JPET Fast Forward. Published on August 27, 2004 as DOI: 10.1124/jpet.104.073767

Regulation of M_2 muscarinic acetylcholine receptor expression and signaling by prolonged exposure to allosteric modulators

Lauren T. May, Yvonne Lin, Patrick M. Sexton and Arthur Christopoulos

Department of Pharmacology (L.T.M., Y.L., P.M.S., A.C.) and Howard Florey Institute of Experimental Physiology and Medicine (P.M.S., A.C.), University of Melbourne, Parkville, 3010, Victoria, Australia

Running title: Chronic effects of M_2 muscarinic receptor modulators

Author for correspondence: Dr. Arthur Christopoulos, NHMRC Senior Research Fellow, Department of Pharmacology, University of Melbourne, Grattan St., Parkville, 3010, Victoria, Australia; Ph: +613 8344 8417; Fax: +613 8344 0241; email: arthurc1@unimelb.edu.au

Number of text pages: 39

Number of tables: 4

Number of figures: 6

Number of references: 36

Number of words in Abstract: 245

Number of words in Introduction: 587

Number of words in Discussion: 1499

Nonstandard abbreviations:

C_7/3-phth, heptane, 1,7-bis-(dimethyl-3'-phthalimidopropyl)-ammonium bromide; CCh, carbachol; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified eagles medium; GPCR, G protein-coupled receptor; ECAR, extracellular acidification rate; mAChR, muscarinic acetylcholine receptor; NMS, N-methylscopolamine

e) Recommended section: Cellular & Molecular

Abstract

The effects of prolonged exposure of M_2 muscarinic acetylcholine receptors (mAChRs), stably expressed in CHO cells, to the allosteric modulators gallamine, alcuronium and heptane-1,7-bis (dimethyl-3′-phthalimidopropyl) ammonium bromide (C_7/3′-phth) were compared to the effects of the agonist, carbachol (CCh), and antagonists, atropine and N-methylscopolamine (NMS). Intact cell saturation binding assays using [^{3}H]NMS found that pretreatment of the cells for 24 hr with CCh caused a significant downregulation of receptor number, whereas atropine, NMS and all three allosteric modulators caused receptor upregulation. Functional assays using a cytosensor microphysiometer to measure whole-cell metabolic rate found no acute effects of gallamine on receptor signaling, whereas atropine appeared to behave as an inverse agonist. Pretreatment of the cells with gallamine (20 μM) or atropine (20 nM) resulted in a significant enhancement of the maximal effect evoked by CCh. In contrast, CCh (100 μM) pretreatment resulted in a significant reduction in maximal receptor signaling capacity. Time course experiments revealed that the effects of atropine and gallamine on receptor upregulation are only visualized after at least 12 hr ligand exposure, compared to the more rapid effects of CCh, which achieve steady state downregulation within 90 min. Additional experiments monitoring CCh-mediated M_2 mAChR internalization in the presence of gallamine revealed that part of the mechanism underlying the effects of the modulator on receptor expression may involve a change in receptor internalization properties. These findings suggest that, like orthosteric ligands,

GPCR allosteric modulators are also able to mediate long term effects on receptor regulation.

The majority of all known drugs mediate their therapeutic effects by targeting cell-surface receptors, of which the G protein-coupled receptor (GPCR) superfamily constitutes that largest group (Drews, 2000). The ability to selectively target drugs to various GPCRs has traditionally involved the design of ligands that act at the receptor's orthosteric binding site, that is, the binding site recognized by the endogenous ligand for that receptor (Neubig et al., 2003). However, it is now becoming accepted that many GPCRs can also possess at least one allosteric binding site, that is, a site topographically distinct from the orthosteric site that, when occupied by ligand, can modulate the binding and/or functional properties of orthosteric ligands (Christopoulos and Kenakin, 2002).

Allosteric modulators possess a number of theoretical advantages over orthosteric drugs. For instance, they can either inhibit or potentiate ligand binding affinity and/or function. This is in contrast to orthosteric ligands, which can only act competitively (May and Christopoulos, 2003). Allosteric modulators also have the possibility of displaying greater subtype-selectivity for some receptors by recognizing binding domains that show high sequence divergence between receptor subtypes (Christopoulos, 2002), or by selectively exerting a cooperative effect on the binding of an endogenous orthosteric agonist at a single receptor to the exclusion of other subtypes (Lazareno et al., 2004). Many of these properties of allosteric modulators have been investigated in some detail using the using muscarinic acetylcholine receptor (mAChR) family as a model GPCR system (Lee and El-Fakahany, 1991; Tucek and Proska, 1995; Birdsall et al., 1996; Ellis, 1997;

Christopoulos et al., 1998; Holzgrabe and Mohr, 1998). In particular, the M_2 mAChR is well-established as possessing a high affinity for a variety of structurally diverse modulators that, nevertheless, appear to interact at a common allosteric binding site (Ellis and Seidenberg, 1992; Lanzafame et al., 1997; Tränkle et al., 1998).

In addition to the acute effects of both orthosteric and allosteric drugs on mAChR function, the cellular host system itself can exert a profound effect on the function of the receptor when the latter is exposed to a drug for prolonged periods of time. For example, it is widely accepted that upon prolonged agonist exposure, mAChRs, like most other GPCRs, undergo signal attenuation by mechanisms commonly referred to as desensitization, sequestration and/or downregulation (Bunemann et al., 1999). More recently, inverse agonist ligands have also been demonstrated to exert profound effects on GPCR regulation and expression after prolonged exposure, although in a manner opposite to that seen with agonists; a common observation upon prolonged inverse agonist treatment is an upregulation in GPCR expression levels (Milligan and Bond, 1997). Interestingly, although allosteric modulators of GPCRs are now being recognized as potentially novel therapeutic agents, surprisingly little is known about their long term effects on receptor function, even though this information is vital if these drugs are to be used clinically.

Given that allosteric modulators engender potentially unique receptor conformations by binding to domains that are distinct from those of orthosteric agonists and inverse agonists, the aim of the present study was to use the human M_2

mAChR as a model system to investigate the effects of long-term exposure of allosteric modulators on the cell-surface expression and the signaling properties of this receptor, and to gain some insight into the mechanisms underlying these effects. We report, for the first time, that prolonged exposure to three different allosteric modulators of M_2 mAChRs results in a significant enhancement of cell-surface receptor expression that may reflect the ability of the modulators to promote a receptor conformation that displays modified internalization properties.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), geneticin and trypsin were obtained from GIBCO (Gaithersburg, USA). (-)-[N-methyl-^{3}H]scopolamine methyl chloride (70-87Ci/mmol) was from Du Pont-New England Nuclear (Boston, USA). Bio-Rad Protein Assay Kit was obtained from Bio-Rad (California, USA). Ultima Gold was from Packard (Greningen, Netherland). Alcuronium was a generous gift from Hoffmann-La Roche (Basel, Switzerland), and heptane-1,7-bis-(3'-phthalimidopropyl)-ammonium bromide (C_7/3-phth) was synthesized at the Institute of Drug Technology (IDT; Boronia, Victoria, Australia). All other chemicals and reagents were from Sigma Chemical Company (St Louis, USA).

Cell Culture

CHO-K1 cell lines stably transfected with the human M_2 mAChR (CHO M_2 cells; provided by Dr. Mark Brann, University of Vermont Medical School), were cultured in DMEM supplemented with 10% foetal bovine serum, 20 mM HEPES and 50 μg/ml geneticin. Cells were grown at 37°C in 5% CO_2: 95% O_2. At approximately 90% confluence, cells were harvested by trypsinization followed by centrifugation (400 × g, 3 min) and re-suspension (three times) in HEPES buffer (110 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM glucose, 20 mM HEPES, 58 mM sucrose; pH 7.4 with NaOH). Cells between passages 6 to 20 were used in all experiments, unless otherwise indicated.

Saturation Binding Assays

Two different types of saturation binding assays were performed: (i) Complete saturation binding isotherms, using increasing concentrations of the radiolabeled muscarinic antagonist, [^{3}H]NMS (0.02 nM – 5 nM), were constructed in intact CHO M_2 cells. This assay utilized approximately 10^5 cells per assay tube, made up in a final volume of 1 ml HEPES buffer, with 10 μM atropine used to determine non-specific binding. For initial control experiments, cells were incubated at 37°C for 1 hr in a shaking water bath. In subsequent experiments, cells were pretreated with a fixed concentration (approximately 10 x K_B; see Results) of carbachol (CCh), atropine or gallamine, at 37°C for 24 hr (unless otherwise indicated in Results), prior to extensive washing on ice to remove pretreatment ligand while preventing any receptor cycling. The system was then allowed to equilibrate with radioligand for 3 hr at 4°C, and the reaction was then terminated by rapid vacuum filtration through Whatman GF/C filters using a Brandel Cell Harvester, followed by 3 washes of ice cold 0.9% sodium chloride buffer. Control experiments (not shown) indicated that 3 hr was sufficient for binding equilibrium to be achieved at 4°C. Filters were then dried, placed in scintillation vials, 4 mL/vial of Packard Ultima Gold scintillant was added and the vials were left to sit for at least 3hr. Radioactivity was then determined by scintillation counting. (ii) A "two-point" saturation binding assay was also performed on CHO M_2 cells. This assay is a modified saturation binding assay where only two concentrations, 0.1 nM and 1 nM, of [^{3}H]NMS are used, allowing a calculation of cell surface receptor density ($B_{max;}$ see also Data Analysis). This assay was performed in triplicate and utilized 2×10^5 cells per tube in a total

volume of 1 mL HEPES buffer. Cells were initially pretreated with a fixed concentration (approximately 10 x K_B ; see Results) of orthosteric ligand (CCh, atropine or NMS), allosteric modulator (gallamine, C_7/3-phth or alcuronium), or a combination of NMS and alcuronium, at 37°C for 24 hr (unless otherwise indicated in Results), prior to extensive washing on ice. Due to a loss of activity over time (presumably due to breakdown), the C_7/3-phth and alcuronium were replaced every 6 hr. All other details are as described above for the complete saturation binding experiments.

Inhibition Binding Assays

In initial experiments, approximately 2 × 10^5 CHO M_2 cells in 1 mL HEPES buffer/tube were incubated with a fixed concentration of [^{3}H]NMS (0.2 nM) in the absence or presence of increasing concentrations of CCh (10 nM-3 mM), atropine (3 pM-3 μM) or NMS (0.1 pM-0.1 μM). Reactions were allowed to proceed for 1 hr at 37°C, with non-specific binding defined using 10 μM atropine. Reaction termination and determination of radioactivity were as described in the preceding section.

To determine the equilibrium binding parameters describing the interaction between the agonist, CCh, the allosteric modulator, gallamine, and the radioligand, [^{3}H]NMS, additional combination inhibition binding experiments were undertaken. Cells were incubated with a fixed concentration of [^{3}H]NMS (0.2 nM) in the absence or presence of increasing concentrations of CCh alone (0.1μM–10mM), gallamine alone (10 nM – 1 mM) or gallamine (10 nM – 1 mM) together with fixed concentrations of CCh (30

μM or 200 μM for 3 experiments; 100 μM or 1 μM for 3 experiments). Incubations (10^5 cells per tube; 1 mL total volume) were for 3 hr at 4°C. Reactions were terminated and radioactivity determined as described above.

Receptor Internalization Assay

The experimental protocol used to monitor the internalization of cell-surface M_2 mAChRs was modified from that of Pals-Rylaarsdam et al. (1997). Approximately 10^5 cells/well were distributed in 24-well plates and allowed to reach confluence over 24 hr. Subsequently, cells were exposed to CCh (100 μM), gallamine (20 μM), atropine (20 nM) or a combination of CCh and gallamine for various time points (see Results) at 37°C before being washed 5 times with ice-cold HEPES buffer. Cells were then incubated at 4°C, in order to arrest receptor cycling, with a near-saturating concentration of [^{3}H]NMS (2 nM) for 3 hr to measure the remaining accessible cell-surface receptors. Non specific binding was determined using 10 μM atropine. At the end of the 3 hr incubation period, buffer and drugs were aspirated and the wells washed 3 times. Cells were solubilized from the wells with 2 x 0.5 ml of 0.2 M NaOH, and both fractions were then combined and radioactivity determined by scintillation counting as described above.

Cytosensor Microphysiometer Assay

Approximately 2×10^5 cells were plated into sterile capsule cups (12 mm diameter, 3.0μm pore) in a 12-well plate 24 hr prior to use. At the same time as plating, the cells were exposed to various drug or vehicle pretreatment conditions as indicated in

the Results. At the end of the 24 hr pretreatment, cells were loaded into the sensor chambers of a cytosensor microphysiometer (Molecular Devices, Monlo Park, CA, U.S.A.) and superfused with medium (bicarbonate-free DMEM, pH 7.4) until the cellular acidification rate was steady (~30 min). Increasing concentrations of CCh (1nM to 1 mM) were then superfused across each chamber at a pump speed of 100µl/min in order to construct a cumulative concentration-response curve. Each pump cycle was of 1 min 30 s duration, with drug being perfused for 1 min and then the pump being switched off for the remaining 30 s. Recordings of extracellular pH were made from 1 min 8 s to 1min 28 s of each cycle and the extracellular rate of acidification (ECAR; mV s^{-1}/change in pH units) was calculated using the Cytosoft program (Molecular Devices).

Data Analysis

Both total and non-specific saturation binding datasets were globally fitted to the following equation via nonlinear regression using Prism 4.0 (GraphPad Software, San Diego, CA, USA):

$$Y=\frac{B_{max.}[A]}{K_A+[A]}+NS\cdot[A] \qquad (1)$$

where Y denotes the total binding, [A] the concentration of [^{3}H]NMS, B_{max} is the maximum number of binding sites, K_A is the equilibrium dissociation constant of [^{3}H]NMS, and NS is the fraction of the total binding that represents non-specific binding. This latter parameter was shared between both total and non-specific binding datasets. B_{max} values from the "two-point" saturation binding experiments

were calculated using the following equation (Lazareno and Birdsall, 1995; Christopoulos, 2000):

$$B_{max} = \frac{[B1][B2]([A1]-[A2])}{[A1][B2]-[A2][B1]} \qquad (2)$$

where B_{max} is as previously described, [A1] and [A2] are the low and high concentrations of [^{3}H]NMS used, and [B1] and [B2] are the corresponding specific binding counts. It should be noted that this approach provides reliable estimates of the B_{max}, relative to estimates obtained from complete saturation binding curve analysis, provided that the experimental error is ≤ 10% CV (A. Christopoulos, unpublished), as is normally the case for [^{3}H]NMS binding at mAChRs. These experiments also allowed for a calculation of the equilibrium dissociation constant, K_A, of [^{3}H]NMS after the various treatment conditions using the following equation (Lazareno and Birdsall, 1999):

$$K_A = \frac{[A1][A2]([B2]-[B1])}{[B1][A2]-[B2][A1]} \qquad (3)$$

Inhibition binding assays

Normalized orthosteric ligand competition binding data were fitted to the following equation according to a simple mass-action model for competition for one binding site:

$$Y = \frac{100.([A]+K_A)}{[A]+K_A.\left(1+[B]/K_B\right)} \qquad (4)$$

where [B] denotes the concentration of orthosteric ligand present, K_B the equilibrium dissociation constant for the orthosteric ligand, and Y, [A], K_A are as previously defined.

For the combination experiments between the radioligand, [^{3}H]NMS, allosteric modulator, gallamine, and unlabelled orthosteric ligand, CCh, datasets were globally fitted to the following extended allosteric ternary complex model (Fig. 1; see Christopoulos, 2000; Christopoulos and Kenakin, 2002):

$$Y = \frac{100 \cdot \left([A] + K_A\right)}{[A] + K_{App}} \qquad (5)$$

with

$$K_{App} = \frac{K_A K_B}{\alpha \cdot [B] + K_B} \cdot \left[1 + \frac{[I]^{slope}}{K_I} + \frac{[B]}{K_B} + \frac{\beta \cdot [I]^{slope} \cdot [B]}{K_I \cdot K_B} \right] \qquad (6)$$

where [A], [B] and [I] denote the concentrations, and K_A, K_B and K_I denote the equilibrium dissociation constants, of the radioligand, allosteric modulator and unlabeled orthosteric ligand, respectively, α denotes the cooperativity factor for the allosteric interaction between the radioligand and the allosteric modulator, β denotes the cooperativity factor for the allosteric interaction between the unlabeled orthosteric ligand and the allosteric modulator, and the parameter, *slope,* denotes a logistic slope factor for the binding of the unlabeled orthosteric ligand. According the allosteric ternary complex model (Ehlert, 1988; Lazareno and Birdsall, 1995;

Christopoulos, 2002) the cooperativity factor is a measure of the extent by which the affinity of one ligand is modified by the concomitant binding of another ligand on the same receptor. In this model, values of α and β greater than 1 denote positive cooperativity, whereas values less than 1 denote negative cooperativity. For this analysis, the K_A parameter was fixed to a constant value, as determined separately in [^{3}H]NMS saturation experiments.

For the receptor internalization experiments, the kinetics of loss of cell-surface [^{3}H]NMS binding were analyzed according to the following two-phase exponential decay model:

$$Y=\text{Span1}\cdot e^{-k_1\cdot t}+\text{Span2}\cdot e^{-k_2\cdot t}+\text{Plateau} \qquad (7)$$

where Span1 and Span2 denote the percentage of each phase, Plateau denotes the minimal asymptotic value, and k_1 and k_2 denote the rate constants for the components defined by Span1 and Span2, respectively. An extra-sum-of-squares (F-test) was used to determine whether the data were significantly better fitted to this model as compared to a simpler model characterized by a single Span and k value.

Agonist concentration-response data from the cytosensor assays were consistently characterized by a marked decline in maximal agonist response at high agonist concentrations (see Results). Thus, to obtain a close model fit to the data and derive estimates of agonist potency and maximal response range, the following Gaussian

curve equation was used (Christopoulos et al., 2001; Motulsky and Christopoulos, 2004):

$$E = Basal + Range \cdot e^{-\left[\frac{10^{Log[A]} - midA}{slope}\right]^2} \qquad (8)$$

with

$$midA = LogEC_{50} + slope\sqrt{-\ln(0.5)} \qquad (9)$$

where E denotes effect, Basal denotes the minimum asymptotic effect in the absence of agonist, Log[A] the logarithm of the concentration of agonist, slope, a slope factor, $LogEC_{50}$ the logarithm of the midpoint location parameter, and Range denotes the maximal response range over the Basal value.

In practice, all affinity, potency and cooperativity parameters were determined as logarithms (Christopoulos, 1998). In all instances, results are expressed as mean ± S.E.M. Statistical analyses were performed by one-way ANOVA, with $p < 0.05$ indicating statistical significance.

JPET Fast Forward. Published on August 27, 2004 as DOI: 10.1124/jpet.104.073767
This article has not been copyedited and formatted. The final version may differ from this version.

Results

Determination of ligand binding properties in intact CHO M_2 cells.

Initial saturation binding experiments were performed to determine the affinity of [^{3}H]NMS for the human M_2 mAChR in intact CHO M_2 cells. The data were well described by a standard one-site hyperbolic binding model, with a calculated value of Log K_A being –9.60 ± 0.01 and B_{max} being 5.50 ± 1.10 fmol/10^5 cells (n = 3). Subsequent inhibition binding experiments were performed to determine the affinity (Log K_B) of a variety of test compounds for the M_2 mAChR, as this information was required to inform the design of experiments investigating the effects of prolonged ligand exposure. The results of these characterization experiments for the orthosteric agonist, CCh, and the orthosteric antagonists, atropine and NMS, are shown in Table 1, together with affinity and cooperativity values for the allosteric modulators gallamine, alcuronium and C_7/3-phth derived from a recent study by our group utilizing the same cells under identical experimental conditions (Avlani et al., 2004). It should be noted that the Log K_B value for CCh is only an apparent measure of agonist affinity, as CCh can cause internalization under these assay conditions (see below). In all instances of competition binding, the curve slope factors were not significantly different from unity.

Effects of prolonged ligand exposure on M_2 mAChR cell surface expression.

Fig. 2A illustrates the effects of 24 hr pretreatment of CHO M_2 cells with CCh, atropine or gallamine (at approximately 10 x K_B values from Table 1) on the binding

properties of [^{3}H]NMS. In comparison to vehicle controls, pretreatment of CHO M_2 cells with the agonist, CCh, led to a significant decrease in cell surface expression of the M_2 mAChR (Table 2). In contrast, the orthosteric antagonist atropine and, interestingly, the allosteric modulator, gallamine, each caused a significant increase in the [^{3}H]NMS B_{max} value after 24 hr exposure (Table 2). No effects were observed on radioligand affinity (Table 1). Furthermore, the Log K_A values determined for [^{3}H]NMS from these experiments were not significantly different ($p > 0.05$) between any of the groups and the vehicle controls, indicating that the washing protocol utilized was sufficient to fully remove the pretreatment ligands from the receptor compartment.

Specificity of ligand pretreatment effects on M_2 mAChR cell surface expression.

To investigate the effect of prolonged pretreatment of CHO M_2 cells with a wider variety of allosteric and orthosteric ligands, we utilized a "two-point" saturation binding assay, which specifically allows for the calculation of radioligand B_{max} values without the need to construct complete saturation binding curves. The results of these experiments are shown in Fig. 2B, where significant ($p < 0.05$) increases in receptor cell surface expression were revealed after pretreatment with the potent antagonist, NMS, the allosteric inhibitor, C_7/3'-phth, or the allosteric enhancer, alcuronium, as well as with atropine or gallamine. In addition, the combination of NMS and alcuronium, which is normally characterized by positive binding cooperativity at M_2 mAChRs (e.g., Avlani et al., 2004), also resulted in a significant enhancement of receptor cell surface expression, although this was no greater than

that observed with either ligand used alone. As before, pretreatment with CCh led to a significant decrease in cell surface M_2 mAChR expression. In all instances, there was no significant effect of pretreatment on calculated [^{3}H]NMS Log K_A (see Fig.2B legend).

Effects of prolonged ligand exposure on M_2 mAChR function.

The functional consequence of M_2 mAChR activation after various pretreatment conditions was determined via microphysiometric measurements of ligand-mediated changes on whole cell ECAR. The presence of increasing concentrations of gallamine did not cause any significant change in ECAR, in contrast to the clear stimulation observed with CCh (Fig. 3A). Interestingly, atropine caused a small, but significant ($p < 0.05$), concentration-dependent decline in basal ECAR, with a Log EC_{50} of -7.3 ± 0.4; this was not due to a pH change in the highest concentrations of antagonist used, as this was routinely adjusted to pH 7.4 for all concentrations. In contrast to the effects of acute exposure of the CHO cells to gallamine, 24 hr pretreatment with the modulator prior to washout caused a significant effect on cellular responsiveness, as evidenced by changes in the concentration-response profile for CCh between the various pretreatment groups. These experiments are summarized in Fig. 3B and Table 3. Although no drug pretreatment resulted in a change in CCh potency relative to vehicle-pre-treated cells, significant differences were noted for the maximal agonist response in cells pre-treated with CCh, atropine or gallamine. These effects on response range were in accord with the effects seen on

receptor cell surface expression in the binding assays, with atropine and gallamine causing an increase in agonist responsiveness, whereas pretreatment with CCh caused a significant decrease in subsequent responsiveness.

Time course of ligand effects on M_2 mAChR cell surface expression.

Further experiments were performed to investigate the time course of the effect of pretreatment with CCh, atropine or gallamine on cell-surface M_2 mAChR expression. As shown in Fig. 4, CCh clearly caused maximal reduction of receptor expression within 3 hr, whereas atropine and gallamine only began to cause an enhancement in cell surface expression from 12 hr, which was statistically significant ($p < 0.05$) at 24 hr.

It is possible that the effects of prolonged exposure to gallamine lead to a long-term stabilization of a conformation of the M_2 mAChR on the cell surface that is less prone to (agonist-independent) internalization, as has previously been shown for inverse agonist ligands (e.g., Gether et al., 1997). The fact that gallamine occupies a topographically distinct binding site on the M_2 mAChR to that utilized by CCh provided us with a unique opportunity to further investigate the effects of the modulator on actual agonist-driven internalization, which is not possible when studying classic orthosteric antagonist/inverse agonist effects on receptor trafficking. In order to perform such experiments, however, it was first necessary to determine the occupancy-binding relationships for CCh and gallamine, both alone and combined, at the M_2 mAChR, in order to account for the negative cooperativity

between the two ligands when they concomitantly occupy the receptor. Fig. 5 shows a family of inhibition binding curves that are representative of this type of experiment. The curves of the inhibition of [^{3}H]NMS binding by CCh alone, gallamine alone and gallamine in the presence of two different concentrations of CCh were simultaneously fitted to an extended ternary complex model of allosteric interaction (Equations 3-5) as described in the Methods. The results from these experiments and accompanying analysis are summarized in Table 4. Although the estimates of both CCh and gallamine dissociation constants are approximately half a log unit lower than those shown in Table 1, this is most likely due to the fact that the combination experiments were performed at 4°C, in order to ensure that the estimate of CCh affinity, in particular, is not influenced by any concomitant internalization that the agonist may cause at 37°C. Importantly, these inhibition binding experiments allowed for the determination of β, the co-operativity factor describing the allosteric interaction between CCh and gallamine, which indicated that there was an approx. 200-fold reciprocal reduction in ligand affinity when both CCh and gallamine bound to the M_2 mAChR at the same time.

Based on the results shown in Table 4, 100 μM CCh was calculated to occupy approximately 80% of the M_2 mAChRs, and this concentration was chosen for further receptor internalization studies. In order to measure the effect on internalization of the same level of receptor occupancy by CCh in the presence of gallamine, however, the concentration of CCh that was needed was calculated to be 250 μM when combined with 10 μM gallamine. Under these conditions, the

modulator will occupy approximately 13% of the receptors, which is equivalent to the receptor occupancy of 1 μM gallamine in the absence of CCh. The results of these internalization experiments are shown in Fig. 6. In the presence of 100 μM CCh alone, internalization resulted in an approximately 90% loss of cell-surface [^{3}H]NMS binding within 90 mins, and was characterized by two exponential phases (k_1 = 0.82 ± 0.30 min^{-1}; 34 ± 8%; k_2 = 0.07 ± 0.01 min^{-1}; 56 ± 7%; n = 4), as determined by F-test (F= 8.4; $p < 0.001$). Repeating these experiments using 1 mM CCh resulted in almost identical observations (data not shown), indicating that maximal internalization is attained at 100 μM CCh. In the presence of both CCh and gallamine, internalization appeared to be better described by a monophasic function (0.15 ± 0.02 min^{-1}; n= 4), as determined by F-test (F=2.9; $p > 0.05$); this finding would indicate a significant reduction in the extent of internalized receptors being observed at 45 min onward.

It is also possible, however, that the two phases of internalization observed in the absence of gallamine are retained in the presence of the modulator, but that one or other of the amplitudes is reduced such that the F-test cannot statistically resolve a biphasic fit over a (simpler) monophasic fit. To explore this possibility, the internalization data were re-fitted with the two rate constants constrained to be shared between datasets while the amplitudes (Span values) were allowed to vary; this represents a conservative hypothesis that assumes gallamine has no effect on the rate of CCh internalization. The results of this analysis are also shown in Fig. 6 (dashed lines), and were characterized by the following parameters: k_1 = 0.83 ± 0.37

min^{-1}; k_2 = 0.07 ± 0.02 min^{-1}; Span 1 (Control) = 33 ± 8%; Span 1 (+ Gallamine) = 16 ± 8%; Span 2 (Control) = 56 ± 7%; Span 1 (+ Gallamine) = 59 ± 7%. Thus, in contrast to the initial analysis, the data can also be adequately described by assuming that gallamine had no effect on the rate of CCh internalization, but rather reduced the amplitude of the fast phase (Span 1). A comparison of the two analytical methods by F-test found a statistical preference for the initial model (biphasic internalization converted to a monophasic internalization by gallamine), with F= 2.9 and $p > 0.05$, but it can be seen from visual inspection of Fig.6 that the data do not allow for an unambiguous discrimination between the two models.

Discussion

The major finding of this study is that prolonged exposure to allosteric modulators can cause upregulation of cell-surface M_2 mAChR expression and cellular responsiveness. These effects are independent of the well-documented actions of acute modulator administration on orthosteric ligand binding affinity, since both allosteric antagonists (gallamine, C_7/3-phth) and an enhancer (alcuronium) of NMS binding affinity have the same effect on cell-surface receptor expression. Investigation of the potential mechanism underlying these effects, using gallamine as a prototypical example of an mAChR modulator, suggests that the modulators are able to promote a receptor conformation that possesses modified internalization properties.

The initial experiments described in this study directly assessed cell-surface receptor expression using saturation binding with the hydrophilic antagonist, [^{3}H]NMS. In agreement with previous reports (Pitcher et al., 1998; Tsuga et al; 1998 Bunemann et al., 1999), pretreatment of CHO M_2 cells with the agonist, CCh, for 24 hr significantly downregulated the maximal number of cell-surface receptors. In contrast, pretreatment with the antagonists, atropine or NMS, or the modulators gallamine, alcuronium or C_7/3-phth had the opposite effect, resulting in a significant upregulation of M_2 mAChRs after 24 hr. Interestingly, combination of NMS and alcuronium, which are known to allosterically enhance each other's binding to the M_2 mAChR, did not produce any greater effect than either compound alone; this

may reflect a "ceiling" to the upregulation that can be achieved at high degrees of receptor occupancy. Since both atropine and NMS have previously been shown to possess inverse agonist activity at M_2 mAChRs (Jakubík et al., 1995), the effects of these ligands in our study can most readily be reconciled with the fact that inverse agonists are known to cause GPCR upregulation, presumably by stabilizing a conformational state of the receptor that is less prone to downregulation (Gether et al., 1997; Milligan and Bond, 1997). The effect of the allosteric modulators, however, is novel. By their very nature, these ligands bind to a common site on the M_2 mAChR that is topographically distinct from that recognized by orthosteric ligands, and engender a conformation that is negatively cooperative towards the binding of the endogenous agonist, acetylcholine (Lazareno and Birdsall, 1995; Christopoulos 2000). However, gallamine and alcuronium may also show positive cooperativity towards the binding of G proteins under certain experimental conditions (Jakubík et al., 1996; 1998), although we have not observed any evidence for such an effect in our studies (Fig. 3A). Our current findings now also suggest that these modulator-induced conformations can also be "inverse agonist-like" with respect to their effects on regulation of receptor number. Indeed, Zahn et al. (2002) have previously demonstrated inverse agonism for alcuronium in an assay of M_2 mAChR-mediated [^{35}S]GTPγS turnover. Overall, our observations provide further support to the concept that efficacy at GPCRs encompasses a broader spectrum of receptor behaviors than the classic activation-deactivation paradigm (Kenakin, 2002).

To our knowledge, only one other study has investigated the potential for long term effects of allosteric modulators on Family A GPCRs. Bhattacharya and Linden (1996) found that 24 hr pretreatment of A_1 adenosine receptors with the allosteric enhancer, PD 81,723, had no effect on the maximal density of binding sites recognized by either agonist or antagonist radioligands in membrane binding assays. However, they did note a small downregulation of antagonist-labeled sites in intact cells, which was more pronounced when an agonist radioligand was used. These findings were reconciled with the fact that PD 81,723 appears to have a small propensity to mediate receptor-G protein coupling in its own right, and thus produce "agonist-like" effects on receptor regulation. Since we have found no evidence for acute effects of these allosteric modulators on signaling under our experimental conditions, we have no reason to expect a similar effect to that observed by Bhattacharya and Linden (1996) at the A_1 receptor.

The cytosensor microphysiometer was used in our study to measure integrated whole-cell functional responses as an index of receptor activation, both acutely and after prolonged ligand exposure. As expected, CCh caused an increase in ECAR, although this was characterized by a decline at the highest concentrations of agonist used, presumably due to acute desensitization. Interestingly, atropine was also able to mediate a response, but opposite to that observed with CCh. This observation may be indicative of atropine behaving as an inverse, in agreement with previous studies (Jakubík et al., 1995; Zahn et al., 2002). However, the potency of atropine as an inverse agonist in the cytosenor experiments was approx. 25-fold weaker than its

Log K_B value shown in Table 1. This is at odds with the classic expectation that an inverse agonist's potency should approximate its receptor affinity. It is possible that the reduced potency of atropine as an inverse agonist in our hands reflects nonlinear stimulus-response coupling in the CHO cells, which would be expected to increase agonist potency while decreasing inverse agonist potency.

The cytosensor experiments also addressed the consequences of prolonged cellular exposure to different mAChR ligands on the subsequent ability of the receptor to signal. Pretreatment with CCh resulted in a marked attenuation of receptor signaling, consistent with the findings of the saturation assays and indicative of receptor downregulation. In contrast, pretreatment of the cells with gallamine and atropine caused a significant enhancement in the maximal agonist-mediated response. These experiments thus provided functional evidence for an upregulation of M_2 mAChRs in response to atropine, an inverse agonist, and gallamine, an allosteric modulator.

Collectively, the effects of gallamine, C_7/3-phth or alcuronium pretreatment on saturation binding, and gallamine pretreatment on cellular function, suggested a role for allosteric modulators in regulating receptor trafficking. The final series of experiments in our study were performed in order to gain insight into potential mechanisms mediating the phenomenon. The time for achieving the maximal effect of CCh on receptor expression was much faster than the effects of either atropine or gallamine, which required at least 12 - 24 hr (Fig. 5). This may suggest that the

maximal increase in receptor cell surface expression in the presence of atropine or gallamine reflects a slowing of agonist-independent receptor cycling such that a new steady-state cell surface expression is achieved, with/without additional *de novo* protein synthesis.

In addition to studying the effects of gallamine alone on receptor regulation, the ability to monitor CCh-mediated internalization in the concomitant presence of gallamine was a particularly advantageous experimental manipulation. This was possible due to the lack of mutual exclusivity in binding between orthosteric and allosteric sites. However, because internalization is occupancy-driven, it was first necessary to account for the negative allosteric effect that gallamine and CCh exert on each other's binding affinities in order to ensure that the occupancy of the orthosteric site by the agonist remained the same in internalization assays conducted in the presence of gallamine to those conducted in its absence. The relevant binding parameters for determining these occupancy relationships were obtained from separate inhibition binding experiments. At equilibrium, 100 μM CCh was calculated to occupy approximately 80% of the receptors; in the presence of 10 μM of gallamine, the concentration of CCh thus needed to be increased to 250 μM for equivalent occupancy. Similarly, 10 μM of gallamine in presence of 100 μM of CCh gave the same occupancy (13%) as that calculated for 1 μM gallamine alone. However, there are two caveats to this approach. First, these latter binding experiments were performed at 4°C, whereas the internalization was performed at 37°C. This is an unavoidable consequence of radioligand binding assays using intact

CHO cells, since the receptor will internalize to agonist during the time course of the assay if it were performed at 37°C. Second, it would have been desirable to have attained a higher degree of receptor occupancy by the modulator in the internalization experiments, but the high negative cooperativity between gallamine and CCh made this impracticable due to the high concentrations of CCh that would have been required to maintain 80% receptor occupancy under such conditions. Nevertheless, the results from our internalization experiments are consistent with previous observations that M_2 mAChRs display a biphasic mode of loss of cell-surface receptors in response to agonist. Koening and Edwardson (1996) have demonstrated that this biphasic response reflects the composite effects of receptor internalization and recycling. Our analysis suggests that the ability of gallamine to up-regulate cell-surface receptor expression possibly involved a loss of the initial fast component of agonist-driven internalization, although we are currently unable to conclude whether this is also accompanied by a significant change in the internalization rate.

In conclusion, our results suggest that allosteric modulators change the trafficking properties of M_2 mAChRs by a mechanism that may involve a modification in receptor internalization properties. These changes in receptor trafficking can be manifested at both the level of ligand binding as well as the level of cellular responsiveness. Since allosteric modulators of GPCRs are now being targeted as novel therapeutic entities (Christopoulos, 2002), the present study has provided

important insight into some of the consequences of prolonged exposure to these types of agents, as would be expected, for example, in a chronic dosing regimen.

References

Avlani VA, May LT, Sexton PM and Christopoulos A (2004) Application of a kinetic model to the apparently complex behaviors of negative and positive allosteric modulators of muscarinic acetylcholine receptors. *J Pharmacol Exp Ther* **308**:1062-1072.

Bhattacharya S and Linden J (1996) Effects of long-term treatment with the allosteric enhancer, PD81,723, on Chinese hamster ovary cells expressing recombinant human A_1 adenosine receptors. *Molecular Pharmacology* **50**:104-111.

Birdsall NJ, Lazareno S and Matsui H (1996) Allosteric regulation of muscarinic receptors. *Prog. Brain Res.* **109**:147-151.

Bunemann M, Lee KB, Pals-Rylaarsdam R, Roseberry AG and Hosey MM (1999) Desensitization of G-protein-coupled receptors in the cardiovascular system. *Annu. Rev. Physiol.* **61**:169-192.

Christopoulos A (1998) Assessing the distribution of parameters in models of ligand-receptor interaction: to log or not to log. *Trends in Pharmacological Sciences* **19**:351-357.

Christopoulos A (2000) Quantification of allosteric interactions at G protein-coupled Receptors using radioligand binding assays, in *Current Protocols In Pharmacology* (Enna SJ ed) pp 1.22.21-21.22.40, Wiley and Sons, NY.

Christopoulos A (2002) Allosteric binding sites on cell-surface receptors: novel targets for drug discovery. *Nat Rev Drug Discov* **1**:198-210.

Christopoulos A, Grant MK, Ayoubzadeh N, Kim ON, Sauerberg P, Jeppesen L and El-Fakahany EE (2001) Synthesis and pharmacological evaluation of dimeric muscarinic acetylcholine receptor agonists. *J Pharmacol Exp Ther* **298**:1260-1268.

Christopoulos A and Kenakin T (2002) G Protein-coupled receptor allosterism and complexing. *Pharmacol Rev* **54**:323-374.

Christopoulos A, Lanzafame A and Mitchelson F (1998) Allosteric interactions at muscarinic cholinoceptors. *Clin. Exp. Pharmacol. Physiol.* **25**:184-194.

Drews J (2000) Drug discovery: a historical perspective. *Science* **287**:1960-1964.

Ehlert FJ (1988) Estimation of the affinities of allosteric ligands using radioligand binding and pharmacological null methods. *Mol. Pharmacol.* **33**:187-194.

Ellis J (1997) Allosteric binding sites on muscarinic receptors. *Drug Dev. Res.* **40**:193-204.

Ellis J and Seidenberg M (1992) Two allosteric modulators interact at a common site on cardiac muscarinic receptors. *Mol. Pharmacol.* **42**:638-641.

Gether U, Ballesteros JA, Seifer R, Sanders-Bush E, Weinstein H and Kobilka BK (1997) Structural instability of a constitutively active G protein-coupled receptor. Agonist-independent activation due to conformational flexibility. *J. Biol. Chem.* **272**:2587-2590.

Holzgrabe U and Mohr K (1998) Allosteric modulators of ligand binding to muscarinic acetylcholine receptors. *Drug Disc. Today* **3**:214-222.

Jakubík, J., Bacakova L, Lisa V, El-Fakahany EE and Tucek S (1996) Activation of muscarinic acetylcholine receptors via their allosteric binding sites. *Proc. Natl. Acad. Sci. USA* **93**:8705-8709.

Jakubík J, Bacaková L, El-Fakahany EE and Tucek S (1995) Constitutive activity of the M_1-M_4 subtypes of muscarinic receptors in transfected CHO cells and of muscarinic receptors in the heart cells revealed by negative antagonists. *FEBS Letters* **377**:275-279.

Jakubík J, Haga T and Tucek S (1998) Effects of an agonist, allosteric modulator, and antagonist on guanosine-g-[^{35}S]thiotriphosphate binding to liposomes with varying muscarinic receptor/G_o protein stoichiometry. **54**:899-906.

Kenakin T (2002) Drug efficacy at G protein-coupled receptors. *Annu Rev Pharmacol Toxicol* **42**:349-379.

Koenig JA and Edwardson JM (1996) Intracellular trafficking of the muscarinic acetylcholine receptor: Importance of subtype and cell type. *Mol. Pharmacol.* **49:** 351-359.

Lanzafame A, Christopoulos A and Mitchelson F (1997) Three allosteric modulators act at a common site, distinct from that of competitive antagonists, at muscarinic acetylcholine M_2 receptors. *J. Pharmacol. Exp. Ther.* **282**:278-285.

Lazareno S and Birdsall NJM (1995) Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: interactions of strychnine and acetylcholine at muscarinic receptors. *Mol. Pharmacol.* **48**:362-378.

Lazareno S and Birdsall NJM (1999) Measurement of competitive and allosteric interactions in radioligand binding studies in *G Protein-Coupled Receptors* (Haga T and Berstein G, eds) pp 1-48, CRC Press, Boca Raton, FL.

Lazareno S, Dolezal V, Popham A and Birdsall NJM (2004) Thiochrome enhances acetylcholine affinity at muscarinic M_4 Receptors: Receptor subtype selectivity via cooperativity rather than affinity. *Mol Pharmacol* **65**:257-266.

Lee NH and El-Fakahany EE (1991) Allosteric antagonists of the muscarinic acetylcholine receptor. *Biochem. Pharmacol.* **42**:199-205.

May LT and Christopoulos A (2003) Allosteric modulators of G-protein-coupled receptors. *Curr Opin Pharmacol* **3**:551-556.

Milligan G and Bond RA (1997) Inverse agonism and the regulation of receptor number. *Trends in Pharmacological Sciences* **18**:468-474.

Motulsky HJ and Christopoulos A (2004) *Fitting models to biological data using linear and nonlinear regression. A practical guide to curve fitting.* Oxford University Press, New York.

Neubig RR, Spedding M, Kenakin T and Christopoulos A (2003) International Union of Pharmacology Committee on Receptor Nomenclature and Drug Classification. XXXVIII. Update on Terms and Symbols in Quantitative Pharmacology. *Pharmacol Rev* **55**:597-606.

Pals-Rylaarsdam R, Gurevich VV, Lee KB, Ptasienski JA, Benovic JL and Hosey MM (1997) Internalization of the m2 muscarinic acetylcholine receptor. Arrestin-independent and -dependent pathways. *Journal of Biological Chemistry* **272**:23682-23689.

Pitcher JA, Freedman NJ and Lefkowitz RJ (1998) G protein-coupled receptor kinases. *Annual Review of Biochemistry* **67**:653-692.

Tränkle C, Mies-Klomfass E, Cid MHB, Holzgrabe U and Mohr K (1998) Identification of a [^{3}H]Ligand for the common allosteric site of muscarinic acetylcholine M_2 receptors. **54**:139-145.

Tsuga H, Kameyama K and Haga T (1998) Desensitization of human muscarinic acetylcholine receptor m2 subtypes is caused by their sequestration/internalization. **124**:863-868.

Tucek S and Proska J (1995) Allosteric modulation of muscarinic acetylcholine receptors. *Trends Pharmacol. Sci.* **16**:205-212.

Zahn K, Eckstein N, Trankle C, Sadee W and Mohr K (2002) Allosteric modulation of muscarinic receptor signaling: alcuronium- induced conversion of pilocarpine from an agonist into an antagonist. *J Pharmacol Exp Ther* **301**:720-728

Footnotes

This work was supported by Project Grant No. 251538 of the National Health and Medical Research Council of Australia (NHMRC). Arthur Christopoulos and Patrick Sexton are Senior Research Fellows of the NHMRC. Lauren May is a recipient of a Melbourne Research Scholarship.

Address for reprints

Dr. Arthur Christopoulos, NHMRC Senior Research Fellow, Department of Pharmacology, University of Melbourne, Grattan St., Parkville, 3010, Victoria, Australia; Ph: +613 8344 8417; Fax: +613 8344 0241; email: arthurc1@unimelb.edu.au

Figure Legends

Fig. 1 An extended allosteric ternary complex model. A, B and I denote the radioligand, allosteric modulator and unlabeled orthosteric ligand, respectively, and K_A, K_B and K_I denote their respective equilibrium dissociation constants; α denotes the cooperativity factor for the allosteric interaction between the radioligand and the allosteric modulator and β denotes the cooperativity factor for the allosteric interaction between the unlabeled orthosteric ligand. Values of α and β greater than 1 denote positive cooperativity, whereas values less than 1 denote negative cooperativity.

Fig.2 Cell-surface binding of [^{3}H]NMS to M_2 mAChRs, expressed in intact CHO cells, that had been pre-exposed to a variety of ligands for 24 hr at 37°C, prior to extensive washout on ice. (A) Saturation binding isotherms after pretreatment with vehicle (●), CCh 100 μM (□), atropine 20 nM (▽) or gallamine 20 μM (○). Data points represent the mean ± S.E.M of 3 – 6 experiments performed in triplicate at 4°C for 3 hr. (B) [^{3}H]NMS "two-point" B_{max} determinations after pretreatment with the orthosteric ligands CCh 100 μM, atropine 20 nM or NMS 2.5 nM, the allosteric modulators gallamine 20 μM, C_7/3-phth 20 μM or alcuronium 10 μM, or a combination of NMS 2.5 nM and alcuronium 10 μM. B_{max} values were normalized to those obtained using vehicle pretreated controls (mean = 4.95 ± 0.65 fmol/10^5 cells). Values of [^{3}H]NMS LogK_A were also calculated (see Materials and Methods) for each

of the treatments and were as follows: vehicle (-9.69 ± 0.28), CCh (-9.77 ± 0.23), atropine (-9.66 ± 0.25), NMS (-9.69 ± 0.28), gallamine (-9.54 ± 0.26), C_7/3-phth (-9.79 ± 0.18), alcuronium (-9.83 ± 0.25), NMS + alcuronium (-9.86 ± 0.15). Data represent the mean ± S.E.M of 4-8 experiments performed in triplicate at 4°C for 3 hr.

Fig. 3 (A) Extracellular acidification rates (ECAR) of CHO M_2 cells exposed to vehicle (●) or increasing concentrations of CCh (○), atropine (□) or gallamine (△). Individual pre-stimulated ECAR responses were normalized to 100% and are shown as the mean ± S.E.M of 4 experiments. (B) Effects of CCh on ECAR in CHO M_2 cells that had been pretreated for 24 hr with vehicle (●), 100 μM CCh (○), 20 nM atropine (□) or 20 μM gallamine (△) at 37°C, followed by extensive washout on ice. Data points represent ± S.E.M of 13-17 experiments.

Fig. 4 Time course for change in M_2 mAChR cell surface expression in intact CHO cells. CHO M_2 cells were pretreated with either 100 μM CCh (■), 20 nM atropine (▼) or 20 μM gallamine (▲) for the indicated times at 37°C, followed by extensive washing on ice. B_{max} values were calculated using "two-point" [^{3}H]NMS saturation binding performed at 4°C for 3 hr. Non-specific binding was defined using 10 μM atropine. B_{max} values were normalized to those obtained using vehicle pretreated controls (mean = 2580 ± 258 DPM). Each point represents the mean ± S.E.M of 7-10 experiments conducted in triplicate.

Fig. 5 Determination of the negative cooperativity between gallamine and CCh. CHO M_2 cells were incubated with a fixed concentration of [^{3}H]NMS (0.2 nM) for 3 hr at 4°C in the presence of increasing concentrations of CCh alone (●), gallamine alone (○) or gallamine together with fixed concentrations of CCh (100 μM □ or 1 mM ▽). Data are representative of one out of six such experiments, with each point representing the mean ± S.E.M of triplicate determinations.

Fig. 6 Time course of internalization of M_2 mAChRs in CHO cells following exposure to 100 μM CCh (●) or 250 μM CCh + 10 μM gallamine (○) for various time intervals as shown, prior to extensive washing and assessment of surface receptor density with 2 nM [^{3}H]NMS at 4°C for 3 hr. The increased concentration of CCh in the presence of gallamine was chosen based on the estimated negative cooperativity in binding between the two ligands (Table 4), thus ensuring equivalent levels of receptor occupancy to those observed in the presence of 100 μM CCh alone. Solid curves represent the best fit of either a two-phase (CCh alone) or one-phase (CCh plus gallamine) exponential model, as determined by F-test. Dashed curves represent the best global fit of a two-phase exponential model to both datastets, with the rate constants for each phase shared between the datasets. Data are mean ± S.E.M. of 4 experiments, with incubations performed in duplicates. * Indicates $p<0.05$ for the difference between % internalization between the two treatments.

JPET Fast Forward. Published on August 27, 2004 as DOI: 10.1124/jpet.104.073767
This article has not been copyedited and formatted. The final version may differ from this version.

Table 1 Binding parameters for orthosteric ligands and allosteric modulators against [^{3}H]NMS at human M_2 mAChRs expressed in intact CHO cells. Values are mean ± S.E.M of three experiments conducted in triplicate at 37°C for 60 min.

Ligand	Log K_B [a]	Log α [b]
CCh	-5.10 ± 0.07	NA [c]
Atropine	-8.70 ± 0.05	NA [c]
NMS	-9.70 ± 0.07	NA [c]
Gallamine	-5.70 ± 0.04 [d]	-1.30 ± 0.12 [d] (0.05)
Alcuronium	-6.10 ± 0.07 [d]	0.23 ± 0.07 [d] (1.7)
C_7/3-phth	-5.70 ± 0.23 [d]	-1.02 ± 0.06 [d] (0.1)

[a] Logarithm of the ligand equilibrium dissociation constant at the free receptor, determined using nonlinear regression as outlined in the Methods. For the CCh experiments, this value is only an apparent Log equilibrium dissociation constant due to the fact that CCh internalized the receptor over the time course of the assay.

[b] Logarithm of the cooperativity factor. Antilogarithm (geometric mean) is given in parentheses. All other details as for [a] above.

[c] Not applicable

[d] Data taken from Avlani et al. (2004), where the experiments were performed under identical conditions to those of the current study, except for the incubation time for alcuronium, which was 90 mins.

Table 2 Saturation binding parameters for [^{3}H]NMS at human M_2 mAChRs expressed in intact CHO cells after pretreatment with the indicated ligand for 24 hr. Values are mean ± S.E.M.

Pretreatment (24 hr) [a]	**LogK_A** [b]	**B_{max}** [c] **(fmol/10^5 Cells)**	**n** [d]
Vehicle	-9.73 ± 0.15	4.49 ± 0.94	6
CCh 100 μM	-9.73 ± 0.05	1.34 ± 0.27 **	3
Atropine 20 nM	-9.79 ± 0.14	6.63 ± 0.17 *	3
Gallamine 20 μM	-9.69 ± 0.17	6.42 ± 0.07 *	4

[a] Cells were treated as indicated at 37°C, followed by extensive washout on ice prior to assay.

[b] Logarithm of the radioligand equilibrium dissociation constant.

[c] Maximal density of binding sites.

[d] Number of experiments.

* $p < 0.05$, as determined by one way ANOVA.

** $p < 0.01$, as determined by one way ANOVA.

JPET Fast Forward. Published on August 27, 2004 as DOI: 10.1124/jpet.104.073767
This article has not been copyedited and formatted. The final version may differ from this version.

Table 3 Estimates of CCh potency and maximal response range for M_2 mAChR-mediated increases in whole-cell extracellular acidification rate in CHO cells under varying pretreatment conditions. Values are mean ± S.E.M.

Pretreatment (24 hr) [a]	**LogEC_{50}** [b]	**ECAR (% Basal)** [c]	**n** [d]
Vehicle	-5.81 ± 0.13	36 ± 4	17
CCh 100 μM	-5.40 ± 0.26	18 ± 3 ***	15
Atropine 20 nM	-5.99 ± 0.12	60 ± 7**	16
Gallamine 20 μM	-5.78 ± 0.13	55 ± 5*	13

[a] Cells were treated as indicated at 37°C, followed by extensive washout prior to assay.

[b] Logarithm of CCh EC_{50} value.

[c] Maximal ECAR response range.

[d] Number of experiments.

*** $p < 0.001$ compared to vehicle pretreatment, as determined by one way ANOVA.

** $p < 0.01$ compared to vehicle pretreatment, as determined by one way ANOVA.

* $p < 0.05$ compared to vehicle pretreatment, as determined by one way ANOVA.

JPET Fast Forward. Published on August 27, 2004 as DOI: 10.1124/jpet.104.073767
This article has not been copyedited and formatted. The final version may differ from this version.

Table 4 Binding parameters for interaction according to an extended allosteric ternary complex model between [^{3}H]NMS, gallamine and CCh at human M_2 mAChRs expressed in intact CHO cells. Estimates are the mean ± S.E.M. for six binding assays conducted in triplicate at 4°C for 3 hr.

Model Parameter	Estimates
Log K_A [a]	-9.73
Log K_B [b]	-5.19 ± 0.04
Log α [c]	-1.82 ± 0.15 (α=0.015)
Log K_I [d]	-4.63 ± 0.08
Log β [e]	-2.27 ± 0.55 (β=0.0054)
Slope [f]	0.98 ± 0.01

[a] Logarithm of the radioligand ([^{3}H]NMS) equilibrium dissociation constant. This value was taken from the experiments summarized in Table 2, and fixed as a constant for the current analysis.

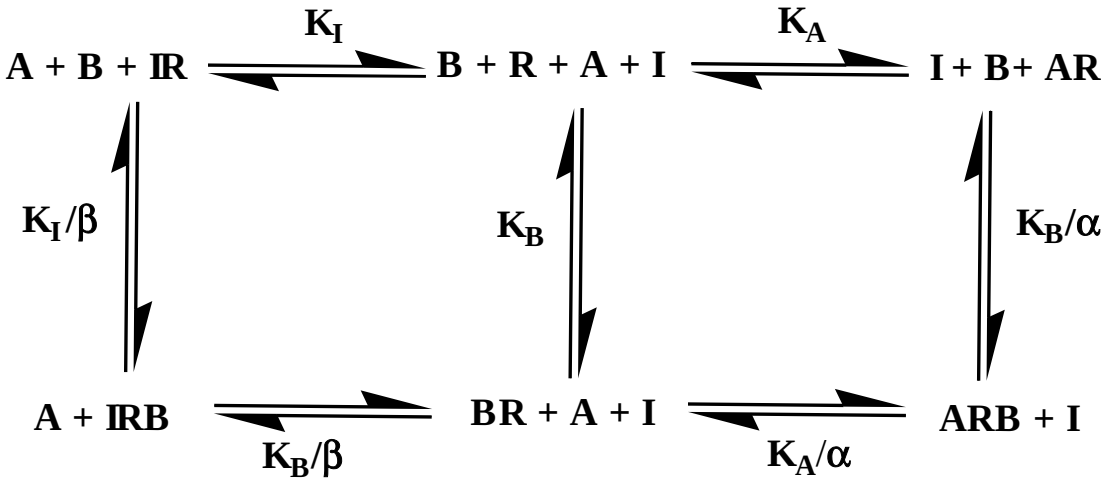
[b] Logarithm of the allosteric modulator (gallamine) dissociation constant.

[c] Logarithm of the cooperativity factor for the interaction between radioligand and modulator. Antilogarithm (geometric mean) is given in parentheses

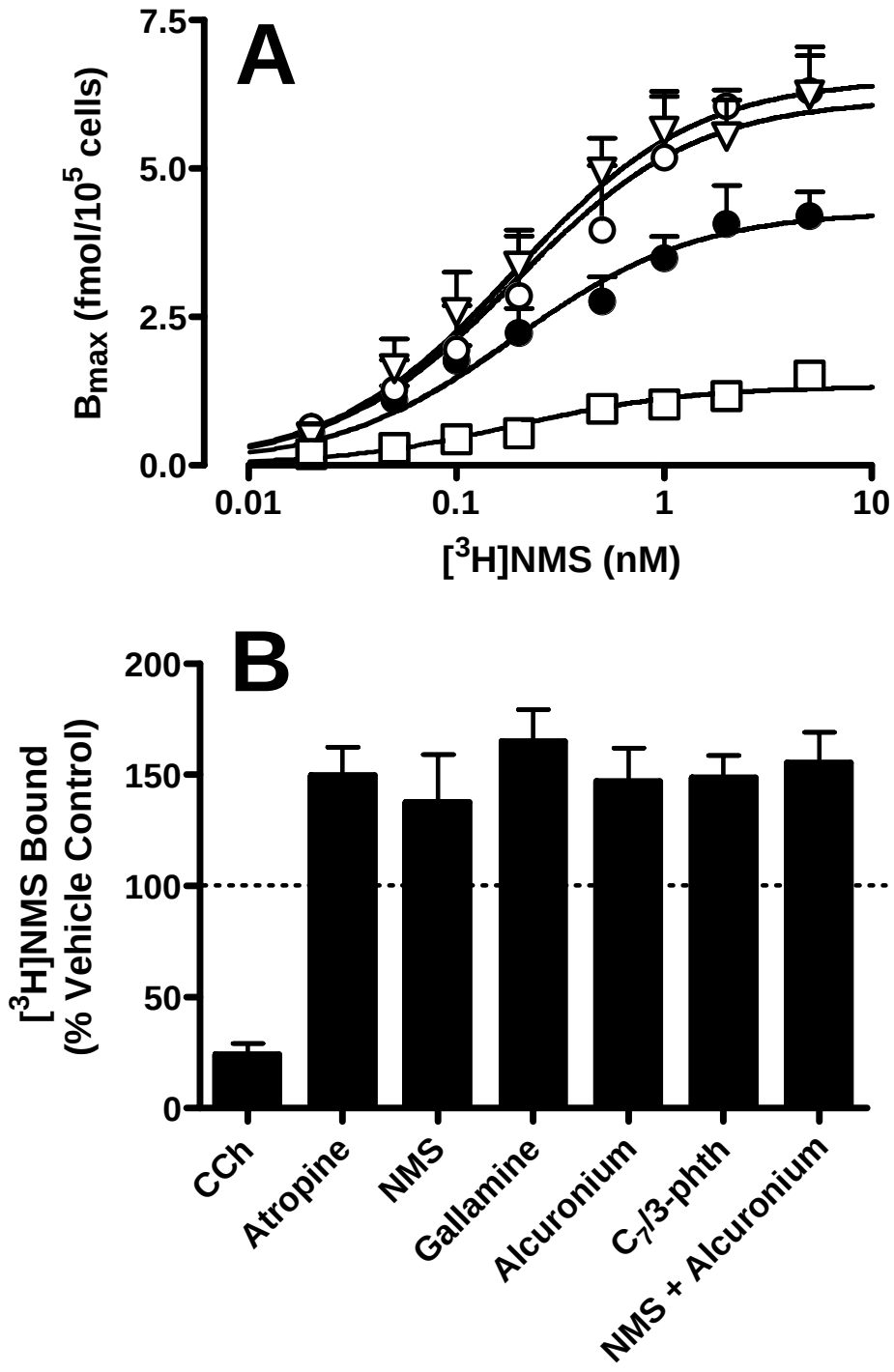
[d] Logarithm of the unlabelled competitive inhibitor (CCh) equilibrium dissociation constant.

[e] Logarithm of the cooperativity factor for the interaction between unlabelled competitive inhibitor and modulator. Antilogarithm (geometric mean) is given in parentheses

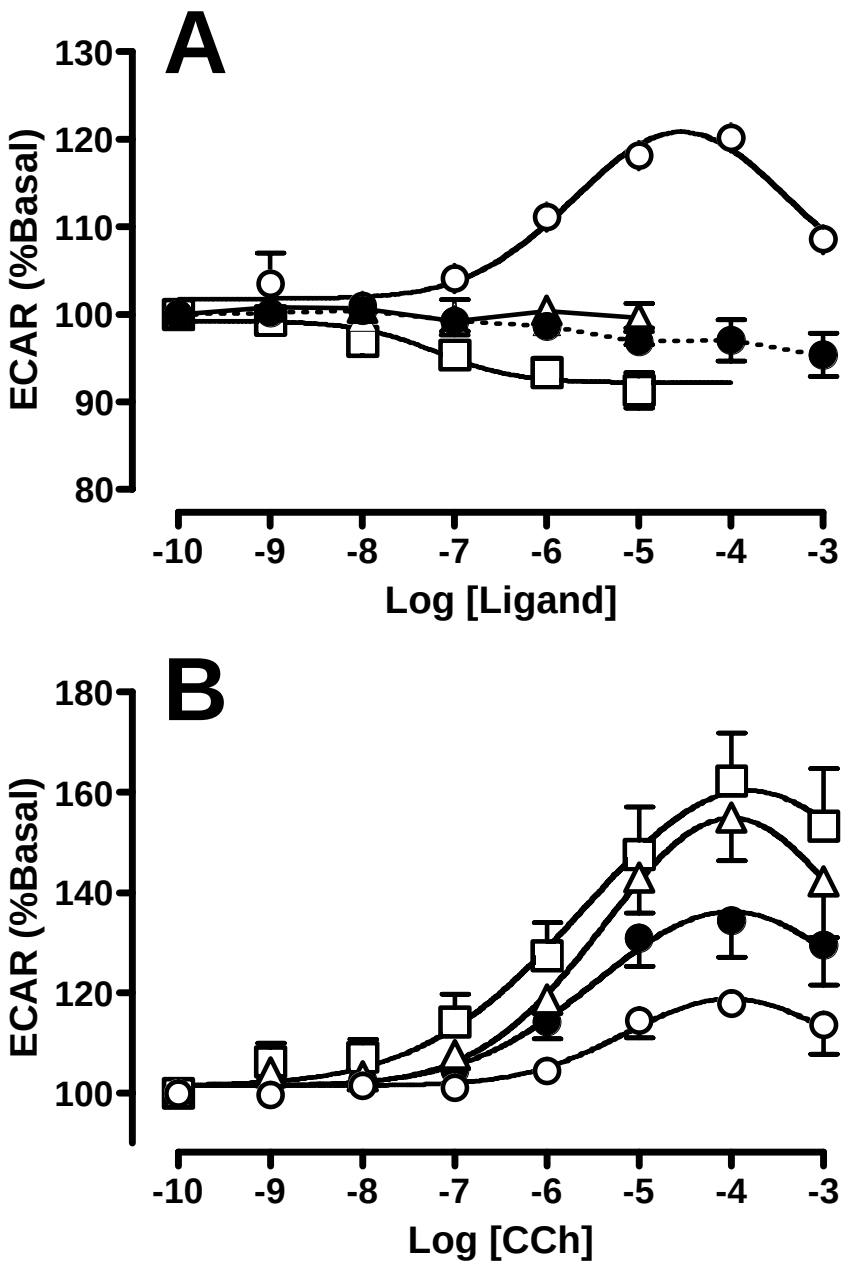
[f] Logistic slope factor for the interaction of the unlabelled competitive inhibitor with the receptor.



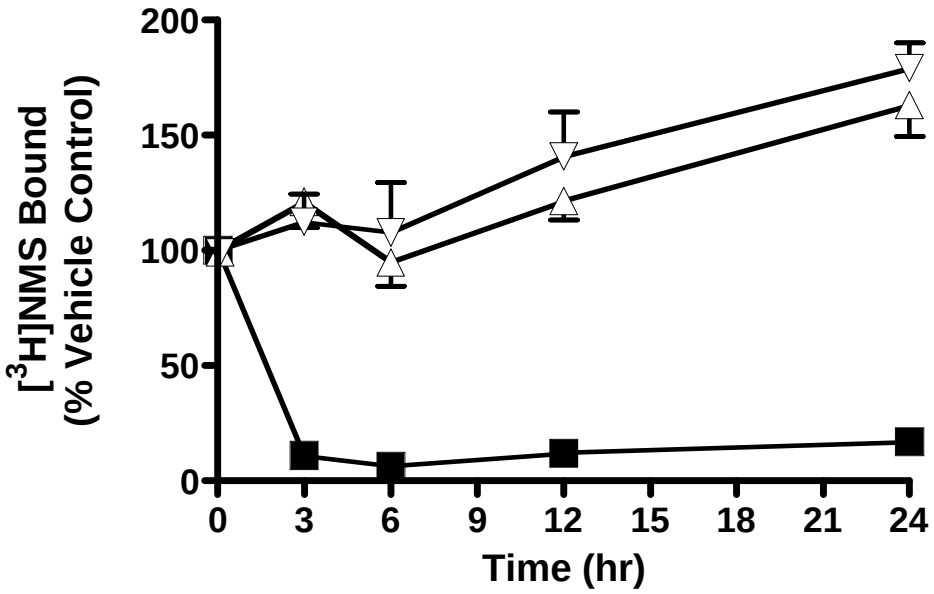
May et al., Figure 1



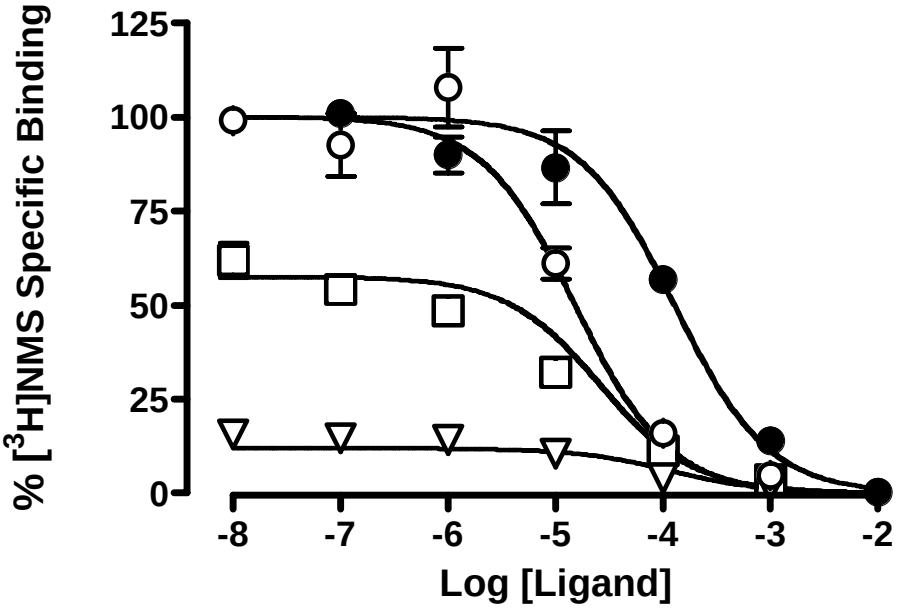
May et al., Figure 2



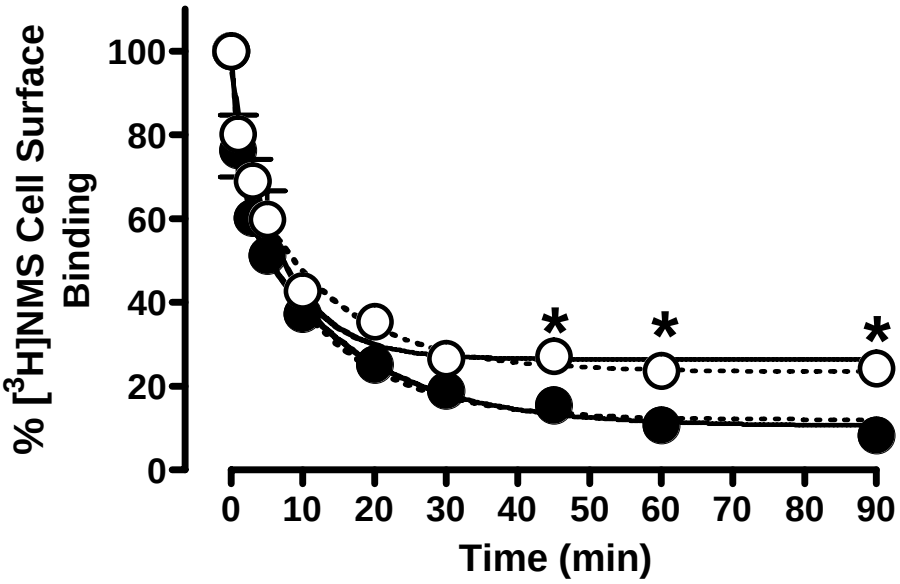
May et al., Figure 3



May et al., Figure 4



May et al., Figure 5



May et al., Figure 6