Molecular Mechanisms of Action of M₅ Muscarinic Acetylcholine Receptor Allosteric Modulators

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ABSTRACT
Recently, the first subtype-selective allosteric modulators of the M₅ muscarinic acetylcholine receptor (mAChR) have been described, but their molecular mechanisms of action remain unknown. Using radioligand-binding and functional assays of inositol phosphate (IP) accumulation and Ca²⁺ mobilization in a recombinant cell line stably expressing the human M₅ mAChR, we investigated the effects of the positive allosteric modulator (PAM), ML380, and negative allosteric modulator, ML375. In functional assays, ML380 caused robust enhancements in the potency of the full agonists, acetylcholine (ACh), carbachol, and oxotremorine-M, while significantly increasing the maximal response to the partial agonist, pilocarpine. In contrast, ML375 displayed negative cooperativity with each of the agonists in a manner that varied with the pathway investigated and progressively reduced the maximal pilocarpine response. Radioligand-binding affinity cooperativity estimates were consistent with values derived from functional assays in some instances but not others, suggesting additional allosteric effects on orthosteric ligand efficacy. For ML375 this was confirmed in IP assays performed after reduction of receptor reserve by the alkylating agent, phenox ybenzamine, as it reduced the maximal ACh response. In contrast, ML380 enhanced only ACh potency after receptor alkylation, with no effect on maximal response, consistent with studies of the M₅ mAChR with the prototypical PAM, BQ212. Interaction studies between ML380 and ML375 also indicated that they most likely used an overlapping allosteric site. Our findings indicate that novel small-molecule modulators of the M₅ mAChR display mixed mechanisms of action compared with previously characterized modulators of other mAChRs.

Introduction

The muscarinic acetylcholine receptors (mAChRs) are prototypical family A guanine nucleotide-binding protein-coupled receptors (GPCRs); the M₁, M₃, and M₅ mAChR subtypes primarily couple to Gα₁₁ proteins to activate the inositol phosphate (IP) pathway, whereas M₂ and M₄ mAChR subtypes primarily couple to Gαq proteins to mediate the inhibition of adenylyl cyclase (Porter et al., 2002; Matsui et al., 2004; Langmead et al., 2008). The mAChRs are widely expressed in the central nervous system (CNS) and periphery, where they control a number of physiologic processes. In the CNS, they have been implicated as important therapeutic targets for a range of disorders, including schizophrenia, Alzheimer’s disease, addiction, and Parkinson’s disease (Wess et al., 2007; Langmead et al., 2008).

To date, the majority of studies focusing on mAChRs in CNS disorders have targeted the M₁ and M₄ subtypes (Bymaster et al., 1997; Shannon et al., 2000; Brady et al., 2008; Chan et al., 2008; Ma et al., 2009; Shirey et al., 2009). However, despite only representing 2% of the total mAChR population in the brain (Yamada et al., 2003), the M₅ mAChR is expressed in highly discrete regions of therapeutic interest. For example, it is the only mAChR subtype for which mRNA transcript can be identified in dopamine-containing neurons of the substantia nigra pars compacta and the ventral tegmental area (Vilaró et al., 1990; Weiner et al., 1990; Yasuda et al., 1993), leading to the hypothesis that the M₅ mAChR may regulate midbrain dopamine transmission and reward mechanisms and thus represent a target for treating drug addiction (Vilaró et al., 1990; Weiner et al., 1990).

The phenotype of M₅ mAChR knockout (KO) mice supports the validity of the receptor as an addiction target; KO mice self-administer less cocaine and morphine, and are unable to develop conditioned place preference to the same drugs, with blunted withdrawal symptoms (Basile et al., 2002; Pink-Jensen et al., 2003; Thomsen et al., 2005). However, until recently, there has been a lack of sufficiently potent and selective tool compounds with which to probe receptor function and augment findings from constitutive M₅ mAChR KO mice.

ABBREVIATIONS: ACh, acetylcholine; CCh, carbachol; CHO, Chinese hamster ovary; CNS, central nervous system; GPCR, guanine nucleotide-binding protein-coupled receptor; IP, inositol phosphate; KO, knockout; mAChR, muscarinic ACh receptor; NAM, negative allosteric modulator; NMS, N-methylscopolamine; oxo-M, oxotremorine-M; PAM, positive allosteric modulator; PBZ, phenox ybenzamine.
A major breakthrough in this regard has been the discovery of small-molecule allosteric modulators for these mAChRs. By targeting topographically distinct binding sites from those used by orthosteric ligands, allosteric modulators can selectively target receptor subtypes to enhance or inhibit the function of acetylcholine (ACh) while maintaining the spatial and temporal nature of endogenous neurotransmission. Allosteric modulators also offer the potential of reduced propensity for off-target mediated toxicity and engendering signal-pathway bias (Gregory et al., 2007, 2010; Leach et al., 2007; Conn et al., 2009; Valant et al., 2012; Kruse et al., 2014). Highly selective positive allosteric modulators (PAMs) have been identified for both the M1 and M4 mAChR subtypes (Chan et al., 2008; Shirey et al., 2008, 2009; Ma et al., 2009), enabling their role in animal models of cognition and schizophrenia to be elucidated.

Most recently, screening and medicinal chemistry studies identified the first M5 mAChR-selective allosteric ligands, including the exemplar PAM, ML380 [1-((1H-indazol-5-yl)sulfonyl)-N-ethyl-N-(2-(trifluoromethyl)benzyl)piperidine-4-carboxamido], and the negative allosteric modulator (NAM), ML375 [(S)-9b-(4-chlorophenyl)-1-((3,4-difluorobenzyloxy)-2,3-dihydro-1H-imidazo[2,1-a]isoindol-5(9bH)-one] (Gentry et al., 2013, 2014) (Fig. 1). However, the molecular mechanisms underlying their activity remain largely unexplored. Given that GPCR allosteric modulators can display a range of activities, and that the type of activity can determine the degree of in vivo efficacy in a context-sensitive manner (Christopoulos, 2014), the aim of the current study was to evaluate the in vivo efficacy in a context-sensitive manner (Christopoulos, 2014), the aim of the current study was to evaluate the in vivo efficacy in a context-sensitive manner.

Materials and Methods

Materials. Flp-In-Chinese hamster ovary (CHO) cells were obtained from Life Technologies (Mulgrave, Australia). Dulbecco’s modified Eagle medium was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum was purchased from ThermoTrace (Melbourne, Australia). [3H]-N-methylscopolamine ([3H]-NMS; specific activity, 84 Ci/mmol) and MicroScint scintillation liquid were purchased from Perkin-Elmer Life Sciences. The IP-One assay kit was purchased from Cisbio (Codolet, France). ACh, carbachol (CCh), pilocarpine, oxotremorine-M (oxo-M), atropine, and phenoxybenzamine (PBZ) were purchased from Sigma-Aldrich (St. Louis, MO). ML380 and ML375 were generous gifts of C. Lindsley (Vanderbilt University, Nashville, TN). BQ212 was synthesized in-house, as described previously (Abdul-Ridha et al., 2014a,b). All other chemicals were purchased from Sigma-Aldrich. For all procedures, purified water (18.2 MΩ cm) from a Milli-Q PF Plus system was used.

Cell Culture. The wild-type human (hM5) mAChR construct was isogenically integrated into Flp-In CHO cells (Invitrogen), and cells were selected in the presence of 600 μg/mL hygromycin B at 37°C, 5% CO2, as previously described for the hM1 mAChR (Abdul-Ridha et al., 2014a). CHO-hM1 and CHO-hM4 cells were subcultured in a 1:3 ratio every 2 days after reaching 80% confluence with 6–10 mL Versene to detach cells and incubated in a humidified atmosphere at 37°C, 5% CO2, for 2–5 minutes. Cells were counted by a hemocytometer to be seeded at 25,000 cells/well in 96-well cell culture plates 24 hours prior to assay, unless otherwise stated.

[3H]-NMS Equilibrium Binding. CHO-hM3 cells were seeded into 96-well Isolates (PerkinElmer Life Sciences) in 100 μL culture media and incubated in a humidified atmosphere at 37°C, 5% CO2, for at least 6 hours. After 6 hours, media was removed and cells were washed with 100 μL phosphate-buffered saline, before physiological HEPES buffer (100 μL, 110 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl2, 1 mM MgSO4, 25 mM glucose, 50 mM HEPES, and 58 mM sucrose, pH 7.4) was added. Binding was determined in the presence of 0.3 nM [3H]-NMS. Nonspecific binding of the radioligand was assessed in the presence of 10 μM atropine and total binding by radioligand in the absence of HEPES buffer alone. Binding assays were terminated after a 2-hour incubation with radioligand at 37°C by removal of any unbound radioligand and the repeated washing of cells (3 times) with 100 μL 0.9% NaCl at 4°C, followed by addition of 100 μL Microscint scintillation liquid. Bound radioactivity was assessed by liquid scintillation counting by a MicroBeta2 Plate Counter (PerkinElmer Life Sciences, Glen Waverley, Australia). For inhibition-binding experiments, varying concentrations of individual (or combinations of) agonists, allosteric modulators, and antagonist in HEPES buffer were added to the cells in the presence of [3H]-NMS in a final assay of volume of 100 μL.

IP Accumulation. The IP-One assay kit (Cisbio) was used for the direct quantitative measurement of myo-inositol 1-phosphate in CHO-hM5 or CHO-hM4 cells, as described previously (Abdul-Ridha et al., 2014a), with the exception that cells were seeded at 25,000 per well onto a transparent 96-well cell culture plate, and the following day cells were stimulated with various concentrations of orthosteric and/or allosteric ligands, and were then incubated for 40 minutes at 37°C, 5% CO2. Responses were normalized to that to a single, maximal concentration of ACh (10 μM).

Calcium Mobilization. CHO-hM3 cells were cultured overnight in 96-well cell culture plates at 50,000 cells/well. The following day, media was removed and replaced with 50 μL 1 μM Fluo-4-AM Ca2+-sensitive dye in assay buffer (Hank’s balanced salt solution supplemented with 1.26 mM CaCl2, 20 mM HEPES, and 2.5 mM propranolol, pH 7.4), and the cells were incubated 45 minutes at 37°C, 5% CO2. Dye was then removed and replaced with 50 μL freshly prepared Fluo-4 dye. Test compounds were added to the cells in a FLEXStation II (Molecular Devices, Sunnyvale, CA). For interaction studies, ligands were added simultaneously to cells. Ca2+/Fluo-4 fluorescence was measured at an emission wavelength of 525 nm for 90 seconds (19 seconds prior to and 71 seconds following the addition of ligand) at room temperature.

Receptor Alkylation Studies. To determine functional affinity and efficacy estimates for the agonists, additional IP accumulation assays were performed, as described above, but on cells pretreated for 30 minutes at 37°C with the irreversible orthosteric-site alkylating agent, PBZ (or vehicle buffer control), followed by three washes with 10% phosphate-buffered saline. PBZ reduces functional receptor availability by irreversible steric hindrance for orthosteric agonists (ACh, CCh, oxo-M) or due to the irreversible formation of an inactive state for which the positive allosteric modulator, ML380, has minimal affinity due to high negative cooperativity.

Fig. 1. Structures of (A) ML380 and (B) ML375.
Data Analysis. GraphPad Prism version 6.04 (San Diego, CA) was used for all statistical analysis and curve fitting. For the empirical analysis of functional concentration–response data, agonist concentration–response curves were analyzed using a standard logistic function to determine measures of potency (as negative logarithms; pEC50) and maximal response (Emax). For direct determination of functional agonist dissociation constants (Ka) and operational efficacies (τ) from the alkylation experiments, agonist IP accumulation concentration–response curves (in the presence or absence of PBZ pretreatment) were globally fitted to the operational model of agonism (Black and Leff, 1983):

\[
Y = \text{Basal} + \left( \frac{(E_m - \text{Basal})}{1 + \left( \frac{K_a}{\text{EC}_{50}} \right)^n} \right) \left( \frac{1}{1 + \left( \frac{1}{\tau_0} + \left( 10^\text{Log}[A] \right)^n \right)} \right)
\]

(1)

where [A] represents the concentration of the agonist, K_a represents the agonist equilibrium dissociation constant, Basal is the response in the absence of ligand, E_m is the maximal system response, τ represents the agonist operational efficacy, which subserves both receptor density and stimulus–response coupling efficiency, and n is the slope of the transducer function linking occupancy to response. A modified form of eq. 1 was also applied to the data for the partial agonist, pilocarpine, using a four-parameter logistic fit of the ACh control (full agonist) concentration–response curve to provide estimates of E_m, transducer slope (n), and Basal for fitting the pilocarpine data. For functional interaction studies between full orthosteric agonists and allosteric ligands in the IP accumulation or calcium assays, the following simplified operational model of allosterism was applied (Leach et al., 2007):

\[
Y = \text{Basal} + \frac{(E_m - \text{Basal})}{\left( \frac{[A]K_b + [B]E_{C50}}{[A][K_b + \alpha[B]] + \tau[B][E_{C50}]} \right)^n} \left( \frac{1}{1 + \left( \frac{1}{\tau_0} + \left( 10^\text{Log}[A] \right)^n \right)} \right)
\]

(2)

where Basal is the response in the absence of ligand, EC_{50} is the midpoint of the full agonist concentration–response curve, K_b is the equilibrium dissociation constant of the allosteric ligand (fixed to the K_a value from alkylation studies for ML380 for the IP assay), τ_0 represents the capacity of the allosteric ligand to exhibit agonism (constrained to 0 for ML375), and α represents the affinity/efficacy cooperativity parameter describing the combined effect of the allosteric modulator on agonist function (both affinity and efficacy). E_m and n are as described above. As this model is only suitable for full agonists that remain full agonists in the presence of allosteric modulator (Aurelio et al., 2009; Leach et al., 2010), the pilocarpine datasets were analyzed according to the complete model of allosterism and agonism (Leach et al., 2007):

\[
Y = \text{Basal} + \frac{(E_m - \text{Basal})}{\left( \frac{[A]K_b + [B]K_d + K_a[B] + a[A][B]}{[A][K_b + a[B]] + \tau[B][K_d] + \tau[B][K_a]} \right)^n} \left( \frac{1}{1 + \left( \frac{1}{\tau_0} + \left( 10^\text{Log}[A] \right)^n \right)} \right)
\]

(3)

where [A] and [B] represent the concentrations of the orthosteric agonist and allosteric ligand, respectively, and K_a and K_d represent their respective equilibrium dissociation constants (constrained as above for ML380). τ_0 and τ represent the relative efficacies of the orthosteric and allosteric ligands, receptively, and n represents the slope of the transducer function that links occupancy to response. α represents the affinity cooperativity between the orthosteric agonist and allosteric modulator, and β represents a scaling factor that describes the magnitude and direction of the effect of the allosteric modulator on orthosteric agonist efficacy. Where required to facilitate convergence of global model fitting, the value of n was constrained to unity and indicated as such.

All interaction radioligand-binding studies were analyzed according to the following adapted form of an allosteric ternary complex model that accounts for the interaction of two orthosteric ligands and one allosteric ligand on a receptor (Christopoulos and Mitchelson, 1997):

\[
Y = \frac{B_{\text{max}}[A]}{\left[ [A][K_a] + \left( \frac{k_a[H] + K_a}{1 + \left( f[K_a] + [B] + \left[ (\alpha[f][B])/(K_a[K_b]) \right] \right) \right] \right)}
\]

(4)

where [A], [B], and [H] represent the concentrations of the radioligand ([3H]-NMS), allosteric ligand, and orthosteric inhibitor, respectively, and K_a, K_d, and K_b represent their respective equilibrium dissociation constants, and B_{max} is as defined above. The value K_a was fixed to 0.3 nM. α_a and α_b represent the affinity cooperativity values between the allosteric ligand and the radioligand or orthosteric inhibitor, respectively; values greater than 1 indicate positive cooperativity; values less than 1 (but > 0) indicate negative cooperativity; and values of unity indicate neutral cooperativity. All potency, affinity, and cooperativity parameters were estimated as logarithms (Christopoulos, 1998). Where appropriate, fitted parameters were compared by extra sum-of-squares F-test (Motulsky and Christopoulos, 2004).

Results

The effects of ML380 and ML375 were studied in canonical G_{q/11}-linked IP accumulation and Ca^{2+} mobilization functional assays. ACh stimulates both IP accumulation (pEC_{50} = 6.68 ± 0.05; n_H = 1.0 ± 0.1; n = 3) and Ca^{2+} mobilization (pEC_{50} = 7.63 ± 0.06; n_H = 1.3 ± 0.2; n = 4) in CHO-hM5 cells (Fig. 2). The PAM, ML375, also robustly stimulated IP accumulation and Ca^{2+} mobilization (pEC_{50} values 5.33 ± 0.06; n_H = 1.4 ± 0.2; n = 5 and 5.71 ± 0.07; n_H = 1.4 ± 0.3; n = 3, respectively; Fig. 2), appearing as a high efficacy agonist in its own right. The NAM, ML375, was inactive on its own in either signaling assay.

To determine the degree of relative intrinsic efficacy of the allosteric agonist, ML380, relative to that of the orthosteric agonists, concentration–response curves to ACh, CCh, oxo-M, or ML380 in IP accumulation assays were re-established after pretreatment (followed by washout) of the CHO-hM5 cells with PBZ to irreversibly reduce receptor accessibility through direct steric hindrance (orthosteric agonists) or high negative cooperativity (ML380). As shown in Fig. 3, this resulted in a reduction in both potencies and maximal responses for all agonists. Application of the operational model of agonism to the resulting families of curves for each agonist (Table 1)

Fig. 2. Effect of ACh, ML380, and ML375 on (A) IP accumulation and (B) Ca^{2+} mobilization in CHO-hM5 cells. Data are expressed as a percentage of maximal ACh response and represent the mean ± S.E.M. of at least four independent experiments performed in triplicate; fitted curves are from a four-parameter logistic equation.
revealed that PBZ reduces the freely accessible receptor population by 48 ± 13% at 1 μM, 88 ± 4.4% at 10 μM, and 99 ± 0.4% at 100 μM. All of the orthosteric and allosteric ligands had similar functional affinities for their respective sites on the receptor (pK_A = 4.8–5.3), but ML380 displayed the lowest operational efficacy (τ = 4) compared with the orthosteric agonists (τ = 20–33; Table 1).

As well as exhibiting intrinsic agonist activity, ML380 potentiated the activity of ACh in both IP and Ca^{2+} mobilization assays (Fig. 4, A and B), thus acting as a PAM agonist (Christopoulos, 2014) with net cooperativity (Log αβ) values of 0.96 ± 0.26 (αβ = 9.1) and 1.69 ± 0.26 (αβ = 49), respectively (Table 2). The stronger positive cooperativity for ML380 in the calcium assay is consistent with its higher degree of stimulus–response coupling, also reflected by the greater intrinsic agonism for ML380 at this endpoint versus the IP assay (Log τ_A = 1.18 ± 0.18; τ_A = 15, versus Log τ_B = 0.69 ± 0.06; τ_B = 4.9). To evaluate potential probe dependence of the PAM activity, interaction studies were also conducted with CCh, oxo-M, and the partial agonist, pilocarpine, in the IP assay. The positive modulation of CCh- and oxo-M–mediated responses by ML380 was similar to that for ACh (Fig. 5; Table 2), indicating little probe dependence with full agonists. Interestingly, ML380 markedly potentiated the potency and maximal response to the partial agonist, pilocarpine, with a Log αβ value of 1.78 ± 0.15 (αβ = 60), which was significantly greater than that observed against the full agonists and not consistent with the predictions of a simple two-state model of allostery as exemplified by prior studies of the related M1 mAChR with PAMs such as BQCA and BQZ12 (Canals et al., 2012; Abdul-Ridha et al., 2014a,b).

The NAM, ML375, produced parallel, rightward shifts in concentration–response curves to ACh in both IP and Ca^{2+} mobilization assays (Fig. 4, C and D). Analysis of the latter yielded estimates of allosteric site affinity (pK_B = 6.62 ± 0.06; n = 4) and saturable negative cooperativity [Log αβ = −1.34 ± 0.04 (αβ = 0.05); n = 4]. In the IP assay, however, the reduction in agonist potency did not appear to reach a ceiling level over the entire concentration range tested, indicative of either a competitive interaction or, more likely, a very high degree of negative cooperativity. As such, the data could be fitted to an allosteric model in which the negative cooperativity could be constrained to a very small value (Log αβ = −3.0) under which the allosteric ternary complex model and a competitive interaction model become indistinguishable (Keov et al., 2014). This yielded an estimate of the ML375 pK_B = 6.22 ± 0.13 (n = 6), which was in excellent agreement with the allosteric ternary complex analysis of the Ca^{2+} mobilization data. As was observed for the ML380 experiments, there was little difference between the observed modulation of ACh-, CCh-, and oxo-M–stimulated IP accumulation, with ML375 causing parallel rightward shifts with high negative cooperativity and similar estimates of affinity irrespective of the agonist tested (Fig. 5; Table 2). Again, as observed with the PAM with ML380, the modulation of pilocarpine activity by the NAM yielded a different profile to the full agonists, with ML375 virtually abolishing the maximal agonist response (Fig. 5; Table 2).

To further understand the mechanisms underlying the positive and negative cooperativity exhibited by ML380 and ML375, respectively, whole-cell ³[H]-NMS–binding studies were subsequently performed to directly determine the cooperative effects on agonist binding alone (α). ³[H]-NMS bound to whole CHO-hM5 cells in a monophasic and saturable manner with an estimated pK_D = 9.64 ± 0.12 (K_D = 0.26 ± 0.07 nM; n = 4) and maximal binding capacity (B_max) of 7.3 ± 1.2 fmol/10^6 cells.
ML380 increased the affinity of ACh, CCh, and oxo-M to inhibit the binding of [3H]-NMS from CHO-hM5 cells with estimates of positive cooperativity (Log α) similar to the Log αβ values determined in the IP accumulation assays (Fig. 6; Table 3). In addition, ML380 exerted weak negative cooperativity with respect to the inverse agonist radioligand, [3H]-NMS, and neutral affinity cooperativity with respect to the weak partial agonist, pilocarpine (Log α = 0.00 ± 0.18; Fig. 6; Table 3).

Similar [3H]-NMS–binding studies revealed that ML375 caused a concentration-dependent decrease in the affinities of ACh, CCh, and, to a lesser extent, oxo-M and pilocarpine (Fig. 7; Table 3). Conversely, ML375 exhibited neutral-to-weak positive cooperativity with respect to [3H]-NMS itself. The affinity (pKB) estimates for ML375 were broadly in line with those generated from the functional assays (Table 2). However, the degree of negative cooperativity with respect to agonist binding was not sufficient to account for the higher negative cooperativity noted in the functional assays (Table 2).

The discrepancies between cooperativity values across binding and functional studies, and the direct effects observed with either PAM or NAM on the maximal effect of the partial agonist, pilocarpine, thus led to the hypothesis that these allosteric modulators may be exerting their overall effects on agonist function not only by modulating orthosteric agonist affinity, but also intrinsic efficacy. As these effects were not apparent in the functional assays with the full orthosteric agonists [because their higher efficacies (Table 1) allowed them to achieve the maximum cellular effect in both the absence and presence of modulator under control conditions], we exploited the ability of PBZ to irreversibly alkylate the mAChRs and effectively reduce the number of spare receptors, thus enforcing conditions in which even high efficacy agonists like ACh would require maximal receptor occupancy to achieve maximal observed effect. Accordingly, pretreatment with PBZ (3 μM), followed by extensive washout, yielded a depression in the ACh maximal response and potency to stimulate IP accumulation in CHO-hM5 cells (control pEC50 = 7.00 ± 0.13, Emax = 98.3 ± 3.2%; PBZ-treated pEC50 = 6.03 ± 0.10, Emax = 47.2 ± 1.9%; Fig. 8A). In the presence of ML380 (10 μM), the potency (pEC50 = 7.09 ± 0.15; P < 0.0001, F-test), but not the maximal response to ACh (49.7 ± 1.8%; P = 0.35, F-test), was significantly increased compared with control. There was a similar lack of effect on the maximal ACh response in the presence of a higher concentration of ML380 (30 μM; data not shown) and also a small elevation in the basal response, reflecting residual ML380 agonist activity (Fig. 8A).

An almost identical profile was seen when the experimental paradigm was repeated using oxo-M as the orthosteric agonist (Fig. 8B). In the presence of a higher concentration of ML380 (30 μM), the potency (pEC50 = 6.03 ± 0.10, Emax = 50.8 ± 3.2%; PBZ-treated pEC50 = 5.35 ± 0.15, Emax = 30.2 ± 1.8%; P < 0.0001, F-test), but not the maximal response to oxo-M, was significantly increased compared with control. There was a similar lack of effect on the maximal oxo-M response in the presence of a higher concentration of ML380 (30 μM; data not shown) and also a small elevation in the basal response, reflecting residual ML380 agonist activity (Fig. 8A).

An almost identical profile was seen when the experimental paradigm was repeated using oxo-M as the orthosteric agonist.
To compare this mechanism with that of other well-studied allosteric modulators of mAChRs, we performed similar alkylation and IP accumulation studies in CHO cells stably expressing the Gq/11-coupled M1 mAChR subtype using BQZ12, a high-affinity congener of the M1 mAChR PAM, BQCA, that has been proposed to act according to a simple two-state model of allostery (Canals et al., 2012; Abdul-Ridha et al., 2014a). As with the M5 mAChR experiments, PBZ pretreatment reduced ACh potency and maximal response to stimulate IP accumulation in CHO-hM5 cells (control pEC50 = 6.66 ± 0.06, Emax = 88.5 ± 1.5%; PBZ-treated pEC50 = 5.18 ± 0.11, Emax = 49.8 ± 2.2%; Fig. 8C). In the presence of BQZ12 (10–30 nM), the ACh potency was significantly enhanced by approximately 50-fold (P < 0.0001, F-test), but there was no effect on the maximal response (P = 0.65, F-test; Fig. 8C), suggesting that ML380 may exert its effects at the M5 mAChR in a similar manner to BQZ12 at the M1 mAChR, that is, primarily by modulation of full agonist affinity.

Interestingly, when profiled under identical PBZ-treated conditions, ML375 (10–30 μM) significantly decreased both potency (control pEC50 = 6.85 ± 0.05, Emax = 98.8 ± 1.1%; PBZ-treated = 5.45 ± 0.09, Emax = 48.2 ± 1.4%; pEC50 = 3.98 ± 0.12 at 10 μM ML375; 3.75 ± 0.17 at 30 μM ML375; P < 0.01, F-test) and maximal response (27.1 ± 2.0% at 10 μM; 22.1 ± 2.0% at 30 μM; P < 0.05, F-test) of ACh to stimulate IP accumulation compared with control (Fig. 8D), indicating that it is a NAM of both ACh affinity and signaling efficacy.

Finally, we investigated whether ML380 and ML375 mediate their PAM and NAM effects, respectively, from a common site on the M5 receptor. Using the IP accumulation assay, we determined the ability of ML375 or the orthosteric antagonist, atropine, to inhibit the agonist response to a single, fixed concentration of ML380 (10 μM). Atropine and ML375 both fully inhibited the response to ML380 (pIC50 values 8.13 ± 0.10 and 5.79 ± 0.21, respectively; Fig. 9). As ML380 is clearly allosteric with respect to ACh and [3H]-NMS (vide supra), the inhibition mediated by atropine is almost certainly allosteric, but characterized by a high degree of negative cooperativity.

Moreover, three-way binding studies monitoring the competition between atropine, a [3H]-NMS, in the absence or presence of ML380 (Supplemental Fig. 1) revealed that atropine has neutral affinity cooperativity with respect to modulator (Log α = 0.00 ± 0.12), suggesting that the inhibition seen in the functional assay must be mediated by high negative efficacy cooperativity.
et al., 2014). ML375 is a highly subtype-selective NAM of muscarinic M5 mAChRs, with negative allosteric modulator effects on ACh-stimulated calcium mobilization at both human and rat muscarinic M5 mAChRs, with a range of orthosteric agonists in assays of IP accumulation and Ca2+ mobilization, with ML375 possessing sub-micromolar affinity for the M5 mAChR and ML380 displaying similar affinity as CCh and oxo-M. Three-way radioligand-binding studies also suggested that ML380 could increase the affinity of ACh for the M5 mAChR (Gentry et al., 2014). ML375 is a highly subtype-selective NAM of ACh-stimulated calcium mobilization at both human and rat muscarinic M5 mAChRs, which was shown to be allosteric by its ability to slow [3H]-NMS dissociation from the receptor (Gentry et al., 2013). Despite these data, relatively little is known about the mechanisms of modulation by these allosteric ligands. The aim of the current studies was to fully characterize the pharmacology of the PAM, ML380, and the NAM, ML375, at the muscarinic M5 mAChR using radioligand-binding and canonical Gq/11-linked signaling assays (IP accumulation and Ca2+ mobilization). By doing so, we reveal that these allosteric ligands have minimal effects on antagonists, but have the potential to modulate both orthosteric agonist affinity and intrinsic efficacy of agonists in a probe-dependent manner.

Our studies confirm that ML380 and ML375 display PAM agonist and NAM behavior, respectively, when interacting with a range of orthosteric agonists in assays of IP accumulation and Ca2+ mobilization, with ML375 possessing sub-micromolar affinity for the M5 mAChR and ML380 displaying similar affinity as CCh and oxo-M. Three-way radioligand-binding studies with the inverse agonist radioligand, [3H]-NMS and ACh, CCh, or oxo-M suggest that ML380 and ML375 exert their effects in a manner broadly consistent with a two-state mechanism (Fig. 2; Table 2), as previously described for PAMs of the muscarinic M1 receptor, Bridges and Gentry, 2010; Gentry et al., 2013, 2014). ML380 and ML375 exert their effects in a manner broadly consistent with a two-state mechanism (Fig. 2; Table 2), as previously described for PAMs of the muscarinic M1 receptor, Bridges and Gentry, 2010; Gentry et al., 2013, 2014).

### Discussion

Recent screening and medicinal chemistry efforts have resulted in the first subtype-selective small-molecule positive and negative allosteric modulators of muscarinic M5 mAChRs (Bridges et al., 2010; Gentry et al., 2013, 2014). ML380 has been described as a PAM of ACh-stimulated calcium mobilization at both human and rat muscarinic M5 mAChRs, with moderate selectivity versus the M1 and M3 mAChR subtypes. Radioligand-binding studies also suggested that ML380 could increase the affinity of ACh for the M5 mAChR (Gentry et al., 2014). ML375 is a highly subtype-selective NAM of ML380 and ML375, with a range of orthosteric agonists in assays of IP accumulation and Ca2+ mobilization, with ML375 possessing sub-micromolar affinity for the M5 mAChR and ML380 displaying similar affinity as CCh and oxo-M. Three-way radioligand-binding studies with the inverse agonist radioligand, [3H]-NMS and ACh, CCh, or oxo-M suggest that ML380 and ML375 exert their effects in a manner broadly consistent with a two-state mechanism (Fig. 2; Table 2), as previously described for PAMs of the muscarinic M1 receptor, Bridges and Gentry, 2010; Gentry et al., 2013, 2014).

### Table 3

<table>
<thead>
<tr>
<th>Agonist</th>
<th>$pK_a$</th>
<th>$pK_B$</th>
<th>$\log \alpha$ (a)</th>
<th>$\log \alpha$ (b)</th>
<th>$\log \alpha$ (c)</th>
<th>$pK_B$</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACh</td>
<td>4.64 ± 0.07</td>
<td>4.79</td>
<td>0.87 ± 0.12 (7.4)</td>
<td>−0.17 ± 0.06 (0.67)</td>
<td>4.94 ± 0.09</td>
<td>−1.37 ± 0.12 (0.04)</td>
</tr>
<tr>
<td>CCh</td>
<td>4.32 ± 0.06</td>
<td>4.79</td>
<td>0.74 ± 0.11 (5.5)</td>
<td>−0.36 ± 0.06 (0.44)</td>
<td>4.43 ± 0.13</td>
<td>−1.52 ± 0.18 (0.03)</td>
</tr>
<tr>
<td>Oxo-M</td>
<td>4.86 ± 0.06</td>
<td>4.79</td>
<td>1.06 ± 0.11 (12)</td>
<td>−0.40 ± 0.06 (0.40)</td>
<td>4.94 ± 0.09</td>
<td>−0.85 ± 0.13 (0.14)</td>
</tr>
<tr>
<td>Pilocarpine</td>
<td>4.83 ± 0.05</td>
<td>4.79</td>
<td>0.00 ± 0.18 (1.0)</td>
<td>−0.57 ± 0.05 (0.43)</td>
<td>4.88 ± 0.09</td>
<td>−0.57 ± 0.11 (0.27)</td>
</tr>
</tbody>
</table>

Estimated parameters represent the mean ± S.E. of three experiments performed in duplicate. Three-way binding interactions were analyzed according to eq. 4 (Materials and Methods).}

**Fig. 7.** Three-way radioligand-binding studies to demonstrate the effect of ML375 (0.3–30 μM) on (A) ACh-, (B) CCh-, (C) oxo-M-, and (D) pilocarpine-mediated inhibition of [3H]-NMS binding in whole CHO-hM5 cells. Cells were incubated with radioligand for 2 h at 37°C; data are expressed as a percentage of control specific binding and represent the mean ± S.E.M. of at least three independent experiments performed in triplicate. Fitted curves are from global analysis of datasets according to eq. 4 (in Materials and Methods) with parameter estimates shown in Table 3.
BQA and BQZ12 (Canals et al., 2012; Abdul-Ridha et al., 2014a). However, the modulation of the partial agonist, pilocarpine, by both ML380 and ML375 indicated that additional mechanisms might contribute to their pharmacology. ML380 displayed neutral affinity cooperativity with respect to pilocarpine, yet potentiated its function in IP accumulation assays by approximately 60-fold (Tables 2 and 3). In addition, the PAM agonist displayed a steeper concentration–response relationship when mediating IP accumulation in both the absence and presence of orthosteric agonists (Figs. 3 and 5), suggesting that it may bind cooperatively with respect to itself or that it has the potential to change the sensitivity of the coupling of the receptor to the G_{q/11} pathway. Finally, ML375 displayed only weak negative affinity cooperativity with pilocarpine, yet ablates the functional response to this agonist (Tables 2 and 3). These effects cannot be accommodated within a simple model allowing only for affinity modulation and indicate that both ML380 and ML375 might additionally modulate the signaling efficacy of the M₅ mAChR.

As effects of allosteric modulators on relative efficacy are often difficult to detect in functional assays (due to the use of full agonists that reach the maximal system response and/or assays with high degrees of stimulus–response coupling), we used the receptor-alkylating agent, PBZ, to reduce levels of receptor reserve. Pretreatment with PBZ reduced the potency and maximal response of ACh to stimulate IP accumulation in CHO-hM₅ cells and opened a window to visualize both negative and positive effects on the maximal response to the agonist. Under these conditions, ML380 clearly increased the potency of both ACh and oxo-M, but did not increase the maximal response (Fig. 8), suggesting that it modulates only the affinity of full agonists. Similar studies with the M₁ mAChR verified that this PAM effect was conserved across mAChRs; under identical assay conditions, the M₁ mAChR PAM, BQZ12, caused only a leftward shift in the concentration–response curve to ACh, with no change in maximal agonist response (Fig. 8). However, the NAM, ML375, clearly decreased the maximal response to ACh, respectively (Fig. 8), suggesting that it engenders changes in the ability of the M₅ mAChR to couple to G protein(s) as well as modulating the affinity of agonist binding. The effect of ML380 on pilocarpine signaling efficacy is also a clear indicator that the PAM can exert probe-dependent effects on receptor–effector coupling as well.

Although the location of allosteric sites on the M₅ mAChR has not been extensively studied, mutagenesis studies, as well as computational and structural insights, support the notion that there is at least one common allosteric binding site in the extracellular vestibule of mAChRs (Huang et al., 2005; Dror et al., 2013; Kruse et al., 2013). Thus, despite topographical similarities, these data raise the potential of differential molecular modes of allosteric modulation within the same receptor family. Across the wider GPCR superfamilies, different modes of allosteric modulation have already been observed: at the family C mGlu1 and mGlu5 receptors, NAMs such as CPCCOEt and MPEP do not alter the ability of orthosteric agonists to bind to the receptor, but exclusively negatively modulate agonist efficacy (Litschig et al., 1999; Bradley et al., 2011). At the other end of the spectrum are the prototypical mAChR NAMs, gallamine and C₇/3-phth, and the M₁ mAChR-selective PAMs, BQCA and BQZ12, which appear to modulate only ACh affinity (Canals et al., 2012; Abdul-Ridha et al., 2014a). Many allosteric modulators of GPCRs exert a mixed mechanism with respect to agonist affinity and efficacy, including muscarinic M₂ and M₄ receptor PAMs (Valant et al., 2012; Croy et al., 2014) and mGlu4 and mGlu5 receptor...
PAMs (Mathiessen et al., 2003; Bradley et al., 2011). The studies described in this work indicate that positive modulation of the M₅ mACHR can occur via regulation of either affinity (for ACh and oxo-M) or efficacy (as for pilocarpine) and that negative modulation is mediated by changes in both properties.

If, as our findings suggest, this is achieved via interaction with a common allosteric site, it would be interesting for future studies to determine whether this site represents the prototypical allosteric site proposed for other mACHRs in the extracellular vestibule (Krusz et al., 2014), or whether the M₅ mACHR modulators interact with a hitherto unidentified site on this receptor. Moreover, this may have implications to the ultimate use of such compounds and how their effects translate into native tissue or in vivo studies. For instance, a recent investigation by Foster et al. (2014) using an earlier generation M₅ mACHR PAM (ML129) (Bridges et al., 2009) found that somatodendritic M₅ mACHRs increase sub-

References


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