G Protein Coupling and Signaling Pathway Activation by M₁ Muscarinic Acetylcholine Receptor Orthosteric and Allosteric Agonists

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ABSTRACT

The M₁ muscarinic acetylcholine (mACh) receptor is among a growing number of G protein-coupled receptors that are able to activate multiple signaling cascades. AC-42 (4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine) is an allosteric agonist that can selectively activate the M₁ mACh receptor in the absence of an orthosteric ligand. Allosteric agonists have the potential to stabilize unique receptor conformations, which may in turn cause differential activation of signal transduction pathways. In the present study, we have investigated the signaling pathways activated by AC-42, its analog 77-LH-28-1 (1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)-quinolinone), and a range of orthosteric muscarinic agonists [oxotremorine-M (oxo-M), arecoline, and pilocarpine] in Chinese hamster ovary cells reconstituting the human M₁ mACh receptor. Each agonist was able to activate Ga₁₁₁-dependent signaling, as demonstrated by an increase in guanosine 5′-O-(3-thiotriphosphate) ([35S]GTPγS) binding to Gaα₁₁₁ proteins and total [3H]inositol phosphate accumulation assays in intact cells. All three orthosteric agonists caused significant enhancements in [35S]GTPγS binding to Gaα₁₁₁ subunits over basal; however, neither allosteric ligand produced a significant response. In contrast, both orthosteric and allosteric agonists are able to couple to the Gαₛ,cAMP pathway, enhancing forskolin-stimulated cAMP accumulation. These data provide support for the concept that allosteric and orthosteric mACh receptor agonists both stabilize receptor conformations associated with Gαₛ and Gαₛ-dependent signaling; however, AC-42 and 77-LH-28-1, unlike oxo-M, arecoline, and pilocarpine, do not seem to promote M₁ mACh receptor-Gα₁₁₁ coupling, suggesting that allosteric agonists have the potential to activate distinct subsets of downstream effectors.

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ABBREVIATIONS: mACh, muscarinic acetylcholine; GPCR, G protein-coupled receptor; AC-42, 4-n-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine; 77-LH-28-1, 1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1H)-quinolinone; oxo-M, oxotremorine-M; GTPγS, guanosine 5′-O-(3-thiotriphosphate); NMS, N-methylscopolamine; CHO, Chinese hamster ovary; KHB, Krebs-Henseleit buffer; [3H]InsP₃, [3H]inositol mono-, bis-, and trisphosphate; Ins(1,4,5)P₃, inositol 1,4,5-trisphosphate; eGFP-PH, enhanced green fluorescent protein tagged to the pleckstrin homology domain of phospholipase C; AFU, arbitrary fluorescence unit(s); PLC, phospholipase C.
Lazareno, 2005; Gregory et al., 2007). These loci within the receptor are less well conserved than critical regions within the transmembrane core and represent sites within the receptor that could be exploited to develop subtype-specific ligands (Hulme et al., 2003; Gregory et al., 2007).

Spalding et al. (2002) have reported that a novel compound, AC-42, is able to activate selectively the M1 mACh receptor. By constructing chimeric receptors, this group was able to demonstrate that AC-42 binds to a site (proposed to consist of residues in the N terminus and transmembrane 1 along with residues in transmembrane 7 and the third extracellular loop) distinct from the orthosteric site (Spalding et al., 2002). The suggested “ectopic” location of the binding site was further supported by the discovery that an Y381A point mutation in the acetylcholine binding site was sufficient to abolish receptor activation by an orthosteric agonist but had no effect on the functional response to AC-42 (Spalding et al., 2002). Subsequent work has demonstrated that AC-42 acts allosterically (Langmead et al., 2006); consequently, we use the term “allosteric agonist” here to describe AC-42.

Although it is well recognized that ligand binding to M1 mACh receptors initiates G_{i/o}11-dependent activation of phospholipase C and consequent generation of inositol 1,4,5-trisphosphate and 1,2-diacylglycerol, the ability of this receptor subtype to influence additional downstream effector pathways (via distinct G protein subtypes) has also been reported. Thus, the M2 mACh receptor has been reported to activate G_{i/o} and G_{o}11 proteins (Offermanns et al., 1994; Akam et al., 2001) and to activate adenyl cyclase activity via a G protein-dependent mechanism (Burford and Nahorski, 1996). The ability of the M1 mACh receptor to activate multiple downstream effectors via distinct signaling pathways allows the possibility that allosteric agonists like AC-42 may not only display functional mACh receptor subtype selectivity but also may activate all or a subset of the pathways activated by orthosteric agonists. This phenomenon, referred to variously as “agonist-directed trafficking of receptor stimulus,” “functional selectivity,” and most recently as “ligand-induced differential signaling” (Kenakin, 2003; Urban et al., 2003), has been reported for a number of receptors including pituitary adenyl cyclase-activating protein receptors (Spergel et al., 1993), 5-hydroxytryptamine_{2c} receptors (Berg et al., 1998), and CB1 cannabinoid receptors (Bonhaus et al., 2002). Subsequent work has demonstrated that AC-42 acts allosterically (Langmead et al., 2006); consequently, we use the term “allosteric agonist” here to describe AC-42.

Materials and Methods
Materials. All chemicals and reagents were purchased from Sigma Chemical (Poole, Dorset, UK) unless otherwise stated. Tissue culture reagents and Lipofectamine2000 transfection reagent were from Invitrogen (Paisley, UK). [35S]GTPγS, [3H]AMP, [3H]NMS, [3H]inositol, and protein A-Sepharose CL-4B were from GE Healthcare (Chalfont St. Giles, UK). Charcoal and borosilicate cover slips were from BDH (Poole, Dorset, UK). Primary antibody for G_{o}11 was generated against the common 11-mer C terminus sequence LQNLKKKEYNLV as described previously (Akam et al., 2001). Primary antibody for G_{o} was generated by Cambridge Research Biochemicals (Northwich, UK) against the common G_{o}112 C-terminal 10-mer sequence KENLKDCCGLF. AC-42 and 77-LH-28-1 were synthesized “in-house” by GlaxoSmithKline (Harlow, UK).

Cell Culture. Chinese hamster ovary cells stably expressing the recombinant human M1 mACh receptor (CHO-M1 cells), originally obtained from Dr. Noel Buckley (then at National Institutes for Medical Research, London, UK), were grown in minimal essential medium supplemented with fetal bovine serum (10%), penicillin (50 units/ml), streptomycin (50 μg/ml), and amphotericin B (2.5 μg/ml). Cells were maintained at 37°C in a humidified atmosphere of 5% CO2 in air.

Cell Membrane Preparation. Confluent monolayers of CHO-M1 cells were rapidly washed with 10 μM HEPES, 0.9% NaCl, and 0.2% EDTA, pH 7.4 (HBS-EDTA) before incubation with HBS-EDTA for 15 min to lift cells. Cells were centrifuged (400 g, 4 min), and the pellet was resuspended in 10 μM HEPES and 10 μM EDTA, pH 7.4, homogenized using a Polytron homogenizer (3 × 5-s bursts; Kinematica AG, Littau, Switzerland) and centrifuged (40,000 g, 15 min, 4°C). The cell pellet was resuspended in 10 μM HEPES and 0.1 mM EDTA, pH 7.4, and rehomogenized and centrifuged as described above. The final pellet was resuspended in the 10 μM HEPES and 10 μM EDTA, pH 7.4, buffer to give a final protein concentration of 2 mg/ml and either used immediately or snap-frozen and stored at −80°C.

[3H]NMS Binding. [3H]NMS inhibition binding assays were carried out as described previously (Mistry et al., 2005). In general, CHO-M1 cells were seeded at a density of 75,000 cells/well in 24-well plates. The next day, cells were washed three times with warmed KHB (composition: 118 mM NaCl, 8.5 mM HEPES, 4.7 mM KCl, 4 mM NaHCO3, 1.3 mM CaCl2, 1.2 mM MgSO4·7H2O, 1.2 mM KH2PO4, and 11.7 mM glucose, pH 7.4) before being maintained on ice in a total assay volume of 1 ml of ice-cold KHB containing appropriate concentrations of agonists and approximately 0.2 nM [3H]NMS. After a 5-h incubation on ice-cold KHB before the addition of 0.1 M NaOH (500 μl). After cell solubilization, SafeFluor scintillation fluid was added, and samples were counted.

[35S]GTPγS Binding and Immunoprecipitation of Go Subunits. [35S]GTPγS binding and immunoprecipitation of Go subunits was performed as described previously (Akam et al., 2001). In general, CHO-M1 membranes were diluted in assay buffer (10 μM HEPES, 100 mM NaCl, and 10 mM MgCl2, pH 7.4) to give a final protein concentration of 75 μg per 50 μl. Membranes were then added to assay buffer containing final concentrations of 1 nM [35S]GTPγS and either 1 or 10 μM GDP (see Results). Membranes were incubated at 30°C for 2 or 5 min (see Results) with agonists before reactions were terminated by the addition of 1 ml of ice-cold assay buffer and immediate transfer to an ice bath. Samples were centrifuged (20,000g, 6 min, 4°C), and membrane pellets were solubilized by the addition of 50 μl of ice-cold solubilization buffer (100 mM Tris/HCl, 200 mM NaCl, 1 mM EDTA, 1.25% Igepal, and 0.2% SDS, pH 7.4) and regular vortex mixing over 30 min. After complete protein resolubilization, 50 μl of solubilization buffer without SDS was added to 20 μl of 1:10 diluted polyclonal antibody against common Go subunits, 50 μl of 20 μl of 1:10 diluted polyclonal antibody against common Go subunits.
was added. Solubilized protein was precleared using normal rabbit serum at a dilution of 1:100 and 3% (w/v) protein A-Sepharose beads in TE buffer (10 mM Tris/HCl and 10 mM EDTA, pH 8.0) added for 60 min at 4°C. Protein A-Sepharose beads and insoluble material were collected by centrifugation (20,000g, 6 min, 4°C), and 100 µl of the supernatant was transferred to fresh tubes containing G-specific anti-serum. Samples were vortex-mixed and rotated at 4°C for 90 min before being centrifuged (20,000g, 6 min, 4°C). Supernatants were aspirated, and the protein A-Sepharose beads were washed three times with ice-cold solubilization buffer (without SDS). Recovered beads were then mixed with 1 ml of Fliscint-IV scintillation cocktail and counted by liquid scintillation spectrometry.

**cAMP Accumulation Assay.** CHO-M1 cells seeded at 100,000 cells/well in 24-well plates 2 days before assay were washed twice before the addition of 450 µl of Ca²⁺-free KHB containing 100 µM EGTA. Cells were incubated at 37°C with agonists for 10 min before termination by aspiration of buffer and addition of ice-cold 0.5 M trichloroacetic acid (400 µl). After addition of 50 µl of 10 mM EDTA, samples were mixed with 500 µl of 1,1,2-trichlorofluoroethane:tri-n-octylamine (1:1, v/v). Samples were centrifuged at 16,000g for 4 min, and 200 µl of the upper phase was removed. Sample pH was brought to approximately 7.5 by the addition of 50 µl of 60 mM NaHCO₃. cAMP concentrations were determined using a binding assay as described previously (Mistry et al., 2005).

**Total [3H]inositol Phosphate Accumulation Assay.** CHO-M1 cells were seeded at 100,000 cells/well in 24-well plates and incubated in fresh medium containing 2.5 µCi/ml [3H]inositol for 48 h. Confluent cell monolayers were washed twice in KHB, and then 275 µl of KHB containing 10 mM LiCl was added. Cells were incubated for 30 min at 37°C before the addition of agonist or vehicle for 15 min. Reactions were terminated by the addition of ice-cold trichloroacetic acid (1 M), and samples were neutralized using the freon/tri-n-octylamine method as described above. The [3H]inositol mono-, bis-, and trisphosphate ([3H]InsP₃) fraction was recovered by anion exchange chromatography. Dowex-1 (formate form) columns were regenerated with 10 ml of ammonium formate (2 M)/formic acid (0.1 M) before being washed extensively with distilled water. Samples were washed onto the columns with 5 ml of distilled water. Columns were initially washed with 10 ml of ammonium formate (60 mM)/sodium tetraborate (5 mM). The [3H]InsP₃ fraction was then eluted in 10 ml of ammonium formate (0.75 M)/formic acid (0.1 M). A 5-ml aliquot of the collected eluate was mixed with 10 ml of FastFluor scintillation cocktail, and radioactivity was determined by liquid scintillation counting.

**Ins(1,4,5)P₃ Mass Assay.** CHO-M1 cells seeded at 100,000 cells/well in 24-well plates 2 days before assay were washed twice, and 450 µl of warmed KHB was added for 30 min at 37°C before the addition of maximal agonist concentrations for the times indicated. Reactions were terminated by the addition of ice-cold trichloroacetic acid (0.5 M) and extracted using the freon/tri-n-octylamine method as described above. Determination of Ins(1,4,5)P₃ concentrations was by a competition binding assay described previously (Challiss et al., 1988).

**Single-Cell Imaging of Ins(1,4,5)P₃.** CHO-M1 cells were seeded onto 25-mm glass coverslips and transiently transfected with the enhanced green fluorescent protein tagged to the pleckstrin homology domain of phospholipase C (eGFP-PH) construct (Stauffer et al., 1998) using Lipofectamine2000 according to the manufacturer’s instructions. After 48 h, translocation of the eGFP-PH biosensor was visualized using an Olympus FV500 confocal microscope (Olympus, Tokyo, Japan), with cells maintained at 37°C and perfused with KHB (5 ml/min). Changes in the cell localization of eGFP-PH were examined of a proximal signaling event in membrane preparations from a number of cells/tissues (Friedman et al., 1993; Lazareno and Birdsall, 1993; Akam et al., 2001). This technique has been optimized in a CHO cell background.

**Results**

Both AC-42 and 77-LH-28-1, a structural homolog, have been reported previously to have an allosteric mechanism of action (Langmead et al., 2006; Spalding et al., 2006; May et al., 2007). In preliminary experiments, both ligands were shown to possess similar allosteric properties. Thus, they each significantly slowed [3H]NMS dissociation from the M₁ mACh receptor in CHO-M1 membranes, and analysis of the abilities of these compounds to displace specific [3H]NMS binding at different concentrations of [3H]NMS (0.2, 2, and 5 nM) was consistent with an allosteric mechanism of action (see Langmead et al., 2006; Spalding et al., 2006).

**Agonist Affinity Estimates for M₁ mACh Receptors.** M₁ mACh receptor binding affinities for oxo-M, AC-42, 77-LH-28-1, arecoline, and pilocarpine were determined by [3H]-NMS competition binding in intact CHO-M1 cell monolayers. Initial [3H]NMS saturation binding analysis determined a B₅₀ value of 4.84 ± 0.31 pmol/mg protein and a Kᵦ value of 0.29 ± 0.05 nM in intact CHO-M1 cells (n = 4; data not shown). CHO-M₁ cell monolayers were incubated with an approximate Kᵦ value of [3H]NMS in the presence of varying agonist concentrations at 4°C for 5 h (to achieve equilibrium binding without causing receptor internalization). The apparent binding affinity (pKᵦ) and Hill slope for each agonist are summarized in Table 1. 77-LH-28-1 displayed a significantly higher binding affinity than AC-42, oxo-M, arecoline, and pilocarpine (P < 0.05).

**Analysis of M₁ mACh Receptor-Gs/11 Coupling.** Agonist stimulation in the presence of [35S]GTPγS and subsequent immunoprecipitation of specific Go subunits allows examination of a proximal signaling event in membrane preparations from a number of cells/tissues (Friedman et al., 1993; Lazareno and Birdsall, 1993; Akam et al., 2001). This technique has been optimized in a CHO cell background.

<table>
<thead>
<tr>
<th>pKᵦ</th>
<th>Hill Slope</th>
</tr>
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<tbody>
<tr>
<td>oxo-M</td>
<td>5.30 ± 0.07</td>
</tr>
<tr>
<td>AC-42</td>
<td>5.52 ± 0.04</td>
</tr>
<tr>
<td>77-LH-28-1</td>
<td>6.00 ± 0.04</td>
</tr>
<tr>
<td>Arecoline</td>
<td>5.06 ± 0.20</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>4.95 ± 0.10</td>
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</table>

Data are the means ± S.E.M. for at least three separate experiments performed in duplicate.
to show that the concentration-dependent increase in [35S]GTPγS binding, stimulated by the human M1 mACh receptor, is optimal in the presence of 1 μM GDP after 2-min incubations at 30°C (Akam et al., 2001). Maximal activation of the M1 mACh receptor by oxo-M caused an approximate 30-fold increase in [35S]GTPγS binding to Goq11 with an EC50 value of approximately 1 μM (Fig. 1; Table 2). The M1-selective allosteric agonists, AC-42 and 77-LH-28-1, were partial agonists with respect to oxo-M, eliciting approximately 30 and 60% of the oxo-M response, respectively (Fig. 1A). 77-LH-28-1 was more potent than AC-42, causing an increase in [35S]GTPγS-Goq11 binding with an EC50 value of approximately 0.4 compared with 1.6 μM for AC-42 (Table 2). Pilocarpine and arecoline also behaved as partial agonists with respect to oxo-M, causing approximately 35 and 60% of the maximal response (Fig. 1B). Thus, AC-42 displays comparable relative efficacy to the orthosteric agonist pilocarpine in activating Goq11 subunits, whereas 77-LH-28-1 is comparable with the orthosteric agonist arecoline. Time courses for agonist-stimulated increases in Goq11-[35S]GTPγS binding are shown as insets to Fig. 1, A and B. In agreement with the previous observation of Akam et al. (2001), 2-min incubations are sufficient to cause maximal increases in Goq11-[35S]GTPγS binding over basal values.

**Characterization of [3H]InsPx Accumulation in CHO-M1 Cells.** As an index of PLC activation, agonist-stimulated accumulation of total [3H]InsPx was assessed in the presence of Li+. Maximal stimulation with oxo-M caused a 15-fold increase in [3H]InsPx accumulation (1,198,704 ± 293,374 dpm/mg protein over a basal value of 77,064 ± 18,779 dpm/mg protein) with an EC50 of 0.1 μM (Fig. 2; Table 2). This represents a 10-fold leftward shift in the concentration dependence for the [3H]InsPx accumulation versus Goq11-[35S]GTPγS binding for the full, orthosteric agonist (Table 2). Both AC-42 and 77-LH-28-1 behaved as partial agonists in this assay with respect to the oxo-M response, causing approximately 55 and 87% of the full agonist response, respectively. The respective relative efficacy (Rmax) values for Fig. 1. Concentration dependencies of agonist-stimulated [35S]GTPγS binding to Goq11 subunits in CHO-M1 cell membranes. CHO-M1 cell membranes were incubated with the indicated concentrations of oxo-M, AC-42, and 77-LH-28-1 (A) or oxo-M, arecoline, and pilocarpine (B) in the presence of 1 μM GDP and 1 nM [35S]GTPγS for 2 min at 30°C. Incubations were terminated, and Goq11 subunits were immunoprecipitated as described under Materials and Methods. Inset figures, A and B, time courses for increases in Goq11-[35S]GTPγS binding stimulated by agonist or vehicle additions: oxo-M (100 μM), AC-42 (30 μM), 77-LH-28-1 (10 μM), arecoline (1 mM), or pilocarpine (1 mM). Concentration dependence and time course data are presented as means ± S.E.M. for at least three separate experiments performed in duplicate (see Table 2).
arecoline and pilocarpine were 101 and 90% (Fig. 2; Table 2).

**TABLE 2**

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$pEC_{50}$</th>
<th>$R_{\text{max}}$</th>
<th>$n$</th>
<th>$pEC_{50}$</th>
<th>$R_{\text{max}}$</th>
<th>$n$</th>
<th>$pEC_{50}$</th>
<th>$R_{\text{max}}$</th>
<th>$n$</th>
<th>cAMP ($G_s^{\text{max}}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxo-M</td>
<td>6.07 ± 0.14</td>
<td>100</td>
<td>4</td>
<td>7.01 ± 0.16</td>
<td>100</td>
<td>6</td>
<td>5.24 ± 0.12</td>
<td>100</td>
<td>6</td>
<td>5.71 ± 0.02</td>
</tr>
<tr>
<td>AC-42</td>
<td>5.79 ± 0.08</td>
<td>29.6 ± 2.4</td>
<td>5</td>
<td>5.73 ± 0.07</td>
<td>55.5 ± 2.3</td>
<td>6</td>
<td>8.7 ± 5.0</td>
<td>5</td>
<td>5.06 ± 0.15</td>
<td>13.2 ± 1.0</td>
</tr>
<tr>
<td>77-LH-28-1</td>
<td>6.40 ± 0.07</td>
<td>58.0 ± 7.1</td>
<td>5</td>
<td>6.61 ± 0.10</td>
<td>87.0 ± 2.4</td>
<td>4</td>
<td>N.D.</td>
<td>9.8 ± 4.1</td>
<td>6.01 ± 0.08</td>
<td>42.0 ± 2.9</td>
</tr>
<tr>
<td>Arecoline</td>
<td>5.53 ± 0.08</td>
<td>57.0 ± 2.1</td>
<td>3</td>
<td>5.79 ± 0.15</td>
<td>101.0 ± 9.0</td>
<td>3</td>
<td>4.61 ± 0.18</td>
<td>43.4 ± 3.4</td>
<td>4.61 ± 0.14</td>
<td>39.2 ± 2.8</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>5.62 ± 0.10</td>
<td>34.3 ± 3.8</td>
<td>3</td>
<td>5.40 ± 0.29</td>
<td>90.0 ± 2.3</td>
<td>3</td>
<td>4.56 ± 0.33</td>
<td>36.7 ± 4.2</td>
<td>4.41 ± 0.24</td>
<td>26.5 ± 2.2</td>
</tr>
</tbody>
</table>

N.D., could not be derived.

Thus, $R_{\text{max}}$ values for all of the orthosteric and allosteric partial agonists increased with respect to $[^{3}\text{H}]\text{InsP}_x$ accumulation compared with $\text{G}_{\alpha q11-[^{35}\text{S}]}\text{GTP}\gamma\text{S}$ binding; however, significant differences between $EC_{50}$ values for these two readouts were only observed for the full agonist (Table 2).

**Characterization of $\text{Ins}(1,4,5)\text{P}_3$ Responses in CHO-M1 Populations and in Single Cells.** Although the Li$^+$-blocked, $[^{3}\text{H}]\text{InsP}_x$ accumulation assay provides an index of PLC activity, we also conducted experiments to investigate the time course of $\text{Ins}(1,4,5)\text{P}_3$ accumulation in CHO-M1 cells to compare further the activation profiles of allosteric and orthosteric agonists with respect to $\text{G}_{\alpha q11}$-coupled downstream signaling. Basal levels of $\text{Ins}(1,4,5)\text{P}_3$ were $44 ± 3$ pmol/mg protein ($n = 12$) in cell population experiments. oxo-M (100 $\mu$M) caused a biphasic rise in $\text{Ins}(1,4,5)\text{P}_3$ levels, with an initial peak of $703 ± 37$ pmol/mg protein after 15 s, falling to a plateau level ($278 ± 20$ pmol/mg protein) sustained from 60 s onwards (Fig. 3). In contrast, activation of CHO-M1 cells by either arecoline (1 mM) or pilocarpine (1 mM) caused monophasic $\text{Ins}(1,4,5)\text{P}_3$ accumulation profiles, with maxima being reached at around 60 s (Fig. 3B). It was notable that the “steady-state” $\text{Ins}(1,4,5)\text{P}_3$ accumulations caused by oxo-M, arecoline, and pilocarpine between 60 and 300 s were similar (approximately 300 pmol/mg protein).

The allosteric agonist 77-LH-28-1 (10 $\mu$M) caused a similar monophasic profile of $\text{Ins}(1,4,5)\text{P}_3$ accumulation to the orthosteric partial agonists, achieving a similar steady-state value (Fig. 3A). In contrast, stimulation with AC-42 (100 $\mu$M) produced a modest, monophasic increase in $\text{Ins}(1,4,5)\text{P}_3$ accumulation (reaching $122 ± 9$ pmol/mg protein at 120 s; Fig. 3A), which was significantly lower than steady-state values for all of the other agonists examined ($P < 0.01$).

Complementary experiments were performed using the fluorescent biosensor eGFP-PH to visualize real-time changes in $\text{Ins}(1,4,5)\text{P}_3$ levels in single CHO-M1 cells. At rest, eGFP-PH is maintained at the plasma membrane because of its affinity for phosphatidylinositol 4,5-bisphosphate. On PLC activation, the biosensor translocates to the cytoplasm because of its greater affinity for $\text{Ins}(1,4,5)\text{P}_3$. Therefore, increases in cytoplasmic fluorescence provide an index of the changes in $\text{Ins}(1,4,5)\text{P}_3$ and/or phosphatidylinositol 4,5-bisphosphate levels and, hence, PLC activity. This experimental technique was used specifically to assess the relative rates of activation of phosphoinositide turnover caused by the different orthosteric and allosteric agonists (Fig. 4). Stimulation with oxo-M (100 $\mu$M, 30 s) induced a rapid increase in $\text{Ins}(1,4,5)\text{P}_3$ accumulation, which peaked at 15 to 30 s and immediately declined toward baseline on agonist washout (Fig. 4A). Consistent with the cell population time courses, arecoline (1 mM), pilocarpine (1 mM), and 77-LH-28-1 (10 $\mu$M) caused smaller peak $\text{Ins}(1,4,5)\text{P}_3$ accumulations. To analyze the initial rates of second messenger generation, basal and peak fluorescence levels were defined, and the
change between 10 and 90% of peak Ins(1,4,5)P3 accumulation was determined and expressed as a change in cytoplasmic fluorescence [which is directly proportional to Ins(1,4,5)P3 accumulation] per unit time (see Materials and Methods). Using this index of the rate of increase in Ins(1,4,5)P3 (expressed as arbitrary fluorescence units per second), we found that oxo-M caused the most rapid response \((23.6 \pm 8.4 \text{ AFU/s})\), with arecoline \((8.2 \pm 3.3 \text{ AFU/s})\), pilocarpine \((8.0 \pm 1.0 \text{ AFU/s})\), and 77-LH-28-1 \((7.1 \pm 3.8 \text{ AFU/s})\) giving essentially similar, but slower, rates. In comparison with oxo-M, Ins(1,4,5)P3 accumulations stimulated by arecoline, pilocarpine, and 77-LH-28-1 declined quite slowly over a 300- to 500-s period (Fig. 4, A and B). Cell-to-cell variation in responses to AC-42 \((100 \mu M)\) was greater than for the other agonists studied, and in some cells (see Fig. 4B), AC-42 was able to cause a peak in Ins(1,4,5)P3 accumulation comparable with the other partial agonists (Fig. 4B); however, the rate of rise evoked by AC-42 was always much slower than for the other agonists \((1.5 \pm 0.5 \text{ AFU/s})\).

**Analysis of Receptor-Gα1/2 Coupling.** Although the M2 and M4 mACh receptor subtypes couple preferentially to G\(_{\alpha}\) proteins (Caulfield and Birdsall, 1998), it has been demonstrated that the M1 and M3 mACh receptor subtypes are also capable of activating G\(_{\alpha}\) subunits in addition to G\(_{\alpha}\)/11 subunits (Offermanns et al., 1994; Akam et al., 2001). To investigate whether the M1 mACh receptor-selective allosteric agonists can cause the M1 mACh receptor to activate multiple G protein-dependent pathways, we first assessed the agonist-dependent activation of G\(_{\alpha}\) subunits in CHO-M1 membranes.

In initial experiments designed to optimize the \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/12 immunoprecipitation assay, oxo-M caused a marked \((272 \pm 74\% \text{ over basal})\) and concentration-dependent \((\text{pEC}_{50} \text{ (M)} = 7.14 \pm 0.06 \text{ M})\) increase in \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/12 binding in CHO-M2 membranes. Similar optimal binding conditions were obtained for agonist-stimulated increases in \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/12 binding in CHO-M1 membranes \((10 \mu M \text{ GDP}, 2\text{-min incubations at } 30°C; \text{ see Fig. 5A, inset})\). Under these conditions, oxo-M stimulated a more modest \([\text{basal } 15,120 \pm 707 (15); +\text{oxo-M, } 23,240 \pm 1280 (15) \text{ cpm/mg protein}]\) yet highly significant \((P < 0.001)\) increase in \[^{35}\text{S} \]GTP\(_{S}\) binding. The increase in \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/12 binding stimulated by oxo-M was concentration-dependent \((\text{pEC}_{50} \text{ (M)} = 5.13 \pm 0.08 \text{ M})\) and lay approximately 10-fold to the right of the oxo-M-stimulated \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/11 binding curve (see Fig. 5B; Table 2). Arecoline and pilocarpine both stimulated significant increases in \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/12 binding over basal (Fig. 5, A and B), with their respective concentration-response curves also lying approximately 10-fold to the right of their respective \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/11 binding responses (Table 2). In contrast, neither allosteric agonist \((\text{at concentrations up to } 30 \mu M \text{ (77-LH-28-1) or } 300 \mu M \text{ (AC-42)}\) was able to cause a significant increase in \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/12 binding in CHO-M1 membranes (Fig. 5A; Table 2). These data suggest that the allosteric agonists are less able to facilitate M1 mACh receptor-dependent GTP/GDP exchange on G\(_{\alpha}\)/12 proteins compared with orthosteric partial agonists that are equipotential with respect to other signaling readouts.

**Analysis of Receptor-Gαq Coupling.** To explore the potential coupling of the M1 mACh receptor to G\(_{\alpha}\) in the CHO cell background (Burford and Nahorski, 1996), we initially assessed the ability of orthosteric and allosteric agonists to stimulate \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/11 binding in CHO-M1 membranes. To first validate the \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/11 immunoprecipitation assay, we showed that stimulation of CHO-β_{2} cell membranes \((\beta_{2}-adrenoceptor density, approximately 300 fmol/mg protein)\) with isoprenaline \((10 \mu M)\) resulted in a 4-fold increase in \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/11 binding over basal \((\text{basal, 6253} \pm 853; +\text{isoprenaline, 27,227} \pm 920 \text{ cpm/mg protein})\) under optimized conditions \([\text{membrane preincubation with } 1 \mu M \text{ GDP} ; \text{addition of agonist to membranes for } 5 \text{ min before incubation at } 30°C \text{ with }[^{35}\text{S} \]GTP\(_{S}\)/S (1 \text{ nM})\) (see Carruthers et al., 1999). Under these conditions, none of the mACh receptor agonists caused a detectable increase in \[^{35}\text{S} \]GTP\(_{S}\)-G\(_{\alpha}\)/11 binding.

As an alternate index of mACh receptor-G\(_{\alpha}\)/activation, we measured cAMP accumulation in intact CHO-M1 cells. Addition of oxo-M \((100 \mu M)\) caused a robust 10- to 20-fold increase in cAMP accumulation \((\text{basal, } 11 \pm 3; +\text{oxo-M, } 183 \pm 8 \text{ pmol/mg protein}; \text{pEC}_{50} \text{ (M)} = 5.05 \pm 0.08); however, none of the partial agonists caused a significant increase over basal (data not shown). In contrast, arecoline, pilocarpine, AC-42, and 77-LH-28-1 significantly enhanced the increase in cAMP accumulation caused by forskolin \((1 \mu M)\). Previous studies have
found that under these conditions, receptor activation in CHO cells can indirectly modulate forskolin-stimulated adenyl cyclase activity through activation of Ca\textsuperscript{2+} entry and Ca\textsuperscript{2+}-dependent effects on isoforms of adenyl cyclase (Cooper et al., 1995). Therefore, in these assays, Ca\textsuperscript{2+} was omitted from the medium, and EGTA (100 μM) was added to remove the possibility of Ca\textsuperscript{2+} entry-driven effects on M₁ mACH receptor-mediated cAMP responses. Under these conditions, all agonists induced concentration-dependent, monophasic enhancements of forskolin-stimulated cAMP accumulation (Fig. 6). oxo-M caused a 7.5-fold enhancement of the forskolin-stimulated response (+forskolin, 151 ± 10; +forskolin/oxo-M, 1130 ± 66 pmol/mg protein). Arecoline, pilocarpine, AC-42, and 77-LH-28-1 all behaved as partial agonists with respect to the maximal oxo-M response (Fig. 6, A and B; Table 2). The rank order of potency with respect to cAMP accumulation was 77-LH-28-1 (6.01) > oxo-M (5.71) > AC-42 (5.06) > arecoline (4.61) = pilocarpine (4.41).

**Discussion**

The present study provides a profile of the proximal signaling pathways coupled to an allosteric agonist of the M₁ mACH receptor, AC-42 (Spalding et al., 2002), and a closely related compound, 77-LH-28-1 (May et al., 2007; Langmead et al., 2008). In general, we have focused on the ability of orthosteric and allosteric mACH receptor agonists to activate Gαq/11-dependent signaling events and explored the ability of these agonists to stimulate M₁ mACH receptor coupling to alternative Gq, and Gι, protein-dependent signaling pathways. Although orthosteric and allosteric agonists share many properties, we have demonstrated that there exist subtle differences in the signaling outcomes these two subclasses of agonist are able to activate.

Initial assessment of the abilities of orthosteric and allosteric agonists to promote M₁ mACH receptor-Gαq/11 protein coupling in the CHO-M₁ membranes used the [35S]GTPγS binding assay (Hilf et al., 1989; Lazareno and Birdsell, 1993) adapted to distinguish [35S]GTPγS binding to specific G protein subpopulations (Friedman et al., 1993; Akam et al., 2001). Consistent with previous work (Spalding et al., 2002, 2006; Langmead et al., 2006, 2008), AC-42 and 77-LH-28-1 were able to activate the M₁ mACH receptor in the absence of an orthosteric ligand, causing robust increases in [35S]GTPγS-Gαq/11 binding, with 77-LH-28-1 exhibiting a greater intrinsic activity and higher potency than AC-42 with respect to this proximal signaling readout. Nevertheless, recent data indicate that AC-42 is sufficiently efficacious to stimulate significant M₁ mACH receptor-stimulated [35S]GTPγS-Gαq/11 binding in native systems (Salah-Uddin et al., 2008).

A consequence of M₁ mACH receptor-Gαq/11 coupling is the activation of PLC. In the presence of lithium, it is possible to assess [3H]InsP₃ accumulation as an index of PLC activity in cells labeled to equilibrium with [3H]inositol. Using this assay, it was observed that the EC₅₀ for the full agonist oxo-M was approximately 10-fold left-shifted for [3H]InsP₃ accumulation in intact cells compared with the membrane-based [35S]GTPγS-Gαq/11 binding response. In contrast, EC₅₀ values for these responses were similar for both orthosteric and allosteric partial agonists, but increases in relative efficacy for [3H]InsP₃ accumulation compared with [35S]GTPγS-Gαq/11 binding responses were seen in all cases (Table 2).

To gain a more kinetic perspective on the actions of the
different mACh receptor agonists on PLC activity, temporal profiles for Ins(1,4,5)P3 accumulation were also investigated. Ins(1,4,5)P3 response profiles vary both with respect to the initiating GPCR and the pharmacological properties of the stimulating agonist (Willars and Nahorski, 1995; Nash et al., 2002; Bartlett et al., 2005). Here, only the full agonist oxo-M stimulated a rapid, peak-and-plateau Ins(1,4,5)P3 response. Monophasic increases in Ins(1,4,5)P3, reaching similar plateau levels to those seen for oxo-M after 60 to 120 s, were seen for arecoline, pilocarpine, and 77-LH-28-1. In contrast, AC-42, which yielded similar pEC50 and Rmax values to pilocarpine with respect to stimulating [35S]GTPγS binding to Goi1/2, stimulated a monophasic increase in Ins(1,4,5)P3 that was 50% of that achieved by the other partial agonists. Further differences between the agonists used were illustrated by single-cell studies of Ins(1,4,5)P3 generation, using eGFP-PH (Stauffer et al., 1998; Nash et al., 2002). Application of oxo-M caused a rapid increase in eGFP-PH translocation from the plasma membrane to the cytoplasm, which rapidly reversed on agonist washout. Arecoline, pilocarpine, and 77-LH-28-1 gave broadly similar responses to each other, with slower increases, lower maxima, and slower reversibility on washout than oxo-M. Greater variation in individual responses was seen with respect to eGFP-PH translocation after AC-42 application (delivered either by perfusion or direct bath application). Although the eventual extent of eGFP-PH translocation caused by AC-42 in some cells approached that caused by 77-LH-28-1, the rate of rise was always slow (10-fold slower relative to oxo-M). These data clearly indicate kinetic differences between the ago-
nists, but these seem to be more related to efficacy differences between agents than whether they bind at an orthosteric or allosteric site on the receptor.

Agonists can bind to GPCRs and stabilize or induce specific conformations that allow the activated receptor to regulate downstream signaling cascades, a phenomenon referred to as agonist-directed trafficking of receptor stimulus, functional selectivity, and “ligand bias” (Kenakin, 2003; Urban et al., 2007; Violin and Lefkowitz, 2007). Unambiguous determination of agonist-selective conformations can best be deduced at the level of the receptor-G protein interaction.

We initially investigated agonist-stimulated M1 mACh receptor-Gαq coupling because in a previous report, we showed that the M1 mACh receptor is capable of activating Gαq/11 subunits in a CHO cell background, and the degree of coupling to Gαq/11 is agonist-dependent (Akam et al., 2001). Here, we were able to confirm the activation of M1 mACh receptor-stimulated Gαq protein GTP/GDP exchange using a Gαq/11 antibody to immunoprecipitate [35S]GTPγS-Gαq11/2 complexes. The orthosteric agonists oxo-M, arecoline, and pilocarpine all stimulated significant increases in [35S]GTPγS-Gαq11/2 binding, and pEC50 values could be obtained for these agonists, which were approximately 10-fold right-shifted relative to those determined for receptor-stimulated [35S]GTPγS-Gαq11/2 binding (see Table 2). In contrast, both allosteric agonists failed to cause a significant increase in [35S]GTPγS-Gαq11/2 binding, and as a consequence, pEC50 values could not be determined. These data suggest that M1 mACh receptor conformations favoring coupling to Gαq/11 proteins are better stabilized by oxo-M, arecoline, and pilocarpine compared with AC-42 and 77-LH-28-1.

Gαq/11-coupled receptors have often been reported to stimulate cAMP accumulation in a variety of cell types. Although in some cases this has been shown to involve Gi protein-independent coupling via a Ca2+- and/or PKC-dependent mechanism (Felder et al., 1988; Cooper et al., 1995), in the CHO cell background, direct Gi coupling has been demonstrated for the α1B-adrenergic (Horie et al., 1995) and M1 mACh (Burford and Nahorski, 1996) receptors. Assessment of receptor-Gi coupling using a [35S]GTPγS-Gαi1/2 binding/immunoprecipitation assay (Carruthers et al., 1989) was not possible because significant agonist-dependent increases in [35S]GTPγS-Gαi1/2 binding were not observed in CHO-M1 membranes challenged with any of the mACh receptor agonists. This apparent lack of M1 mACh receptor-Gi coupling should be interpreted cautiously, because isoprenaline only elicited a maximal 4-fold increase in [35S]GTPγS-Gαs binding in CHO-β2 membranes. In intact CHO-β2 cells, isoprenaline causes a profound increase in cAMP accumulation (>100-fold), which is at least an order of magnitude greater than that elicited by oxo-M in intact CHO-M1 cells (see Results). To compare M1 mACh receptor-Gαq coupling stimulated by the orthosteric and allosteric agonists, we looked at the ability of each agonist to enhance cAMP accumulation stimulated by a submaximal concentration of forskolin (1 μM). Indirect effects on adenylyl cyclase isoforms because of activation of a Gαs/11/PLC/Ca2+-pathway can be abolished by performing experiments under Ca2+-free conditions (emission of Ca2+-free conditions (see Materials and Methods)). Under these conditions, cAMP accumulation is unaffected by pertussis toxin treatment or protein kinase C inhibitors (R. Mistry and R. A. J. Challiss, unpublished data) and is a result of Gαs activation. All agonists studied were able to stimulate monophasic enhancements of forskolin-stimulated cAMP accumulation. oxo-M stimulated the greatest increase, with a pEC50 value ≥ 10-fold right-shifted relative to that for the [3H]InsP3 response (Table 2). In contrast to the data obtained for M1 mACh receptor-Gαq coupling, 77-LH-28-1 and AC-42 were able to increase cAMP accumulation with EC50 values only 4- to 5-fold right-shifted compared with those obtained for [3H]InsP3 accumulation, suggesting they are able to stabilize an M1 mACh receptor conformation capable of interacting with the Gαs subunits.

Although much of the relevant literature focuses on M1 mACh receptor signaling via Gαq/11, there are reports of M1 mACh receptors linking to distal cellular outputs via Gs and Gαq. For example, although it is well established that M1 mACh receptors inhibit the M-type potassium current via Gαq/11 (Delmas and Brown, 2005), a pertussis toxin-sensitive component to M-current inhibition has also been reported (Haley et al., 2000; Lechner et al., 2003). Likewise, it has been reported that M1 mACh receptor stimulation of cAMP response element-driven luciferase reporter gene expression in JEG-3 cells occurs via a PLC/Ca2+-independent, Gαq-dependent pathway (Migeon and Nathanson, 1994). Thus, it is possible that Gαq-, Gαs-, and Gαq/11-dependent signaling contribute to at least a subset of M1 mACh receptor-mediated responses in different cell backgrounds and under particular (patho)physiological conditions.

In conclusion, although AC-42 and its structural homolog 77-LH-28-1 clearly interact with the M1 mACh receptor different from conventional orthosteric agonists (Spalding et al., 2002, 2006; Langmead et al., 2006), they nevertheless cause activation of Gαq/11 signaling that is functionally indistinguishable from orthosteric agonists. In addition, AC-42 and 77-LH-28-1 are much less able to facilitate receptor-Gαq/11 coupling than are orthosteric agonists of comparable intrinsic activity. Thus, in addition to the potential for much greater mACh receptor subtype selectivity of allosteric agonists, it is possible that these agents will activate only a subset of signaling pathways permitting further specificity of action.

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