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G Protein Coupling and Signaling Pathway Activation by M_1 Muscarinic Acetylcholine Receptor Orthosteric and Allosteric Agonists

Rachel L. Thomas, Rajendra Mistry, Christopher J. Langmead, Martyn D. Wood, and R. A. John Challiss

Department of Cell Physiology and Pharmacology, University of Leicester, Leicester, United Kingdom (R.L.T., R.M., R.A.J.C.); and Psychiatry Centre of Excellence in Drug Discovery, GlaxoSmithKline, Harlow, United Kingdom (C.J.L., M.D.W.)

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ABSTRACT

The M_1 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 M_1 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(1*H*)-quinolinone), and a range of orthosteric muscarinic agonists [oxotremorine-M (oxo-M), arecoline, and pilocarpine] in Chinese hamster ovary cells recombinantly expressing the human M_1 mACh receptor. Each agonist was able to activate $G\alpha_{q/11}$-dependent signaling, as demonstrated by an increase in guanosine 5′-*O*-(3-thiotriphosphate) ([^{35}S]GTPγS) binding to $G\alpha_{q/11}$ proteins and total [^{3}H]inositol phosphate accumulation assays in intact cells. All three orthosteric agonists caused significant enhancements in [^{35}S]GTPγS binding to $G\alpha_{i1/2}$ subunits over basal; however, neither allosteric ligand produced a significant response. In contrast, both orthosteric and allosteric agonists are able to couple to the $G\alpha_s$/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 $G\alpha_{q/11}$- and $G\alpha_s$-dependent signaling; however, AC-42 and 77-LH-28-1, unlike oxo-M, arecoline, and pilocarpine, do not seem to promote M_1 mACh receptor-$G\alpha_{i1/2}$ coupling, suggesting that allosteric agonists have the potential to activate distinct subsets of downstream effectors.

Muscarinic acetylcholine (mACh) receptors are family A members of the G protein-coupled receptor (GPCR) superfamily and are responsible for mediating the metabotropic effects of the neurotransmitter acetylcholine. In humans and other mammals, five subtypes of mACh receptor have been identified, termed M_1 to M_5 (Bonner et al., 1987), and these have been further subdivided into M_1/M_3/M_5 and M_2/M_4 subgroupings based on sequence similarities and their respective $G_{q/11}$ and $G_{i/o}$ signal transduction coupling preferences (Caulfield and Birdsall, 1998). Pharmacological manipulation of mACh receptor activity has the potential for positive therapeutic intervention in a variety of psychiatric and neurological diseases, including schizophrenia and Alzheimer's disease. It is unfortunate that despite much effort, relatively few truly subtype-selective mACh receptor agonists/antagonists have been reported. This lack of success has been attributed to the high level of sequence conservation among the amino acid residues within transmembrane domains III, V, VI, and VII, which form the orthosteric binding pocket of M_1 to M_5 mACh receptors (Hulme et al., 2003). However, mACh receptors also possess allosteric binding sites distinct from the orthosteric binding site. Residues at the extracellular interface with transmembrane domain VII and within the second and third extracellular loops have been shown to be important for binding allosteric ligands (see Birdsall and

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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(1*H*)-quinolinone; oxo-M, oxotremorine-M; GTPγS, guanosine 5′-*O*-(3-thiotriphosphate); NMS, *N*-methylscopolamine; CHO, Chinese hamster ovary; KHB, Krebs-Henseleit buffer; [^{3}H]InsP_x, [^{3}H]inositol mono-, bis-, and trisphosphate; Ins(1,4,5)P_3, inositol-1,4,5-trisphosphate; eGFP-PH, enhanced green fluorescent protein tagged to the pleckstrin homology domain of phospholipase Cδ; 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 M_1 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 M_1 mACh receptors initiates $G_{q/11}$-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 M_1 mACh receptor has been reported to activate $G_{i/o}$ and $G_{q/11}$ proteins (Offermanns et al., 1994; Akam et al., 2001) and to activate adenylyl cyclase activity via a G_s protein-dependent mechanism (Burford and Nahorski, 1996). The ability of the M_1 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., 2007), has been reported for a number of receptors including pituitary adenylyl cyclase-activating protein receptors (Spengler et al., 1993), 5-hydroxytryptamine$_{2C}$ receptors (Berg et al., 1998), and CB_1 cannabinoid receptors (Bonhaus et al., 1998). Previous work exploring $G_{i/o}$ coupling through the M_1 and M_3 mACh receptors revealed differences between methacholine- and pilocarpine-stimulated [^{35}S]GTPγS binding to $G_{i/o}$ proteins (Akam et al., 2001), and this orthosteric agonist-specific activation was also observed at M_2 and M_4 mACh receptors, where pilocarpine seems to selectively activate G_{i3} over G_{i1} and/or G_{i2} proteins (Akam et al., 2001).

AC-42 has been shown to stimulate both phosphoinositide hydrolysis and a Ca^{2+} response consistent with activation of $G\alpha_{q/11}$ subunits (Spalding et al., 2002, 2006; Langmead et al., 2006, 2008). However, investigation of alternative signaling pathways activated by allosteric agonists at the M_1 mACh receptor remains largely uncharacterized. Therefore, in this study, we have investigated the actions of AC-42, its analog 77-LH-28-1 (Langmead et al., 2008), and orthosteric mACh receptor agonists [oxotremorine-M (oxo-M), arecoline, and pilocarpine] to determine whether these compounds differentially affect receptor-G protein coupling, assessing these effects at the level of receptor recognition, G protein coupling, and signal transduction pathway activation.

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). [^{35}S]GTPγS, [^{3}H]cAMP, [^{3}H]NMS, myo-[^{3}H]inositol, and protein A-Sepharose CL-4B were from GE Healthcare (Chalfont St. Giles, UK). Charcoal and borosilicate coverslips were from BDH (Poole, Dorset, UK). Primary antibody for $G\alpha_{q/11}$ was generated against the common 11-mer C terminus sequence LQLNLKEYNLV as described previously (Akam et al., 2001). Primary antibody for $G\alpha_i$ was generated by Cambridge Research Biochemicals (Northwich, UK) against the common $G\alpha_{i1/2}$ C-terminal 10-mer sequence KENLKDCGLF. 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 M_1 mACh receptor (CHO-M_1 cells), originally obtained from Dr. Noel Buckley (then at National Institutes for Medical Research, London, UK), were grown in minimal essential medium-α 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% CO_2 in air.

Cell Membrane Preparation. Confluent monolayers of CHO-M_1 cells were rapidly washed with 10 mM 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 mM HEPES and 10 mM 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 mM HEPES and 0.1 mM EDTA, pH 7.4, and rehomogenized and centrifuged as described above. The final pellet was resuspended in the 10 mM HEPES and 10 mM 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.

[^{3}H]NMS Binding. [^{3}H]NMS inhibition binding assays were carried out as described previously (Mistry et al., 2005). In general, CHO-M_1 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 $NaHCO_3$, 1.3 mM $CaCl_2$, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 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 [^{3}H]NMS. After a 5-h incubation at 4°C cells were washed three times with 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.

[^{35}S]GTPγS Binding and Immunoprecipitation of Gα Subunits. [^{35}S]GTPγS binding and immunoprecipitation of Gα subunits was performed as described previously (Akam et al., 2001). In general, CHO-M_1 membranes were diluted in assay buffer (10 mM HEPES, 100 mM NaCl, and 10 mM $MgCl_2$, 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 [^{35}S]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 (20,000*g*, 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. 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 (20,000*g*, 6 min, 4°C), and 100 μl of the supernatant was transferred to fresh tubes containing G protein-specific anti-serum. Samples were vortex-mixed and rotated at 4°C for 90 min before being centrifuged (20,000*g*, 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 FloScint-IV scintillation cocktail and counted by liquid scintillation spectrometry.

cAMP Accumulation Assay. CHO-M_1 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 Ca^{2+}-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 16,000*g* 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 $NaHCO_3$. cAMP concentrations were determined using a binding assay as described previously (Mistry et al., 2005).

Total [^{3}H]Inositol Phosphate Accumulation Assay. CHO-M_1 cells were seeded at 100,000 cells/well in 24-well plates and incubated in fresh medium containing 2.5 μCi/ml [^{3}H]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 [^{3}H]inositol mono-, bis-, and trisphosphate ([^{3}H]InsP_x) 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 [^{3}H]InsP_x 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 SafeFluor scintillation cocktail, and radioactivity was determined by liquid scintillation counting.

Ins(1,4,5)P_3 Mass Assay. CHO-M_1 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)P_3 concentrations was by a competition binding assay described previously (Challiss et al., 1988).

Single-Cell Imaging of Ins(1,4,5)P_3. CHO-M_1 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 Cδ (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 expressed as a change in cytosolic fluorescence relative to basal as described previously (Nash et al., 2002). Drugs were applied through a perfusion line for the times indicated.

Data Analysis. All data are expressed as mean ± S.E.M. for the indicated number of experiments or as a representative trace of at least three independent experiments. Radioligand binding data and agonist concentration-response curves were analyzed using Prism 4.0 (GraphPad Software Inc., San Diego, CA). IC_{50} values derived from agonist displacement of [^{3}H]NMS binding were converted to K_b values using the method of Cheng and Prusoff (1973). For calculation of the initial rate of Ins(1,4,5)P_3 accumulation in eGFP-PH fluorescent imaging experiments, basal and peak fluorescence values were defined within an area of interest in the cytoplasm (see Bartlett et al., 2005); the time taken for the fluorescence to increase from 10 to 90% of the (peak − basal) value was determined, as was the magnitude of the 10 → 90% fluorescence change; this allowed the calculation of an initial rate as arbitrary fluorescence units (AFU) per second. Statistical differences between multiple data sets were assessed by one-way analysis of variance followed by Bonferroni's multiple-range test at $P < 0.05$ using Prism 4.0 (GraphPad Software Inc.). For agonist potency ranking, > represents a statistically significant difference, and ≈ represents no difference.

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 [^{3}H]NMS dissociation from the M_1 mACh receptor in CHO-M_1 membranes, and analysis of the abilities of these compounds to displace specific [^{3}H]NMS binding at different concentrations of [^{3}H]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 M_1 mACh Receptors. M_1 mACh receptor binding affinities for oxo-M, AC-42, 77-LH-28-1, arecoline, and pilocarpine were determined by [^{3}H]-NMS competition binding in intact CHO-M_1 cell monolayers. Initial [^{3}H]NMS saturation binding analysis determined a B_{max} value of 4.84 ± 0.31 pmol/mg protein and a K_D value of 0.29 ± 0.05 nM in intact CHO-M_1 cells (n = 4; data not shown). CHO-M_1 cell monolayers were incubated with an approximate K_d value of [^{3}H]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 (pK_B) 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 M_1 mACh Receptor-G$\alpha_{q/11}$ Coupling. Agonist stimulation in the presence of [^{35}S]GTPγS and subsequent immunoprecipitation of specific Gα-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 1

Comparison of binding affinity constant (pK_B) and Hill slope estimates for mACh receptor agonists at intact CHO-M_1 cells

Data are the means ± S.E.M. for at least three separate experiments performed in duplicate.

	pK_B	Hill Slope
oxo-M	5.30 ± 0.07	0.80 ± 0.04
AC-42	5.52 ± 0.04	1.07 ± 0.07
77-LH-28-1	6.00 ± 0.04	0.98 ± 0.02
Arecoline	5.06 ± 0.20	0.87 ± 0.15
Pilocarpine	4.95 ± 0.10	0.93 ± 0.06

to show that the concentration-dependent increase in [^{35}S]-GTPγS-G$\alpha_{q/11}$ binding, stimulated by the human M_1 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 M_1 mACh receptor by oxo-M caused an approximate 30-fold increase in [^{35}S]GTPγS binding to G$\alpha_{q/11}$ with an EC_{50} value of approximately 1 μM (Fig. 1; Table 2). The M_1-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 [^{35}S]GTPγS-G$\alpha_{q/11}$ binding with an EC_{50} 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 G$\alpha_{q/11}$ subunits, whereas 77-LH-28-1 is comparable with the orthosteric agonist arecoline. Time courses for agonist-stimulated increases in G$\alpha_{q/11}$-[^{35}S]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 G$\alpha_{q/11}$-[^{35}S]GTPγS binding over basal values.

Characterization of [^{3}H]InsP_x Accumulation in CHO-M_1 Cells. As an index of PLC activation, agonist-stimulated accumulation of total [^{3}H]InsP_x was assessed in the presence of Li^+. Maximal stimulation with oxo-M caused a 15-fold increase in [^{3}H]InsP_x accumulation (1,198,704 ± 293,374 dpm/mg protein over a basal value of 77,064 ± 18,779 dpm/mg protein) with an EC_{50} of 0.1 μM (Fig. 2; Table 2). This represents a 10-fold leftward shift in the concentration dependence for the [^{3}H]InsP_x accumulation versus G$\alpha_{q/11}$-[^{35}S]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 (R_{max}) values for

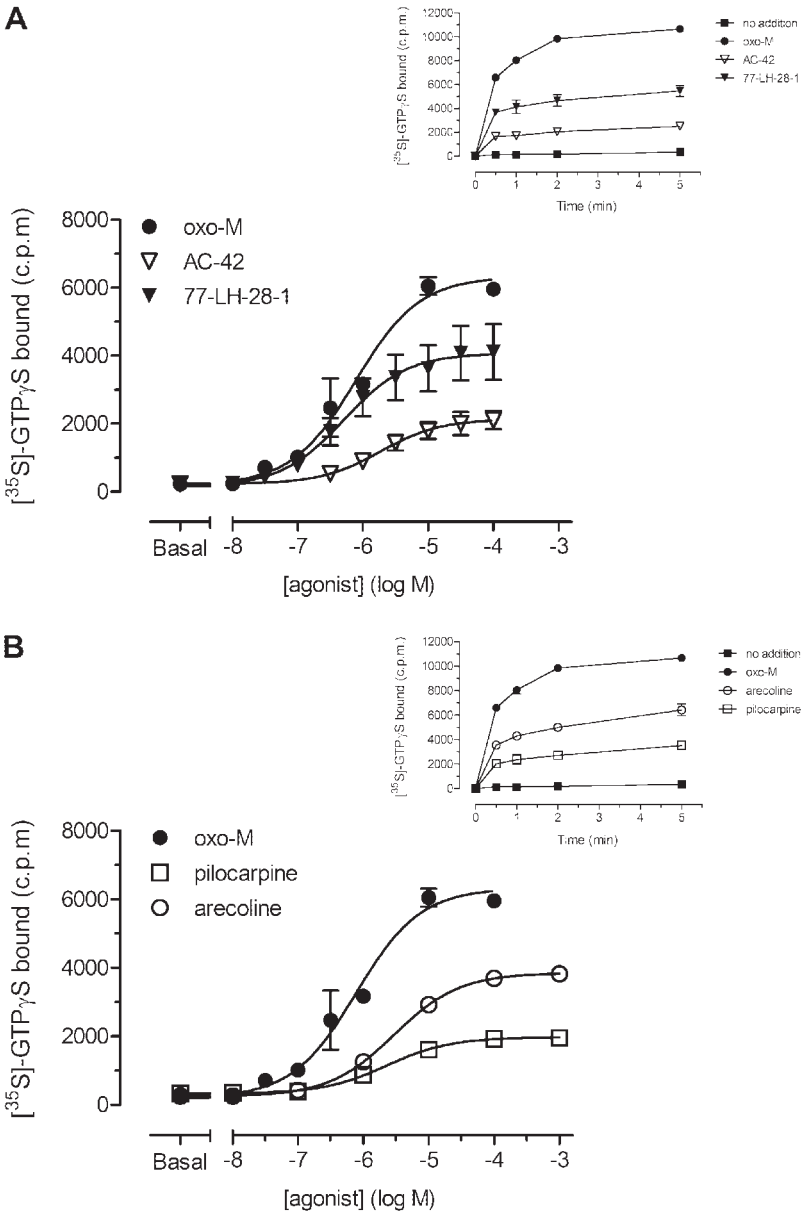

Fig. 1. Concentration dependencies of agonist-stimulated [^{35}S]GTPγS binding to G$\alpha_{q/11}$ subunits in CHO-M_1 cell membranes. CHO-M_1 cell membranes were incubated with the indicated concentrations of oxo-M, AC-42, and 77-LH-28-1 (A) or oxo-M, arecoline, and pilocarpine (B) in the presence of 1 μM GDP and 1 nM [^{35}S]GTPγS for 2 min at 30°C. Incubations were terminated, and G$\alpha_{q/11}$ subunits were immunoprecipitated as described under *Materials and Methods*. Inset figures, A and B, time courses for increases in G$\alpha_{q/11}$-[^{35}S]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).

TABLE 2

Comparison of pEC_{50} and R_{max} values for different M_1 mACh receptor-mediated responses

pEC_{50} values are given as $-\log$ (M) values, and R_{max} values are given as a percentage of the maximal response to oxo-M. Statistical analyses of rank order of potency are presented in the main text. Data are the mean ± S.E.M. of at least three separate experiments performed in duplicate.

Agonist	$G\alpha_{q/11}$-[^{35}S]GTPγS			[^{3}H]$InsP_x$			$G\alpha_{i1/2}$-[^{35}S]GTPγS			cAMP (G_s)		
	pEC_{50}	R_{max}	n	pEC_{50}	R_{max}	n	pEC_{50}	R_{max}	n	pEC_{50}	R_{max}	n
oxo-M	6.07 ± 0.14	100	4	7.01 ± 0.16	100	6	5.24 ± 0.12	100	6	5.71 ± 0.02	100	4
AC-42	5.79 ± 0.08	29.6 ± 2.4	5	5.73 ± 0.07	55.5 ± 2.3	6	N.D.	8.7 ± 5.0	5	5.06 ± 0.15	13.2 ± 1.0	5
77-LH-28-1	6.40 ± 0.07	58.0 ± 7.1	5	6.61 ± 0.10	87.0 ± 2.4	4	N.D.	9.8 ± 4.1	6	6.01 ± 0.08	42.0 ± 2.9	5
Arecoline	5.53 ± 0.08	57.0 ± 2.1	3	5.79 ± 0.15	101.0 ± 9.0	3	4.61 ± 0.18	43.4 ± 3.4	4	4.61 ± 0.14	39.2 ± 2.8	4
Pilocarpine	5.62 ± 0.10	34.3 ± 3.8	3	5.40 ± 0.29	90.0 ± 2.3	3	4.56 ± 0.33	36.7 ± 4.2	6	4.41 ± 0.24	26.5 ± 2.2	3

N.D., could not be derived.

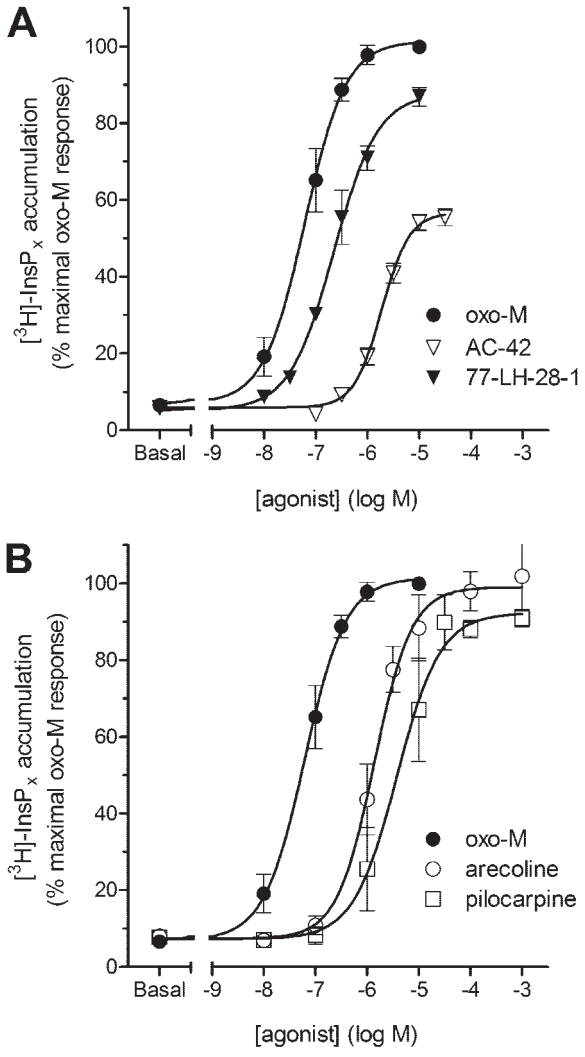

Fig. 2. Concentration dependencies of agonist-stimulated [^{3}H]inositol phosphate accumulation in CHO-M_1 cells. CHO-M_1 cells labeled with [^{3}H]inositol (2.5 μCi/ml; 48 h) were preincubated at 37°C for 30 min with 10 mM LiCl before agonist addition. Incubations were terminated after 15 min by medium removal and the rapid addition of ice-cold trichloroacetic acid (0.5 M) and analyzed to determine [^{3}H]$InsP_x$ accumulation as described under *Materials and Methods*. Data for AC-42 and 77-LH-28-1 (A) and arecoline and pilocarpine (B) are expressed as a percentage of the maximal oxo-M response (1,198,704 ± 293,374 dpm/mg protein) over basal values (77,064 ± 18,779 dpm/mg protein) and 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). Thus, R_{max} values for all of the orthosteric and allosteric partial agonists increased with respect to [^{3}H]$InsP_x$ accumulation compared with $G\alpha_{q/11}$-[^{35}S]GTPγS binding; however, significant differences between EC_{50} values for these two readouts were only observed for the full agonist (Table 2).

Characterization of Ins(1,4,5)P_3 Responses in CHO-M_1 Populations and in Single Cells. Although the Li^+-blocked, [^{3}H]$InsP_x$ accumulation assay provides an index of PLC activity, we also conducted experiments to investigate the time course of Ins(1,4,5)P_3 accumulation in CHO-M_1 cells to compare further the activation profiles of allosteric and orthosteric agonists with respect to $G\alpha_{q/11}$-coupled downstream signaling. Basal levels of Ins(1,4,5)P_3 were 44 ± 3 pmol/mg protein (n = 12) in cell population experiments. oxo-M (100 μM) caused a biphasic rise in Ins(1,4,5)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-M_1 cells by either arecoline (1 mM) or pilocarpine (1 mM) caused monophasic Ins(1,4,5)P_3 accumulation profiles, with maxima being reached at around 60 s (Fig. 3B). It was notable that the "steady-state" Ins(1,4,5)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 μM) caused a similar monophasic profile of Ins(1,4,5)P_3 accumulation to the orthosteric partial agonists, achieving a similar steady-state value (Fig. 3A). In contrast, stimulation with AC-42 (100 μM) produced a modest, monophasic increase in Ins(1,4,5)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 Ins(1,4,5)P_3 levels in single CHO-M_1 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 Ins(1,4,5)P_3. Therefore, increases in cytoplasmic fluorescence provide an index of the changes in Ins(1,4,5)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 μM, 30 s) induced a rapid increase in Ins(1,4,5)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 μM) caused smaller peak Ins(1,4,5)P_3 accumulations. To analyze the initial rates of second messenger generation, basal and peak fluorescence levels were defined, and the

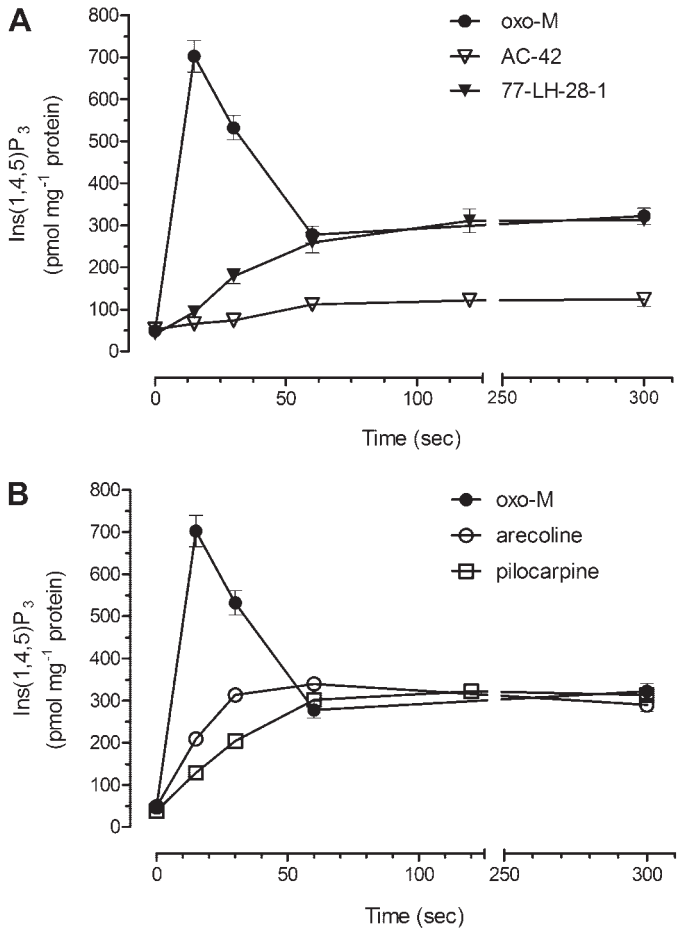

Fig. 3. Time courses of agonist-stimulated Ins(1,4,5)P_3 accumulation in CHO-M_1 cells. Confluent monolayers of CHO-M_1 cells were stimulated for the times indicated with oxo-M (100 μM), AC-42 (30 μM), or 77-LH-28-1 (10 μM) (A) or oxo-M (100 μM), arecoline (1 mM), or pilocarpine (1 mM) (B). Incubations were terminated at the indicated time points, and samples were analyzed for Ins(1,4,5)P_3 levels as described under *Materials and Methods*. Data are presented as means ± S.E.M. for four (AC-42, 77-LH-28-1, pilocarpine) or five (oxo-M, arecoline) separate experiments performed in duplicate.

change between 10 and 90% of peak Ins(1,4,5)P_3 accumulation was determined and expressed as a change in cytoplasmic fluorescence [which is directly proportional to Ins(1,4,5)P_3 accumulation] per unit time (see *Materials and Methods*). Using this index of the rate of increase in Ins(1,4,5)P_3 (expressed as arbitrary fluorescence units per second), we found that oxo-M caused the most rapid response (23.6 ± 8.4 AFU/s), with arecoline (8.2 ± 3.3 AFU/s), pilocarpine (8.0 ± 1.0 AFU/s), and 77-LH-28-1 (7.1 ± 3.8 AFU/s) giving essentially similar, but slower, rates. In comparison with oxo-M, Ins(1,4,5)P_3 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 μM) was greater than for the other agonists studied, and in some cells (see Fig. 4B), AC-42 was able to cause a peak Ins(1,4,5)P_3 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 ± 0.5 AFU/s).

Analysis of Receptor-G$\alpha_{i1/2}$ Coupling. Although the M_2 and M_4 mACh receptor subtypes couple preferentially to $G_{i/o}$ proteins (Caulfield and Birdsall, 1998), it has been demonstrated that the M_1 and M_3 mACh receptor subtypes are also capable of activating Gα_i subunits in addition to G$\alpha_{q/11}$ subunits (Offermanns et al., 1994; Akam et al., 2001). To investigate whether the M_1 mACh receptor-selective allosteric agonists can cause the M_1 mACh receptor to activate multiple G protein-dependent pathways, we first assessed the agonist-dependent activation of Gα_i subunits in CHO-M_1 membranes.

In initial experiments designed to optimize the [^{35}S]-GTPγS-G$\alpha_{i1/2}$ immunoprecipitation assay, oxo-M caused a marked (272 ± 74% over basal) and concentration-dependent [pEC_{50} (M) = 7.14 ± 0.06] increase in [^{35}S]GTPγS-G$\alpha_{i1/2}$ binding in CHO-M_2 membranes. Similar optimal binding conditions were obtained for agonist-stimulated increases in [^{35}S]GTPγS-G$\alpha_{i1/2}$ binding in CHO-M_1 membranes (10 μM GDP, 2-min incubations at 30°C; see Fig. 5A, inset). Under these conditions, oxo-M stimulated a more modest [basal binding, 15,120 ± 707 (15); +oxo-M, 23,240 ± 1280 (15) cpm/mg protein] yet highly significant ($P < 0.001$) increase in [^{35}S]GTPγS binding. The increase in [^{35}S]GTPγS-G$\alpha_{i1/2}$ binding stimulated by oxo-M was concentration-dependent [pEC_{50} (M) = 5.13 ± 0.08 M] and lay approximately 10-fold to the right of the oxo-M-stimulated [^{35}S]GTPγS-G$\alpha_{q/11}$ binding curve (see Fig. 5B; Table 2). Arecoline and pilocarpine both stimulated significant increases in [^{35}S]GTPγS-G$\alpha_{i1/2}$ 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}S]GTPγS-G$\alpha_{q/11}$ binding responses (Table 2). In contrast, neither allosteric agonist [at concentrations up to 30 μM (77-LH-28-1) or 300 μM (AC-42)] was able to cause a significant increase in [^{35}S]GTPγS-G$\alpha_{i1/2}$ binding in CHO-M_1 membranes (Fig. 5A; Table 2). These data suggest that the allosteric agonists are less able to facilitate M_1 mACh receptor-dependent GTP/GDP exchange on G$\alpha_{i1/2}$ proteins compared with orthosteric partial agonists that are equiefficacious with respect to other signaling readouts.

Analysis of Receptor-Gα_s Coupling. To explore the potential coupling of the M_1 mACh receptor to Gα_s in the CHO cell background (Burford and Nahorski, 1996), we initially assessed the ability of orthosteric and allosteric agonists to stimulate [^{35}S]GTPγS-Gα_s binding in CHO-M_1 membranes. To first validate the [^{35}S]GTPγS-Gα_s immunoprecipitation assay, we showed that stimulation of CHO-β_2 cell membranes (β_2-adrenoceptor density, approximately 300 fmol/mg protein) with isoprenaline (10 μM) resulted in a 4-fold increase in [^{35}S]GTPγS-Gα_s binding over basal (basal, 6253 ± 853; +isoprenaline, 27,227 ± 920 cpm/mg protein) under optimized conditions [membrane preincubation with 1 μM GDP; addition of agonist to membranes for 5 min before incubation at 30°C with [^{35}S]GTPγS (~1 nM)] (see Carruthers et al., 1999). Under these conditions, none of the mACh receptor agonists caused a detectable increase in [^{35}S]-GTPγS-Gα_s binding.

As an alternate index of mACh receptor-Gα_s activation, we measured cAMP accumulation in intact CHO-M_1 cells. Addition of oxo-M (100 μM) caused a robust 10- to 20-fold increase in cAMP accumulation (basal, 11 ± 3; +oxo-M, 183 ± 8 pmol/mg protein; pEC_{50}, 5.05 ± 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 μM). Previous studies have

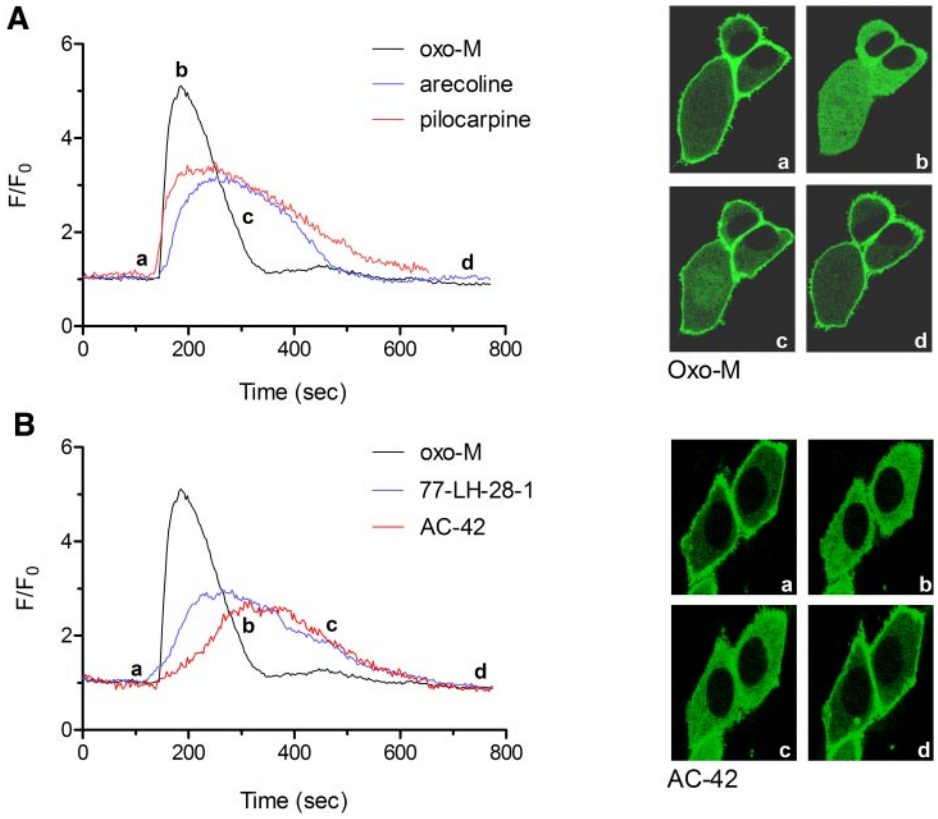

Fig. 4. Assessment of changes in Ins(1,4,5)P_3 in response to orthosteric and allosteric agonists in single CHO-M_1 cells using confocal fluorescence imaging. CHO-M_1 cells were transfected with the fluorescent biosensor eGFP-PH, and after 48 h, Ins(1,4,5)P_3 production was measured by profiling the translocation of the eGFP-PH probe from the plasma membrane to the cytoplasm. Representative images and time course traces from single CHO-M_1 cells stimulated for 30 s with oxo-M (100 μM), arecoline (1 mM), or pilocarpine (A) or oxo-M (100 μM), AC-42 (30 μM), or 77-LH-28-1 (10 μM) (B), followed by washout. In each panel, images are shown corresponding to a to d labels on the time course traces. Data are expressed as the change in fluorescence intensity from the basal fluorescence level (F/F_0). The traces shown are representative of five to 10 cells analyzed per coverslip over three separate experiments.

found that under these conditions, receptor activation in CHO cells can indirectly modulate forskolin-stimulated adenylyl cyclase activity through activation of Ca^{2+} entry and Ca^{2+}-dependent effects on isoforms of adenylyl cyclase (Cooper et al., 1995). Therefore, in these assays, Ca^{2+} was omitted from the medium, and EGTA (100 μM) was added to remove the possibility of Ca^{2+} entry-driven effects on M_1 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 M_1 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 $G\alpha_{q/11}$-dependent signaling events and explored the ability of these agonists to stimulate M_1 mACh receptor coupling to alternative $G_{i/o}$ and G_s 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 M_1 mACh receptor-$G\alpha_{q/11}$ protein coupling in the CHO-M_1 membranes used the [^{35}S]GTPγS binding assay (Hilf et al., 1989; Lazareno and Birdsall, 1993) adapted to distinguish [^{35}S]GTPγS binding to specific Gα 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 M_1 mACh receptor in the absence of an orthosteric ligand, causing robust increases in [^{35}S]-GTPγS-$G\alpha_{q/11}$ 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 M_1 mACh receptor-stimulated [^{35}S]-GTPγS-$G\alpha_{q/11}$ binding in native systems (Salah-Uddin et al., 2008).

A consequence of M_1 mACh receptor-$G\alpha_{q/11}$ coupling is the activation of PLC. In the presence of lithium, it is possible to assess [^{3}H]InsP_x accumulation as an index of PLC activity in cells labeled to equilibrium with [^{3}H]inositol. Using this assay, it was observed that the EC_{50} for the full agonist oxo-M was approximately 10-fold left-shifted for [^{3}H]InsP_x accumulation in intact cells compared with the membrane-based [^{35}S]GTPγS-$G\alpha_{q/11}$ binding response. In contrast, EC_{50} values for these responses were similar for both orthosteric and allosteric partial agonists, but increases in relative efficacy for [^{3}H]InsP_x accumulation compared with [^{35}S]GTPγS-$G\alpha_{q/11}$ binding responses were seen in all cases (Table 2).

To gain a more kinetic perspective on the actions of the

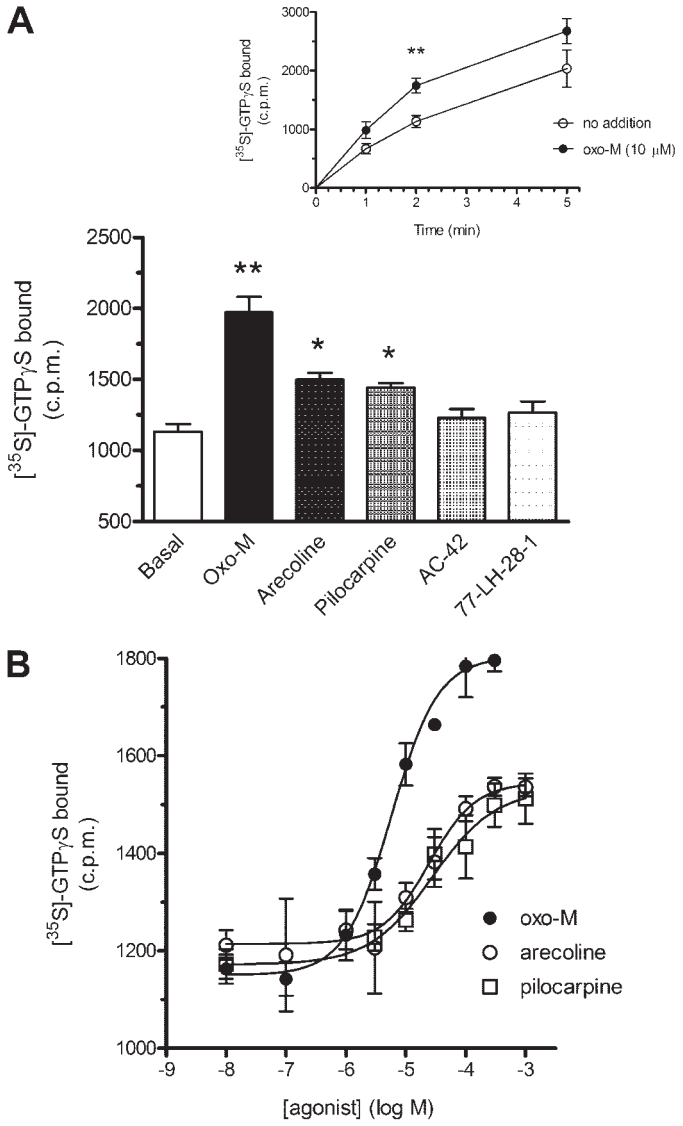

Fig. 5. Concentration-dependent agonist-stimulated [^{35}S]GTPγS binding to $G\alpha_{i1/2}$ subunits in CHO-M_1 cell membranes. CHO-M_1 cell membranes were incubated with oxo-M (100 μM; $n = 7$), arecoline (1 mM; $n = 5$), pilocarpine (1 mM; $n = 7$), AC-42 (100 μM; $n = 8$), or 77-LH-28-1 (30 μM; $n = 7$) in the presence of 10 μM GDP and 1 nM [^{35}S]GTPγS for 2 min at 30°C (A). Incubations were terminated after this time, and specific Gα subunits were immunoprecipitated as described under *Materials and Methods*. Statistically significant differences are indicated as follows: ∗, $P < 0.05$; and ∗∗, $P < 0.01$. A, inset, time course of change in [^{35}S]GTPγS-$G\alpha_{i1/2}$ binding in the absence and presence of oxo-M (10 μM). Concentration-dependent increases in [^{35}S]GTPγS-$G\alpha_{i1/2}$ binding stimulated by oxo-M ($n = 5$), arecoline ($n = 4$), or pilocarpine ($n = 6$) are shown in B. All data are presented as means ± S.E.M. for the indicated number of separate experiments (see Table 2) performed in duplicate.

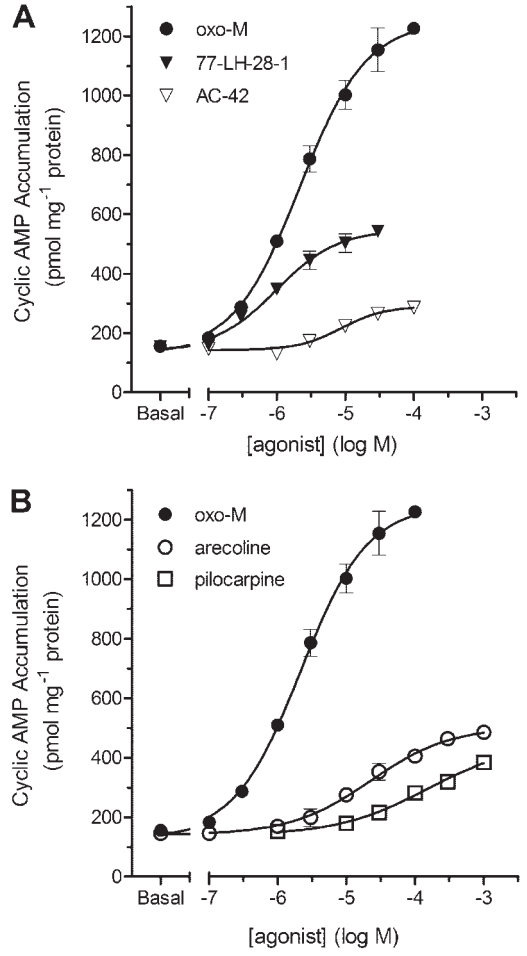

Fig. 6. Concentration-dependent effects of mACh receptor agonists on forskolin-stimulated cAMP accumulation. Confluent CHO-M_1 cell monolayers were washed twice in Ca^{2+}-free KHB before incubation in the presence of the indicated concentrations of mACh receptor agonists for 10 min before the addition of forskolin (1 μM) for 10 min. After this time, incubations were terminated, and cAMP levels were determined as described under *Materials and Methods*. Data are shown for AC-42 and 77-LH-28-1 (A) and arecoline and pilocarpine (B); oxo-M data are reproduced in both panels. Data are presented as means ± S.E.M. for the indicated number of separate experiments (see Table 2) performed in duplicate.

different mACh receptor agonists on PLC activity, temporal profiles for Ins(1,4,5)P_3 accumulation were also investigated. Ins(1,4,5)P_3 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)P_3 response. Monophasic increases in Ins(1,4,5)P_3, 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 pEC_{50} and R_{max} values to pilocarpine with respect to stimulating [^{35}S]GTPγS-$G\alpha_{q/11}$ binding (see Table 2), stimulated a monophasic increase in Ins(1,4,5)P_3 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)P_3 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 variously 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 M_1 mACh receptor-$G\alpha_i$ coupling because in a previous report, we showed that the M_1 mACh receptor is capable of activating $G\alpha_{i/o}$ subunits in a CHO cell background, and the degree of coupling to $G\alpha_{i/o}$ is agonist-dependent (Akam et al., 2001). Here, we were able to confirm the activation of M_1 mACh receptor-stimulated $G\alpha_i$ protein GTP/GDP exchange using a $G\alpha_{i1/2}$ antibody to immunoprecipitate [^{35}S]GTPγS-$G\alpha_{i1/2}$ complexes. The orthosteric agonists oxo-M, arecoline, and pilocarpine all stimulated significant increases in [^{35}S]-GTPγS-$G\alpha_{i1/2}$ binding, and pEC_{50} values could be obtained for these agonists, which were approximately 10-fold right-shifted relative to those determined for receptor-stimulated [^{35}S]GTPγS-$G\alpha_{q/11}$ binding (see Table 2). In contrast, both allosteric agonists failed to cause a significant increase in [^{35}S]GTPγS-$G\alpha_{i1/2}$ binding, and as a consequence, pEC_{50} values could not be determined. These data suggest that M_1 mACh receptor conformations favoring coupling to $G\alpha_{i1/2}$ proteins are better stabilized by oxo-M, arecoline, and pilocarpine compared with AC-42 and 77-LH-28-1.

$G_{q/11}$-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 G_s protein-independent coupling via a Ca^{2+}- and/or PKC-dependent mechanism (Felder et al., 1989; Cooper et al., 1995), in the CHO cell background, direct G_s coupling has been demonstrated for the α_{1B}-adrenergic (Horie et al., 1995) and M_1 mACh (Burford and Nahorski, 1996) receptors. Assessment of receptor-G_s coupling using a [^{35}S]GTPγS-$G\alpha_s$ binding/immunoprecipitation assay (Carruthers et al., 1999) was not possible because significant agonist-dependent increases in [^{35}S]GTPγS-$G\alpha_s$ binding were not observed in CHO-M_1 membranes challenged with any of the mACh receptor agonists. This apparent lack of M_1 mACh receptor-G_s coupling should be interpreted cautiously, however, because isoprenaline only elicited a maximal 4-fold increase in [^{35}S]GTPγS-$G\alpha_s$ binding in CHO-β_2 membranes. In intact CHO-β_2 cells, isoprenaline causes a profound increase in cAMP accumulation (>100-fold), which is at least an order of magnitude greater that that elicited by oxo-M in intact CHO-M_1 cells (see *Results*). To compare M_1 mACh receptor-$G\alpha_s$ 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 $G_{q/11}$/PLC/Ca^{2+} pathway can be abolished by performing experiments under Ca^{2+}-free conditions (omission of Ca^{2+} from the KHB and addition of EGTA; 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 $G\alpha_s$ activation. All agonists studied were able to stimulate monophasic enhancements of forskolin-stimulated cAMP accumulation. oxo-M stimulated the greatest increase, with a pEC_{50} value $\geq$ 10-fold right-shifted relative to that for the [^{3}H]$InsP_x$ response (Table 2). In contrast to the data obtained for M_1 mACh receptor-$G\alpha_i$ coupling, 77-LH-28-1 and AC-42 were able to increase cAMP accumulation with EC_{50} values only 4- to 5-fold right-shifted compared with those obtained for [^{3}H]$InsP_x$ accumulation, suggesting they are able to stabilize an M_1 mACh receptor conformation capable of interacting with the $G\alpha_s$ subunits.

Although much of the relevant literature focuses on M_1 mACh receptor signaling via $G\alpha_{q/11}$, there are reports of M_1 mACh receptors linking to distal cellular outputs via $G\alpha_i$ and $G\alpha_s$. For example, although it is well established that M_1 mACh receptors inhibit the M-type potassium current via $G\alpha_{q/11}$ (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 M_1 mACh receptor stimulation of cAMP response element-driven luciferase reporter gene expression in JEG-3 cells occurs via a PLC/Ca^{2+}-independent, $G\alpha_s$-dependent pathway (Migeon and Nathanson, 1994). Thus, it is possible that $G\alpha_i$-, $G\alpha_s$-, and $G\alpha_{q/11}$-dependent signaling contribute to at least a subset of M_1 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 M_1 mACh receptor differently from conventional orthosteric agonists (Spalding et al., 2002, 2006; Langmead et al., 2006), they nevertheless cause activation of $G_{q/11}$ signaling that is functionally indistinguishable from orthosteric agonists. In addition, AC-42 and 77-LH-28-1 are able to enhance forskolin-induced cAMP stimulation, as are orthosteric agonists. In contrast, AC-42 and 77-LH-28-1 are much less able to facilitate receptor-$G_{i1/2}$ 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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References

Akam EC, Challiss RA, and Nahorski SR (2001) $G_{q/11}$ and $G_{i/o}$ activation profiles in CHO cells expressing human muscarinic acetylcholine receptors: dependence on agonist as well as receptor-subtype. *Br J Pharmacol* **132:**950–958.

Bartlett PJ, Young KW, Nahorski SR, and Challiss RAJ (2005) Single cell analysis and temporal profiling of agonist-mediated inositol 1,4,5-trisphosphate, Ca^{2+}, diacylglycerol, and protein kinase C signaling using fluorescent biosensors. *J Biol Chem* **280:**21837–21846.

Berg KA, Maayani S, Goldfarb J, Scaramellini C, Leff P, and Clarke WP (1998) Effector pathway-dependent relative efficacy at serotonin type 2A and 2C receptors: evidence for agonist-directed trafficking of receptor stimulus. *Mol Pharmacol* **54:**94–104.

Birdsall NJ and Lazareno S (2005) Allosterism at muscarinic receptors: ligands and mechanisms. *Mini Rev Med Chem* **5:**523–543.

Bonhaus DW, Chang LK, Kwan J, and Martin GR (1998) Dual activation and inhibition of adenylyl cyclase by cannabinoid receptor agonists: evidence for ago-

nist-specific trafficking of intracellular responses. *J Pharmacol Exp Ther* **287:**884–888.
Bonner TI, Buckley NJM, Young AC, and Brann MR (1987) Identification of a family of muscarinic acetylcholine receptor genes. *Science* **237:**527–532.
Burford NT and Nahorski SR (1996) Muscarinic m1 receptor-stimulated adenylate cyclase activity in Chinese hamster ovary cells is mediated by $G_s\alpha$ and is not a consequence of phosphoinositidase C activation. *Biochem J* **315:**883–888.
Carruthers AM, Warner AJ, Michel AD, Feniuk W, and Humphrey PP (1999) Activation of adenylate cyclase by human recombinant SST_5 receptors expressed in CHO-K1 cells and involvement of $G_s\alpha$ proteins. *Br J Pharmacol* **126:**1221–1229.
Caulfield MP and Birdsall NJ (1998) International Union of Pharmacology: XVII. Classification of muscarinic acetylcholine receptors. *Pharmacol Rev* **50:**279–290.
Challiss RA, Batty IH, and Nahorski SR (1988) Mass measurements of inositol 1,4,5-trisphosphate in rat cerebral cortex slices using a radioreceptor assay: effects of neurotransmitters and depolarization. *Biochem Biophys Res Commun* **157:**684–691.
Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 percent inhibition (I_{50}) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.
Cooper DM, Mons N, and Karpen JW (1995) Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature* 374:421–424.
Delmas P and Brown DA (2005) Pathways modulating neural KCNQ/M (Kv7) potassium channels. *Nat Rev Neurosci* **6:**850–862.
Felder CC, Kanterman RY, Ma AL, and Axelrod J (1989) A transfected m1 muscarinic acetylcholine receptor stimulates adenylate cyclase via phosphatidylinositol hydrolysis. *J Biol Chem* **264:**20356–20362.
Friedman E, Butkerait P, and Wang HY (1993) Analysis of receptor-stimulated and basal guanine nucleotide binding to membrane G proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Anal Biochem* **214:**171–178.
Gregory KJ, Sexton PM, and Christopoulos A (2007) Allosteric modulation of muscarinic acetylcholine receptors. *Curr Neuropharmacol* **5:**157–167.
Haley JE, Delmas P, Offermanns S, Abogadie FC, Simon MI, Buckley NJ, and Brown DA (2000) Muscarinic inhibition of calcium current and M current in $G\alpha_q$-deficient mice. *J Neurosci* **20:**3973–3979.
Hilf G, Gierschik P, and Jakobs KH (1989) Muscarinic acetylcholine receptor-stimulated binding of guanosine 5′-O-(3-thiotriphosphate) to guanine-nucleotide-binding proteins in cardiac membranes. *Eur J Biochem* **186:**725–731.
Horie K, Itoh H, and Tsujimoto G (1995) Hamster α_{1B}-adrenergic receptor directly activates G_s in the transfected Chinese hamster ovary cells. *Mol Pharmacol* **48:**392–400.
Hulme EC, Lu ZL, and Bee MS (2003) Scanning mutagenesis studies of the M_1 muscarinic acetylcholine receptor. *Receptors Channels* **9:**215–228.
Kenakin T (2003) Ligand-selective receptor conformations revisited: the promise and the problem. *Trends Pharmacol Sci* **24:**346–354.
Langmead CJ, Austin NE, Branch CL, Brown JT, Buchanan KA, Davies CH, Forbes IT, Fry VA, Hagan JJ, Herdon HJ, et al. (2008) Characterization of a CNS penetrant, selective M_1 muscarinic receptor agonist, 77-LH-28-1. *Br J Pharmacol* **154:**1104–1115.
Langmead CJ, Fry VA, Forbes IT, Branch CL, Christopoulos A, Wood MD, and Herdon HJ (2006) Probing the molecular mechanism of interaction between 4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine (AC-42) and the muscarinic M_1 receptor: direct pharmacological evidence that AC-42 is an allosteric agonist. *Mol Pharmacol* **69:**236–246.
Lazareno S and Birdsall NJ (1993) Pharmacological characterization of acetylcholine-stimulated [^{35}S]-GTPγS binding mediated by human muscarinic m1–m4 receptors: antagonist studies. *Br J Pharmacol* **109:**1120–1127.
Lechner SG, Mayer M, and Boehm S (2003) Activation of M_1 muscarinic receptors triggers transmitter release from rat sympathetic neurons through an inhibition of M-type K^+ channels. *J Physiol* **553:**789–802.
May LT, Avlani VA, Langmead CJ, Herdon HJ, Wood MD, Sexton PM, and Christopoulos A (2007) Structure-function studies of allosteric agonism at M_2 muscarinic acetylcholine receptors. *Mol Pharmacol* **72:**463–476.
Migeon JC and Nathanson NM (1994) Differential regulation of cAMP-mediated gene transcription by m1 and m4 muscarinic acetylcholine receptors: preferential coupling of m4 receptors to Gi alpha-2. *J Biol Chem* **269:**9767–9773.
Mistry R, Dowling MR, and Challiss RAJ (2005) An investigation of whether agonist-selective receptor conformations occur with respect to M_2 and M_4 muscarinic acetylcholine receptor signalling via $G_{i/o}$ and G_s proteins. *Br J Pharmacol* **144:**566–575.
Nash MS, Schell MJ, Atkinson PJ, Johnston NR, Nahorski SR, and Challiss RAJ (2002) Determinants of metabotropic glutamate receptor-5-mediated Ca^{2+} and inositol 1,4,5-trisphosphate oscillation frequency: receptor density versus agonist concentration. *J Biol Chem* **277:**35947–35960.
Offermanns S, Wieland T, Homann D, Sandmann J, Bombien E, Spicher K, Schultz G, and Jakobs KH (1994) Transfected muscarinic acetylcholine receptors selectively couple to G_i-type G proteins and $G_{q/11}$. *Mol Pharmacol* **45:**890–898.
Salah-Uddin H, Thomas DR, Davies CH, Hagan JJ, Wood MD, Watson JM, and Challiss RAJ (2008) Pharmacological assessment of M_1 muscarinic acetylcholine receptor-$G_{q/11}$ protein coupling in membranes prepared from *post mortem* human brain tissue. *J Pharmacol Exp Ther* **325:**869–874.
Spalding TA, Ma JN, Ott TR, Friberg M, Bajpai A, Bradley SR, Davis RE, Brann MR, and Burstein ES (2006) Structural requirements of transmembrane domain 3 for activation by the M_1 muscarinic receptor agonists AC-42, AC-260584, clozapine, and *N*-desmethylclozapine: evidence for three distinct modes of receptor activation. *Mol Pharmacol* **70:**1974–1983.
Spalding TA, Trotter C, Skjaerbaek N, Messier TL, Currier EA, Burstein ES, Li D, Hacksell U, and Brann MR (2002) Discovery of an ectopic activation site on the M_1 muscarinic receptor. *Mol Pharmacol* **61:**1297–1302.
Spengler D, Waeber C, Pantaloni C, Holsboer F, Bockaert J, Seeburg PH, and Journot L (1993) Differential signal transduction by five splice variants of the PACAP receptor. *Nature* **365:**170–175.
Stauffer TP, Ahn S, and Meyer T (1998) Receptor-induced transient reduction in plasma membrane $PtdIns(4,5)P_2$ concentration monitored in living cells. *Curr Biol* **8:**343–346.
Urban JD, Clarke WP, von Zastrow M, Nichols DE, Kobilka B, Weinstein H, Javitch JA, Roth BL, Christopoulos A, Sexton PM, et al. (2007) Functional selectivity and classical concepts of quantitative pharmacology. *J Pharmacol Exp Ther* **320:**1–13.
Violin JD and Lefkowitz RJ (2007) β-Arrestin-biased ligands at seven-transmembrane receptors. *Trends Pharmacol Sci* **28:**416–422.
Willars GB and Nahorski SR (1995) Quantitative comparisons of muscarinic and bradykinin receptor-mediated $Ins(1,4,5)P_3$ accumulation and Ca^{2+} signalling in human neuroblastoma cells. *Br J Pharmacol* **114:**1133–1142.

Address correspondence to: Dr. R.A. John Challiss, Department of Cell Physiology and Pharmacology, University of Leicester, Room 4/04, Henry Wellcome Building, Lancaster Road, Leicester LE1 9HN, UK. E-mail: jc36@leicester.ac.uk