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SCH-202676: An Allosteric Modulator of Both Agonist and Antagonist Binding to G Protein-Coupled Receptors

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ABSTRACT

A novel thiadiazole compound, SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5-(2*H*)-ylidene)methanamine), has been identified as an inhibitor of both agonist and antagonist binding to G protein-coupled receptors (GPCRs). SCH-202676 inhibited radioligand binding to a number of structurally distinct, heterologously expressed GPCRs, including the human μ-, δ-, and κ-opioid, α- and β-adrenergic, muscarinic M_1 and M_2, and dopaminergic D_1 and D_2 receptors, but not to the tyrosine kinase epidermal growth factor receptor. SCH-202676 had no direct effect on G protein activity as assessed by [^{35}S]guanosine-5′-*O*-(γ-thio)triphosphate binding to purified recombinant $G_{o\alpha}$- or $G_{\beta\gamma}$-stimulated ADP-ribosylation of $G_{o\alpha}$ by pertussis toxin. In addition, SCH-202676 inhibited antagonist binding to the β_2-adrenergic receptor expressed in *Escherichia coli*, a system devoid of classical heterotrimeric G proteins. SCH-202676 inhibited radiolabeled agonist and antagonist binding to the α_{2a}-adrenergic receptor with an IC_{50} value of 0.5 μM, decreased the B_{max} value of the binding sites with a slight increase in the K_D value, and inhibited agonist-induced activation of the receptor. The effects of SCH-202676 were reversible. Incubation of plasma membranes with 10 μM SCH-202676 did not alter subsequent radioligand binding to the α_{2a}-adrenergic receptor and the dopaminergic D_1 receptor. Taken together, our data suggest that SCH-202676 has the unique ability to allosterically regulate agonist and antagonist binding to GPCRs in a manner that is both selective and reversible. The scope of the data presented suggests this occurs by direct interaction with a structural motif common to a large number of GPCRs or by activation/inhibition of an unidentified accessory protein that regulates GPCR function.

G protein-coupled receptors (GPCRs) are a family of structurally related membrane-bound proteins that play a central role in the recognition and signal transduction of hormones and neurotransmitters. GPCRs mediate the response for a variety of sensory stimuli such as vision, smell, and pain, and for many hormonal regulatory systems. Both small-molecule natural products and synthetically designed therapeutic agents exert their effects on GPCRs by acting either as agonists that mimic the function of the endogenous ligand for its receptor or as antagonists that block the effect of such ligands. The binding of an agonist to its receptor results in a change in the conformation of the receptor that leads to the activation of specifically associated heterotrimeric G proteins. In turn, this activation initiates a cascade of signaling events within the cell. Alternatively, antagonist binding stabilizes an inactive conformation of the receptor and blocks agonist-induced conformational changes and signal transduction (Gether and Kobilka, 1998).

GPCRs share general structural motifs, including seven transmembrane helices connected by intra- and extracellular loops, an extracellular amino terminus and a cytoplasmic carboxyl terminus. Based on amino acid sequence, ligand pharmacology, and receptor function, there have been over 100 distinct members of this receptor superfamily identified to date (Ji et al., 1998). Conclusions drawn from a large number of studies suggest that general themes apply to the molecular interactions between GPCRs and their cognate ligands (Ji et al., 1998). For example, small ligands generally bind to sites within the hydrophobic core of the transmembrane α-helices, whereas the binding sites for larger peptides and proteins are comprised of the amino terminus and the extracellular, hydrophilic loops (Gether and Kobilka, 1998). Furthermore, there is enough structural diversity among GPCRs to design selective agonists and antagonists for different receptor subtypes.

We report herein that a novel thiadiazole, SCH-202676,

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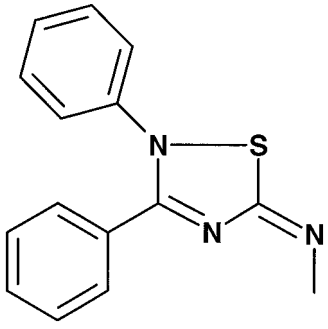
ABBREVIATIONS: GPCRs, G protein-coupled receptors; EGF, epidermal growth factor; CHO, Chinese hamster ovary; GTPγS, guanosine-5′-*O*-(γ-thio)triphosphate; DTT, dithiothreitol.

inhibits agonist and antagonist binding to a wide variety of unrelated GPCRs. A number of compounds that inhibit agonist binding via modulation of G protein function have been described previously (Herrmann and Jakobs, 1988; Anand-Srivastava, 1989; Huang et al., 1990; Beindl et al., 1996; Freissmuth et al., 1996). In contrast, data detailed in this report imply that SCH-202676 modulates the binding of both agonists and antagonists in a G protein-independent manner. The data support the notion that SCH-202676 interacts with a structurally conserved, allosteric regulatory site on GPCRs or, alternatively, with a common accessory modulator of GPCR function.

Experimental Procedures

Chemistry. Figure 1, below, shows the structure of SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5-(2*H*)-ylidene)methanamine). SCH-202676 (M_r 267.29) was purchased from Sigma-Aldrich, St Louis, MO (special chemical collection) and was prepared as described previously by Goerdeler and Eggers (1986).

Radiolabel Ligand Binding Assays. Radiolabeled agonist and antagonist binding to muscarinic (Gattu et al., 1995), α-adrenergic (Huang et al., 1990), β-adrenergic (Strader et al., 1987), dopaminergic (Tice et al., 1994); δ- (Malatynska et al., 1995), κ- (Zhu et *al.*, 1995), and μ-opioid (Wang et al., 1994); and epidermal growth factor (EGF) (Dittadi et al., 1990) receptors were performed as described by each cited reference. Human clones of the receptors expressed in Chinese hamster ovary (CHO) cells or other cell lines were used for the assays. SCH-202676 was tested in a concentration range of 0.01 to 100 μM. IC_{50} values for SCH-202676 inhibition of the binding were determined by curve fitting the data with GraphPad Prism (GraphPad Software, San Diego, CA). Radiolabeled ligand concentrations used in all assays approximated the K_D value of the receptors. However, K_i values were not derived from the IC_{50} values, because a competitive interaction of SCH-202676 with the receptors could not be proved.



SCH-202676

Fig. 1. Structure of SCH-202676 (*N*-(2,3-diphenyl-1,2,4-thiadiazol-5(2*H*)-ylidene)methanamine, M_r 267.29).

Expression of Human β_2-Adrenergic Receptor in DH5α Bacterial Cells. The β_2-adrenergic receptor was expressed in *Escherichia coli* by a modification of methods described previously by Marullo et al. (1988) and Freissmuth et al. (1991). The human β_2-adrenergic receptor (cDNA obtained from Dr. R. Lefkowitz, GenBank accession number 4501968) was amplified using polymerase chain reaction primers designed to incorporate an *Eco*RI site at the 5′-end, and *Sal*I at the 3′-end (upper primer: 5′-CTTGAATTCGGGGCAACCCGGGAACGG-3′, lower primer: 5′-TCTGTCGACTTACAGCAGTGAGTCATT-3′, respectively). After digestion with the corresponding restriction enzymes, the polymerase chain reaction product was ligated into a pFLAG-1 vector (Eastman Kodak Co., Rochester, NY). The nucleotide sequence of the pFLAG-1 β_2-adrenergic receptor cDNA was verified using the dRhodamine Terminator Cycle Sequencing Reaction system (PE Biosystems, Foster City, CA) and analyzed on an ABI PRISM 377 automated DNA sequencer (PE/ABI, Foster City, CA). Transformation of the purified plasmid into *E. coli* strain DH5α was performed using the standard commercial protocol provided with the DH5α competent cells (Life Technologies, Gaithersburg, MD). The pFLAG/β_2-adrenergic receptor-positive DH5α transformants were cultured at 37°C in ampicillin-containing (100 μg/ml) Luria broth culture to an optical density of 500 (λ= 600 nm) at which point 0.5 mM isopropylthio-β-D-galactoside was added. After additional incubation for 2.5 h at 23°C, membranes were isolated as described previously (Stanasila et al., 1999). Membrane pellets were resuspended in 1 ml of cold 50 mM Tris-HCl, pH 7.4, containing 10% glycerol and 1% BSA. Aliquots were frozen in liquid nitrogen and stored at −80°C. Protein determinations were made before the BSA addition using the micro bicinchoninic acid assay (BCA; Pierce, Rockford, IL). Competition binding of [^{125}I]iodocyanopindolol to 50 μg of pFLAG-1/β_2-adrenergic receptor DH5α membranes in 500 μl of buffer containing 75 mM Tris-HCl, pH 7.4, 12.5 mM $MgCl_2$, 2 mM EDTA, and protease inhibitors (Complete+, EDTA; Boehringer Mannheim) was performed as described before (Perkin Elmer Life Sciences, Norwalk, CT).

Human α_{2a}-Adrenergic Receptor Binding Assays. [^{3}H]Yohimbine and [^{3}H]UK-14,304 binding to human HT-29 adenocarcinoma cell membranes expressing the α_{2a}-adrenergic receptor were

TABLE 2

Effect of SCH-202676 on [^{125}I]EGF binding to A431 cell membranes

[^{125}I[EGF binding to A431 cell membranes expressing the EGF receptor in absence (control) and presence of 10 μM SCH-202676 was performed as described under *Experimental Procedures*. Data shown are mean values ± S.E.M. of triplicates.

Treatment	[^{125}I]EGF Specific Bound
	cpm
Control	12,054 ± 518
10 μM SCH-202676	14,551 ± 785

TABLE 1

Effect of SCH-202676 on antagonist binding to GPCRs

Binding assays were performed as described under *Experimental Procedures* using recombinant human receptors. Data shown are mean ± S.E. *n* = number of observations.

Receptor	G-Protein Coupling	Radiolabeled Ligand	IC_{50}	*n*
			μM	
δ-Opioid	G_i	[^{3}H]Diprenorphine	0.3 ± 0.04	3
κ-Opioid	G_i	[^{3}H]Diprenorphine	1.7 ± 0.09	4
μ-Opioid	G_i	[^{3}H]Diprenorphine	1.8 ± 0.02	4
M_1	G_q	[^{3}H]*N*-Methylscopolamine	0.4	2
M_2	G_i	[^{3}H]*N*-Methylscopolamine	0.4	2
α_{2a}-Adrenergic	G_i	[^{3}H]Yohimbine	0.5 ± 0.1	4
β_1-Adrenergic	G_s	[^{125}I]Idocyanopindolol	1.2 ± 0.03	4
D_1	G_s	[^{3}H]SCH-23390	0.1 ± 0.0	4
D_2	G_i	[^{3}H]Methylspiperone	0.1 ± 0.0	4

carried out as described previously (Turner et al., 1985). Briefly, 40 μg of HT-29 cell membranes was incubated with 1 to 1.2 nM [^{3}H]yohimbine or about 0.4 nM [^{3}H]UK-14,304 in 200 μl of buffer containing 50 mM Tris-HCl. pH 7.4, 10 mM $MgCl_2$, 1 mM EGTA, and 1 mg/ml BSA. Assays were carried out for 60 min at room temperature and terminated by rapid filtration over GF/B filters presoaked in 0.3% polyethylenimine. Samples were washed seven times with 2 ml of cold (4°C) 10 mM Tris-HCl, pH 7.4, and radioactivity retained on the filters was quantified using a scintillation counter. Nonspecific binding was determined in the presence of 10 μM yohimbine.

Saturation binding assays were performed using 0.1 to 10 nM [^{3}H]yohimbine and 0.1 to 20 nM [^{3}H]UK-14,304. K_D and B_{max} values were derived from the binding data by the method of Scatchard (1949).

GTPγS Binding Assays. [^{35}S]GTPγS binding to purified recombinant $G_{o\alpha}$ was performed using a modified method described by Sternweis and Robishaw (1984). The binding assay was initiated by addition of 2 μM [^{35}S]GTPγS (2500 cpm/pmol) to purified recombinant $G_{o\alpha}$ in buffer containing 50 mM Na-HEPES, pH 8.0, 1 mM EDTA, 1 mM DTT, 25 mM $MgCl_2$, and 0.1% polyoxyethylene-10-lauryl ether. Reactions were performed at 10°C and terminated by a 40-fold dilution in ice-cold (4°C) buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 25 mM $MgCl_2$. The $G_{o\alpha}$ was harvested by rapid filtration over BA85 nitrocellulose filters (Schleicher and Schuell, Keene, NH) followed by extensive washes with buffer. Filters were then suspended in liquid scintillation cocktail, and the amount of [^{35}S]GTPγS bound to the protein was quantified using liquid scintillation spectrometry.

Agonist-induced increase in [^{35}S]GTPγS binding was used to evaluated functional activation of the receptor. Assays were performed on 20 μg of CHO cell membranes expressing the α_{2a}-adrenergic receptor. Briefly, reactions were performed in buffer containing 50 mM Na-HEPES, pH 7.4, 120 mM NaCl, 10 mM $MgCl_2$, 0.2 mM EGTA, 10 μM GDP, and 1 mg/ml BSA. Membranes were incubated with compounds for 1 h at room temperature, and assays were initiated by addition of [^{35}S]GTPγS (400–500 pM). Reactions were carried out at room temperature for 30 min and were terminated by rapid filtration over GF/B filters. Filters were washed seven times with cold (4°C) buffer containing 20 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 20 mM $MgCl_2$. Filters were then suspended in liquid scintillation cocktail, and the amount of [^{35}S]GTPγS bound to the membrane was quantified using liquid scintillation spectrometry.

Pertussis Toxin-Stimulated ADP-Ribosylation of Recombinant $G_{o\alpha}$. ADP-ribosylation of $G_{o\alpha}$ was performed as described by Casey et al. (1989). Purified recombinant $G_{o\alpha}$ and $G_{\beta1\gamma2}$ were prepared in a reaction mixture containing 50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM DTT, 2 mM $MgCl_2$, 5 μM NAD, [adenylate-^{32}P]NAD, 200 μM GDP, 1 mM dimyristoylphosphatidylcholine, and 0.1% polyoxyethylene-10-lauryl ether. Assays were initiated by the addition of 200 ng of pertussis toxin preactivated for 60 min at 30°C in buffer containing 10 mM Tris-HCl, pH 7.5, 5 mM DTT, and 5 mM ATP. Reactions were carried out at room temperature and subsequently terminated by a 40-fold dilution into 2% (w/v) SDS with 50 μM NAD. Samples were precipitated by addition of trichloroacetic acid to a final concentration of 15% (w/v). Proteins were then harvested by rapid filtration over BA85 nitrocellulose filters (Schleicher and Schuell) and washed extensively with 6% trichloroacetic acid. Filters were suspended in liquid scintillation cocktail, and the amount of [adenylate-^{32}P]NAD incorporation was quantified using liquid scintillation spectrometry.

All experiments were performed three to five times. Results shown

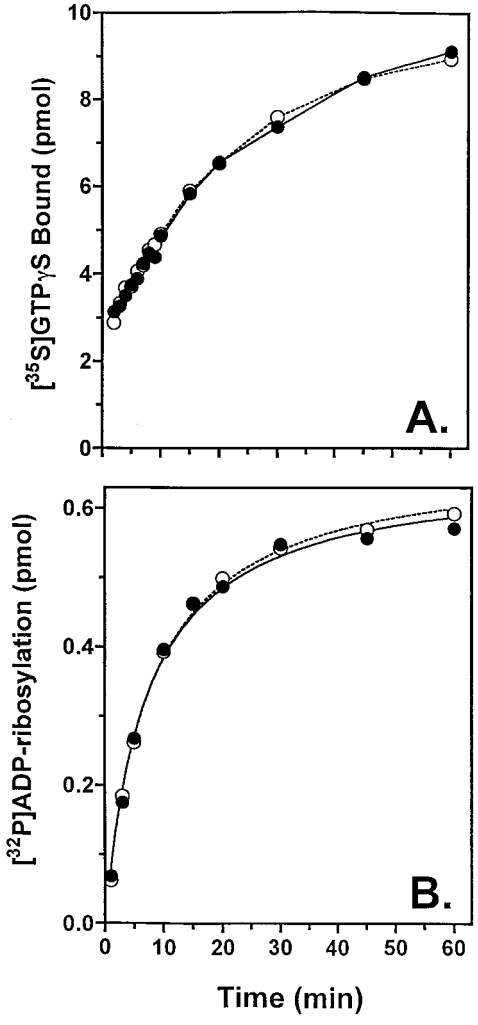


Fig. 2. Effect of 10 μM SCH-202676 on [^{35}S]GTPγS binding to $G_{o\alpha}$ (A) and $\beta\gamma$-stimulated ADP-ribosylation of $G_{o\alpha}$ by pertussis toxin (B). A, [^{35}S]GTPγS (2307 cpm/pmol) binding to $G_{o\alpha}$ (10 pmol) in the absence (●) or presence (○) of 10 μM SCH-202676 was performed at 10°C as described under *Experimental Procedures*. B, $\beta1\gamma2$ (0.08 pmol)-stimulated ADP-ribosylation of $G_{o\alpha}$ (2 pmol) by pertussis toxin in the absence (●) or presence (○) of 10 μM SCH-202676 using [^{32}P]NAD at 17,884 cpm/pmol was performed at 30°C as described under *Experimental Procedures*. ●, control; ○, 10 μM SCH-202676.

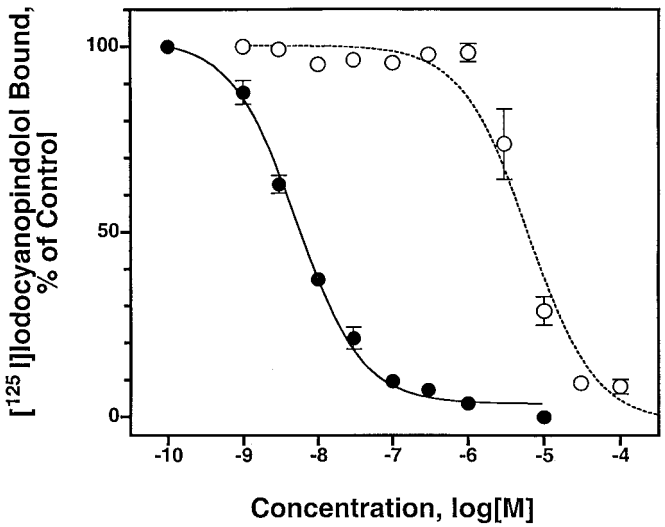


Fig. 3. SCH-202676 inhibition of [^{125}I]iodocyanopindolol binding to recombinant human β_2-adrenergic receptor expressed in *E. coli*. Concentration-dependent inhibition of [^{125}I]iodocyanopindolol binding to *E. coli* membranes expressing recombinant human β_2-adrenergic receptor by propranolol (●) and SCH-202676 (○) was performed as described under *Experimental Procedures*. Average total and nonspecific binding in the assay was 8176 and 1383 cpm, respectively.

in Tables 2 and 3, and Figs. 2 through 7 (see below) are from a representative study. Data points in the figures are mean values of duplicate determinations or mean values ± S.E. from triplicate determinations in each assay.

Protein concentrations were determined using the micro bicinchoninic acid assay (BCA; Pierce, Rockford, IL) with bovine serum albumin as a standard.

Materials. [^{3}H]Yohimbine (74.5 Ci/mmol), [^{3}H]UK-14,304 (27.3 Ci/mmol), [^{3}H]diprenorphine (60–80 Ci/mmol), [^{3}H]*N*-methylscopolamine (82 Ci/mmol), [^{125}I]iodocyanopindolol (2000 Ci/mmol), [^{3}H]SCH-23390 (70 Ci/mmol), [^{3}H]methylspiperone (84 Ci/mmol), [^{35}S]GTPγS (1500 Ci/mmol), [α-^{32}P]NAD$^+$ (800 Ci/mmol), and ^{125}I-EGF (murine, 1020 Ci/mmol) were obtained from Perkin Elmer. Pertussis toxin (islet-activating protein) from *Bordella pertussis* and naloxone were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant human EGF was obtained from Bachem (Torrance, CA).

CHO cell membranes expressing recombinant human δ- and μ-opioid, and κ-opioid receptor expressed in human embryonic kidney 293 cells were obtained from Receptor Biology, Inc. (Beltsville, MD). CHO cells expressing recombinant human M_1 and M_2 muscarinic receptors were obtained from Tom I. Bonner (National Institutes of Health, Bethesda, MD), and CHO cells expressing recombinant human D_1 and D_2 dopamine receptors were obtained from David K. Grandy (Vollum Institute, Oregon Health Sciences Institute, Portland, OR). CHO cell membranes expressing the human α_{2a}-adrenergic receptor were obtained from Euroscreen (Brussels, Belgium). Recombinant human β_1-adrenergic receptor expressed in SF9 insect cells was obtained from Perkin Elmer Life Sciences. Membrane preparations of A431 cells endogenously expressing the human EGF receptor and human HT-29 adenocarcinoma cells expressing the α_{2a}-adrenergic receptor were obtained from Receptor Biology, Inc. Purified recombinant G protein α and $\beta\gamma$ subunits were prepared as previously described (Linder and Gilman, 1991; Lee et al., 1994).

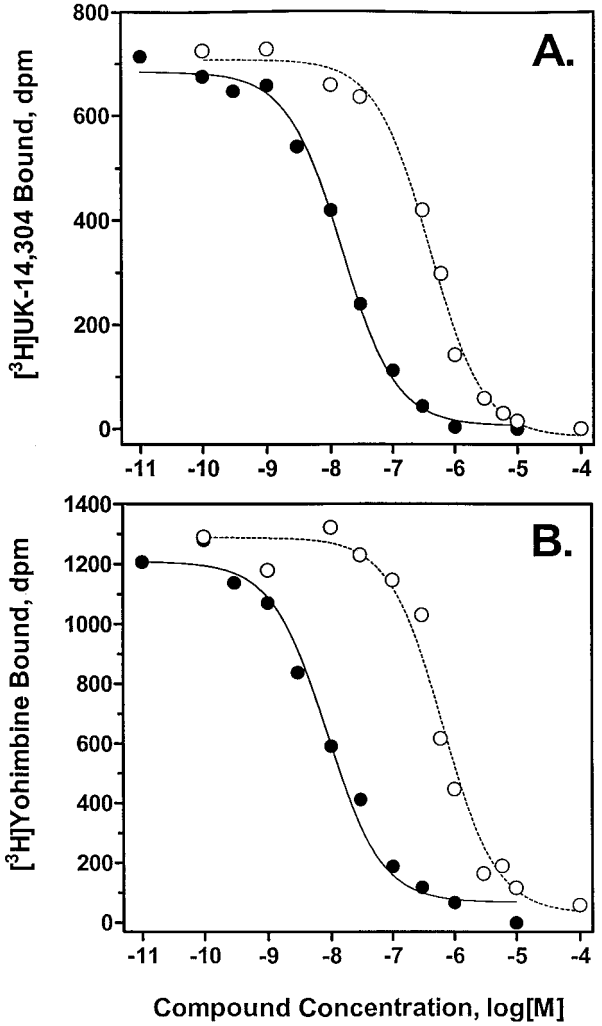


Fig. 4. SCH-202676 inhibits agonist and antagonist binding to the α_{2a}-adrenergic receptor. Competition binding of yohimbine (●) and SCH-202676 (○) with the agonist [^{3}H]UK-14,304 (A) and the antagonist [^{3}H]yohimbine (B) to HT-29 cell membranes. Binding assays were performed as described under *Experimental Procedures*. Nonspecific binding in the assay for [^{3}H]UK-14,304 (A) and [^{3}H]yohimbine (B) binding were 92 and 194 dpm, respectively. Data presented show specific binding in dpm.

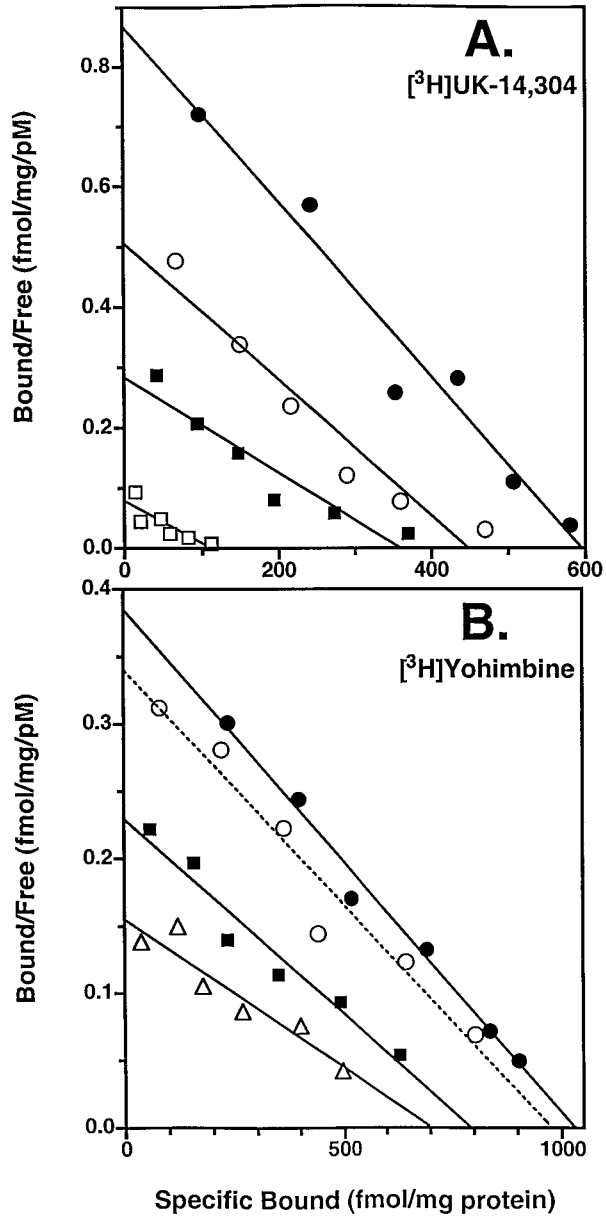


Fig. 5. Effect of SCH-202676 on the characteristics of agonist and antagonist binding to the α_{2a}-adrenergic receptor. A, Scatchard analysis of the agonist [^{3}H]UK-14,304 binding to HT-29 cell membranes in absence (●) and presence of 0.3 μM SCH-202676 (○), 0.5 μM SCH-202676 (■), or 100 μM GTPγS (□). B, Scatchard analysis of the antagonist [^{3}H]yohimbine binding to HT-29 cell membranes in absence (●) and presence of 0.1 (○), 0.3 (■), or 0.5 μM (△) SCH-202676. Saturation binding assays were performed as described under *Experimental Procedures*.

Results

SCH-202676 is a synthetic thiadiazole compound with a molecular mass of 267.29 Da (Fig. 1). As shown in Table 1, SCH-202676 inhibited radiolabeled antagonist binding to many GPCRs. SCH-202676 displayed no preference, inhibiting radioligand binding to receptors that couple to either the G_s, G_i/G_o, or G_q family of G proteins with IC_{50} values ranging from 0.1 to 1.8 μM. At 10 μM, SCH-202676 fully inhibited radiolabeled antagonist binding to all receptor subtypes evaluated. In contrast to its action on GPCRs, SCH-202676 (10 μM) had no effect on ^{125}I-EGF binding to A431 cell membranes expressing the human EGF receptor, a membrane-bound receptor tyrosine kinase (Table 2).

To elucidate the site of action of SCH-202676, its ability to modulate G protein function was studied. SCH-202676 at 10 μM (a concentration sufficient to completely inhibit radiolabeled antagonist binding to GPCRs) had no effect on the rate or extent of [^{35}S]GTPγS binding to purified recombinant $G_{o\alpha}$ (Fig. 2A). Pertussis toxin-mediated ADP-ribosylation of G_α is catalyzed by the interaction of G_α with $G_{\beta\gamma}$ (Casey et al., 1989; Fawzi et al., 1991). This assay was used to evaluate the effect of SCH-202676 on the function of $G_{\beta\gamma}$. At 10 μM, SCH-202676 did not alter the rate or extent of pertussis toxin catalyzed ADP-ribosylation of recombinant $G_{o\alpha}$ (Fig. 2B).

To evaluate the direct action of SCH-202676 on GPCRs, its ability to inhibit radioligand binding to the human β_2-adrenergic receptor expressed in *E. coli* was studied. This system lacks heterotrimeric G proteins (Marullo et al., 1988). As shown in Fig. 3, SCH-202676 completely inhibited binding of the antagonist [^{125}I]iodocyanopindolol to the β_2-adrenergic receptor expressed in *E. coli* with an IC_{50} value of 6.2 μM.

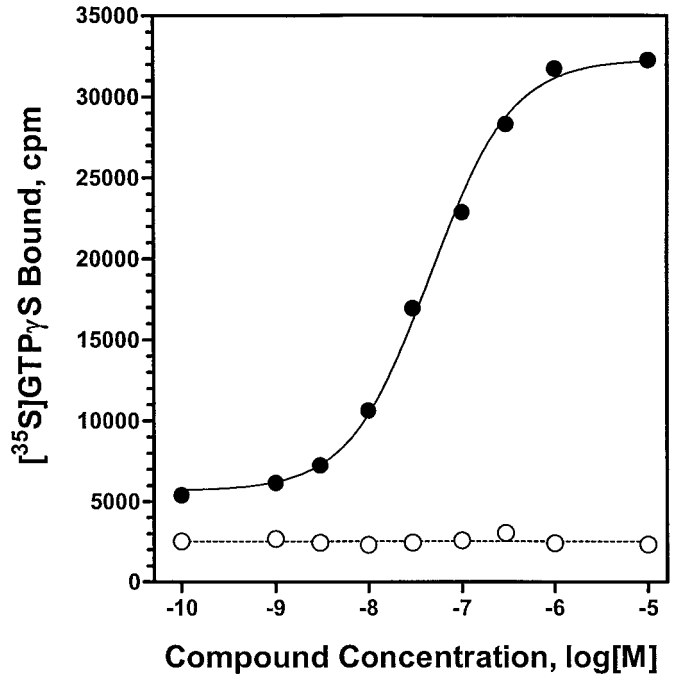


Fig. 6. SCH-202676 blocks the agonist UK-14,304-induced functional activation of the α_{2a}-adrenergic receptor. [^{35}S]GTPγS incorporation into G proteins was used to determine the functional role of SCH-202676 on agonist-induced receptor activation of the α_{2a}-adrenergic receptor expressed in CHO cells. UK-14,304-induced increase in [^{35}S]GTPγS binding in CHO cell membranes (●) was blocked by 6 μM SCH-202676 (○). [^{35}S]GTPγS functional binding assays were performed as described under *Experimental Procedures*.

The action of SCH-202676 on the human α_{2a}-adrenergic receptor expressed in HT-29 cell membranes was studied to gain further insight into the molecular interactions of SCH-202676 with GPCRs. Figure 4 shows that SCH-202676 inhibited the binding of the agonist [^{3}H]UK-14,304 (Fig. 4A) and the antagonist [^{3}H]yohimbine (Fig. 4B) to the human α_{2a}-adrenergic receptor in a concentration-dependent manner with IC_{50} values of 0.4 and 0.7 μM, respectively. Saturation binding and Scatchard analysis of both [^{3}H]UK-14,304 (Fig. 5A) and [^{3}H]yohimbine binding (Fig. 5B) to the α_{2a}-adrenergic receptor in the presence of SCH-202676 showed a decrease in the B_{max} value and an increase in the K_D value of the ligand binding (Fig. 5), suggesting that SCH-202676 is a noncompetitive inhibitor of the binding. As anticipated, a decrease in the B_{max} value of [^{3}H]UK-14,304 binding was observed in the presence of 100 μM GTPγS (Fig. 5A). Agonist-induced increase in [^{35}S]GTPγS incorporation into G proteins was used to evaluate functional activation of the α_{2a}-adrenergic receptor. Figure 6 shows that SCH-202676 (6 μM) blocked the agonist UK-14,304 induced activation of the α_{2a}-adrenergic receptor.

Kim and Neubig (1985, 1987) have shown that alkaline treatment of platelet membranes removes G proteins, but not receptor, from the membranes. To evaluate the role of G proteins in the action of SCH-202676 on the α_{2a}-adrenergic receptor, HT-29 cell membranes were treated with alkali (pH 11.6) for 10 min before the binding assays, as described by Kim and Neubig (1985, 1987). Control membranes were

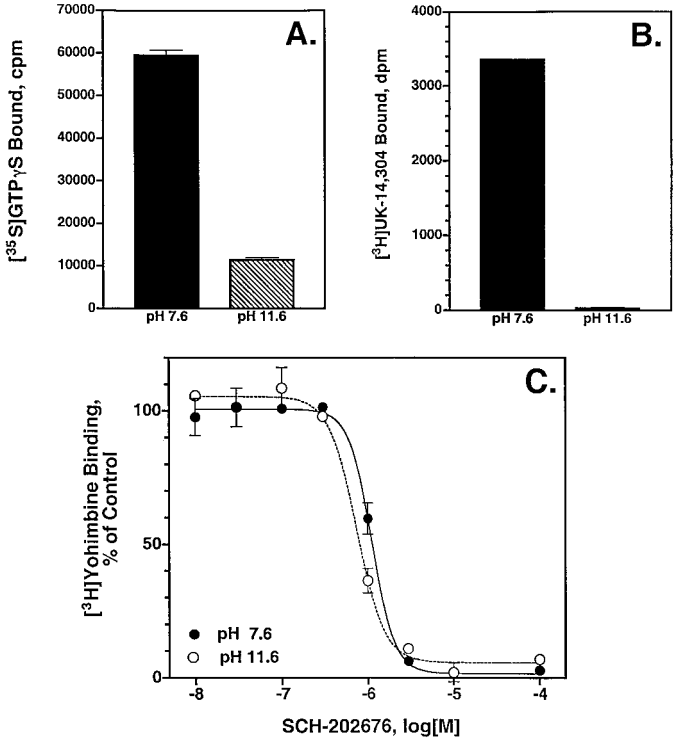


Fig. 7. Inactivation of G proteins after alkaline treatment of HT-29 cell membranes did not alter sensitivity to SCH-202676. HT-29 cell membranes were incubated in pH 11.6 buffer for 10 min as described by Kim and Neubig (1985, 1987) to inactivate G proteins. Control membranes were incubated in pH 7.6 buffer. A, [^{35}S]GTPγS binding to alkaline-treated membranes was reduced by 83% compared with control. B, agonist [^{3}H]UK-14,304 binding was abolished by alkaline treatment. C, concentration-dependent inhibition of [^{3}H]yohimbine binding by SCH-202676 was not altered by alkaline treatment.

treated under identical conditions at pH 7.6. As shown in Fig. 7, alkaline treatment of HT-29 cell membranes resulted in an 83% decrease in [^{35}S]GTPγS binding to the membrane (Fig. 7A) and a total loss of the agonist [^{3}H]UK-14,304 binding (Fig. 7B). However, alkaline treatment had no effect on the K_D value of [^{3}H]yohimbine binding but reduced the B_{max} value by 28% (data not shown). As shown in Fig. 7C, alkaline treatment of the membranes had no effect on SCH-202676 potency in inhibition of [^{3}H]yohimbine binding to the membrane preparation.

To determine whether the action of SCH-202676 is caused by a covalent incorporation into target proteins, the reversibility of its action on the α_{2a}-adrenergic and the D_1 dopaminergic receptors was evaluated. HT-29 cell membranes were treated with 10 μM SCH-202676 for 1 h at room temperature followed by removal of the compound by repeated washing of the membranes. As shown in Table 3, such pretreatment with 10 μM SCH-202676 did not alter binding characteristics of the agonist [^{3}H]UK-14,304 or the antagonist [^{3}H]yohimbine to the human α_{2a}-adrenergic receptor. In addition, identical treatment of CHO cell membranes expressing the D_1 dopaminergic receptor with 10 μM SCH-202676 had no effect on the binding characteristics of the D_1 receptor antagonist [^{3}H]SCH-23390 (Table 3). These data indicate that the inhibitory effect of SCH-202676 on radioligand binding is reversible.

Discussion

We have identified SCH-202676 as a small molecule that inhibits agonist and antagonist binding to a number of unrelated GPCRs. As shown in Table 1, SCH-202676 inhibited radiolabeled antagonist binding to a number of structurally diverse GPCRs that couple to the G_i/G_o, G_s, or G_q G protein families. This lack of selectivity for the subtype of G protein coupling suggests that the action of SCH-202676 is related to a receptor property common to many GPCRs. To gain insight into the molecular site of action of SCH-202676, its effect on the α_{2a}-adrenergic receptor was studied as a model for other GPCRs. Scatchard analysis of agonist and antagonist binding to the α_{2a}-adrenergic receptor shows that the B_{max} value of the binding sites is decreased, whereas the K_D value is slightly increased in the presence of SCH-202676. In addition, SCH-202676 blocked agonist-induced activation of the α_{2a}-adrenergic receptor. Actions of SCH-202676 on the α_{2a}-adrenergic receptor show that the compound is acting in a noncompetitive manner and allosterically modulating receptor conformation. In the new conformation, the receptor has a lower affinity for both agonists and antagonists.

We tested the reversibility of SCH-202676's actions on the α_{2a}-adrenergic and the D_1 dopaminergic receptors to test the possibility that SCH-202676 alters the function of GPCRs through a nonselective covalent modification of GPCRs or solubilization of the lipid bilayer. Treatment of HT-29 membranes expressing the α_{2a}-adrenergic receptor with 10 μM SCH-202676 for 1 h followed by repeated washing and removal of the compound from the membrane showed that such pretreatment with SCH-202676 does not alter the binding characteristics of agonist or antagonist to the α_{2a}-adrenergic receptor. In addition, similar treatment of CHO cell membranes expressing the dopaminergic D_1 receptor with 10 μM SCH-202676 did not alter antagonist binding to the receptor. These results indicate that the actions of SCH-202676 on the α_{2a}-adrenergic and the D_1 dopaminergic receptors are reversible and could not be caused by a covalent incorporation into its target site of action or solubilization of the lipid bilayer.

Alternatively, SCH-202676 could be interfering with GPCR function in a nonselective manner by a generalized alteration of membrane-associated proteins. To evaluate this possibility we have studied the effect of SCH-202676 on ^{125}I-EGF binding to the EGF receptors, a membrane-associated receptor tyrosine kinase, which binds EGF as a dimer (Cochet et al., 1988). The studies revealed that SCH-202676 has no effect on ^{125}I-EGF binding to the EGF receptors. These results indicate that SCH-202676's effect could not be caused by a nonselective action on membrane-associated proteins or by a detergent-like property of the compound.

Several lines of evidence suggest that the action of SCH-202676 is independent of heterotrimeric G proteins. First, evaluation of the direct interaction between SCH-202676 and G proteins showed that SCH-202676 had no effect on the rate or extent of GTPγS binding to purified recombinant $G_{o\alpha}$, nor did it affect $G_{\beta\gamma}$-catalyzed ADP-ribosylation of $G_{o\alpha}$ by pertussis toxin. Second, SCH-202676 inhibited radiolabeled antagonist binding to cloned β_2-adrenergic receptor expressed in *E. coli* that lack heterotrimeric G proteins. Third, removal of G proteins from cell membranes harboring α_{2a}-adrenergic receptors had no effect on the action SCH-202676. Limited alkaline treatment of plasma membranes strips many proteins, including heterotrimeric G proteins, from the plasma membrane (Citri and Schramm, 1980; Kim and Neubig 1985, 1987). The loss of G protein is reflected in an inhibition of agonist binding with no effect on antagonist binding. Alkaline treatment of HT-29 cell membranes resulted in a decrease in GTPγS binding and the loss of agonist binding with no effect on antagonist binding. In contrast, SCH-202676 was equipotent in inhibiting radiolabeled antagonist binding to

TABLE 3

Reversibility of SCH-202676's action on the α_{2a}-adrenergic and the D_1 dopaminergic receptors

HT-29 cell membranes expressing the α_{2a}-adrenergic receptor and CHO cell membranes expressing the dopaminergic D_1 receptor were incubated with 10 μM SCH-202676 or vehicle (control) for 1 h at room temperature in buffer containing 50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$, and 1 mM EGTA. Then, reaction mixtures were centrifuged for 30 min at 100,000*g* and pellets were washed twice with same buffer. Final pellets were resuspended in assay buffer (50 mM HEPES, pH 7.4, 10 mM NaCl, 1 mM $MgCl_2$, and 2.5 mM $CaCl_2$) and were used for saturation binding and protein determination assays as described under *Experimental Procedures*.

Treatment	α_{2a}-Adrenergic Receptor				D_1 Dopaminergic Receptor, [^{3}H]SCH-23390	
	[^{3}H]Yohimbine		[^{3}H]UK-14,304			
	K_D	B_{max}	K_D	B_{max}	K_D	B_{max}
	nM	*fmol/mg*	*nM*	*fmol/mg*	*nM*	*pmol/mg*
Control	5.5	642	0.5	725	0.9	1.6
SCH-202676	5.4	655	0.6	636	1.1	2.1

both control and alkaline-treated membrane preparations. Both SCH-202676 inhibition of antagonist binding to the α_{2a}-adrenergic receptor and its unaltered potency to inhibit antagonist binding to alkaline-treated membrane preparations indicate that the site of action of SCH-202676 is independent of G proteins.

The lack of selectivity of SCH-202676 on GPCRs indicates that if the compound is acting on a site(s) located on the receptor, then this site(s) must be conserved among the receptor subtypes tested. In addition, the site(s) of action of SCH-202676 must have the potential for allosteric modulation of the receptor conformation. The well-characterized DRY motif at the cytoplasmic side of transmembrane 3 is an example of a highly conserved domain in members of the rhodopsin GPCR family (Gether and Kobilka, 1998). Other conserved residues include AsnI:18 (Schwartz nomenclature; Schwartz, 1994), AspII:10, TrpIV:06, ProV:16, ProVI:15, and ProVII:17 (Gether and Kobilka, 1998). It is interesting to speculate that SCH-202676 binds at or near conserved site(s) on the receptors, leading to a change in the orientation of transmembrane domains of GPCRs. Such alterations in the orientation of the transmembrane helices have been reported for agonist binding to the β_2-adrenergic receptor (Gether and Kobilka, 1998).

Another mode of action for SCH-202676 is interference with receptor oligomerization. A growing body of genetic and biochemical data support the concept that GPCRs exist as homo- and heterooligomers. Recently, two laboratories have established that GPCRs form constitutive oligomers in living cells using fluorescence resonance energy transfer techniques (Angers et al., 2000; Overton and Blumer, 2000). Receptor oligomerization may have important functional consequences, including facilitation of signaling (Overton and Blumer, 2000) and regulation of ligand binding (Jordan and Devi, 1999). Heterodimerization influences the ligand binding properties of GABA(B) (Jones et al., 1998; White et al., 1998) and M3 muscarinic receptors (Zeng and Wess, 1999). It is conceivable that oligomerization impacts the ligand binding properties of homodimers and could thereby be affected by SCH-202676.

Alternatively, SCH-202676 could exert its effects on a regulatory protein tightly associated with the receptor. G proteins are the best-characterized GPCR regulatory proteins. However, our results rule out G proteins as targets of SCH-202676's actions. Other regulatory proteins of GPCR function include arrestins (Lefkowitz, 1998), GPCR kinase (Lefkowitz, 1998), and receptor activity-modifying proteins (McLatchie et al., 1998). GPCR kinase has a high affinity for agonist bound receptors, and arrestins bind phosphorylated receptors after agonist binding (Lefkowitz, 1998). Neither protein is likely to be the target of SCH-202676, because this compound's function is independent of agonist binding to the receptor. Receptor activity-modifying proteins are also unlikely to be the target of SCH-202676, because these proteins regulate the receptor at the level of cell surface expression (McLatchie et al., 1998). The target of SCH-202676 is unlikely to be a peripheral membrane-associated protein, because alkaline treatment, which inactivates and removes such proteins, does not alter receptor sensitivity to the compound. Nevertheless, the possibility that a novel GPCR-associated regulatory protein is the site of action of SCH-202676 could not be ruled out.

A number of compounds that allosterically modulate the function of GPCRs have been reported by other investigators (Herrmann and Jakobs, 1988; Anand-Srivastava, 1989; Huang et al., 1990; Beindl et al., 1996; Freissmuth et al., 1996). Unlike SCH-202676, these compounds either act directly on G proteins or modulate receptor coupling to G proteins. In addition, unlike Suramin (Freissmuth et al., 1996), SCH-202676 is a small molecule (M_r 267.29). In contrast to small molecule allosteric modulators of the muscarinic acetylcholine receptor (Holzgrabe and Mohr, 1998; Lazareno et al., 1998), SCH-202676 lacks receptor specificity. Na^+ is a well known allosteric modulator of GPCRs (see e.g., Horstman et al., 1990) affecting agonist binding to most if not all GPCRs. Unlike Na^+, SCH-202676 inhibits both agonist and antagonist binding to GPCRs. Structurally, SCH-202676 is devoid of functionally reactive moieties and does not seem to have a detergent-like action.

In summary, we report herein the discovery of SCH-202676, a novel small molecule that interacts with a number of structurally unrelated GPCRs. The ability of SCH-202676 to interact with multiple receptor subtypes combined with its lack of direct action on G protein function suggest that this molecule interacts with a structurally conserved domain on GPCRs or on an unidentified accessory protein that regulates GPCR function. SCH-202676 is a unique tool that could help identify and characterize allosteric site(s) that regulate the conformation and function of GPCRs.

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