Investigation of the Interaction of a Putative Allosteric Modulator, \(N-(2,3\text{-diphenyl-1,2,4-thiadiazole-5-(2H)-ylidene})\) Methanamine Hydrobromide (SCH-202676), with \(M_1\) Muscarinic Acetylcholine Receptors

Alfred Lanzafame and Arthur Christopoulos

Department of Pharmacology, University of Melbourne, Parkville, Victoria, Australia

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ABSTRACT

The interaction between a novel G protein-coupled receptor modulator, \(N-(2,3\text{-diphenyl-1,2,4-thiadiazole-5-(2H)-ylidene})\) methanamine hydrobromide (SCH-202676), and the \(M_1\) muscarinic acetylcholine receptor (mAChR) was investigated. In contrast to the prototypical mAChR allosteric modulator, heptane 1,7-bis-(dimethyl-3\text{-phthalimidopropyl})-ammonium bromide (C\text{7/3-phth}), SCH-202676 had no effect on the dissociation kinetics of \[^{3}\text{H}]\text{N}-\text{methylscopolamine (}[^{3}\text{H}]\text{NMS)} at \(M_1\) mAChRs stably expressed in Chinese hamster ovary (CHO) cell membranes. However, SCH-202676 completely inhibited the binding of \[^{3}\text{H}]\text{NMS in membrane preparations, with a Hill slope significantly greater than unity, indicative of positive cooperativity in the binding of the inhibitor. Moreover, SCH-202676 caused dextral shifts of the \[^{3}\text{H}]\text{NMS saturation binding curve that were greater than expected for a competitive interaction. The addition of C\text{7/3-phth (100 \mu M) had no significant effect on the inhibitory potency of SCH-202676. In contrast to the findings in cell membranes, the interaction between SCH-202676 and \[^{3}\text{H}]\text{NMS in intact M1 CHO cells yielded saturation and inhibition isotherms that were compatible with the predictions for a competitive interaction. Intact cell assays of acetylcholine-mediated phosphoinositide hydrolysis in the absence or presence of SCH-202676 revealed a mixed competitive/noncompetitive mode of interaction that was dependent on the concentration of SCH-202676. These data reveal that the nature of the interaction between SCH-202676 and the \(M_1\) mAChR is dependent on whether it is studied using intact versus broken cell preparations. It is proposed that SCH-202676 uses a dual mode of ligand-receptor interaction involving both extra- and intracellular attachment points on the \(M_1\) mAChR that are distinct from the allosteric binding site recognized by prototypical mAChR modulators such as C\text{7/3-phth}.}

Ligand binding behavior that deviates from the predictions of simple bimolecular mass action kinetics is invariably classed as either “noncompetitive” and/or “allosteric”. Often, these terms are used as empirical descriptors of an unknown mechanism that may or may not reflect a true nonclassical mode of drug action. The ability to test whether a drug interacts allosterically at G protein-coupled receptors (GPCRs) is important due to the recognition that many GPCRs contain allosteric binding sites for endogenous and/or synthetic ligands (Christopoulos and Kenakin, 2002). These ligands, or allosteric modulators, interact at binding sites on the receptor distinct from that of classic, orthosteric ligands (agonists and competitive antagonists). However, the manifestations of allosterism are many and varied, and therefore a mechanistic framework is required to characterize and quantify the effects of such ligands. The simplest model applied to the study of allosteric interactions at GPCRs is the ternary complex model (TCM), as shown in Fig. 1 (Stockton et al., 1983; Ehlert 1988). Upon binding to the allosteric site, ligands that act according to the TCM induce conformational changes at the orthosteric site of the receptor that either enhance or inhibit the binding of an orthosteric ligand; this interaction is reciprocal at equilibrium and leads to positive

**ABBREVIATIONS:** GPCR, G protein-coupled receptor; TCM, ternary complex model; mAChR, muscarinic acetylcholine receptor; SCH-202676, \(N-(2,3\text{-diphenyl-1,2,4-thiadiazole-5-(2H)-ylidene})\) methanamine hydrobromide; NMS, \(N\)-methylscopolamine; C\text{7/3-phth, heptane, 1,7-bis-(dimethyl-3\text{-phthalimidopropyl})-ammonium bromide; DMEM, Dulbecco’s modified Eagle’s medium; PI, phosphoinositide; ACh, acetylcholine; C-R, concentration-response.
One of the best studied model systems for investigation of allosteric ligands has been the muscarinic acetylcholine receptors (mAChRs; Lee and El-Fakahany, 1991; Tucek and Proksa, 1995; Ellis, 1997; Christopoulos et al., 1998), and it is currently believed that at least one allosteric site on these receptors is located at a more extracellular level to the orthosteric site (Leppik et al., 1994; Matsui et al., 1995; Christopoulos et al., 1998; Buller et al., 2002). Because the extracellular regions of mAChRs, as well as other GPCRs, tend to show less sequence homology across receptor subtypes than the orthosteric domains, targeting allosteric sites is a logical approach to defining more selective ligands for such GPCRs.

Recently, a novel thiadiazole compound, N-(2,3-diphenyl-1,2,4-thiadiazole-5-(2H)-yliidene)methanamine (SCH-202676; Fig. 2), was identified as an allosteric modulator of a variety of GPCRs, including mAChRs (Fawzi et al., 2001). This finding was based on radioligand binding assays performed on membrane preparations derived from cells transfected to individual cells transfected to different GPCRs. In particular, SCH-202676 was found to cause a reduction in both agonist and antagonist maximal binding capacity ($B_{max}$) with only a slight effect on radioligand affinity when investigated via saturation binding assays at $\alpha_2$ adrenoceptors. Additional inhibition binding assays also demonstrated an antagonistic effect on the binding of [3H]N-methylscopolamine ([3H]NMS) to M1 and M2 mAChRs, although this was not studied in any further detail (Fawzi et al., 2001). Given that the simple TCM (Fig. 1) only predicts changes in orthosteric ligand affinity and not $B_{max}$, the nature of the interaction between SCH-202676 and its target GPCRs is likely to be different from that of classic allosteric modulators that act in accordance with the TCM and/or is different between one GPCR and another.

Therefore, the aim of the present study was to investigate the mode of interaction of SCH-202676 with the M1 mAChR using radioligand binding and functional assays. For comparative purposes, some experiments used the prototypical and well characterized mAChR allosteric modulator heptane, 1,7-bis-(dimethyl-3-phthalimidopropyl)-ammonium bromide (C7/3-phth; Fig. 2) (Christopoulos et al., 1999). We show that the nature of the interaction between SCH-202676 and the M1 mAChR is highly dependent on whether it is studied using intact versus broken cell preparations. The noncompetitive behavior of SCH-202676 cannot be explained by the simple TCM and suggests that SCH-202676 may possess a dual mode of ligand-receptor interaction involving both extra- and intracellular attachment points on the M1 mAChR.

### Materials and Methods

**Materials.** Drugs and chemicals were obtained from the following sources: [3H]NMS was from PerkinElmer Life Sciences (Boston, MA); [3H]Hymo-inositol was from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK); SCH-202676 was from Tocris Cookson Inc. (Ballwin, MO); C7/3-phth was synthesized by the Institute of Drug Technology (Boronia, Victoria, Australia); Dulbecco’s modified Eagle’s medium (DMEM) and genetin were from Invitrogen (Carlsbad, CA); fetal bovine serum was from ThermoTrace, (Melbourne, Victoria, Australia), and DOWEX AG1-X8 ion-exchange resin was obtained from Bio-Rad (Hercules, CA). All other materials were obtained from Sigma-Aldrich (St. Louis, MO).

**Cell Culture.** Chinese hamster ovary (CHO-K1) cells, stably transfected with the human M1 mAChR (M1 CHO cells), were kindly provided by Dr. M. Brann (University of Vermont Medical School, Burlington, VT). Cells were grown and maintained in DMEM containing 20 mM HEPES, 10% fetal bovine serum, and 50 μg/ml genetin for 4 days at 37°C in a humidified incubator containing 5% CO₂, 95% O₂ before harvesting by trypsinization followed by centrifugation (300g, 3 min) and resuspension of the pellet in DMEM.

**Cell Membrane Preparation.** M1 CHO cells were grown, harvested, and centrifuged as described above, with the final pellet resuspended in 5 ml of ice-cold Tris-HCl buffer (50 mM Tris, 3 mM MgCl₂, and 0.2 mM EGTA; pH 7.4 with HCl) and then homogenized using a Polytron homogenizer for three 10 s intervals at maximum setting with 30 s cooling periods used between each burst. The cell homogenate was centrifuged (1000g, 10 min, 25°C), the pellet discarded, and the supernatant was recentrifuged at 30,000g for 30 min at 4°C. The resulting pellet containing cell membrane was resuspended in 5 ml of Tris-HCl buffer and protein content was determined using the method of Bradford (Bradford, 1976). The homogenate was then aliquoted into 1-ml amounts and stored frozen at −80°C until required for radioligand binding assays.

**Saturation Binding Assays.** In these assays, M1 CHO cell membranes (10 μg/assay tube) or intact CHO cells (200,000 cells/assay tube) were incubated in a total volume of 1 ml of HEPES buffer (110

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**Fig. 1.** Ternary complex model, where A represents orthosteric ligand, B represents allosteric ligand, $K_A$ and $K_B$ represent equilibrium dissociation constants for the binding of ligands A and B, respectively, and $\alpha$ represents the cooperativity factor for the allosteric interaction between ligand B and ligand A. In this model, $\alpha > 1$ denotes positive cooperativity, $\alpha < 1$ denotes negative cooperativity, and $\alpha = 1$ denotes neutral cooperativity.

**Fig. 2.** Structures of SCH-202676 and C7/3-phth.
mM NaCl, 5.4 mM KCl, 1.5 mM CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, 25 mM glucose, 50 mM HEPES, and 58 mM sucrose; pH 7.4) with varying concentrations of <sup>[3H]</sup>NMS (10 PM–20 nM) in the absence and presence of different concentrations of SCH-202676 (1 nM to 37°C). The reaction was then terminated by rapid filtration through Whatman GF/F filters using a cell harvester (Brandel Inc., Gaithersburg, MD). Non-specific binding was determined using 10 μM atropine. Filters were washed three times with 4-ml aliquots of ice-cold saline and dried before radioactivity (dpm) was measured using liquid scintillation counting.

**Inhibition Binding Assays.** Initial experiments were performed in a total volume of 1 ml of HEPES buffer using M<sub>1</sub> CHO cell membrane homogenates (10 μg/assay tube) or intact cells (100,000 cells/assay tube), incubated with <sup>[3H]</sup>NMS (0.2 or 2 nM), and various concentrations of SCH-202676 (30 nM–300 μM) for 1 h at 37°C, unless otherwise indicated. Additional SCH-202676 experiments were conducted in M<sub>1</sub> CHO membranes in the presence of 100 μM C<sub>7</sub>/3-phth. Non-specific binding, reaction termination, and radioactivity determination were as described above.

**Radioligand Dissociation Kinetic Assays.** <sup>[3H]</sup>NMS dissociation kinetic binding curves were performed in HEPES buffer, using a reverse time protocol. In these experiments, M<sub>1</sub> CHO membranes (10 μg/assay tube in a total volume of 1 ml) were added to tubes containing <sup>[3H]</sup>NMS (0.5 nM) in a time staggered approach so that each replicate was allowed to equilibrate for 1 h at 37°C. Once the receptors and radioligand had equilibrated, atropine (10 μM) was added at appropriate time intervals to prevent reassociation of <sup>[3H]</sup>NMS to the M<sub>1</sub> mAChR in the absence or presence of C<sub>7</sub>/3-phth or SCH-202676, as indicated under Results. Non-specific binding was determined using atropine (10 μM). In some experiments, SCH-202676 (1 μM) was added alone after <sup>[3H]</sup>NMS-receptor equilibration to prevent reassociation of the radioligand. Non-specific binding, reaction termination, and radioactivity determination were as described above.

**Phosphoinositide (PI) Hydrolysis Assays.** M<sub>1</sub> CHO cells were loaded overnight with <sup>[3H]</sup>myo-inositol (1 μCi/ml) at 37°C in a humidified incubator containing 5% CO<sub>2</sub>, 95% O<sub>2</sub>. On the day of the experiment, cells were harvested and centrifuged (300g, 3 min), and the final pellet was resuspended in 5 ml of HEPES buffer and used for cell counting. The cell suspension was then diluted in HEPES buffer containing 10 mM LiCl, distributed into assay tubes (~500,000 cells/tube), and allowed to incubate for 15 min at 37°C. After this preincubation period, ACh, SCH-202676, or appropriate vehicle controls (see below) were added to the remaining assay tubes and the reaction was allowed to proceed for a further 30 min before being terminated by stop solution (methanol/chloroform, 2:1). Assay tubes were centrifuged at 450g for 5 min at room temperature, and total inositol phosphates were separated by ion exchange chromatography on Dowex AG-1-X8 resin. <sup>[14C]</sup>inositol-1-phosphate was used as an internal recovery standard. The amount of radioactivity (dpm) in each sample was measured using a liquid scintillation counter and values were corrected for recovery determined for each sample. Initial experiments involved the construction of concentration kinetic binding (C-R) curves to increasing concentrations of ACh (10 nM–1 mM) in the absence or presence of SCH-202676 (1, 10, or 100 μM), which was preincubated for 15 min before agonist addition. Further experiments involved the establishment of antagonist inhibition curves using increasing concentrations of SCH-202676 (30 nM–0.1 mM), in the presence of ACh (1 μM), which was preincubated for 15 min before addition of SCH-202676. In these latter experiments, a complete C-R curve to ACh was also established in parallel as a measure of the responsiveness of the system to agonist stimulation.

**Data Analysis.** Data sets of total and nonspecific binding, derived from each complete saturation binding assay, were analyzed according to the following equation using Prism 4.0 (GraphPad Software Inc., San Diego, CA):

\[
\text{Binding} = \frac{B_{\text{max}}[A]}{[A] + K_a} + \text{NS}[A] 
\]

where \(B_{\text{max}}\) denotes the maximal density of binding sites; \(K_a\) the radioligand equilibrium dissociation constant; and NS, the fraction of nonspecific binding. The hyperbolic term in eq. 1 was not used when fitting the nonspecific binding data, whereas the parameter NS was shared between both total and nonspecific binding data sets. Radioligand inhibition binding isotherms were analyzed according to the following logistic function:

\[
Y = \frac{(\text{Top} - \text{Bottom})(II)^{\text{n}}}{[B]^{\text{n}} + IC_{50}^{\text{n}}} 
\]

where Y denotes the percentage of specific binding; Top and Bottom denote the maximal and minimal asymptotes, respectively; \(\text{II}\) denotes the inhibitor concentration; IC<sub>50</sub> denotes the inhibitor potency (midpoint location) parameter; and \(\text{n}\) denotes the Hill slope factor. Where appropriate, and assuming simple competition, IC<sub>50</sub> values were converted to \(K_i\) values (inhibitor equilibrium dissociation constant) using the Cheng and Prusoff (1973) equation.

For the dissociation kinetic experiments, complete dissociation curves for the radioligand, in the absence and presence of the highest concentration of applied modulator were evaluated by nonlinear regression using the following equation:

\[
B_t = B_0 e^{-\frac{k_{\text{off}} t}{[A]}} 
\]

where \(B_t\) denotes the specific binding of radioligand at time t, \(B_0\) denotes the specific binding of radioligand at equilibrium (time = 0) and \(k_{\text{off}}\) denotes the observed radioligand dissociation rate constant. For the C<sub>7</sub>/3-phth/<sup>[3H]</sup>NMS dissociation kinetic experiments, data sets were globally fitted to the eq. 3 with the parameter \(k_{\text{off}}\) defined as follows:

\[
k_{\text{off}} = \frac{[B]^{\text{top}} k_{\text{off}}}{K_{gp} + k_{\text{off}}} 
\]

where \(k_{\text{off}}\) denotes the rate constant for dissociation of the radioligand from the free (unoccupied) receptor, \(k_{\text{off}}\) denotes the rate constant for the dissociation of the radioligand from the modulator-occupied receptor, and \(K_{gp}\) denotes the dissociation constant of the modulator for the radioligand-occupied receptor (Lazareno and Birdsell, 1995; Christopoulos et al., 1999). The parameter slope denotes an empirical slope factor; a value not significantly different from unity may be taken as presumptive evidence of a simple, one-to-one, mass-action relationship between the allosteric modulator and its binding site on the receptor.

For the functional assays, agonist C-R curves in the absence and presence of antagonist, as well as antagonist inhibition curves, were globally fitted to the following equation using Prism 4 (Motulsky and Christopoulos, 2003):

\[
\text{Response} = \frac{(\text{Top} - \text{Bottom})}{1 + \left(\frac{[A]}{IC_{50}^{\text{n}}}ight)^{n_{\text{Hill}}}} 
\]

where Top represents the maximal asymptote of the C-R curves, Bottom represents the lowest asymptote of the C-R curves, LogEC<sub>50</sub> represents the logarithm of the agonist EC<sub>50</sub> in the absence of antagonist, \(\text{A}\) represents the concentration of the agonist, \(B\) represents the concentration of the antagonist, \(n_{\text{Hill}}\) represents the Hill slope of the agonist curve, \(s\) represents the Schild slope for the competitive antagonist, and \(pA_2\) represents the negative logarithm of the concentration of antagonist that shifts the agonist EC<sub>50</sub> by a factor of 2. If the Schild slope was not significantly different from unity, it was
Results

Dissociation Kinetic Assays. A hallmark of many prototypical allosteric modulators of mACHRs is the ability to retard orthosteric ligand dissociation (Kostenis and Mohr, 1996). As shown in Fig. 3A, C7/3-phth produced a concentration-dependent retardation of [3H]NMS dissociation at the M₁ mACHR in CHO cell membranes, indicative of an allosteric mode of action. The dissociation curves for the radioligand in the absence and presence of the highest concentration of C7/3-phth on the apparent radioligand dissociation rate were assessed using a two-point experimental design (Kostenis and Mohr, 1996). A constrained simultaneous analysis of each complete family of experimental data obtained using intact cells, with the LogEC₅₀ term being constrained as such and the estimate of -pA₂ represented the LogKᵦ. Equation 5 was also used to analyze the saturation binding data obtained using intact cells, with the LogEC₅₀ term being replaced by LogKᵦ and the value of nᵦ being constrained to 1.

In all cases, potency and affinity values were estimated as logarithms (Christopoulos, 1998). Data shown are the mean ± S.E.M. Comparisons between mean values were performed by unpaired t tests. Unless otherwise stated, values of p < 0.05 were taken as statistically significant.

In contrast, kinetic assays examining [3H]NMS dissociation in M₁ CHO cell membranes in the absence and presence of SCH-202676 found no effect of the latter compound on radioligand dissociation (Fig. 3B). The kₐff value of 0.29 ± 0.03 min⁻¹ (n = 4) observed in the presence of 1 μM SCH-202676 was not significantly different (p > 0.05) from the control kₐff value determined in its absence (0.26 ± 0.02 min⁻¹; n = 4). Interestingly, the addition of SCH-202676 alone also resulted in [3H]NMS dissociation (kₐff of 0.23 ± 0.02 min⁻¹; n = 4) that was indistinguishable from that observed in the presence of atropine alone.

Inhibition Binding Assays Using M₁ CHO Membranes. In M₁ CHO cell homogenates, SCH-202676 completely inhibited the binding of 0.2 nM [3H]NMS, with an nᵦᵣ significantly greater than unity (p < 0.05; Fig. 4A; Table 1). Increasing the concentration of [3H]NMS from 0.2 to 2 nM did not change the location or shape of the SCH-202676 inhibition curve; the LogIC₅₀ value being −6.80 ± 0.09 (n = 4) and nᵦᵣ being 2.52 ± 0.35 in the presence of 2 nM [3H]NMS (Fig. 4A).

To investigate whether the steep inhibition curves observed with SCH-202676 were due to possible nonequilibrium artifacts, additional inhibition binding assays used a...
longer equilibration time (2 h). The LogIC50 value of \(-6.87 \pm 0.04\) (\(n = 3\)) obtained for the 2-h incubation data (data not shown) was not significantly different (\(p > 0.05\)) from that derived from 1-h equilibration studies.

If SCH-202676 were indeed an allosteric modulator of mACHRs, the dissociation kinetic experiments suggested that it may not mediate its effects via an interaction with the classic allosteric site recognized by C7/3-phth. To further investigate this hypothesis, inhibition binding assays were repeated with [3H]NMS versus SCH-202676 in the absence and presence of C7/3-phth (100 \(\mu\)M). As shown in Fig. 4B, there were no significant changes (\(p > 0.05\)) in the LogIC50 \((-6.63 \pm 0.08; \(n = 3\)) or \(n_H\) (2.73 \(\pm\) 0.49) parameters in the presence of C7/3-phth compared with control (Table 1) values, suggesting that SCH-202676 did not share a common site of action with C7/3-phth.

**Saturation Binding Assays Using M1 CHO Membranes.** To gain further insight into the possible mechanisms underlying the steep curves observed in the inhibition binding experiments, complete [3H]NMS saturation binding assays were performed. As shown in Fig. 5A, a narrow range of concentrations of SCH-202676 caused a profound dextral shift of the radioligand saturation curve such that [3H]NMS occupancy could not be fully determined in the presence of 0.2 and 0.3 \(\mu\)M SCH-202676. In the presence of the lowest concentration of SCH-202676 (0.1 \(\mu\)M), it was noted that there was a significant (\(p < 0.05\)) shift in the [3H]NMS apparent Log\(K_A\) from \(-9.18 \pm 0.06\) (\(n = 4\)) to \(-8.68 \pm 0.10\) (\(n = 3\)), but no effect on \(B_{max}\).

**Saturation Binding Assays Using M1 CHO Cells.** It is possible that the noncompetitive mode of interaction of SCH-202676 described above reflects its binding to an intracellular domain of the M1 mACHR that is accessible due to the broken cell nature of the assays. Additional saturation experiments were thus performed using intact M1 CHO cells, where it was found that SCH-202676 was less potent at inhibiting [3H]NMS saturation binding than in membrane-based experiments (Fig. 5B). Importantly, there was no apparent effect of SCH-202676 on the \(B_{max}\) of the radioligand, with only the \(K_A\) being altered. Assuming a simple competitive mode of interaction, analysis of the data in Fig. 5B according to eq. 5 yielded a Schöld slope of 1.21 \( \pm \) 0.24, which was not significantly different from unity (\(p > 0.05\); F test). Constraining this value to 1 yielded an estimated log \(K_H\) of \(-7.23 \pm 0.15\) for the interaction between SCH-202676 and [3H]NMS in the intact cells.

**Inhibition Binding Assays Using M1 CHO Cells.** The difference in the binding properties of SCH-202676 at M1 mACHRs between membranes and intact cells was further illustrated using [3H]NMS inhibition binding assays in intact cells. Figure 6 and Table 1 show that the inhibition binding curve for SCH-202676 was significantly shallower in intact cells compared with membranes. Because the Hill slope ob-

### Table 1

**Inhibition binding parameters for SCH-202676 against 0.2 nM [3H]NMS at M1 mACHRs expressed in CHO cell membranes versus intact cells, determined using an equilibrium binding assay**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Membranes</th>
<th>Intact Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\log IC_{50})</td>
<td>-6.78 ± 0.04</td>
<td>-6.54 ± 0.13</td>
</tr>
<tr>
<td>(n_H)</td>
<td>2.58 ± 0.25</td>
<td>1.06 ± 0.04</td>
</tr>
</tbody>
</table>

* Student’s \(t\) test found a significant (\(p < 0.05\)) difference between experiments using membranes versus intact cells.

* Logarithm of the midpoint potency parameter for SCH-202676 (\(n = 4–6\)).

### Table 2

**Concentration-response curve parameters for ACh in the absence and presence of SCH-202676 at M1 mACHRs expressed in CHO cells, determined using a functional PI hydrolysis assay**

Values are the mean ± S.E.M. of 3 to 11 experiments conducted in triplicate.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SCH-202676 ((\mu)M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(E_{max})</td>
<td>100 ± 1</td>
</tr>
<tr>
<td>(\log EC_{50})</td>
<td>-6.78 ± 0.06</td>
</tr>
<tr>
<td>(n_H)</td>
<td>1.79 ± 0.15</td>
</tr>
</tbody>
</table>

* Student’s \(t\) test found a significant (\(p < 0.05\)) difference between control and SCH-202676-treated groups.
tained from SCH-202676 binding in the intact cells were not significantly different from 1 ($p > 0.05$; F test) the IC$_{50}$ value was converted to a $K_I$ value, yielding a Log$K_I$ estimate for the interaction between SCH-202676 and [3H]NMS of $-6.71 \pm 0.14$ ($n = 4$) in intact cells. This value was not significantly different ($p > 0.05$) from the Log$K_B$ value determined above from the intact cell saturation binding assays.

**PI Hydrolysis Assays.** To determine the effects of SCH-202676 on cellular function, PI hydrolysis experiments were conducted in intact M$_1$ CHO cells under similar assay conditions to those used in the radioligand binding experiments. As shown in Fig. 7, ACh was able to mediate a robust response in PI accumulation. In contrast, SCH-202676 alone (up to 100 $\mu$M) had no effect on basal PI hydrolysis (data not shown). The presence of increasing concentrations of SCH-202676 led to progressive dextral displacements of the ACh C-R curves (Fig. 7A). Although no significant effect was noted on the maximal agonist response in the presence of 1 $\mu$M SCH-202676, as expected for a competitive interaction, higher concentrations caused a significant reduction in ACh $E_{\text{max}}$ values ($p < 0.05$; Fig. 7A; Table 2). Without prejudice to mechanism, the shift of the ACh concentration-response curve observed in the presence of 1 $\mu$M SCH-202676 was used to derive an apparent Log$K_B$ value against ACh, which was $-6.27 \pm 0.23$ ($n = 5$).

To examine a larger range of SCH-202676 concentrations on ACh function, antagonist inhibition curves were constructed against the response to a fixed (1 $\mu$M) concentration of ACh (Fig. 7B). Analysis of the data using eq. 5 gave a Schild slope of $0.95 \pm 0.08$ ($n = 3$), which was not significantly different from unity using an F test ($p > 0.05$). Constraining the Schild slope to 1 yielded a log$K_B$ value of $-6.28 \pm 0.15$ ($n = 3$).

**Discussion**

The development of selective allosteric compounds is currently of great therapeutic relevance, especially in the field of GPCRs (Christopoulos, 2002). The present study used the M$_1$ mAChR to investigate the pharmacology of a recently identified GPCR allosteric ligand. Although some of the findings reported herein confirm and extend a previous (and thus far the only) report of the effect of SCH-202676 as a noncompetitive inhibitor of GPCR binding and function (Fawzi et al., 2001), they also highlight striking differences in the pharmacological properties of the compound when studied in intact cells compared with broken cell preparations. SCH-202676 may possess a dual mode of interaction at the M$_1$ mAChR that uses both extracellular and intracellular receptor attachment points. Furthermore, the interaction of this compound with the M$_1$ mAChR does not seem to involve the well defined allosteric site recognized by more specific mAChR modulator ligands, such as C$_2$-3-phth.

A key theoretical advantage of allosteric modulators of GPCRs that act according to the TCM (Fig. 1) is the potential for greater selectivity of action. This can arise due to modulator specificity for receptor binding domains that are not conserved across receptor subtypes and/or due to differential degrees of cooperativity between orthosteric and allosteric sites at a particular receptor subtype compared with others (Lazareno et al., 1998; Birdsall et al., 1999; Christopoulos and Kenakin, 2002). Although SCH-202676 possesses the desirable property of being a synthetic small molecule ligand, there are a number of experimental observations to suggest that it does not act according to the TCM. First, unlike C$_2$-3-phth, which is a prototypical mAChR modulator (Chris-
topoulos and Mitchelson, 1994; Lanzafame et al., 1996; Christopoulos et al., 1999), SCH-202676 does not promote a change in receptor conformation that manifests as an alteration in orthosteric radioligand dissociation (Fig. 3). Second, membrane-based inhibition binding assays between SCH-202676 and [3H]NMS were characterized by Hill slopes significantly greater than unity (Fig. 4, Table 1), which is not predicted by the simple TCM and not observed with C3/3-phth under similar assay conditions (Christopoulos and Mitchelson, 1998; Christopoulos et al., 1999). Third, the TCM predicts that negative allosteric modulators can display a progressive inability to fully inhibit specific radioligand binding when the concentration of the radioligand is increased (Christopoulos and Kenakin, 2002); increasing the concentration of [3H]NMS by a factor of 10 had no effect whatsoever on the shape or location of the SCH-202676 inhibition curve (Fig. 4A), in contrast to previous observations with C3/3-phth (Christopoulos and Mitchelson, 1998; Christopoulos et al., 1999). Finally, the inhibition binding isotherm for SCH-202676 was not significantly modified by the addition of C3/3-phth (Fig. 4B), indicating that these two ligands do not compete for the same topographically distinct binding domain on the mACr.

Another aspect of the pharmacology of SCH-202676 that is not in accord with the TCM was reported by Fawzi et al. (2001) during studies of agonist and antagonist binding to α2 adrenoceptors, namely, a significant inhibition by SCH-202676 of radioligand Bmax values with only a minimal effect on radioligand affinity. In the present study, similar membrane-based binding experiments at the M1 mACr using [3H]NMS in the presence of increasing concentrations of SCH-202676 revealed a different pattern of behavior, that is, a marked change in apparent radioligand affinity with no clear effect on radioligand Bmax (Fig. 5A). Unfortunately, these experiments were limited by the lack of concentrations of [3H]NMS large enough to define full radioligand-receptor occupancy in the presence of all concentrations of SCH-202676 that were tested. Nevertheless, it is clear that the apparent shifts of the [3H]NMS saturation curves are much greater than expected for simple competition and may be indicative of positive cooperativity in the binding of SCH-202676 to the M1 mACr. This type of cooperative interaction would also be expected to yield steep radioligand inhibition curves, as was indeed observed in this study (Fig. 4).

Given the possibility of cooperativity in the binding of SCH-202676, and the previous suggestion by Fawzi et al. (2001) that the compound may be using an intracellular attachment point conserved across different GPCRs, subsequent experiments in our study focused on the pharmacology of the modulator in intact cells. In addition to being more physiologically relevant than membrane-based studies, it was anticipated that the intact cell assays would provide further insight into the mode of action of SCH-202676 at the M1 mACr. Significant differences were indeed noted for the interaction between SCH-202676 and [3H]NMS in whole CHO cells compared with homogenates; both intact cell saturation and inhibition assays yielded binding curves that were in accord with the predictions of simple competitive antagonism, such as parallel dextral shifts of the [3H]NMS saturation curve with no change in Bmax (Fig. 5B) and a Hill slope factor not significantly different from unity (Fig. 6; Table 1). The assumption of a competitive interaction in the intact cell binding studies allowed for the estimation of apparent SCH-202676 LogKr values that were not significantly different from one another. This finding suggests that, in intact cells, SCH-202676 may not readily access the intracellular milieu but, rather, bind to an extracellular attachment point on the mACr and interact in a pseudocompetitive manner with orthosteric ligands such as [3H]NMS.

The ability of SCH-202676 to antagonize ACh-mediated PI hydrolysis (Fig. 7) is also in agreement with the finding by Fawzi et al. (2001) that the inhibitory effects of SCH-202676 extend to GPCR agonists, as well as antagonists. Importantly, the assay used in the present study utilized intact CHO cells, in contrast to the membrane-based [35S]guanosine 5′-O-(3-thio)triphosphate functional studies of Fawzi et al. (2001). The observation that concentrations of SCH-202676 less than 10 μM seem to interact competitively with ACh supports the findings in the intact cell binding assays. However, higher concentrations of the modulator resulted in a profound inhibition of the maximal attainable agonist effect. There are at least four possibilities to account for this effect of SCH-202676. The first is that the apparent noncompetitive inhibition of ACh-mediated responses at the highest concentrations of SCH-202676 reflect a nonspecific, toxic effect. Although we cannot totally rule out this possibility, we deem it unlikely as SCH-202676 alone (up to 100 μM) had no effect on basal (receptor-independent) PI hydrolysis. The second possibility is that, in intact cells, SCH-202676 binds in a pseudoirreversible manner to the orthosteric site on the M1 mACr and that the progressive decline in ACh maximal responsiveness represents the progressive diminution of receptor reserve by increasing concentrations of SCH-202676. However, this is also unlikely because binding assays using prolonged equilibration times found no evidence for an enhanced antagonistic effect of SCH-202676. The third possibility is that the effects of SCH-202676 are purely noncompetitive and mediated entirely via an allosteric site on the M1 mACr that is topographically distinct from the better characterized allosteric site recognized by modulators such as C3/3-phth. Although possible, this cannot account for the markedly different effects of the inhibitor when assayed in membranes compared with intact cells if the same allosteric site mediates the inhibition in each instance. A final possibility is that SCH-202676 recognizes more than one site on the mACr, with an extracellular attachment point that preferentially mediates the effects seen in intact cells, and an intracellular attachment point that contributes to the effects seen in membranes; the noncompetitive inhibition of agonist function noted with high micromolar concentrations of SCH-202676 may then represent a concentration gradient-driven accumulation of some of the compound in the proximity of or at the intracellular attachment point. This dual binding-mode mechanism can also account for the apparent cooperativity in binding noted for SCH-202676 in the membrane assays, where, presumably, both attachment points on the receptor are readily accessible to the modulator.

Irrespective of the mechanism of action of SCH-202676, the current findings highlight important issues in the study of
allosteric modulators of GPCRs. For instance, as suggested by Fawzi et al. (2001) and supported by some of the findings of the present study, SCH-202676 may recognize a conserved regulatory domain that is located in the intracellular-facing regions of the receptor. In terms of drug discovery, such a site is a relatively unattractive target for two reasons. First, intracellular targets are generally not as readily accessible as extracellular targets, and antagonizing intracellular GPCR domains may impair receptor coupling to accessory cellular proteins in addition to modulating ligand binding. Second, the conserved nature of such a regulatory site implies that ligands acting at this site will lack receptor specificity; allosteric modulators that target less conserved domains on GPCRs are preferable therapeutic candidates. Another important issue highlighted by the present study is that the nature of the assay (e.g., broken cell versus intact cell) can reveal marked differences in the apparent mode of action of modulator ligands. Given that SCH-202676 represents a useful small molecule tool with which to study certain aspects of GPCR allosteric modulation, it would be interesting to determine whether it displays more than one possible mode of interaction at other GPCRs.

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References


Address correspondence to: Dr. Arthur Christopoulos, Department of Pharmacology, University of Melbourne, Grattan St., Parkville, 3010, Victoria, Australia. E-mail: arthurc@unimelb.edu.au

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