Contrasting Effects of Allosteric and Orthosteric Agonists on M₁ Muscarinic Acetylcholine Receptor Internalization and Down-regulation

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

Department of Cell Physiology and Pharmacology, University of Leicester, Henry Wellcome Building, Leicester, United Kingdom (R.L.T., R.A.J.C.); and Neurosciences Centre of Excellence for Drug Discovery, GlaxoSmithKline, New Frontiers Science Park, Harlow, United Kingdom (C.J.L., M.D.W.)

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ABSTRACT

A new class of subtype-selective muscarinic acetylcholine (mACh) receptor agonist that activates the receptor through interaction at a site distinct from the orthosteric acetylcholine binding site has been reported recently. Here, we have compared the effects of orthosteric (oxotremorine-M, arecoline, pilocarpine) and allosteric [4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine (AC-42); 1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1*H*)-quinolinone (77-LH-28-1)] agonists on M₁ mACh receptor internalization and down-regulation, as well as functional coupling in a Chinese hamster ovary (CHO) cell line. In contrast to full and partial orthosteric agonists, which cause significant receptor internalization and down-regulation, prolonged exposure to AC-42 did not significantly alter either cell-surface or total cellular M₁ mACh receptor expression. 77-LH-28-1, an AC-42 homolog, did cause some receptor internalization, but not down-regulation. The presence of atropine completely

prevented the orthosteric agonist-induced adaptive changes in receptor populations; however, in contrast, the copresence of atropine and AC-42 significantly increased both cell-surface receptor and total M₁ mACh receptor expression. Maximal phosphoinositide hydrolysis responses to the partial agonist arecoline were similar in CHO-M₁ cells pretreated for 24 h with either AC-42 or vehicle; in contrast, these responses were markedly reduced when cells were pretreated with oxotremorine-M or pilocarpine. These data indicate that, whereas AC-42 binding to the M₁ mACh receptor can initiate signal transduction, the AC-42-liganded receptor is resistant to the usual mechanisms regulating receptor internalization and down-regulation. In addition, our data suggest unusual interactions between allosteric agonists and orthosteric antagonists to regulate cell-surface and total cellular receptor expression.

Muscarinic acetylcholine (mACh) receptors are G protein-coupled receptors (GPCRs) responsible for the metabotropic actions of the neurotransmitter acetylcholine. Five mACh receptor subtypes, M₁ to M₅, have been identified in humans and other mammals, with the M₁/M₃/M₅ subtypes coupling preferentially to G_{q/11} proteins, leading to the activation of the phospholipase C/inositol 1,4,5-trisphosphate (IP₃)/Ca²⁺ signaling pathway, and the M₂/M₄ subtypes coupling to G_{i/o}

proteins and inhibition of adenylyl cyclase activity (Caulfield and Birdsall, 1998). The mACh receptors are widely distributed throughout the periphery and central nervous system, and have been implicated as potential therapeutic targets in an array of diseases, including chronic obstructive pulmonary disease, irritable bowel syndrome, Alzheimer's disease, and schizophrenia (see Eglen et al., 1999; Felder et al., 2000; Langmead et al., 2008b).

A major obstacle in the development of successful cholinergic therapies has been the lack of mACh receptor subtype-specific ligands, and the potentially serious side-effect profile of agents that interact with multiple mACh receptor subtypes (Bymaster et al., 1998; Eglen et al., 1999; Bartolomeo

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ABBREVIATIONS: 77-LH-28-1, 1-[3-(4-butyl-1-piperidinyl)propyl]-3,4-dihydro-2(1*H*)-quinolinone; AC-42, 4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl] piperidine; CHO, Chinese hamster ovary; ERK, extracellular signal-regulated kinase; GPCR, G protein-coupled receptor; IP₃, inositol phosphate (inositol mono-, bis-, and trisphosphate fraction); mACh, muscarinic acetylcholine; NMS, *N*-methylscopolamine; oxo-M, oxotremorine-M; QNB, quinuclidinyl benzilate; AC260584, 4-[3-(4-butylpiperidin-1-yl)-propyl]-7-fluoro-4*H*-benzo[1,4]oxazin-3-one; AMN082, *N,N'*-dibenzhydrylethane-1,2-diamine dihydrochloride; G418, (2*R*,3*S*,4*R*,5*R*,6*S*)-5-amino-6-[(1*R*,2*S*,3*S*,4*R*,6*S*)-4,6-diamino-3-[(2*R*,3*R*,4*R*,5*R*)-3,5-dihydroxy-5-methyl-4-methylaminooxan-2-yl]oxy-2-hydroxycyclohexyl]oxy-2-(1-hydroxyethyl)oxane-3,4-diol.

et al., 2000). The high degree of sequence conservation among the amino acid residues that form the orthosteric binding pocket is likely to account for the difficulty in discovering subtype-selective drugs (Hulme et al., 2003). An alternative approach is to target allosteric binding sites on mACh receptors, which are likely to be less well conserved than critical orthosteric ligand binding sites within the transmembrane core (Hulme et al., 2003; Gregory et al., 2007). This has resulted in a new generation of subtype-selective mACh receptor ligands that either modulate or stimulate specific receptors in a highly subtype-selective manner (Spalding et al., 2002; Jones et al., 2008; Shirey et al., 2008; Ma et al., 2009; Marlo et al., 2009).

In addition to offering the potential for mACh receptor subtype-specific effects, allosteric agonists may stabilize distinct receptor conformations, relative to orthosterically directed ligands, and therefore instigate a distinct repertoire of receptor signaling and receptor regulatory properties. Indeed, we have recently reported that the M_1 mACh receptor allosteric agonists, AC-42 and 77-LH-28-1, activate a subtly different subset of G proteins compared with equi-efficacious orthosteric ligands (Thomas et al., 2008). Although attention has so far focused on the acute signaling effects and disease-modifying potential of the M_1 mACh receptor allosteric agonists, little is presently known about the receptor-regulatory actions of this new class of receptor-active agent. Such information is vital if the ultimate aim is to develop these agents for clinical application. It is well established that prolonged exposure of GPCRs to orthosteric agonists results in a reduction in receptor-mediated responses through regulatory mechanisms that involve phosphorylation, G protein uncoupling, internalization, and down-regulation (Wess, 1996; Ferguson, 2001; van Koppen and Kaiser, 2003), whereas, at least under some circumstances, inverse agonists can up-regulate receptor expression (Smit et al., 1996; Milligan and Bond, 1997; Dowling et al., 2006). The effects of allosteric ligands on GPCR regulation are poorly defined at present and appear to be receptor subtype-specific. Thus, previous work exploring long-term exposure to allosteric modulators of the M_2 mACh receptor revealed an up-regulation of receptor numbers (May et al., 2005), whereas chronic exposure to AC260584, an allosteric agonist structurally related to AC-42 (Spalding et al., 2002), resulted in no significant changes in M_1 mACh receptor expression (Davis et al., 2005). In contrast, and looking beyond the mACh receptor subfamily, activation of the type 7 metabotropic glutamate receptor by the allosteric agonist AMN082 caused receptor internalization in a manner similar to that elicited by the orthosteric ligand, L-AP4 (Pelkey et al., 2007). Here, we have compared the regulatory effects of longer-term exposure to allosteric versus orthosteric agonists on M_1 mACh receptor internalization, down-regulation, and signal transduction properties.

Materials and Methods

Materials. All chemicals and reagents were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. Tissue culture reagents were from Invitrogen (Paisley, UK). *N*-Methyl-[3 H]scopolamine ([3 H]NMS), [3 H]quinuclidinyl benzilate ([3 H]QNB), *myo*-[3 H]inositol, and enhanced chemiluminescence reagent were from GE Healthcare (Little Chalfont, Buckinghamshire, UK). Antibodies against total ERK1/2, phospho-ERK1/2, and horseradish peroxidase-conjugated secondary antibodies were from New England Biolabs

(UK) Ltd. (Hitchin, Hertfordshire, UK). 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) were grown in minimal essential medium- α supplemented with fetal bovine serum (10%), penicillin (50 units ml^{-1}), streptomycin (50 $\mu\text{g ml}^{-1}$) amphotericin B (2.5 $\mu\text{g ml}^{-1}$), and G418 (500 $\mu\text{g ml}^{-1}$). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air.

[3 H]NMS Binding. [3 H]NMS inhibition binding assays were carried out as described previously (Mistry et al., 2005). Specifically, CHO- M_1 cells were seeded at a density of 75,000 cells per well in 24-well plates. The next day, cells were washed three times with warmed Krebs-Henseleit buffer (KHB; composition: 118 mM NaCl, 4.7 mM KCl, 4 mM NaHCO_3 , 1.3 mM CaCl_2 , 1.2 mM MgSO_4 , 1.2 mM KH_2PO_4 , 11.7 mM glucose, 8.5 mM HEPES, pH 7.4) before being transferred to an ice bath and 1 ml of ice-cold KHB containing appropriate concentrations of agonists and approximately 0.2 nM [3 H]NMS added. 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, 4 ml of SafeFluor scintillation fluid (PerkinElmer Life and Analytical Sciences, Groningen, The Netherlands) was added, and radioactivity was determined by scintillation counting.

Cell Lysate Preparation for Determination of ERK Activity. CHO- M_1 , - M_2 or - M_3 cells were seeded at 150,000 cells per well in 12-well plates 48 h before assay. Cells were washed twice and incubated with KHB for 1 h at 37°C. Drug additions were made directly to the KHB at the concentrations indicated (see figure legends) for 5 min. Incubations were terminated by transfer of the plates to ice and rapid aspiration of agonist-containing KHB. Cells were washed twice with ice-cold KHB before the addition of 150 μl of SDS sample buffer (62.5 mM Tris/HCl, 2% SDS, 10% glycerol, 0.1% bromophenol blue, and 50 mM dithiothreitol, pH 7.4). Cells were scraped from the plate and transferred to centrifuge tubes. Cell lysates were sonicated for 20 s to reduce sample viscosity, boiled for 5 min, and then centrifuged at 20,000g for 1 min at 4°C.

Western Blotting Analysis. Cell lysate samples (25 μl) prepared as described above were separated by 10% SDS-polyacrylamide gel electrophoresis by use of the Bio-Rad mini-gel system (Bio-Rad Laboratories, Hercules, CA). Proteins were electrotransferred to nitrocellulose membranes with use of the Bio-Rad semidry blotter apparatus according to the manufacturer's instructions. After transfer, membranes were blocked for nonspecific binding with 5% nonfat milk in Tris-buffered saline (50 mM Tris/HCl, 150 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were washed for 3 $\times$ 5 min with TBS-T before incubation with the anti-phospho-pERK1/2 antibody (1:1000 in 5% bovine serum albumin in TBS-T) overnight at 4°C. Membranes were washed for 3 $\times$ 5 min to remove excess antibody before detection with a horseradish peroxidase-conjugated secondary antibody (goat anti-rabbit, 1:3000 in 5% milk in TBS-T) for 1 h at room temperature. Membranes were washed 3 $\times$ 5 min with TBS-T before chemiluminescence detection by use of enhanced chemiluminescence reagent (GE Healthcare) and exposure to film (Eastman Kodak, Rochester, NY). Equal protein loading was confirmed by submerging the membrane in stripping buffer (62.5 mM Tris/HCl, 100 mM 2-mercaptoethanol, 2% SDS, pH 6.7) and incubation for 30 min at 50°C. Membranes were then washed with TBS-T before reprobing with an anti-total ERK antibody and detection as described above.

Receptor Regulation Assay. CHO- M_1 , - M_2 , or - M_3 cells were seeded at 75,000 cells per well in 24-well plates for 24 h before being washed twice with warmed phosphate-buffered saline and incubated with serum-free medium at 37°C in a humidified atmosphere of 5% CO_2 in air. Drug additions were made at the indicated time points at 37°C (see Results) before cells were transferred to an ice bath and subjected to six washes with ice-cold KHB to remove the ligand. Cells were then incubated for 16 h at 4°C with either 3 nM [3 H]NMS (to quantify cell-surface receptors), or with 2 nM [3 H]QNB (to quantify

the total receptor population). In both cases, nonspecific binding was determined in the presence of 10 μM atropine. At the end of the incubation period cells were washed four times with ice-cold KHB to remove unbound radioligand, solubilized with 2×0.25 ml of 0.1 M NaOH, and transferred to scintillation vials. Samples were mixed with 4 ml of SafeFluor scintillation fluid before radioactivity was determined by scintillation counting.

[^3H]Inositol Phosphate Accumulation Assay. CHO- M_1 cells were seeded at 100,000 cells per well in 24-well plates for 24 h. Cells were washed twice with warmed phosphate-buffered saline and incubated for 48 h in serum-free medium containing 2.5 $\mu\text{Ci ml}^{-1}$ [^3H]inositol. Agonists were added to the cells for the last 24 h of the labeling period. Confluent cell monolayers were washed five times to remove ligand before incubation in KHB containing a final concentration of 10 mM LiCl for 30 min at 37°C. Agonists or buffer were added for 20 min before reactions were terminated by the addition of an equal volume of ice-cold 1 M trichloroacetic acid and transfer to an ice bath. After extraction (~ 30 min) samples were neutralized by use of the Freon/tri-*n*-octylamine method and the [^3H]inositol mono-, bis-, and triphosphate ([^3H]IP $_x$) fraction recovered by anion exchange chromatography with use of Dowex-1 (formate form) columns. Columns were initially regenerated with 10 ml of 2 M ammonium formate/0.1 M formic acid before being washed extensively with distilled water. Samples were washed onto the columns with 5 ml of distilled water. Columns were then washed with 10 ml of 60 mM ammonium formate/5 mM sodium tetraborate and the [^3H]IP $_x$ fraction eluted in 10 ml of 0.75 M ammonium formate/0.1 M formic acid. A 5-ml aliquot of the collected eluate was mixed with 10 ml of Ultima-Flo AF scintillation cocktail (PerkinElmer, Beaconsfield, Buckinghamshire, UK), and radioactivity was determined by liquid scintillation counting.

Data Analysis. All data are expressed as means $\pm$ S.E.M. for the indicated number of experiments. Radioligand binding data and agonist concentration-response curves were analyzed by use of Prism 5 (GraphPad Software Inc., San Diego, CA). IC $_{50}$ values derived from agonist-[^3H]NMS competition binding experiments were converted to K_B values by the method of Cheng and Prusoff (1973). Statistical differences between multiple data sets were assessed by one-way analysis of variance followed by Bonferroni's multiple-range test at $P < 0.05$ with use of Prism 5 (version 5.5).

Results

Determination of Agonist Binding Affinities at M_1 , M_2 , and M_3 mACh Receptors. [^3H]NMS saturation binding experiments were performed to determine B_{max} and K_D values at the human M_1 , M_2 , and M_3 mACh receptors stably expressed in CHO cells. Receptor densities were 4.66 ± 0.11 , 1.29 ± 0.08 , and 4.09 ± 0.39 pmol mg^{-1} protein in CHO- M_1 , CHO- M_2 , and CHO- M_3 cells with respective K_D values of 0.24 ± 0.02 , 0.16 ± 0.02 , and 0.24 ± 0.02 nM. To estimate agonist binding affinities, CHO- M_1 - M_2 - M_3 cell monolayers were incubated with an approximate K_D value of 0.2 to 0.3 nM [^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 (pK_B) for each agonist is summarized in Table 1. Consistent with previous reports, both AC-42 and 77-LH-28-1 are able to displace specific [^3H]NMS binding from M_2 and M_3 mACh receptors (Spalding et al., 2002; May et al., 2007; Langmead et al., 2008a) with 77-LH-28-1 and AC-42 apparent affinities at the M_2 subtype being comparable with those observed at the M_1 mACh receptor (Table 1).

Effects of Chronic Agonist Exposure on Cell-Surface and Total Cellular Levels of the M_1 mACh Receptor. The effects of a 24-h pretreatment with an orthosteric or

TABLE 1

Comparison of apparent binding affinities of muscarinic agonists at the human M_1 , M_2 , and M_3 mACh receptors recombinantly expressed in CHO cells

Affinity estimates (pK_B) are taken from Thomas et al. (2008) for the M_1 mACh receptor, or as indicated (#) from Mistry et al. (2005).

Ligand	M_1	M_2	M_3
oxo-M	5.30 ± 0.07	5.18 ± 0.04	3.97 ± 0.21
AC-42	5.52 ± 0.04	5.75 ± 0.01	4.82 ± 0.05
77-LH-28-1	6.00 ± 0.04	5.79 ± 0.04	4.83 ± 0.04
Arecoline	5.06 ± 0.20	$4.40 \pm 0.14^{\#}$	N.D.
Pilocarpine	4.95 ± 0.10	$3.70 \pm 0.27^{\#}$	N.D.

All data are presented as means $\pm$ S.E.M. of at least three separate experiments performed in duplicate. N.D., not determined.

allosteric agonist on the expression of the M_1 mACh receptor at the cell surface was investigated by use of a radioligand binding approach. Because of the limiting solubility of AC-42 and the potential toxicity of the dimethyl sulfoxide solvent/vehicle, the maximum concentration that could be used in these experiments was 10 μM AC-42, which results in approximately 75% occupancy of the M_1 mACh receptors. Therefore, the concentrations of all other ligands used were chosen to give an equivalent 75% receptor occupancy. Initial saturation analysis with [^3H]NMS and [^3H]QNB revealed that the M_1 mACh receptor expression levels are 4.66 ± 0.11 and 5.07 ± 0.15 pmol mg^{-1} protein, respectively, indicating that approximately 90% of the receptors in CHO- M_1 cells are at the cell surface. Incubation with oxotremorine-M (oxo-M; 15 μM) for 24 h caused $69 \pm 3\%$ ($P < 0.01$) internalization of the cell-surface M_1 mACh receptor population (Fig. 1A). The orthosteric partial agonists arecoline (30 μM) and pilocarpine (35 μM) also induced significant M_1 mACh receptor internalization (Fig. 1A). Incubation for 24 h with the allosteric agonist 77-LH-28-1 (3 μM) caused $32 \pm 5\%$ ($P < 0.05$) internalization, whereas AC-42 (10 μM) caused an insignificant ($<10\%$) internalization of the cell-surface M_1 mACh receptor population (Fig. 1A).

The internalized receptor can either be processed for recycling to the cell surface (resensitization) or degraded, resulting in a decrease in the total cellular complement of the receptor (down-regulation). To assess this, we used [^3H]QNB to quantify changes in the total M_1 mACh receptor population. Pretreatment for 24 h with the full agonist oxo-M (15 μM) caused the greatest decrease in M_1 mACh receptor level ($\geq 40\%$, $P < 0.05$; Fig. 1B). Of the other orthosteric or allosteric agonists used, only pilocarpine (35 μM) pretreatment resulted in a statistically significant down-regulation. Again, it was noteworthy that, although 77-LH-28-1 caused at most a very small down-regulation ($11 \pm 7\%$; $P > 0.05$), AC-42 actually caused an apparent (15–20%) increase in the total M_1 mACh receptor population (Fig. 1B).

Similar, contrasting effects of oxo-M and AC-42 were also seen in a different CHO- M_1 clone with a lower human M_1 mACh receptor expression level (~ 300 fmol mg^{-1} protein). In this cell-line incubation with oxo-M (15 μM) for 24 h caused $53 \pm 5\%$ ($P < 0.05$) and $35 \pm 14\%$ decreases in cell-surface ([^3H]NMS) and total cellular ([^3H]QNB) M_1 mACh receptor expression. In contrast, 24 h incubation with AC-42 (10 μM) caused no decrease in cell-surface expression ($104 \pm 12\%$) and a marked up-regulation ($234 \pm 58\%$; $P < 0.05$) of total M_1 mACh receptor expression (R. L. Thomas and R. A. J. Challiss, unpublished data).

Fig. 1. Effects of 24 h of treatment with orthosteric and allosteric ligands on cell-surface and total cellular expression of M_1 mACh receptors. CHO- M_1 cells were seeded at 75,000 cells per well and treated with a concentration of agonist calculated to occupy 75% of receptors (oxo-M, 15 μ M; AC-42, 10 μ M; 77-LH-28-1, 3 μ M; arecoline, 30 μ M; pilocarpine, 35 μ M). After 24 h at 37°C plates were transferred to ice, and cell monolayers were washed extensively before incubation for 16 h at 4°C with either 3 nM [³H]NMS to measure cell-surface receptors (A), or 2 nM [³H]QNB to assess total cellular levels of receptor (B). Data are expressed as a percentage of control binding ([³H]NMS, 4.66 ± 0.11 ; [³H]QNB, 5.07 ± 0.15 pmol mg^{-1} protein) and are presented as means $\pm$ S.E.M. for at least four experiments performed in triplicate. Statistically significant differences are indicated as *, $P < 0.05$; **, $P < 0.01$ for drug treatment versus vehicle treatment groups.

Time-Courses of Agonist-Induced Changes in Cell Surface and Total Cellular Levels of M_1 mACh Receptors. Time course studies were next performed to profile agonist-induced changes in M_1 mACh receptor expression both at the cell surface and in the cell as a whole. Pretreatment with oxo-M (15 μ M) caused a time-dependent monoexponential decrease in cell-surface expression of the M_1 mACh receptor. After 60 min of oxo-M treatment, $29 \pm 5\%$ ($P < 0.01$) internalization of the M_1 mACh receptor population was detected (Fig. 2A). Oxo-M-induced M_1 mACh receptor down-regulation occurred more slowly and only reached statistical significance after 21 h of agonist treatment ($P < 0.01$; Fig. 2A).

Arecoline (30 μ M) and pilocarpine (35 μ M) caused similar monoexponential patterns of M_1 mACh receptor internalization with their effects reaching significance early in the time course (after 1–3 h; see Fig. 2B). Investigation of arecoline- and pilocarpine-induced M_1 mACh receptor down-regulation revealed that each ligand was able to induce significant receptor degradation by 16 h ($P < 0.01$; Fig. 2C). Despite their

lower relative efficacies toward acute signaling events compared with oxo-M (e.g., [³⁵S]guanosine 5'-O-(3-thio)triphosphate loading of $G\alpha_{q/11}$, phospholipase C activation; see Thomas et al., 2008), pilocarpine and arecoline caused comparable degrees of receptor down-regulation relative to the full agonist through the 6- to 24-h time period (Fig. 2C).

In contrast to the orthosteric ligands, the profiles for AC-42 and 77-LH-28-1 regulation of M_1 mACh receptor internalization/down-regulation were notably different. Neither allosteric agonist caused receptor internalization over the initial 6 h of exposure (Fig. 2D). Although 77-LH-28-1 did cause a significant M_1 mACh receptor internalization on prolonged exposure ($37 \pm 7\%$, $P < 0.05$; loss of cell-surface receptor at 16 h; Fig. 2D), AC-42 did not significantly affect cell-surface receptor expression at any time point studied (Fig. 2D). Neither AC-42 nor 77-LH-28-1 caused a significant down-regulation of the M_1 mACh receptor; for both agents there seemed to be an acute *increase* in total receptor number peaking at 6 h (Fig. 2E). Whereas for 77-LH-28-1 there was a subsequent decrease in total M_1 mACh receptor number (by $20 \pm 8\%$, $P > 0.05$; after 21 h; Fig. 2E), the initial increase in receptor number (to $122 \pm 3\%$ of the initial expression level at 6 h; Fig. 2E) in the presence of AC-42 was maintained throughout the 24-h time course.

Stability of Orthosteric and Allosteric Ligands. The contrasting effects of orthosteric and allosteric agonists on M_1 mACh receptor internalization/down-regulation prompted us to assess the stability of each of the ligands used. Ligands were added to monolayers of CHO- M_1 cells (at the concentrations used in the studies reported above) and incubated for 24 h at 37°C. Medium from cell incubations and matched concentrations of each agonist freshly prepared in identical culture medium were added to myo-[³H]inositol-prelabeled CHO- M_1 cells. Incubations were terminated and processed to determine the [³H]IP_x accumulation stimulated by each agonist addition. No significant differences in the [³H]IP_x responses stimulated by each ligand were observed, indicating that all of the ligands used here are stable under the conditions used in the receptor regulation studies (Fig. 3).

Antagonism of Agonist-Induced Receptor Internalization and Down-Regulation. To confirm that the observed changes in receptor internalization and down-regulation are mediated by M_1 mACh receptor occupancy we investigated whether the agonist-stimulated effects were prevented by preaddition of the mACh receptor antagonist atropine. Atropine (0.3 μ M) addition alone caused a small, insignificant increase in both [³H]NMS (Fig. 4A) and [³H]QNB (Fig. 4B) binding. Preaddition of atropine (0.3 μ M) prevented the changes induced by oxo-M (15 μ M), arecoline (30 μ M), pilocarpine (35 μ M), or 77-LH-28-1 (3 μ M). In contrast, the copresence of atropine and AC-42 (10 μ M) *increased* cell-surface M_1 mACh receptor levels to $151 \pm 3\%$ ($P < 0.01$; Fig. 4A) and total M_1 mACh receptor expression to $161 \pm 7\%$ ($P < 0.01$; Fig. 4B) compared with control levels (= 100%).

M_2 and M_3 mACh Receptor Regulation by AC-42 and 77-LH-28-1. Because both AC-42 and 77-LH-28-1 bind to M_2 and M_3 mACh receptors, we assessed the effects of prolonged exposure to these ligands on the internalization and/or down-regulation of these receptor subtypes. Pretreatment of CHO- M_2 cells with 20 μ M oxo-M (a concentration determined from binding data to occupy approximately 75% of the recep-

Fig. 2. Time courses of ligand-induced changes in cell-surface and total cellular expression of M_1 mACh receptors. CHO cells were seeded at 75,000 cells per well and treated with a concentration of agonist calculated to occupy 75% of receptors [oxo-M, 15 μ M (A–E); arecoline, 30 μ M; pilocarpine, 35 μ M (B and C); AC-42, 10 μ M; 77-LH-28-1, 3 μ M (D and E)]. After the exposure times indicated (0–24 h) plates were transferred to ice and cell monolayers were washed extensively before incubation for 16 h at 4°C with either 3 nM [3 H]NMS to measure cell-surface receptors (A, B, and D), or 2 nM [3 H]QNB to assess total cellular levels of receptor (A, C, and E). Data are expressed as a percentage of control binding (see *Results*) and are presented as means $\pm$ S.E.M. for at least four experiments performed in triplicate.

tors) for 24 h resulted in a $77 \pm 2\%$ decrease in specific [3 H]NMS binding (internalization), and a $53 \pm 1\%$ decrease in [3 H]QNB binding (down-regulation) (Fig. 5A). These values are broadly similar to those obtained for oxo-M-stimulated changes in M_1 mACh receptors in the CHO cell background (see Fig. 1). Contrasting data were obtained for the allosteric mACh receptor agonists; whereas AC-42 (5 μ M) caused no change, the presence of 77-LH-28-1 (5 μ M) resulted in a $60 \pm 5\%$ increase in cell-surface expression of the

M_2 mACh receptor (Fig. 5A). More marked adaptive changes in the total cellular M_2 mACh receptor expression level were observed after 24 h of treatment with either AC-42 or 77-LH-28-1. Thus, AC-42 and 77-LH-28-1 each caused, respectively, highly significant 179 ± 2 and $100 \pm 6\%$ increases in M_2 mACh receptor levels (Fig. 5A).

The effects of chronic exposure to the allosteric mACh receptor agonists on M_3 mACh receptor expression were also assessed. As mentioned earlier, the solubility limits of AC-42

Fig. 3. All agonists are metabolically stable under these incubation conditions. CHO-M₁ cells were seeded at 120,000 cells per well in 24-well plates and incubated with 2.5 $\mu\text{Ci ml}^{-1}$ *myo*-[³H]inositol for 48 h at 37°C. Cells were incubated for 30 min in medium containing 10 mM LiCl and either freshly prepared ligand (fresh), or ligand that had been exposed (preconditioned) to CHO-M₁ cells for 24 h at 37°C (see *Materials and Methods*) added. Ligand concentrations used were: oxo-M, 15 μM ; AC-42, 10 μM ; 77-LH-28-1, 3 μM ; arecoline, 30 μM ; pilocarpine, 35 μM . [³H]IP_x accumulation data are expressed as dpm mg⁻¹ protein and are presented as means $\pm$ S.E.M. for three separate experiments performed in duplicate.

and 77-LH-28-1 are such that the maximum concentration that can be studied in chronic experiments is 10 μM . At the M₃ mACh receptor it can be calculated that use of 10 μM AC-42 will result in approximately 40% receptor occupancy (Table 1). Pretreatment for 24 h with oxo-M (75 μM , a concentration calculated to give approximately 40% receptor occupancy) caused $50 \pm 3\%$ internalization and $23 \pm 6\%$ down-regulation of the M₃ mACh receptor (Fig. 5B). Pretreatment with either AC-42 (10 μM) or 77-LH-28-1 (10 μM) caused no significant changes in either cell-surface or total cell expression of the M₃ mACh receptor (Fig. 5B).

Signaling Properties of AC-42 and 77-LH-28-1 at Human M₂ and M₃ mACh Receptors. Recent studies have reported actions of AC-42 and 77-LH-28-1 at non-M₁ mACh receptor subtypes (May et al., 2007; Langmead et al., 2008a). Here, we have shown that both allosteric agonists can displace specific [³H]NMS binding to the M₂ and M₃ mACh receptors (Table 1). AC-42 and 77-LH-28-1 have been reported to act allosterically at the M₂ mACh receptor (May et al., 2007), but a similar mechanism of action has not been reported at the M₃ receptor (Langmead et al., 2008a). Oxo-M stimulated significant increases ERK1/2 phosphorylation ($P < 0.001$) via human M₁, M₂, and M₃ mACh receptor subtypes stably expressed in CHO cells (Fig. 6, A–C). AC-42 and 77-LH-28-1 caused, respectively, $71 \pm 14\%$ and $67 \pm 16\%$ of the phorbol-12,13-dibutyrate-stimulated ERK1/2 phosphorylation response at the M₁ mACh receptor, consistent with their activation of other signaling pathways through this subtype (Thomas et al., 2008). At the M₂ mACh receptor, neither AC-42 nor 77-LH-28-1 caused a significant increase in ERK1/2 phosphorylation at agonist concentrations that elicit significant activation via the M₁ mACh receptor (Fig. 6B). At the M₃ mACh receptor, AC-42 was without effect, whereas 77-LH-28-1 caused a statistically insignificant increase in ERK1/2 phosphorylation, which was 20% of the

Fig. 4. Effects of the preaddition of atropine on agonist-induced M₁ mACh receptor internalization and down-regulation. CHO cells seeded at 75,000 cells per well were preincubated with either KHB (–) or 300 nM atropine (+) for 30 min before the addition of ligand (oxo-M, 15 μM ; arecoline, 30 μM ; pilocarpine, 35 μM ; AC-42, 10 μM ; 77-LH-28-1, 3 μM) for 24 h at 37°C. Plates were then transferred to ice, and cell monolayers were washed extensively before incubation for 16 h at 4°C with either 3 nM [³H]NMS to measure cell-surface receptors (A), or 2 nM [³H]QNB to assess total cellular levels of receptor (B). Data are expressed as a percentage of control specific binding ([³H]NMS, $22,084 \pm 701$; [³H]QNB, $16,644 \pm 686$ dpm per well) and are presented as means $\pm$ S.E.M. for at least three separate experiments performed in triplicate. Statistically significant differences are shown as **, $P < 0.01$ for plus versus minus atropine treatment, and as ##, $P < 0.01$ for drug versus vehicle treatment.

response stimulated by phorbol-12,13-dibutyrate (PDBu) (Fig. 6C). All orthosteric and allosteric agonist effects were prevented by preincubation of M₁/M₂/M₃ mACh receptor-expressing CHO cell-lines with atropine (300 nM) for 30 min.

Effects of Chronic Ligand Exposure on the Function of M₁ mACh Receptors. The lack of effect of AC-42 on M₁ mACh receptor internalization and down-regulation despite its ability to activate signal transduction cascades prompted the question of whether any index of receptor desensitization is altered by chronic exposure to this allosteric agonist. Perhaps the most relevant is to assess how prior treatment with AC-42 affects the ability of the M₁ mACh receptor to transduce agonist binding into a (functional) cellular response. To do this we used the [³H]IP_x accumulation assay with the

Fig. 5. Effects of 24 h of treatment with oxo-M or allosteric agonists on cell-surface and total cellular expression of M_2 and M_3 mACh receptors. CHO- M_2 (A) or CHO- M_3 (B) cells were plated at 75,000 cells per well and stimulated for the indicated agonists for 24 h. Agonist concentrations used were: Oxo-M (20 or 75 μ M), AC-42 (5 or 10 μ M), and 77-LH-28-1 (5 or 10 μ M) for CHO- M_2 and CHO- M_3 cells, respectively. Cells were subject to extensive washout with ice-cold KHB before incubation with either 3 nM [³H]NMS ($\square$) or 2 nM [³H]QNB ($\blacksquare$) for 16 h at 4°C. Data are expressed as a percentage of control binding (see *Results*) and are the mean $\pm$ S.E.M. for three separate experiments performed in triplicate. Statistical differences between treatments and control are indicated by ***, $P < 0.001$ as determined by one-way analysis of variance.

partial agonist arecoline as the receptor stimulus. Under control conditions arecoline stimulated a 2- to 3-fold increase in [³H]IP_x levels (basal, 94 218 $\pm$ 23 261; stimulated, 201 540 $\pm$ 19 656 dpm mg⁻¹ protein) in CHO- M_1 cells with an EC₅₀ value of 3.6 μ M (Fig. 7, Table 2). Pretreatment with oxo-M (15 μ M) or pilocarpine (35 μ M) resulted, respectively, in 68 $\pm$ 2 and 44 $\pm$ 14% decreases in the maximal [³H]IP_x response stimulated subsequently by arecoline (Fig. 7, Table 2). In contrast, pretreatment with AC-42 (10 μ M) for 24 h did not decrease the maximal [³H]IP_x response to arecoline and was essentially identical to that seen in control CHO- M_1 cells (Fig. 7; Table 2). These data are in excellent agreement with the previous data obtained from radioligand binding assays, and highlight the contrasting effects of AC-42 versus an equiefficacious orthosteric partial agonist (pilocarpine) on functional and radioligand-based receptor desensitization readouts.

Discussion

Despite the considerable therapeutic potential of drugs that can act selectively at mACh receptor subtypes, relatively few agonists or antagonists have been reported that possess a high degree (>10-fold) of mACh receptor subtype selectivity (Caulfield and Birdsall, 1998; Eglen et al., 1999; Langmead et al., 2008b). The advent of new classes of drug that act via allosteric (as opposed to orthosteric) binding sites on the mACh receptor has provided new opportunities to

Fig. 6. Allosteric agonist activation of ERK in CHO cells stably expressing the M_1 , M_2 , or M_3 mACh receptor. CHO- M_1 (A), CHO- M_2 (B), and CHO- M_3 (C) cells were seeded at 150,000 cells per well in 12-well plates for 48 h before assay. Cell monolayers were preincubated at 37°C for 60 min in KHB before addition of KHB (Basal), oxo-M (100 μ M), AC-42 (10 μ M), or 77-LH-28-1 (10 μ M) for 5 min. Plates were then transferred to ice, washed twice with ice-cold KHB, and solubilized (see *Materials and Methods*). Data are expressed as a percentage of the response to phorbol 12,13-dibutyrate (1 μ M, 5 min) and are presented as means $\pm$ S.E.M. for three separate experiments. Representative phospho-ERK and total-ERK immunoblots are shown below each histogram with each lane representing the respective condition shown in the histogram. Statistically significant differences between basal and drug treatments are indicated as **, $P < 0.01$; ***, $P < 0.001$.

Fig. 7. Concentration dependencies for arecoline-stimulated [^3H]IP $_x$ accumulations in CHO-M $_1$ cells after 24 h of pretreatment with muscarinic agonists. CHO-M $_1$ cells were seeded at 120,000 cells per well in 24-well plates and incubated with 2.5 $\mu\text{Ci ml}^{-1}$ myo-[^3H]inositol for 48 h at 37°C. For the final 24 h of this labeling period, cells were incubated with either KHB, oxo-M (15 μM), AC-42 (10 μM), or pilocarpine (35 μM). At the end of this period cells were washed extensively with warmed KHB and preincubated with 10 mM LiCl for 20 min before addition of the indicated concentrations of arecoline. Data are expressed as dpm mg^{-1} protein for [^3H]IP $_x$ accumulation and are presented as means $\pm$ S.E.M. for three separate experiments performed in duplicate.

TABLE 2

Comparison of pEC_{50} and R_{max} values for arecoline-stimulated [^3H]IP $_x$ accumulation in CHO-M $_1$ cells after a 24-h pretreatment with oxo-M, AC-42, pilocarpine, or vehicle

CHO-M $_1$ cells were treated as described in the legend to Fig. 7. pEC_{50} values are shown as $-\log \text{M}$ values, and R_{max} values are given as a percentage of the maximal response to arecoline in vehicle-treated control cells.

24-h Pretreatment	pEC_{50}	R_{max}
KHB	5.44 ± 0.07	100
oxo-M (15 μM)	$4.89 \pm 0.05^{**}$	$32 \pm 2^{**}$
AC-42 (10 μM)	$5.07 \pm 0.07^*$	107 ± 9
Pilocarpine (35 μM)	$5.10 \pm 0.06^*$	56 ± 14

Data are presented as means $\pm$ S.E.M. for three separate experiments performed in duplicate. Statistically significant differences between drug treatments and vehicle-treated control cells are shown as *, $P < 0.05$; **, $P < 0.01$.

achieve subtype selectivity of action (Birdsall and Lazareno, 2005; Langmead and Christopoulos, 2006; Gregory et al., 2007). AC-42 (Spalding et al., 2002) and its structurally related and more potent analog 77-LH-28-1 (Langmead et al., 2008a) bind to the M $_1$ mACh receptor at a site distinct from the acetylcholine binding site. Although initial studies with M $_1$ /M $_5$ mACh receptor chimeras suggested that AC-42 binding involves N-terminal/transmembrane domains 1 and 7 (Spalding et al., 2002), a recent study by Hulme and colleagues highlighted roles for specific amino acid residues within transmembrane domains 2, 3, and 7, and proposed a "Trp (tryptophan)-flip" mechanism for M $_1$ mACh receptor activation by AC-42 and 77-LH-28-1 (Lebon et al., 2009).

AC-42 and 77-LH-28-1 binding to the M $_1$ mACh receptor initiate a number of cell signaling events, including phosphoinositide hydrolysis and Ca^{2+} mobilization (Spalding et al., 2002, 2006; Langmead et al., 2006, 2008a). In addition, we have recently shown that AC-42 and 77-LH-28-1 (like orthosteric full and partial mACh receptor agonists) can activate M $_1$ mACh receptor-G $_{q/11}$ and -G $_{s}$ -coupling, but, in contrast to orthosterically acting agonists, these allosteric ligands do not

facilitate significant G $_{i/o}$ -coupling in the CHO-M $_1$ cell line used in the present study (Thomas et al., 2008). These data suggest that there may be subtle differences in the receptor conformations stabilized by orthosteric and allosteric agonists, and this prompted the present investigation into whether AC-42 and 77-LH-28-1 cause comparable adaptive changes in the M $_1$ mACh receptor population to orthosteric ligands.

As in the vast majority of GPCRs examined to date, M $_1$ mACh receptor internalization involves phosphorylation, arrestin binding, and endocytosis via a clathrin- and dynamin-dependent mechanism (Ferguson, 2001; van Koppen and Kaiser, 2003). The M $_1$ mACh receptor can be phosphorylated by a number of protein kinases, including G protein-coupled receptor kinases (Haga et al., 1996; Waugh et al., 1999; Willets et al., 2005) to cause acute desensitization. Evidence for arrestin (β -arrestin1/2) involvement is presently inconclusive, but recombinant M $_1$ mACh receptors recruit endogenous β -arrestin2 (but not β -arrestin1) in RBL-2H3 cells (Santini et al., 2000), whereas endogenous M $_1$ mACh receptor desensitization is increased in hippocampal neurons recombinantly expressing β -arrestin2 (Willets et al., 2005). In addition, dominant-negative constructs of β -arrestin (V53D) and dynamin (K44A) inhibit M $_1$ mACh receptor internalization in human embryonic kidney cells (Vögler et al., 1999).

Irrespective of the precise molecular mechanisms of M $_1$ mACh receptor regulation, the cellular location of mACh receptors can be monitored in intact cells by use of [^3H]NMS and [^3H]QNB to quantify, respectively, cell-surface and total mACh receptor subpopulations (Maloteaux et al., 1983; Lamah et al., 1992). This radioligand binding approach has been widely used to demonstrate that a variety of factors, including exposure to agonist, can alter the distribution and total number of mACh receptors present in cells (van Koppen and Kaiser, 2003). In the present study, we have used a CHO cell background devoid of endogenous mACh receptors, and exposed CHO cells stably expressing human M $_1$ mACh receptors to orthosteric and allosteric mACh receptor agonists for periods of up to 24 h at concentrations calculated to give equivalent 75% receptor occupancy. The orthosteric agonist oxo-M caused a time-dependent decrease in [^3H]NMS binding that approached an apparent maximum ($\sim 70\%$ loss of cell-surface receptors) at 21 to 24 h. Time courses for pilocarpine- and arecoline-induced decreases in [^3H]NMS binding were similar in shape, but reached apparent maxima with more cell-surface M $_1$ mACh receptors remaining (see Fig. 2). In marked contrast, neither AC-42 nor 77-LH-28-1 caused any loss of cell-surface receptors in the initial 6-h exposure period. After 24 h cell-surface receptor expression was essentially 100% of the control value in CHO-M $_1$ cells exposed to AC-42, but had declined (by $\sim 30\%$) in cells exposed to 77-LH-28-1. In a preliminary report, chronic (16 h) treatment with AC260584, another AC-42-series analog, also failed to cause M $_1$ mACh receptor down-regulation despite evoking a robust Ca^{2+} response (Davis et al., 2005). The relative lack of effect of the allosteric agonists was not attributable to compound instability, because bioassay (using the [^3H]inositol phosphate assay) revealed that 24 h of exposure to cells and culture medium did not compromise the biological activity of any of the agonists used in this study.

Internalized M $_1$ mACh receptors can either be dephosphorylated and returned to the plasma membrane or degraded

(van Koppen and Kaiser, 2003; Mosser et al., 2008). Quantification of the total M_1 mACh receptor population (by use of [3H]QNB binding) indicated that none of the agonists caused down-regulation during the first 6 h of exposure, and only oxo-M and pilocarpine caused statistically significant down-regulations after a 24-h exposure period. Incubation of CHO- M_1 cells with AC-42 resulted in a modest up-regulation of the M_1 mACh receptor (see Fig. 2). In addition, whereas preaddition of atropine (300 nM added 30 min before agonist) completely prevented receptor internalization and down-regulation for the orthosteric agonists tested, 24 h of treatment with AC-42 in the presence of atropine caused significant increases in both the total ($61 \pm 7\%$; $P < 0.01$) and cell-surface ($51 \pm 3\%$; $P < 0.01$) M_1 mACh receptor populations. This represents a substantial quantitative increase in the cell-surface receptor population (approximately 2.5×10^5 receptors per cell) over this time period. It is possible for this AC-42/atropine effect to be explained by a complex allosteric interaction, where AC-42 has positive cooperativity with respect to atropine in terms of efficacy, with the functional output in this case being a decrease in receptor internalization/degradation. Both AC-42 and atropine have been independently shown to increase cell-surface mACh receptor expression (May et al., 2005), indicating that both are "agonists" with respect to this endpoint. Even if AC-42 and atropine are highly negatively cooperative with respect to binding (although this has not been directly measured here), if they exhibit a sufficiently positive efficacy cooperativity, then the decrease in constitutive receptor recycling mediated by atropine may be facilitated in the presence of AC-42.

Previous studies in the CHO cell background have shown that, in the presence of 300 nM atropine, [3H]IP_x and Ca^{2+} responses to AC-42 (10 μ M) are partially inhibited (by approximately 90 and 40%, respectively; see Langmead et al., 2006). Furthermore, Langmead et al. (2006) have presented evidence that AC-42 and atropine can simultaneously occupy the M_1 mACh receptor, albeit in a highly negatively cooperative manner. However, these data were obtained by use of Ca^{2+} mobilization as a functional measurement and to date there have been no studies investigating the interaction of AC-42 and atropine at the level of receptor regulation where the interaction may not be comparable. It is also interesting to note that chronic treatment of CHO- M_2 , but not CHO- M_3 , cells with AC-42 or 77-LH-28-1 (at concentrations adjusted to give equivalent occupancies of the M_2/M_3 mACh receptor populations) also caused increases in the total cellular M_2 mACh receptor population and, in the case of 77-LH-28-1, a significant increase in cell-surface receptor expression (Fig. 6). Allosteric modulators of the M_2 mACh receptor have been reported to cause receptor up-regulation in a CHO cell background (May et al., 2005). AC-42 and 77-LH-28-1 can bind to non- M_1 mACh receptor subtypes (Spalding et al., 2002; May et al., 2007; Langmead et al., 2008a), but generally lack efficacy (but see May et al., 2007; Langmead et al., 2008a) (Fig. 5). However, to date, no studies are investigating the consequences of occupancy by these allosteric agonists on receptor regulation, and it is possible that these ligands influence receptor regulation.

Our data indicate that chronic occupation of the M_1 mACh receptor by AC-42 and, to at least some extent, 77-LH-28-1 causes much less receptor internalization and down-regulation than in the presence of similarly efficacious orthosteric

agonists, where studies have been specifically designed to match receptor occupancy levels by the different agonists. This conclusion is supported and extended by our observation that after chronic treatment with AC-42, the ability of an orthosteric partial agonist (arecoline) to stimulate phospholipase C activity is largely unaffected, in contrast to the marked reductions in maximal responses seen after chronic treatments with oxo-M or pilocarpine. It is interesting that a very recent report has shown, with use of a β -galactosidase complementation approach, that AC-42 does not cause significant β -arrestin recruitment to the M_1 mACh receptor in a CHO cell background (Ma et al., 2009). These and our data suggest that early events in receptor desensitization are likely to be spared during M_1 mACh receptor stimulation with AC-42 and perhaps other allosterically acting agonists. These data therefore suggest that, in addition to offering considerable potential advantage with respect to receptor subtype specificity and functional selectivity toward signaling readouts (Thomas et al., 2008), allosteric agonists may induce receptor conformations less favorable to receptor desensitization/internalization programs than orthosteric ligands.

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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, United Kingdom. E-mail: jc36@leicester.ac.uk