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A Nanomolar-Potency Small Molecule Inhibitor of Regulator of G-Protein Signaling Proteins

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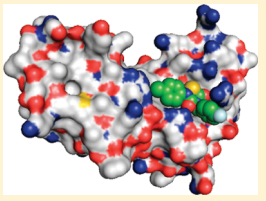
Supporting Information

ABSTRACT: Regulators of G-protein signaling (RGS) proteins are potent negative modulators of signal transduction through G-protein-coupled receptors. They function by binding to activated (GTP-bound) Gα subunits and accelerating the rate of GTP hydrolysis. Modulation of RGS activity by small molecules is an attractive mechanism for fine-tuning GPCR signaling for therapeutic and research purposes. Here we describe the pharmacologic properties and mechanism of action of CCG-50014, the most potent small molecule RGS inhibitor to date. It has an IC_{50} for RGS4 of 30 nM and is >20-fold selective for RGS4 over other RGS proteins. CCG-50014 binds covalently to the RGS, forming an adduct on two cysteine residues located in an allosteric regulatory site. It is not a general cysteine alkylator as it does not inhibit activity of the cysteine protease papain at concentrations >3000-fold higher than those required to inhibit RGS4 function. It is also >1000-fold more potent as an RGS4 inhibitor than are the cysteine alkylators *N*-ethylmaleimide and iodoacetamide. Analysis of the cysteine reactivity of the compound shows that compound binding to Cys^{107} in RGS8 inhibits Gα binding in a manner that can be reversed by cleavage of the compound−RGS disulfide bond. If the compound reacts with Cys^{160} in RGS8, the adduct induces RGS denaturation, and activity cannot be restored by removal of the compound. The high potency and good selectivity of CCG-50014 make it a useful tool for studying the functional roles of RGS4.



G-Protein-coupled receptors (GPCRs) are important drug targets with profound clinical relevance. While direct modulation of GPCR activity with traditional pharmacological agents (orthosteric agonists and antagonists) has proven incredibly successful, compounds that provide more subtle modulation of receptor signaling may provide advantages over traditional GPCR-targeting drugs. Several approaches to this goal have been proposed, including pathway-biased GPCR ligands,[1] allosteric receptor modulators,[2] and compounds that interfere with specific downstream modulators of G-protein signaling.[3] In this report, we describe the properties and mechanism of a novel small molecule compound that potently inhibits a regulator of G-protein signaling (RGS), an important modulator of GPCR signal transduction cascades.

The RGS proteins are negative modulators of many G-protein-coupled receptor (GPCR) signaling pathways.[4] RGS proteins bind directly to the GTP-bound Gα subunit of activated heterotrimeric G-proteins and increase the rate of GTP hydrolysis.[5] By this mechanism, RGS proteins rapidly dampen GPCR signal transduction at the level of the active G-protein subunits. This accelerated turn-off of activated G-proteins provides a cellular mechanism for enhanced temporal and spatial resolution of GPCR signaling.[6]

Genetic ablation of RGS activity either by deletion of a particular RGS gene or by expression of RGS-insensitive Gα subunits has dramatic physiological consequences (for a review, see ref 14). For example, RGS4-deficient mice display increased sensitivity to carbachol-potentiated glucose-stimulated insulin release.[7] Deletion of RGS9 produces a variety of neurological effects, including sensitization to morphine analgesia and reward with decreased tolerance, deficits in working memory, and motor coordination defects.[8,9] The physiological effects of pan-RGS inhibition have also been studied. A knock-in mouse model that expresses a $G\alpha_{i2}$ subunit with a single amino acid mutation (G184S) that prevents functional interactions with RGS proteins has been developed.[22] These mice show dramatic phenotypes, including spontaneous antidepressant-like effects as well as resistance to diet-induced obesity.[10,15] Thus, modulating the activity of specific RGS proteins may provide significant therapeutic benefit.[3,14,18] To this end, we have developed several classes of small molecule RGS inhibitors for use as pharmacological tools and as potential therapeutics.[27,29]

Inhibiting protein−protein interactions, such as the one between an RGS and a Gα subunit, is particularly challenging.[3,26,27] This is predominantly due to the lack of a suitable small molecule binding pocket at the protein−protein interaction interface. However, the GTPase accelerating activity of RGS4 is regulated by phosphatidylinositol 3,4,5-triphosphate at a site far removed from the Gα interaction interface.[16,17] Targeting this allosteric site[18] might be a more tractable approach to inhibiting the RGS−Gα protein−protein interaction than attempting to orthosterically occlude the protein−protein interaction.

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CCG-50014 was discovered in a high-throughput biochemical screen designed to identify inhibitors of five different RGS proteins.[19] This compound was the most potent inhibitor from this screen with a nanomolar IC_{50} value. In this work, we confirm the potent and selective RGS inhibitory activity of this compound and describe its biochemical mechanism of action. Furthermore, we provide evidence that CCG-50014 is able to inhibit the RGS4−$G\alpha_o$ protein−protein interaction in a living cell. This compound represents the first of a class of small molecule RGS inhibitors that function in a cellular environment.

EXPERIMENTAL PROCEDURES

Materials. Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) and were reagent grade or better. [γ-^{32}P]GTP (10 mCi/mL) and [^{35}S]GTPγS (12.5 mCi/mL) were obtained from Perkin-Elmer Life and Analytical Sciences (Boston, MA) and were isotopically diluted before use. Amylose resin was purchased from New England Biolabs (Ipswich, MA). Ni-NTA resin was purchased from Qiagen (Valencia, CA). Avidin-coated microspheres were purchased from Luminex (Austin, TX). CCG-50014 {4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione} and analogues were purchased from the Maybridge compound collection (Fisher Scientific, Waltham, MA) or were as described below using a variation of previously published methods.[20]

Synthesis. A Bruker Avance III 400 MHz NMR spectrometer was used to record ^{1}H and ^{13}C NMR spectra. ESI mass spectra were recorded using a Bruker micrOTOF. Column chromatography was performed with a Combiflash Rf Companion, using Redisep Rf disposable columns containing 40−60 μm silica.

CCG-50014 [4-(4-fluorobenzyl)-2-p-tolyl-1,2,4-thiadiazolidine-3,5-dione]. *N*-Chlorosuccinimide (4.0 g, 30.0 mmol) was added to a solution of 4-fluorobenzyl isothiocyanate (1.25 g, 7.5 mmol) and *p*-tolyl isocyanate (1.0 g, 7.5 mmol) in $CHCl_3$ (70 mL) under nitrogen at room temperature. This solution was stirred for 18 h, exposed to the air, and stirred for an additional 30 min. The reaction mixture was diluted with Et_2O (30 mL) and filtered through a sintered funnel. The residue was washed with further Et_2O (15 mL), and solvent was removed under reduced pressure. The crude product was purified by column chromatography (20% ethyl acetate/hexane), resulting in a pale yellow solid (970 mg, 41%) with spectral data identical to those of the Maybridge sample: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.35 (s, 3H), 4.87 (s, 2H), 7.01−7.05 (m, 2H), 7.20−7.22 (m, 2H), 7.35−7.38 (m, 2H), 7.48−7.51 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.9, 45.3, 115.5, 115.7, 123.7, 130.0, 130.9, 131.0, 131.1, 133.0, 137.3, 151.0, 161.5, 163.9, 165.1.

CCG-203778 [3-(4-methylbenzyl)-1-p-tolylimidazolidine-2,4-dione]. Ethyl bromoacetate (3.3 g, 20 mmol) was added to a solution of *p*-toluidine (2.1 g, 20 mmol) and sodium acetate (2.1 g, 26 mmol) in ethanol (26 mL). The resulting solution was warmed to 80 °C and stirred for 1 h before being cooled to room temperature. The reaction was quenched with water (20 mL) and the aqueous fraction extracted with ethyl acetate (3 × 20 mL). The organic fractions were combined, dried ($MgSO_4$), and filtered, and the solvent was removed under reduced pressure. Silica chromatography (0−20% ethyl acetate/hexane) provided ethyl 2-(*p*-tolylamino)acetate as a pale oil (1.9 g, 50%).

Ethyl 2-(*p*-tolylamino)acetate (260 mg, 1.4 mmol) was dissolved in toluene (5 mL). Methyl benzylisocyanate (200 mg, 1.4 mmol) was added to this solution. The mixture was heated to reflux and stirred for 5 h, until TLC analysis demonstrated that the reaction was complete. The reaction mixture was then cooled to room temperature, and the solvent was removed under reduce pressure. Silica chromatography (0−20% ethyl acetate/hexane) provided ethyl 2-[3-(4-methylbenzyl)-1-*p*-tolylureido]acetate as a pale oil (455 mg, 98%).

Ethyl 2-[3-(4-methylbenzyl)-1-*p*-tolylureido]acetate (455 mg, 1.3 mmol) was dissolved in THF (10 mL) and added dropwise to a solution of NaH (68 mg, 2.8 mmol) in THF (10 mL) at 0 °C. The reaction mixture was allowed to warm to room temperature slowly and stirred for 18 h before the reaction was quenched with water (5 mL). The aqueous fraction was then extracted with dichloromethane (3 × 20 mL). The organic fractions were combined, dried ($MgSO_4$), and filtered, and the solvent was removed under reduced pressure. Column chromatography (0−20% ethyl acetate/hexane) provided CCG-203778 (360 mg, 93%) as a white solid: ^{1}H NMR (400 MHz, $CDCl_3$) δ 2.33 (s, 6H), 4.25 (s, 2H), 4.71 (s, 2H), 7.13−7.15 (m, 2H), 7.16−7.18 (m, 2H), 7.36−7.38 (m, 2H), 7.40−7.43 (m, 2H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.7, 21.1, 42.4, 50.0, 118.5, 128.9, 129.4, 129.8, 132.9, 134.2, 135.0, 137.9, 154.1; HRMS (ESI) $[M + H]^+$ found 295.1448, $C_{18}H_{19}N_2O_2$ requires 295.1446.

Protein Expression and Purification. With the exception of RGS8 and mutants thereof, all RGS and G-proteins were prepared as previously described.[21] Briefly, an N-terminally truncated (Δ51) form of RGS4 was expressed with an N-terminal maltose binding protein fusion tag and a 10-histidine tag from the pMALC2H10 vector. Full-length RGS16 and a C-terminally truncated form of RGS19 (ΔC11) were also expressed as MBP/10-histidine tag fusions using the pMALC2H10 vector. The RGS7 homology domain was expressed as a GST fusion as previously described.[22] The RGS8 homology domain was expressed as an N-terminal six-histidine tag from the pQE-80 vector and was purified as previously described.[23] This construct contains two cysteine residues from RGS8, at positions 107 and 160. For the RGS8 cysteine → serine mutants, site-directed mutagenesis was performed using primers for 107C RGS8 (C160S) (sense, 5′-GCAGGAGCCATCCCTGACTAGCTTTGACCAAG-3′; antisense, 5′-CGTCCTCGGTAGGGACTGATCGAAACTGGTTC-3′) and 160C RGS8 (C107S) (sense, 5′-TGGAATTCTGGTTGGCCAGTGAGGAGTTCAAGAAG-3′; antisense, 5′-ACCTTAAGACCAACCGGTCACTCCTCAAGTTCTTC-3′). Mutagenesis was performed using the QuickChange Multisite Directed Mutagenesis kit (Agilent, La Jolla, CA). Mutants were sequenced at the University of Michigan DNA Sequencing Core. $G\alpha_o$ was expressed with an N-terminal six-histidine tag as previously described.[24] G-Protein activity was determined by [^{35}S]GTPγS binding.[25] In all cases, proteins were purified to >90% homogeneity before use. See note [a] for a concise description of RGS8 cysteine mutant nomenclature.

Chemical Labeling of Purified $G\alpha_o$ and RGS Proteins. RGS proteins were chemically biotinylated on free amines, and $G\alpha_o$ was labeled with AlexaFluor-532 on free thiols as previously described.[26]

Flow Cytometry Protein Interaction Assay (FCPIA) Dose−Response and Reversibility Experiments. FCPIA was performed as previously described using chemically biotinylated RGS proteins and AlexaFluor-532-labeled $G\alpha_o$.[26,27] In brief,

biotintylated RGS proteins were immobilized on LumAvidin microspheres (Luminex) and treated with compound or vehicle control for 15 min at room temperature. The beads were then mixed with fluorescently labeled Gα in the presence of guanosine diphosphate and aluminum fluoride and assessed for association with the RGS protein using a Luminex 200 flow cytometer.

Single-Turnover GTPase Measurements. Compounds were tested for the ability to inhibit the RGS4- and RGS8-stimulated increase in the level of GTP hydrolysis by $G\alpha_o$ as previously described.[11,27] Data are presented as the rate constants (k, in inverse seconds) of GTP hydrolysis.

Thermal Stability Measurements. The thermal denaturation of RGS4, RGS8, and $G\alpha_o$ was assessed using a ThermoFluor Instrument (Johnson & Johnson, Langhorne, PA). Protein (10 μM RGS4 or 5 μM RGS8 or $G\alpha_o$) was incubated with CCG-50014 or vehicle control for 15 min at room temperature in 50 mM HEPES (pH 8.2), 500 mM NaCl, and 5% glycerol in a volume of 15 μL in a black 384-well PCR microtiter plate (ThermoFisher catalog no. TF-0384/K). To this mixture was added 200 μM 1-anilinonaphthalene-8-sulfonic acid. The samples were overlaid with 5 μL of silicone oil and subjected to a temperature ramp with intervening measurements at 25 °C. The experiments were performed using the following parameters: ramp temperature range of 30−90 °C, temperature increment of 1 °C, image collection temperature of 25 °C, and temperature holds of 30 s for ramp temperature and 15 s for image collection temperature. Melting temperatures (T_m) were calculated from the fluorescence data using the sigmoidal fitting procedure in ThermoFluor^{++} (version 1.3.7).

Analysis of the Protein Adduct of RGS by ESI-LC/MS. The molecular mass of the RGS protein was analyzed by ESI-LC/MS using a LCQ ion-trap mass spectrometer (Thermo Scientific, Waltham, MA). RGS8 wild-type or mutant proteins were diluted to 2 μM in 50 mM potassium phosphate buffer (pH 7.4), and CCG-50014 or an equivalent volume of DMSO was added to the sample. Following treatment with CCG-50014, an aliquot (~50 μL) of the protein solution was applied to a reverse-phase Zorbax 300-SB C3 column (2 × 150 mm, 5 μm) (Agilent). The RGS protein was subjected to high-performance liquid chromatography with a binary solvent system consisting of 0.1% TFA in water (solvent A) and 0.1% TFA in acetonitrile (solvent B) using the following gradient: 30% B for 5 min, linear increase to 90% B over 20 min, and 90% B for 30 min. The flow rate was 0.25 mL/min. The mass spectrometer was tuned with horse heart cytochrome *c*, and the following instrumental settings for the mass spectrometer were used: spray voltage of 3.5 kV, capillary temperature of 220 °C, sheath gas flow of 80 (arbitrary units), and auxiliary gas flow of 20 (arbitrary units). The molecular masses of the unmodified and inhibitor-modified RGS proteins were determined by deconvolution of the apoprotein charge envelopes using Bioworks (Thermo Scientific).

Papain Activity Assay. Papain (Sigma-Aldrich) activity was monitored by the increase in fluorescence produced by the liberation of fluorescein from autoquenched fluorescein-conjugated casein (FITC-casein, AnaSpec, San Jose, CA). Papain (0.625 unit) was diluted into a final reaction volume of 100 μL in 20 mM sodium acetate (pH 6.5) supplemented with 2 mM EDTA. The enzyme was treated with iodoacetamide, *N*-ethylmaleimide, CCG-50014, or vehicle control for 30 min at ambient temperature. To this was added FITC-casein to a final concentration of 250 nM. The reaction was allowed to proceed at ambient temperature in the dark. At various times, the fluorescence intensity (excitation at 485 nm and emission at 520 nm) was measured using a Victor II plate reader (Perkin-Elmer). As a control, CCG-50014 was tested using FCPIA at pH 6.5, and it retains full inhibitory activity against the RGS4−$G\alpha_o$ protein−protein interaction.

Docking of CCG-50014 to RGS8. The energy-based docking software Autodock (version 4.0) was used to explore potential binding sites of CCG-50014 on RGS8. The coordinates of RGS8 were obtained from the Protein Data Bank (PDB) (entry 2IHD). Water and a chloride ion were removed from the structure prior to docking. The coordinates of the CCG-50014 ligand were built using the ChemBioOffice 2008 software suite (CambridgeSoft, Cambridge, MA), and the geometry of CCG-50014 was optimized using the semiempirical quantum PM3 method included in the ChemBioOffice 2008 software suite. For unbiased docking, the grid box of the RGS was set at 60 Å × 60 Å × 60 Å to encompass the entire RGS protein. The flexible CCG-50014 ligand was docked to the rigid RGS using a Lamarckian Genetic Algorithm with the following parameters: mutation rate of 0.02, crossover rate of 0.8, and 2.7×10^5 as the maximal number of generations.

RGS4 Membrane Translocation. HEK-293T cells were grown to 80−90% confluency in six-well dishes in DMEM supplemented with 10% fetal bovine serum and penicillin (100 units/mL) and streptomycin (100 μg/mL) under 5% CO_2 at 37 °C. RGS and $G\alpha_o$ expression was induced by transient cotransfection with 250 ng of full-length human RGS4 with an N-terminal GFP tag (RGS4pEGFP-C1) and 250 ng of pcDNA3.1 or pcDNA3.1 encoding wild-type human $G\alpha_o$ or both. Cells were seeded onto poly-D-lysine-coated glass coverslips and cultured for 24−48 h before live cell imaging. Images were acquired on an Olympus Fluoview 500 confocal microscope with a 60 × 1.40 numerical aperture oil objective. Images were obtained by taking a series of stacks every 0.5 μm through the cell. The light source for the fluorescent studies was a 488 nm laser with a 505−525 nm bandpass filter. Images were quantified using NIH ImageJ version 1.43r and GraphPad Prism version 5.04.

RESULTS

FCPIA Characterization of RGS Inhibitory Activity. CCG-50014 (Figure 1A) was originally identified as an inhibitor of RGS4, -8, and -16 in a polyplex high-throughput screen for identifying inhibitors of the RGS−Gα interaction.[19] Here we confirm that CCG-50014 is highly potent at inhibiting RGS4 (IC_{50} = 30 nM) and show that it fully inhibits several other RGS proteins, including RGS8, -16, and -19, but did not have activity on RGS7 (Figure 1 and Table 1). Furthermore, the compound has no activity on a mutated form of RGS4 that lacks cysteine residues in the RGS homology domain (RGS4Cys$^-$), suggesting that CCG-50014 might be thiol-reactive (Table 1).

CCG-50014 Inhibits the Catalytic GTPase Accelerating Activity of RGS8 and RGS4. In a single-turnover GTPase assay, CCG-50014 inhibited the GTPase accelerating activity of RGS8 and RGS4 on $G\alpha_o$ (Figure 1C−F). Under these assay conditions, RGS8 and RGS4 accelerate the rate of $G\alpha_o$-mediated GTP hydrolysis by approximately 5- and 10-fold, respectively. CCG-50014 inhibited the activity of both RGS proteins. At a maximal concentration (100 μM), CCG-50014 did not alter the intrinsic rate of GTP hydrolysis by $G\alpha_o$, proving that the compound does not act by altering the enzymatic activity of the G-protein under

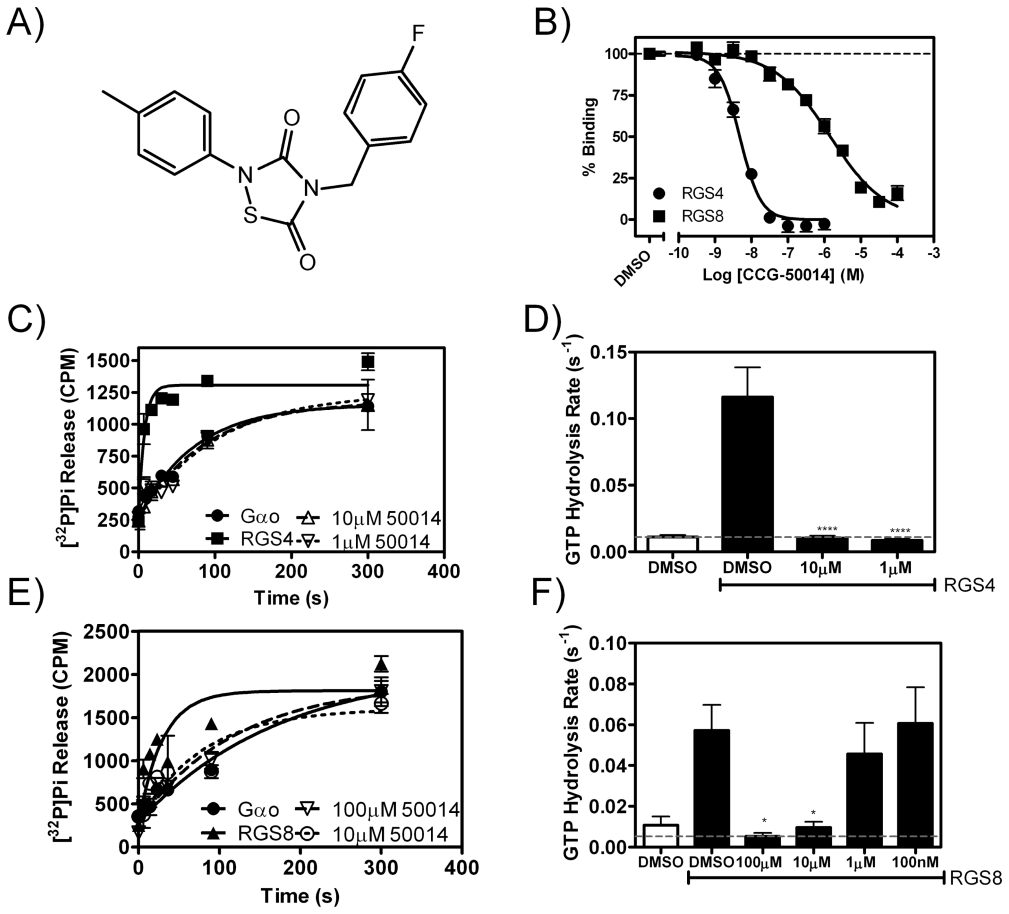

Figure 1. Chemical structure and RGS inhibitory activity of CCG-50014. (A) Chemical structure of CCG-50014 {4-[(4-fluorophenyl)methyl]-2-(4-methylphenyl)-1,2,4-thiadiazolidine-3,5-dione}. B) CCG-50014 concentration-dependently inhibits the binding between aluminum fluoride-activated $G\alpha_o$ and RGS4 or RGS8. Data shown are averages of three independent experiments. This experiment has been independently repeated 28 times, producing average IC_{50} values of 30 ± 6 nM against RGS4 and 11 ± 2 μM against RGS8. (C−F) CCG-50014 also inhibits the GAP activity of (C and D) RGS4 and (E and F) RGS8. Using a single-turnover GAP assay, CCG-50014 concentration-dependently inhibits the GAP activity of both RGS4 and RGS8. *P < 0.05; ***P < 0.0001. All experiments were independently repeated a minimum of three times.

Table 1. RGS Specificity for Inhibition of Gα−RGS Binding[a]

RGS	IC_{50} (μM) ± SEM	Hill slope
RGS4 wild type	0.030 ± 0.006	−1.53
$RGS4Cys^{-}$[b]	N/A[c]	N/A[c]
RGS7	N/A[c]	N/A[c]
RGS8	11 ± 2	−0.99
RGS16	3.5 ± 2.4	−1.33
RGS19	0.12 ± 0.02	−0.61

[a] IC_{50} values in the FCPIA assay show that CCG-50014 is >4-fold more potent for RGS4 than other RGS proteins. Data are presented as mean IC_{50} values ± SEM from at least three independent experiments (for RGS4 and RGS8, n > 28). [b] $RGS4Cys^{-}$ is a mutated form of RGS that contains no cysteine residues in the RGS homology domain.[21] [c] No inhibition below the aqueous solubility limit of the compound (~200 μM).

single-turnover conditions (Figure S1 of the Supporting Information).

CCG-50014 Binds to RGS Proteins but Not to $G\alpha_o$. The melting temperature of a protein can be influenced by the binding of small molecules.[12,13] The thermal denaturation of RGS4, RGS8, and $G\alpha_o$ was characterized in the presence and absence of CCG-50014 using a ThermoFluor instrument. CCG-50014 concentration-dependently destabilized RGS4 and RGS8 (Figure 2A,C,D) but had no effect on $G\alpha_o$ (Figure 2B,E). This suggests that the compound is interacting exclusively with the RGS protein. Compound binding to RGS8 was further confirmed by liquid chromatography−mass spectral (LC-MS) analysis as described below.

CCG-50014 Irreversibly Inhibits RGS Proteins. FCPIA-based reversibility experiments were performed to probe the mechanism of action of the compound on both RGS4 and RGS8 (Figure 3). RGS-coated polystyrene beads were incubated with a saturating concentration (100 μM) of CCG-50014 for 15 min before being thoroughly washed by repeated centrifugation and resuspension. These beads were then analyzed for $G\alpha_o$ binding using FCPIA. Washing of the beads did not restore $G\alpha_o$ binding activity by the RGS proteins. This irreversible inhibition was partially overcome by washing the beads with buffer containing 1 mM dithiothreitol (DTT), suggesting that the mechanism of reactivity could be through sulfhydryl modification, a mechanism in common with the previously described RGS inhibitor, CCG-4986.[21,27]

CCG-50014 Is a Covalent Sulfhydryl Modifier of RGS8. The data thus far suggest that CCG-50014 covalently modifies both

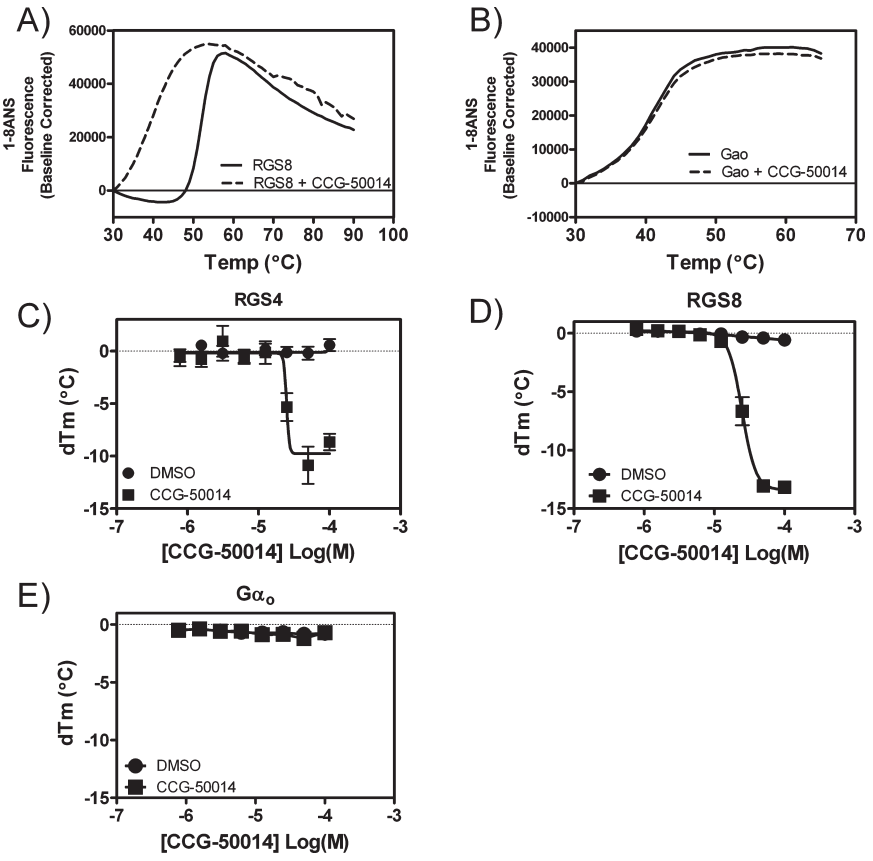

Figure 2. CCG-50014 thermally destabilizes RGS8 in a concentration-dependent manner but has no effect on the thermal stability of $G\alpha_o$. Representative melting traces of (A) RGS8 and (B) $G\alpha_o$ in the absence (—) and presence (---) of 100 μM CCG-50014. Concentration−response curves showing the thermal destabilization effects of CCG-50014 on (C) RGS4, (D) RGS8, and (E) $G\alpha_o$. Data are presented as the means ± SEM of three independent experiments.

RGS4 and RGS8. To test this hypothesis directly, we performed high-performance liquid chromatography−mass spectral analysis on RGS8 samples treated with CCG-50014 (Figure 4A). RGS8 was chosen to simplify the analysis because it contains only two cysteine residues in the RGS homology (RH) domain. After compound treatment, there was a peak shift of the RGS8 corresponding to a full mass adduct of CCG-50014. When wild-type (WT) RGS8 was treated with high concentrations of CCG-50014 (100 μM), a second minor peak corresponding to two full adducts was also observed. To confirm that this action was via cysteine reactivity, the mutant RGS8 in which the two cysteines in the RGS homology (RH) domain were mutated to serine (RGS8cys$^-$) was also analyzed, and no adduct was observed (Figure 4B).

CCG-50014 Depends on Cysteine Residues To Inhibit the AlF_4-$G\alpha_o$−RGS Interaction. To identify the potential cysteine targets of CCG-50014, we studied the compound's effects on RGS8. This protein contains only two cysteines in the RGS homology domain, Cys^{107} and Cys^{160}, making it a more tractable model system than RGS4. Each cysteine from the RGS8 RH domain was mutated to serine, and the activity of the compound was analyzed via FCPIA (Figure 5). These mutants have been named according to the cysteine residue that they retain.[a] Neither cysteine was required for sensitivity to the compound, but mutating both cysteines to serine (RGS8Cys$^-$) reduced the potency of CCG-50014 by >100-fold. The insensitivity of RGS8Cys$^-$ as well as the insensitivity of the RGS4 cysteine null mutant (Table 1) suggests a similar mechanism of action of CCG-50014 on the two proteins.

To determine the relative contribution of each cysteine residue in the RGS8 RH domain in the mechanism of action of CCG-50014, WT RGS8 and the two individual cysteine mutants were treated with a saturating concentration (100 μM) of CCG-50014 before removal of the compound and analysis of the treated protein by gel filtration chromatography and FCPIA (Figure 6). CCG-50014 treatment of WT RGS8 induced a minor mobility shift of the protein on gel filtration, and the recovered protein (with its covalently attached CCG-50014) was analyzed for its ability to compete with untreated bead-bound RGS8 for binding to $G\alpha_o$. In these experiments, untreated RGS8-coated beads were incubated with increasing amounts of the vehicle- or CCG-50014-treated protein that was recovered via gel filtration chromatography. To this mixture was added fluorescently tagged $G\alpha_o$, and the mixture was allowed to equilibrate with the RGS mixtures. If CCG-50014 was forming a stable, irreversible complex with the RGS, it would be expected to compete for $G\alpha_o$ binding less robustly than the vechicle-treated protein. Under these conditions, wild-type RGS8 that was treated with CCG-50014 was 14-fold less potent at competing for Gα binding than was vehicle-treated protein, consistent with residual inhibition of >90%. The CCG-50014- and vehicle-treated 107C RGS8 migrated through the column in a manner identical to that of WT

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RGS8, and no discernible difference in competition for $G\alpha_o$ binding was observed. The 160C RGS8 mutant, however, formed aggregates upon treatment with CCG-50014, and no monomeric, soluble protein was recovered.

To further probe the mechanism of action of this compound, we studied the time course of the development of irreversible inhibition of the RGS8 cysteine mutants. Bead-bound proteins were treated with 20 μM CCG-50014 and then probed for Gα binding using FCPIA. These experiments revealed that the effect of CCG-50014 on 160C RGS8 was completely irreversible (Figure 7A), while the effect on 107C RGS8 could be partially reversed by washing away the compound (Figure 7B).

Because Cys^{160} is buried in the core of RGS8 and Cys^{107} is closer to the surface of the protein, we hypothesized that the compound might interact more rapidly with Cys^{107} than Cys^{160} in the context of wild-type protein. Capitalizing on the fact that there are differential effects of CCG-50014 (partially reversible inhibition vs protein aggregation) depending on which cysteine is labeled, we tested this hypothesis using FCPIA reversibility experiments. The experiment was designed to monitor the development of irreversible inhibition of wild-type RGS8 and the two RGS8 mutants by CCG-50014 in a time-dependent manner (Figure 7C). Wild-type, 107C, or 160C RGS8 was immobilized on beads and treated for varying periods of time with 20 μM CCG-50014 before extensive washing. The beads were then probed for $G\alpha_o$ binding using FCPIA and compared to RGS-coated beads that had been treated with DMSO alone. At this concentration of CCG-50014, binding of 107C RGS8 to Gα was inhibited by ~20% and binding of 160C RGS8 to Gα was inhibited by ~50% at all time points tested. In both cases, the compound rapidly exerted its effect on the RGS protein. The wild-type protein exhibited a delayed development of irreversible inhibition; at early time points, the level of inhibition was ~20% and increased to, but did not exceed, ~50% over 30 min. This suggests that there is a differential mechanism of action of the compound on the two individual mutants that is combined in the wild-type protein with a kinetic lag that is only seen when both cysteines are present.

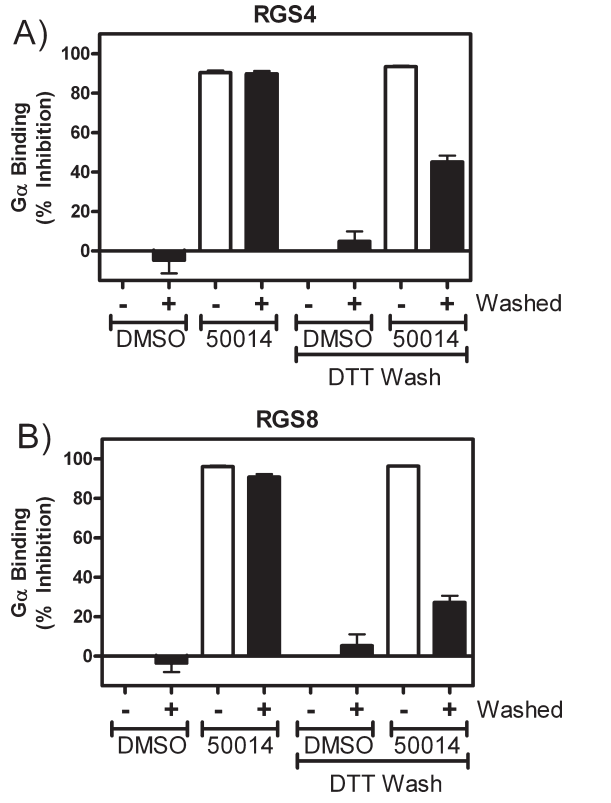

Figure 3. CCG-50014 is an irreversible inhibitor of RGS4 and RGS8, and its effects are partially reversed by the thiol reductant DTT. (A) RGS4- and (B) RGS8-containing beads were treated for 15 min with 100 μM CCG-50014 prior to vigorous washing to remove any unbound compound. To determine if the compound was reacting in a thiol-sensitive manner, washing was performed in the absence or presence of 1 mM DTT. Data are presented as the means ± SEM from at least three independent experiments.

CCG-50014 Is Not a General Cysteine Alkylator. Cysteine-reactive compounds might be expected to have more off-target effects than nonreactive compounds. To determine if this compound could bind to and inhibit reactive cysteines in non-RGS proteins, we tested the ability of CCG-50014 and a known cysteine alkylator [iodoacetamide (IA)] to inhibit the cysteine protease papain (Figure 8). IA inhibited the proteolytic activity of papain in a concentration-dependent manner. However, even at high concentrations (100 μM), CCG-50014 had no effect on papain activity. This suggests that there is selectivity of this class of compounds for cysteines in the RGS over other reactive cysteines.

General Cysteine Alkylators Do Not Inhibit RGS Proteins. One potential explanation for the observed RGS selectivity of

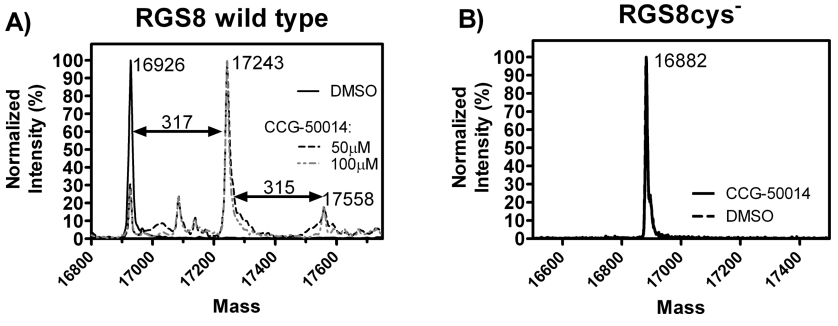

Figure 4. CCG-50014 forms a covalent adduct on RGS8. (A) WT RGS8 protein (2 μM) was treated with the indicated concentrations CCG-50014 before analysis via LC-MS. After treatment with compound, a predominant peak appeared with a mass shift of 317 as compared to that of the vehicle-treated protein, correlating with a full compound mass adduct (CCG-50014 MW of 316.4). A second minor peak with an additional mass shift of 315 was observed, which correlates to the addition of a second adduct of CCG-50014. (B) No adducts are observed on the RGS8Cys^- mutant, in which both C^{107} and C^{160} have been mutated to serine.

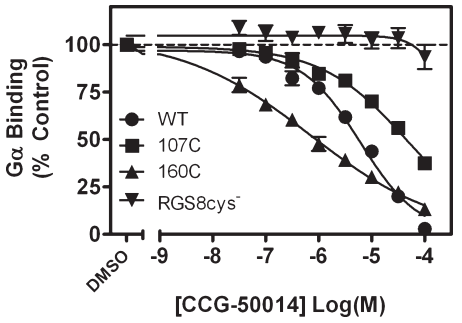

Figure 5. CCG-50014 requires at least one cysteine residue on RGS8 for full activity. Wild-type or mutant RGS8 was biotinylated and loaded on beads, and AlexaFluor-532-Gα_o binding was assessed by FCPIA as described in Experimental Procedures. Mutating both cysteines to serine (RGS8Cys$^-$) produced a protein that was completely insensitive to the effect of CCG-50014. RGS8 mutants with only one cysteine, either Cys^{107} (107C) or Cys^{160} (160C), provided sensitivity to CCG-50014. The inhibition parameters (IC_{50} and Hill coefficient) for CCG-50014 on these proteins were as follows: 6.1 μM and −0.79 for wild-type RGS8 (WT), 46.5 μM and −0.54 for 107C, 0.71 μM and −0.36 for 160C, and >100 μM for RGS8Cys$^-$, respectively. Data are presented as the means ± SEM of three independent experiments.

CCG-50014 could be that RGS proteins are particularly sensitive to thiol modification. We tested the ability of two general cysteine alkylators, *N*-ethylmaleimide (NEM) and IA, to inhibit the RGS−Gα_o interaction (Figure 9). IA had no effect on the binding of Gα_o to any of the RGS proteins tested. At high concentrations (IC_{50} = 30 μM), NEM inhibited RGS4−Gα_o binding; however, it had no effect on RGS8 (Figure 9) or papain (data not shown). These data show that CCG-50014 is more than 3.5 orders of magnitude more potent on RGS4 than either of the general cysteine alkylators tested. This strongly suggests that RGS proteins are not particularly sensitive to cysteine modification and the effect observed by CCG-50014 is more than just random thiol alkylation.

Computational Modeling of the CCG-50014−RGS8 Interaction. To identify potential binding sites for CCG-50014 on RGS8, we performed an unbiased molecular docking simulation over the whole RGS8 molecular surface. Of the 100 docking poses obtained from the simulation, CCG-50014 docked solely in one pocket on the RGS. This is located near the region of the surface of RGS8, the corresponding "B" site of RGS4 (Figure 10). The "B" site[18] has been suggested as the locus of allosteric inhibition of RGS4 family proteins by acidic lipids.[16] This places the compound a considerable distance from the two cysteine

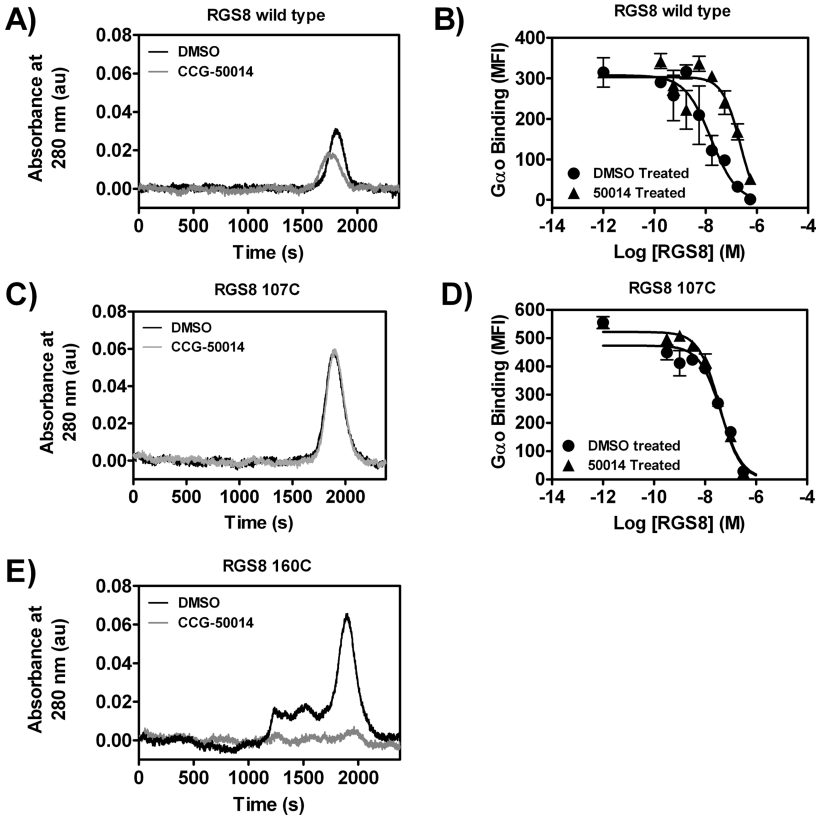

Figure 6. CCG-50014-induced protein aggregation is dependent on the presence of 160C. (A and B) Wild-type, (C and D) 107C, or (E) 160C RGS8 was treated with a 5-fold excess of CCG-50014 before removal of the compound via gel filtration on a 20 mL S75 Superdex column. (A, C, and E) Shown are representative UV chromatogram traces. (B and D) Protein recovered from the peak was tested for Gα_o binding in an FCPIA competition assay with AlexaFluor-532-Ga_o binding to WT RGS8. The wild-type RGS8 chromatogram shows a slightly left shifted and suppressed peak after CCG-50014 treatment, which coincides with a 14-fold decrease in the level of Gα_o binding. The 107C mutant protein exhibited no CCG-50014-induced change in migration on gel filtration, and any inhibition of Gα_o binding activity was reversed by the gel filtration procedure. The 160C mutant protein completely (and visually) aggregates upon compound treatment and is removed by the prefiltration of the samples.

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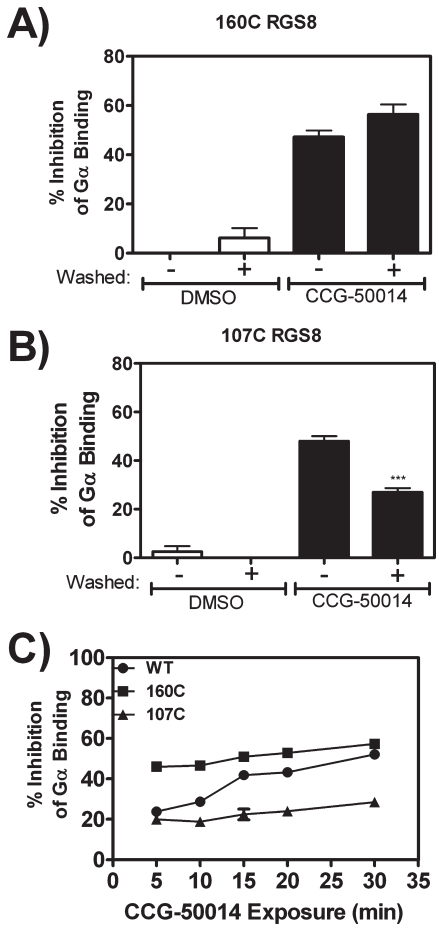

Figure 7. Irreversible inhibition of RGS8 is predominantly mediated by Cys^{160}. Mutant proteins (A) 107C RGS8 and (B) 160C RGS8 were prebound to beads and exposed to 20 μM CCG-50014, after which reversibility experiments were performed. (C) Development of irreversible inhibition after exposure to CCG-50014 differs between the individual cysteine mutants and provides a means of understanding the compound's mechanism of action. Wild-type, 160C, or 107C RGS8 was treated with 20 μM CCG-50014 for the indicated amount of time before the compound was removed by extensive washing. The amount of irreversible inhibition was quantified by comparing the G-protein binding to CCG-50014-treated beads and DMSO-treated beads. The total amount of inhibition (without a wash step) at this concentration of CCG-50014 for each protein was as follows: 64 ± 2% for WT RGS8, 45 ± 2% for 107C RGS8, and 56 ± 1% for 160C RGS8. Data are presented as the means ± SEM from three independent experiments. ***$P < 0.0001$ using an unpaired t test.

residues known to play a role in the compound's inhibitory activity (Figure 10B). It would require a substantial change in the conformation of the protein for the compound to dock at this site and react with a cysteine residue.

Limiting the Reactivity of CCG-50014 Diminishes Potency. To determine the importance of the cysteine reactivity in the mechanism of action of this compound, an analogue of CCG-50014 in which the sulfur was replaced with a methylene was synthesized and tested for activity (Table S1 of the Supporting Information). This compound has limited, if any, activity in the FCPIA assay, suggesting that the main mechanism of action of CCG-50014 is through covalent reactivity with one or more cysteine residues on the RGS.

CCG-50014 Inhibits the $G\alpha_o$-Dependent Membrane Translocation of RGS4 in Living Cells. RGS4 is a cytosolic protein that can be recruited to the plasma membrane by overexpression of certain Gα subunits (Figure 11A−C) or GPCRs.[28] CCG-50014 inhibited the $G\alpha_o$-dependent membrane translocation of GFP-RGS4 in living HEK-293T cells (Figure 11). Treatment of cells that were transiently transfected with GFP-RGS4 and $G\alpha_o$ with vehicle [0.1% DMSO (Figure 11D,E,H)] does not alter the membrane localization of RGS4; however, 100 μM CCG-50014 reversed the membrane localization of the RGS (Figure 11F,G,I). Through line scan analysis, we observed a significant decrease in the extent of membrane localization of the RGS after treatment with CCG-50014. There was also a trend toward an increase in the magnitude of the cytosolic signal of GFP-RGS4 after compound treatment, suggesting the observed phenomenon was not due to nonspecific diminishment of the GFP signal.

DISCUSSION

Molecules disrupting the RGS−Gα interaction should increase the magnitude and/or duration of G-protein signaling responses, leading to pronounced physiological effects. Genetic ablation of RGS expression produces dramatic phenotypes, suggesting that a small molecule RGS inhibitor might provide similar actions in vivo.

CCG-50014 is the most potent small molecule RGS inhibitor identified to date. It inhibits the in vitro interaction between RGS4 and $G\alpha_o$ with an IC_{50} of 30 nM. It is >100-fold selective for RGS4 over two closely related RGS proteins, RGS8 and RGS16 (Figure 1 and Table 1). CCG-50014 is a covalent modifier of cysteine residues (Figure 4), raising concerns about the therapeutic potential of this class of compounds. Even so, this compound has provided significant insight into the mechanism of allosteric RGS inhibition; furthermore, it is the first RGS inhibitor to block the RGS4−$G\alpha_o$ protein−protein interaction in living cells.

The sensitivity of RGS to CCG-50014 requires at least one cysteine residue, a hallmark of a sulfhydryl-reactive irreversible inhibitor.[19] This observation was confirmed by FCPIA reversibility experiments (Figure 3) and subsequent mass spectral analysis of CCG-50014-treated RGS8 (Figure 4). On the basis of the chemical structure of the compound and the full molecular weight adduct observed in the LC-MS experiments, it is likely that the mechanism of reaction of CCG-50014 with a cysteine residue on an RGS protein is by nucleophilic attack of the cysteine thiol on the sulfur atom of the central heterocycle, causing ring opening through cleavage of the sulfur−nitrogen bond. This would be consistent with the reported reactivity of 1,2,4-thiadiazolidine-3,5-diones with triphenylphosphine.[20] The resultant disulfide linkage is sensitive to reductants, which is consistent with the DTT-induced reversibility of CCG-50014 inhibition (Figure 3).

Interestingly, CCG-50014 interacts with cysteine residues in RGS8 that are distant from the Gα interaction interface (Figure 10), suggesting an allosteric mechanism of action similar to previously reported effects of two other RGS inhibitor compounds.[21,29] Unbiased computational modeling predicts that CCG-50014 could bind noncovalently to a site on RGS8 that is near the acidic phospholipid binding site on RGS4.[16,17] Binding in this site would place the reactive group of CCG-50014 within 8−13 Å of the two cysteines in the RGS8 RH domain. While at

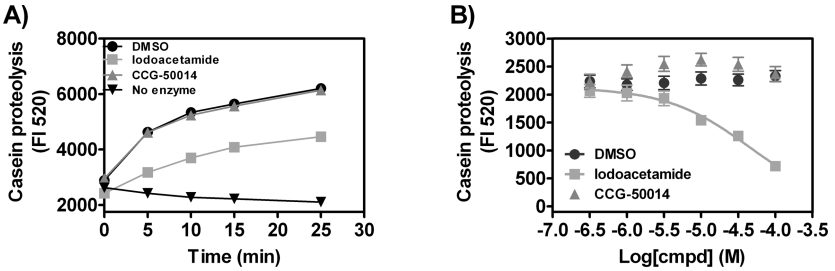

Figure 8. CCG-50014 does not inhibit the cysteine protease, papain. (A) Papain (0.625 unit) was mixed with self-quenching FITC-conjugated casein, and the liberated fluorescence that results from casein-dependent proteolysis was observed as a function of time in the presence of different cysteine alkylators. Iodoacetamide (100 μM) markedly inhibits casein proteolysis by papain, while 100 μM CCG-50014 has no effect. (B) Concentration dependence of the effect of compounds on casein proteolysis (5 min). Data are presented as the means ± SEM from three independent experiments.

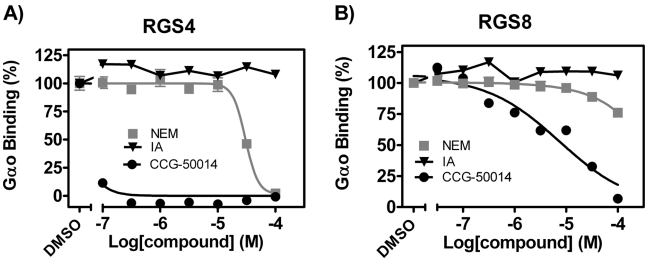

Figure 9. CCG-50014 is a much more potent RGS inhibitor than two general cysteine alkylators, *N*-ethylmaleimide (NEM) and iodoacetamide (IA). Dose−response curves for NEM, IA, and CCG-50014 vs (A) RGS4 and (B) RGS8. The only protein that displayed any sensitivity to the alkylators tested was RGS4, which was inhibited by NEM with an IC_{50} value >3.5 log higher than that of CCG-50014. Data are presented as the means ± SEM from three independent experiments.

this distance, it is unlikely that a covalent bond could be formed; the compound may initially bind to this pocket, and a subsequent conformational change in the protein could provide access to the cysteine thiol. Assuming that the compound docks as modeled, this conformational change is likely to be the fundamental mechanism by which the allosteric modulation of G-protein binding activity is conferred.

The differential sensitivities of the cysteine mutants to CCG-50014 would also be explained by this binding modality. The decreased sensitivity to and increased reversibility of CCG-50014 on 107C RGS8 (Figure 5) are in accord with the fact that Cys^{107} is more solvent accessible and is closer to the hypothesized binding site of the compound. Compound reacting with Cys^{160} causes drastic protein unfolding (Figure 6), which also fits with this model.

The data presented here allow us to postulate a potential mechanism of action for CCG-50014. We propose that the compound initially interacts with Cys^{107}, possibly because the compound may dock at a site close to this residue. Upon reacting with this cysteine, CCG-50014 can trap the RGS in a conformation that is incapable of binding to Gα. Reversal of this reaction is possible, leading to reactivation of the RGS on washing at early times. If the compound interacts with the more deeply buried cysteine (Cys^{160}), it causes a dramatic conformational change in the protein (likely a disruption of the hydrophobic core), leading to protein unfolding. Fitting with this hypothesis, our data suggest that Cys^{107} is labeled more rapidly than Cys^{160} in the wild-type protein (Figure 7C). Then, either the Cys^{107}-bound compound transfers to Cys^{160}, or a second CCG-50014 molecule

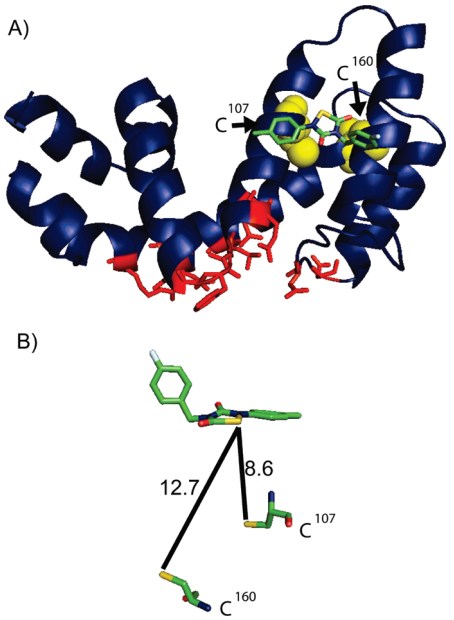

Figure 10. Hypothesized binding site of CCG-50014 on RGS8. (A) CCG-50014 was docked to RSG8 using Autodock (version 4.0) as described in Experimental Procedures, and one docking site showed the greatest predicted affinity (K_i = 18 μM). This site is far from the Gα binding interaction interface and is near RGS4, which is important for RGS regulation by calmodulin and acidic phospholipids. Conserved residues between RGS4 and RGS8 that directly contact $G\alpha_i$ in the RGS4−$G\alpha_{i1}$ structure (PDB entry 1AGR[32]) are colored red. (B) Assuming a static protein, this binding site places the compound close to the two cysteine residues in RGS8, but not within a distance compatible with direct covalent reaction. A conformational change must occur in the RGS to allow intercalation of the compound into the helix bundle. Distances are shown in angstroms. RGS8 structure from PDB entry 2IHD.

binds to Cys^{160} to produce the completely irreversible protein denaturation observed in gel filtration experiments (Figure 6). Therefore, the mechanism behind the irreversible inhibition after labeling of Cys^{160} is likely due to a massive destabilization of the hydrophobic core of the RH domain that would occur by the insertion of CCG-50014.

The development of cysteine-reactive small molecule inhibitors into useful research probes and therapeutic agents is challenging, yet surmountable. There are a few successful therapeutics that function by covalently binding to cysteine residues.

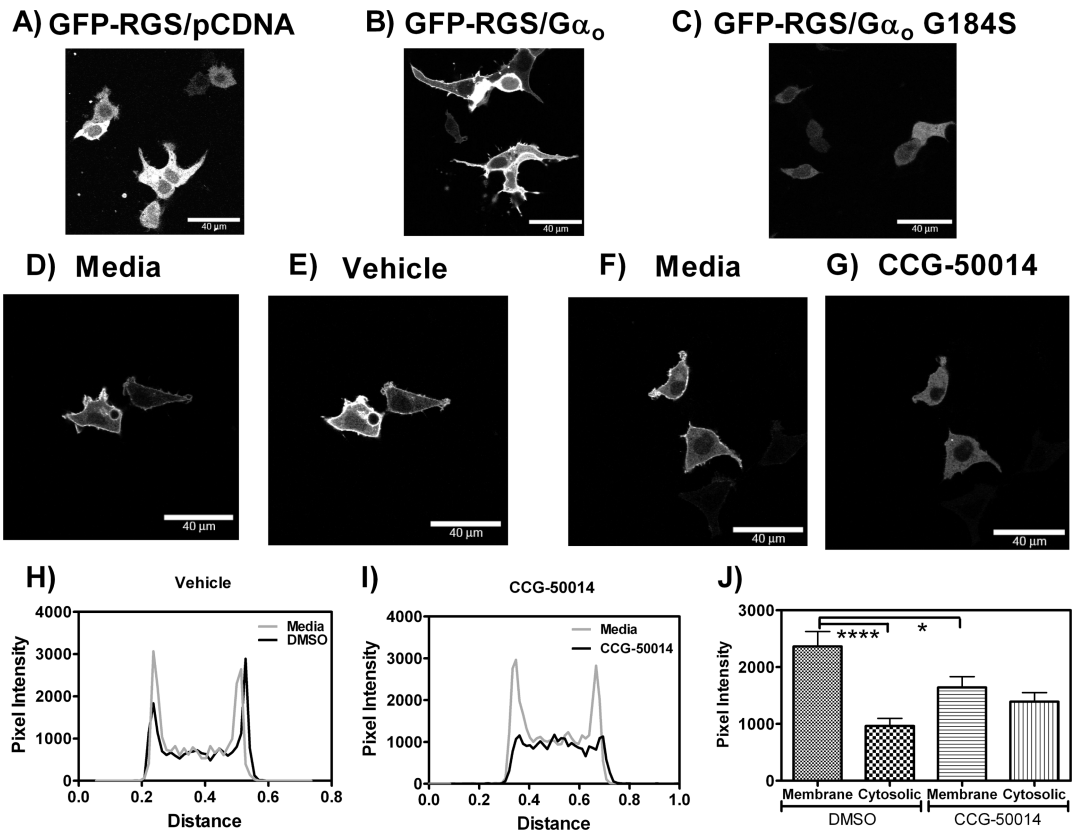

Figure 11. CCG-50014 inhibits the $G\alpha_o$-dependent membrane localization of RGS4. (A) When overexpressed in HEK-293T cells, GFP-RGS4 is localized to the cytosol. (B) Coexpression with $G\alpha_o$ induces a subcellular translocation of GFP-RGS4 to the plasma membrane. (C) This translocation does not occur in response to coexpression with the RGS-insensitive $G\alpha_o$ mutant (G184S). (D and E) Cells expressing $G\alpha_o$ and GFP-RGS4 show no change in the plasma membrane localization of the RGS when treated with vehicle (0.1% DMSO); however, treatment with (F and G) CCG-50014 (100 μM) rapidly induces a loss of the plasma membrane localization of the RGS without diminishing the overall signal. Representative line scans across a cell treated with (H) vehicle and (I) CCG-50014 also show this effect. (J) Quantification of this effect from 10 cells treated with vehicle or CCG-50014 (100 μM) shows a significant decrease in the amount of RGS-GFP located at the membrane after addition of compound. A trend toward an increased level of cytosolic localization after compound treatment is also observed, suggesting that the treatment does not just diminish the GFP signal. $^{****}P < 0.0001$; $^{*}P < 0.05$.

For example, the acid-reflux drug omeprazole operates in the stomach by covalently modifying a proton exchanger.[30] There is also a class of cysteine-reactive irreversible tyrosine kinase inhibitors, typified by CI-1033, that are currently in clinical trials.[31] Cysteine-reactive compounds thus have a place in modern pharmacology; however, they must be closely studied to determine their selectivity profile. While a comprehensive analysis of the effects of CCG-50014 on all cysteine-dependent processes in a cell is clearly impossible, we show that CCG-50014 does not inhibit the activity of the cysteine protease papain at concentrations more than 3000 times higher than that required for RGS inhibition (Figure 8). In contrast, the cysteine alkylator iodoacetamide concentration-dependently inhibited the activity of this protease but had no effect on RGS4 or RGS8. This suggests that there is some selectivity of this class of compounds for cysteines in the RGS over other reactive cysteines. It is possible that the compound cannot enter the active site of papain, and therefore, it would be prudent to extend these studies to a panel of physiologically relevant thiol-dependent processes.

Furthermore, we have shown that CCG-50014 is able to inhibit the $G\alpha_o$-dependent membrane localization of RGS4 in living cells (Figure 11), representing the first small molecule RGS inhibitor with cellular activity. These data suggest that CCG-50014 or related analogues should be useful pharmacological probes for studying the physiological roles of RGS proteins in biology.

In this study, we characterize the mechanism of action of the most potent RGS inhibitor identified to date. While this compound has significant liabilities as a potential drug candidate, it does highlight the fact that it is possible for a small molecule to inhibit the RGS−Gα protein−protein interaction with nanomolar potency. Furthermore, we have shown that CCG-50014 is able to inhibit the RGS4−$G\alpha_o$ protein−protein interaction in living cells. Current work is focused on characterizing the structure−activity landscape of this compound class and adapting these studies to physiological models of RGS activity.

■ ASSOCIATED CONTENT

Supporting Information. A table containing the chemical structure and RGS inhibitory activity of CCG-50014 and CCG-303778, an analogue of CCG-50014 in which the sulfur atom is replaced with a methylene, and a figure depicting the rate constants of GTP hydrolysis by $G\alpha_o$ in the presence and absence

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of 100 μM CCG-50014. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

[a]The RGS8 construct used in this study contains two cysteine residues, C^{107} and C^{160}. Individual cysteine → serine mutants were generated and named on the basis of the cysteine residue that was maintained in the primary sequence. Therefore, C160S RGS8 is called 107C RGS8, and C107S is called 160C RGS8. The double mutant (C160S/C107S) is called RGS8Cys$^-$.

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ABBREVIATIONS

GPCR, G-protein-coupled receptor; RGS, regulator of G-protein signaling; FCPIA, flow cytometry protein interaction assay; DTT, dithiothreitol; IA, iodoacetamide; NEM, *N*-ethylmaleimide; SEM, standard error of the mean; T_m, melting temperature; ESI-LC-MS, electrospray ionization liquid chromatography and mass spectrometry; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid; FITC, fluorescein isothiocyanate; RH, RGS homology.

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