine based on the $k_{act}$ values is 5- to 16-fold, which is closer to the measured range of a 2- to 6-fold decrease in morphine potency. In contrast, using previously published data comparing G$\alpha_{o}^{CGGS}$ and G$\alpha_{o}^{CG}$ (Clark et al., 2003) with the less efficiently coupled CG PTX-insensitive mutation (Bahia et al., 1998; Clark et al., 2006), the measured 8- to 35-fold shift in DAMGO potency was similar to the 16-fold shift predicted from the calculated $k_{act}$ values of 2.1 and 2.3 $s^{-1}$. The measured morphine potency shift of 8-fold was also similar to the 3- to 6-fold shift predicted from the calculated $k_{act}$ values of 0.51 and 0.20 $s^{-1}$. This would suggest that rate constant of hydrolysis in the presence of RGS proteins of 5 $s^{-1}$, which was used for the predictions in this study, seems appropriate in these cells.

## Discussion

This study, using RGS-insensitive mutants of G$\alpha$ proteins, demonstrates that endogenous RGS proteins reduce agonist potency for the inhibition of adenylyl cyclase similarly for full and partial agonists. The maximal agonist response, however, was reduced more by RGS proteins under conditions of slower rates of G protein activation, such as with partial agonists and was not affected by RGS proteins under conditions of faster rates of G protein activation, as with full agonists. These results agree with the predictions of a collision coupling model.

Agonist potency was increased similarly by the RGS-insensitive mutation for G$\alpha_{i2}$- and G$\alpha_{o}$-expressing cells. Previous studies have shown that the rate of GTP hydrolysis is faster with G$\alpha_{o}$ than with G$\alpha_{i2}$ with RGS4 (Posner et al., 1999; Cavalli et al., 2000; Hooks et al., 2003), or with RGS6, -7, -9, or -11 (Hooks et al., 2003). There are several explanations for our finding. There could be RGS proteins present that do not act very efficiently as GAPS at G$\alpha_{o}$, or colocalization of the receptors and G proteins with the RGS proteins may be different. Although RGS proteins that might be considered as prime candidates to interact with $\mu$-opioid signaling in vivo [i.e., RGS4 and RGS9 (Traynor and Neubig, 2005)] are not expressed in C6 cells (Snow et al., 2002), a number of RGS

proteins have been identified (RGS 2, 8, 10, 12, and 14; Snow et al., 2002), and several of these are known to effectively act as GAPS for $G\alpha_{i/o}$ proteins coupled to $\mu$-opioid receptors (reviewed in Xie and Palmer, 2005). Finally, the PTX-insensitive $G\alpha^{CI}$ mutants have faster rates of activation than wild-type $G\alpha$ (Bahia et al., 1998), which may hide differences in the sensitivity of $G\alpha$ proteins to endogenous RGS proteins. Indeed, with the less efficiently coupled $G\alpha^{CG}$ PTX-insensitive mutation, morphine is a partial agonist at inhibition of adenylyl cyclase (Clark et al., 2003), whereas in this study with the $G\alpha^{CI}$ PTX-insensitive mutation, morphine is a full agonist.

The increase in potency with the RGS-insensitive PTX-insensitive $G\alpha_o^{CG}$ was in accordance with the calculations based on the collision coupling model, whereas the potencies in the PTX-insensitive $G\alpha^{CI}$-expressing cells used in this study were not increased to the extent predicted. This may be due to a physical diffusion-controlled limitation on $EC_{50}$ and $k_{act}$. This would limit the differences with and without RGS proteins in a highly efficient $G\alpha^{CI}$ system with an already low $EC_{50}$ to less than the theoretical predictions (see Fig. 2, inset). Alternatively, if RGS proteins do not modulate some of the $G\alpha^{CI}$ pool, the effective $k_{hydrol}$ in the RGS sensitive cells would actually reflect a combination of $k_{hydrol}$ with and without RGS proteins, so the shift in potency with the $G\alpha^{CIGS}$ mutant would be less than predicted.

The maximal effect of the full agonist DAMGO was increased in cells expressing RGS-insensitive $G\alpha_{i2}$ but not $G\alpha_o$ or $G\alpha_{i3}$ proteins. This suggests that the $k_{act}$ for $G\alpha_{i2}$ is lower so that $G\alpha_{i2}$ is more susceptible to the effects of RGS proteins according to the collision coupling model. Using FLAG-tagged $G\alpha$ subunits with similar expression levels, we measured a higher efficiency of coupling for $G\alpha_o$ in the [$^{35}$S]GTP$\gamma$S assay, confirming that the $\mu$-opioid receptor couples more efficiently to $G\alpha_o$ and $G\alpha_{i3}$ than $G\alpha_{i2}$ (Clark et al., 2006). The rate limiting step in the [$^{35}$S]GTP$\gamma$S binding assay is the exchange of GDP for [$^{35}$S]GTP$\gamma$S, so this higher equilibrium level of DAMGO-stimulated [$^{35}$S]GTP$\gamma$S binding at $G\alpha_o$ could be explained by a faster rate of GDP/[$^{35}$S]GTP$\gamma$S exchange compared with the slow off rate of [$^{35}$S]GTP$\gamma$S. Indeed, the rate constant of activation calculated for $G\alpha_o$ was faster. Other studies have shown that $G\alpha_o$ has a faster nucleotide exchange rate than $G\alpha_{i1}$ (Remmers et al., 1999; Kimple et al., 2001) or $G\alpha_{i2}$ (Parker et al., 1991; Zhang et al., 2002). This is also reflected in the higher basal steady state GTPase, which has been observed for $G\alpha_o$ compared with $G\alpha_{i1}$, $G\alpha_{i2}$, or $G\alpha_{i3}$ (Hooks et al., 2003). The endogenous basal rate of GTP hydrolysis, as measured by single turnover assay, has been shown to be similar for $G\alpha_{i2}$ and $G\alpha_o$ (Posner et al., 1999), supporting the suggestion that the faster steady-state GTPase rates for $G\alpha_o$ are due to faster nucleotide exchange rates.

Although we see a more efficient coupling of the $\mu$-opioid receptor with FLAG $G\alpha_o$ than FLAG $G\alpha_{i2}$ or $G\alpha_{i3}$, the degree of inhibition of forskolin-stimulated cAMP accumulation by DAMGO after overnight treatment with PTX was similar across the clones. The difference in the kinetics of the $G\alpha$ subtypes may not be sufficient to significantly affect the maximal response and potency of the full agonist. Alternatively, $G\alpha_o$ could have a lower efficacy than $G\alpha_{i2}$ for inhibiting the adenylyl cyclases present in these cells, despite a faster rate of G protein activation. HEK293 cells are reported to contain ACI, ACIII, and ACVI; $G\alpha_o$ has been shown to inhibit ACI but not ACVI, whereas $G\alpha_i$ is able to inhibit ACI and ACVI (Taussig et al., 1994). There could also be RGS proteins in HEK293 cells that do have a significantly differential effect on $G\alpha_o$ and $G\alpha_{i2}$ (such as RGS6). RGS proteins, receptors, and different adenylyl cyclase subtypes may also colocalize or interact differently with the different $G\alpha$ subtypes, allowing for differential effects within the same cell.

The simple collision coupling model we have used was originally formulated for $G\alpha_s$-mediated stimulation of adenylyl cyclase (Stickle and Barber, 1992; Whaley et al., 1994). The model assumes that the G proteins are closely associated with adenylyl cyclase and that agonist potency is independent of G protein and adenylyl cyclase concentrations (Tolkovsky et al., 1982). On the other hand, there is the possibility that the interaction between the G protein and adenylyl cyclase is transient, so that the kinetics of that encounter need to be considered. A "shuttle mechanism" has been incorporated into the collision coupling model by adding a rate constant for the activation of adenylyl cyclase by G protein and parameters for the number of G proteins and adenylyl cyclase molecules (Krumins et al., 1997). According to this shuttle mechanism, agonist potency at adenylyl cyclase will be increased by increasing the number of G proteins if the rate of activation of adenylyl cyclase by G protein is fast compared with the rate of inactivation (Krumins et al., 1997). In this study, we used clones matched for receptor and $G\alpha$ protein levels to avoid the possible confound of transient G protein and adenylyl cyclase interactions, although data with different FLAG-$G\alpha$ expression levels does suggest that the system is not precoupled. Similar to the present findings, GIRK channel activation by m2 receptor activation of $G\alpha_{i2}$ has also been shown to be consistent with the collision coupling model, whereas m2 activation of GIRK mediated by $G\alpha_o$ was not (Zhang et al., 2002). The authors propose that expression conditions were favorable to formation of a precoupled receptor-$G\alpha_o$-GIRK channel complex. The possibility of precoupled or colocalized receptor and G protein instead of

TABLE 4

Effect of FLAG-$G\alpha_o^{CI}$ expression level on DAMGO stimulation of [$^{35}$S]GTP$\gamma$S binding and inhibition of forskolin-stimulated cAMP accumulation

Expression levels of FLAG-$G\alpha_o^{CI}$ stably expressed in HEK293$\mu$ cells were determined by Western blot analysis as described under *Materials and Methods*. DAMGO stimulation of [$^{35}$S]GTP$\gamma$S binding and inhibition of forskolin-stimulated cAMP accumulation were measured as described under *Materials and Methods*.

| Cells | FLAG/Tubulin Ratio | $\mu$-Opioid Receptors | [$^{35}$S]GTP$\gamma$S Binding | | cAMP Accumulation | |
|---|---|---|---|---|---|---|
| | | | Maximum | $EC_{50}$ | Maximum | $EC_{50}$ |
| | | *fmol/mg protein* | % | *nM* | % | *nM* |
| FLAG-$G\alpha_o^{CI}$/A | 1.0 ± 0.2 | 600 ± 170 | 220 ± 30 | 68 ± 9 | 35 ± 3 | 30 ± 11 |
| FLAG-$G\alpha_o^{CI}$/B | 0.74 ± 0.20 | 850 ± 50 | 230 ± 40 | 68 ± 5 | 27 ± 3 | 42 ± 22 |
| FLAG-$G\alpha_o^{CI}$/C | 0.10 ± 0.08* | 480 ± 140 | 26 ± 4* | 68 ± 32 | 15 ± 2* | 470 ± 160* |

* $P \leq 0.05$ compared with clone A.

collision coupling was also indicated by the lack of relationship between agonist potency for inhibition of adenylyl cyclase and the number of 5-$HT_{1A}$ receptors in NIH-$3T_3$ cells (Varrault et al., 1992). In addition, there is evidence for a continuous interaction between β-adrenergic receptor and G protein for the stimulation of adenylyl cyclase in turkey erythrocyte membranes (Ugur and Onaran, 1997). This led to the suggestion that an equilibrium model of G protein and receptor states, such as the extended ternary complex model (Samama et al., 1993), needs to be considered along with the G protein kinetics of the collision coupling model. This has been done by combining the kinetic parameters of G protein activation and inactivation to the cubic ternary complex model to formulate the cubic ternary complex activation model (Shea et al., 2000). Unlike the collision coupling model, this more comprehensive model will provide predictions for agonist maximal response and potency in the absence or presence of RGS proteins, whether or not the receptor and G protein are precoupled.

In conclusion, this study demonstrates that the degree of effect of RGS proteins is highly system-dependent on the intrinsic efficacy of the agonist and on tissue factors that govern the efficiency of receptor-G protein-effector coupling. Less efficient systems show a much greater positive regulation by inhibition of RGS protein GAP activity and are consequently more susceptible to negative regulation by RGS proteins. Thus, the determinants of the selectivity of action of RGS proteins are not only their differential tissue distribution (Gold et al., 1997) and selectivity for particular receptor types (Ghavami et al., 2004) but also tissue factors such as levels of receptor, their cognate G protein, and effectors. Consequently, specific modulation of agonist activated G protein kinetics by inhibitors of RGS proteins, now being developed in several laboratories (e.g., Roman et al., 2007), may prove to be useful adjuncts in specifically enhancing the actions of agonist drugs and endogenous neurotransmitters.

## Appendix

Here we derive the equations for maximum agonist response and potency for a simple collision coupling model at steady state, as diagrammed in Figure 7. Receptors and ligands are assumed to bind with equilibrium dissociation constant $K_d$. Receptor/ligand complexes exist in active ($LR_a$) or inactive ($LR_i$) forms, referring to their ability to activate G proteins, and these complexes are assumed to distribute according to an equilibrium constant $K_{eq}$ (Kinzer-Ursem et al., 2006). It is assumed that there is no precoupling of (unbound) receptors and G proteins and no constitutive activity. The total numbers of receptors ($R_{TOT}$) and G proteins ($G_{TOT}$) on the cell surface that can participate in signaling are assumed constant (i.e., no internalization, up-regulation, or desensitization). G proteins are activated after diffusion and collision with a ligand-bound receptor with rate constant $k_a$. Thus, the value of $k_a$ depends on the diffusivity of receptors and G proteins in the membrane and also on the number and distribution of G proteins available for activation (which determines the necessary distance that must be traversed via diffusion) (Mahama and Linderman, 1994; Shea et al., 1997). G proteins are inactivated after GTP hydrolysis with rate constant $k_{hydrol}$, the value of which depends on the presence or absence of RGS. At steady state, the rates of G protein activation and deactivation are equal. Thus, the starting equations for this model are simply

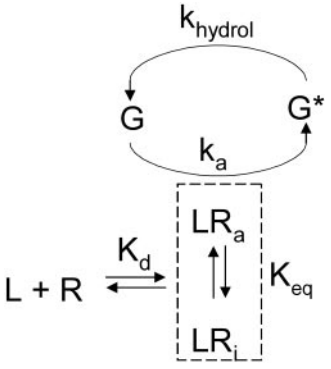



**Fig. 7.** Collision coupling model.

$$K_d = \frac{[L][R]}{[LR]} \tag{4}$$

$$K_{eq} = \frac{LR_a}{LR_i} \tag{5}$$

$$LR = LR_a + LR_i \tag{6}$$

$$k_a[G][LR_a] = k_{hydrol}[G^*] \tag{7}$$

After algebraic manipulation, one can find that the amount of G protein activation is

$$\frac{[G^*]}{G_{TOT}} = \frac{k_a[LR_a]}{k_{hydrol} + k_a[LR_a]} = \frac{k_a R_{TOT} f_{active}\left(\frac{[L]}{K_D + [L]}\right)}{k_{hydrol} + k_a R_{TOT} f_{active}\left(\frac{[L]}{K_D + [L]}\right)} \tag{8}$$

where the fraction of bound receptors in the active form, a measure of the agonist intrinsic efficacy, is

$$f_{active} = \frac{LR_a}{LR_a + LR_i} = \frac{K_{eq}}{K_{eq} = 1} \tag{9}$$

Thus, the fractional activation of G proteins at high ligand concentrations (maximal response), assumed equal in this model to the fractional activation of adenylyl cyclase, is

$$f = \frac{k_a R_{TOT} f_{active}}{k_{hydrol} + k_a R_{TOT} f_{active}} \tag{10}$$

Agonist potency predicted by this model is given by

$$EC_{50} = \frac{k_{hydrol} K_D}{k_{hydrol} + k_a R_{TOT} f_{active}} \tag{11}$$

When all bound receptors are active ($K_{eq} \gg 1$ or $f_{active} = 1$), eqs. 10 and 11 are identical to the results of Whaley et al. (1994).

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