Characterization of the Novel Positive Allosteric Modulator, LY2119620, at the Muscarinic M₂ and M₄ Receptors

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ABSTRACT

The M₂ receptor is a compelling therapeutic target, as this receptor modulates neural circuits dysregulated in schizophrenia, and there is clinical evidence that muscarinic agonists possess both antipsychotic and proconvulsive efficacy. Recent efforts have shifted toward allosteric ligands to maximize receptor selectivity and manipulate endogenous cholinergic and dopaminergic signaling. In this study, we present the pharmacological characterization of LY2119620 (3-amino-5-chloro-N-cyclopropyl-4-methyl-6-[2-(4-methylpiperazin-1-yl)-2-oxoethoxy] thieno[2,3-b]pyridine-2-carboxamide), a M₂/M₄ receptor-selective positive allosteric modulator (PAM), chemically evolved from hits identified through a M₄ allosteric functional assay. Guanosine 5′-[35S]-triphosphate (GTPγS) binding assays revealed evidence of probe dependence in both binding and functional assays. Guanosine 5′-[35S]-triphosphate assays displayed differential potentiation depending on the orthosteric-allosteric pairing, with the largest cooperativity observed for oxotremorine M (Oxo-M) LY2119620. Further [3H]Oxo-M saturation binding, including studies with guanosine-5′-[(β,γ)-imido] triphosphate, suggests that both the orthosteric and allosteric ligands can alter the population of receptors in the active G protein–coupled state. Additionally, this work expands the characterization of the orthosteric agonist, iperoxo, at the M₄ receptor, and demonstrates that an allosteric ligand can positively modulate the binding and functional efficacy of this high efficacy ligand. Ultimately, it was the M₂ receptor pharmacology and PAM activity with iperoxo that made LY2119620 the most suitable allosteric partner for the M₂ active-state structure recently solved (Krusz et al., 2013), a structure that provides crucial insights into the mechanisms of orthosteric activation and allosteric modulation of muscarinic receptors.

Introduction

Muscarinic acetylcholine (ACh) receptors (M₁–M₅) regulate the action of the neurotransmitter ACh, whose signaling roles in the central nervous system include modulation of processes of mood, cognition, exocrine gland function, and smooth muscle control (Wess et al., 2007; Young et al., 2010; Wess, 2012). The demonstrated antipsychotic efficacy of muscarinic agonist agonists in both preclinical and clinical studies makes this G protein–coupled receptor (GPCR) subfamily attractive targets for the treatment of Alzheimer’s disease and schizophrenia (Bodick et al., 1997; Bymaster et al., 2002; Shekhar et al., 2008; reviewed in Felder et al., 2012; Jones et al., 2012; McKinzie and Bymaster, 2012). A key bottleneck to research and therapeutic development efforts for the muscarinic ACh receptor family has been the lack of receptor subtype-selective pharmacological tools to help determine the physiologic relevant family member(s) to target for various indications. However, over the past decade, several muscarinic subtype-selective small molecule ligands have emerged through the successful targeting of unique allosteric binding sites generally located on the exterior surface loops of the receptors [e.g., M₁ (Daval et al., 2013), M₂ (Huang et al., 2005), M₄ (Nawaratne et al., 2010), M₅ (Bridges et al., 2009)]. Within this work, we characterize the allosteric-site ligand LY2119620 (3-amino-5-chloro-N-cyclopropyl-4-methyl-6-[2-(4-methylpiperazin-1-yl)-2-oxoethoxy] thieno[2,3-b]pyridine-2-carboxamide) and demonstrate that it is a high-affinity M₂/M₄ receptor-selective positive allosteric modulator (PAM).

LY2119620 was originally synthesized following hit expansion from a small-molecule screening effort for M₄ receptor–selective allosteric ligands beginning in 1998, a synthetic campaign that also resulted in LY2033298 (3-amino-5-chloro-N-cyclopropyl-6-methoxy-4-methyl-thieno[2,3-b]pyridine-2-carboxamide) built from the same thieno[2,3-b]pyridine core structure.

scaffold (Chan et al., 2004). After the 2004 patent filing by Eli Lilly (Rubio and Hillard, 2006), studies on molecules such as LY2033298 (Chan et al., 2008; Nawaratne et al., 2008, 2010; Leach et al., 2010, 2011; Suratman et al., 2011; Gannon and Millan, 2012; Valant et al., 2012) and others, e.g., VU10010 (Brady et al., 2008; Shirey et al., 2008; Bridges et al., 2010 [http://www.ncbi.nlm.nih.gov/books/NBK143196/]; Lewis et al., 2010; Dencker et al., 2012; Salovich et al., 2012; Huyhn et al., 2013; Le et al., 2013), explored the structure-activity relationship around this scaffold and the in vivo efficacy of such molecules in animal models. Concomitant structure-function work using methods such as site-directed mutagenesis have allowed the mapping of the various ligand binding sites to the M4 receptor and, in one instance, the M2 receptor, and revealed critical regions involved in the receptor activation mechanism (Nawaratne et al., 2008, 2010; Leach et al., 2011; Suratman et al., 2011; Valant et al., 2012). The resultant body of knowledge collected on this M4 receptor-PAM scaffold over the past 15 years allowed us to successfully support the recent breakthrough crystallization efforts of M2 receptor active-state structure (Kruse et al., 2013). Working from the necessity of finding a M2 receptor–selective allosteric binder that could potentiate the already potent agonist, iperoxo, we undertook the characterization of the LY2119620 ligand. The advantage of this ligand over molecules such as LY2033298 was that it had greater M2 receptor PAM activity when paired with ligands like oxotremorine M (Oxo-M). In the present work, we extend the preliminary pharmacological dependence (differences in cooperativity observed with different orthosteric-allosteric pairings); and to positively modulate the potent muscarinic-agonist iperoxo at both the M2 and M4 receptor subtypes.

Materials and Methods
Chinese hamster ovary (CHO) cell lines stably expressing human M1 receptor (Bmax N-methylisopropamine (NMS) = 4.4 pmol/mg membrane; Kd = 0.35 nM), M2 receptor (Bmax NMS = 11.0 pmol/mg membrane; Kd = 0.66 nM), M3 receptor (Bmax NMS = 7.64 pmol/mg membrane; Kd = 0.30 nM), M4 receptor (Bmax NMS = 3.3 pmol/mg membrane; Kd = 0.18 nM), or M2 receptor (Bmax NMS = 4.2 pmol/mg membrane; Kd = 0.33 nM) were obtained from PerkinElmer (Waltham, MA). Chemical and ligands were obtained from the following sources: guanamine-5′-(β-γ-imido)triphosphate (GppNHp) from Axzyara (Farmington, NY); Oxo-M from Tocris (Bristol, UK); ACh from Sigma-Aldrich (St. Louis, MO); iperoxo from Eli Lilly or Monash Institute of Pharmaceutical Sciences (Melbourne, VIC, Australia); LY2119620 from Eli Lilly (Indianapolis, IN); [3H]NMS from GE Healthcare (Piscataway, NJ); [3H]LY2119620 from Vitrex (Placentia, CA); and [3H]Oxo-M and [35S]guanamine-5′-(γ-35S)-triphosphate (GTPγS) from PerkinElmer.

GTPγS-Binding Assays
The level of G protein activation was measured by the amount of nonhydrolyzable GTPγS bound to Gα subunit. The GTPγS-binding was determined using a scintillation proximity assay–bead antibody capture technique (DeLapp et al., 1999). Note for the studies with iperoxo and the M2 receptor that a noncommercial membrane source was used, specifically 25 µg M2 receptor PI membrane preparations of a CHO cell line stably expressing human M2 muscarinic receptor (Bmax = 0.25 ± 0.007 pmol/mg membrane) were used. Otherwise, 20 µg commercial M1-M4 receptor–expressing membranes were used (PerkinElmer). An EC50 value for each response curve was determined by fitting the agonist response data to a three- or four-parameter fit model (GraphPad Prism; GraphPad Software, San Diego, CA).

Agorist-Binding Assays. For the agonist GTPγS-binding curves, 20 µg membranes were incubated in assay buffer (20 mM HEPES, 100 mM NaCl, 0.2 mM EDTA, 1 mM GDP, and 10 mM MgCl2, pH 7.4), 500 pM GTPγS, and varying concentrations of the orthosteric ligands (ACh, iperoxo, McN-A-343 [4-[[3-chlorophenyl]amino]carbonyl]oxy-N,N,N-trimethyl-2-butyln-1-aminium chloride), and pilocarpine, or LY2119620) for 40 minutes at room temperature with mixing. The Go subunits were then captured using anti-rabbit–conjugated scintillation proximity assay beads (PerkinElmer; 1.25 mg/reaction), Guinea antibody (Santa Cruz Biotechnologies, Santa Cruz, CA; 1.3 µg/reaction), and Nonoidet P40 (Roche, 0.1% final concentration) during a 3-hour room temperature incubation. The radioactivity counts of the bound GTPγS were determined by scintillation spectrophotometry (Wallac TriLux; PerkinElmer). An EC50 value was determined by fitting the agonist response data using a three-parameter fit model (GraphPad Prism).

Interaction Experiments. The GTPγS-binding experiment was run as above, except the orthosteric agonist concentration-response curve was measured in the presence of varying concentrations of allosteric ligand LY2119620 (0–10 µM). EC50 values at each concentration of LY2119620 were determined.

Allosteric EC50 Modulation Analysis. The ability of LY2119620 to act as an allosteric modulator was quantified by determining two following: 1) the affinity of LY2119620 for the free receptor (Kd), and 2) the magnitude and direction of the LY2119620 effect on a given orthosteric agonist (as described in Christophoulus and Kenakin, 2002). The Kd and α values were determined by fitting the eight dose-response curves (acquired in the presence of varying concentrations of allosteric ligand LY2119620 (0–10 µM). EC50 values at each concentration of LY2119620 were determined.

Radioligand-Binding Assays
Binding assays were performed in 20 mM HEPES, 100 mM NaCl, and 10 mM MgCl2, pH 7.4 (binding buffer), unless noted. Studies were carried out using commercially prepared CHO membranes expressing the various muscarinic receptor subtypes (PerkinElmer). Reactions were stopped by rapid filtration on a TOMTEC 98-well cell harvester (Tomtec, Hamden, CT). Nonspecific binding was determined using 10 µM atropine. Radioactivity retained on the filter mat was counted on a scintillation spectrophotometry (Wallac 1205 Beta-plate; PerkinElmer). Data were fit to appropriate models using GraphPad Prism 6.7 software (GraphPad Software).

[3H]LY2119620 Saturation-Binding Assays. [3H]LY2119620 equilibrium binding was achieved by incubating 15 µg membranes, orthosteric ligand (100 µM, unless otherwise noted; Oxo-M, ACh, or iperoxo), and various concentrations of [3H]LY2119620 (0.2–60 nM) for 1 hour at 25°C. Association kinetic experiments showed that [3H]LY2119620 reached steady state within 15 minutes; however, a 1-hour incubation was used to allow all ligands within the reaction to reach equilibrium. The specific binding versus time data were fit to a one-site specific binding model, and the Bmax and Kd for the allosteric molecule were calculated for each orthosteric ligand.

[3H]Oxo-M Saturation-Binding Assays. [3H]Oxo-M equilibrium-binding experiments were a two-addition process achieved by first preincubating 15 µg membranes and varying concentrations of [3H]Oxo-M (0.05–15 nM) in the presence or absence of 100 µM GppNHp (GDP-nucleotide analog) for 30 minutes at room temperature, and then adding either 0 or 10 µM allosteric ligand, LY2119620.

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for an additional 30 minutes. The specific binding versus time data were fit to a one-site specific binding model.

**[3H]NMS Kinetic Dissociation Experiments.** The [3H]NMS dissociation kinetic-binding assays were performed using a reverse time protocol. Specifically, 10 μg membrane, 0.2 nM [3H]NMS, and varying concentrations of LY2119620 (0–40 μM; see Fig. 5) were incubated for 1 hour at 30°C in binding buffer. Once equilibrated, 1 μM iperoxo was added in a time-staggered approach to capture 1 to 90 minute time points. The specific binding versus time data were fit to a single exponential decay, and the dissociation rate constant (min⁻¹) was determined for each concentration of LY2119620.

**[3H]NMS Equilibrium Inhibition-Binding Assays.** The displacement of [3H]NMS was performed by adding iperoxo in the absence or presence of 10 μM LY2119620. A concentration of 0.30 nM [3H]NMS was incubated with 15 μg M2/M4 receptor-expressing membranes, 0 or 10 μM LY2119620, and varying concentrations of iperoxo (0–100 μM) and allowed to reach equilibrium for 2 hours at 25°C. The calculated Kᵣ of [3H]NMS was 0.348 nM for the M2 receptor in the absence of LY2119620 and 0.402 nM in the presence of 10 μM LY2119620; the Kᵣ was 0.0878 nM and 0.109 nM for the M4 receptor in the absence and presence of LY2119620, respectively (Supplemental Fig. 2). The Cheng–Prusoff model was used to fit the Kᵣ of iperoxo in the absence and presence of LY2119620 (Cheng and Prusoff, 1973).

**Results**

**Modulation of Receptor G Protein–Coupled Signaling by Allosteric Ligand, LY2119620**

To investigate the selective allosteric modulation of the M₂ and M₄ receptors, we initially used receptor G protein–coupled signaling at all muscarinic subtypes (M₁–M₅). As allosteric ligands such as LY2119620 (Fig. 1) can demonstrate orthosteric probe-dependent binding (Kenakin, 2007; Leach et al., 2010; Valant et al., 2012), studies were carried out in the presence of three orthosteric agonists, iperoxo, Oxo-M, or ACh.

**Characterization of High-Efficacy Agonist, Iperoxo, at the M₄ Receptor**

The agonist, iperoxo, has been reported to have both enhanced affinity and higher efficacy at the M₂ receptor compared with ACh (Langmead and Christopoulos, 2013; Schrage et al., 2013). As this superagonism had not been extensively explored at the M₄ receptor, concentration-response curves for the full agonists ACh, Oxo-M, and iperoxo, and partial-agonist McN-A-343 were carried out using [35S]GTPγS-binding experiments. This assay format was used as it directly measures G protein activation, the first step in GPCR signal transduction. Oxo-M, ACh, and McN-A-343 were selected to calibrate the assay for exploration of the allosteric compound, LY2119620. The potencies of iperoxo at the M₂ receptor (2.12 ± 0.0953 nM) and at the M₄ receptor (8.47 ± 3.00 nM) are much greater than the respective EC₅₀ values of 118 ± 31.7 nM and 514 ± 22.0 nM observed for ACh. Under the conditions tested, no differences in the maximal response elicited by iperoxo, Ach, or Oxo-M were observed (Fig. 2).

**Fig. 1.** The structure of the pharmacological compound LY2119620. The chemical structure of the M₂/M₄ receptor subtype–selective allosteric modulator LY2119620.
Characterization of Allosteric Agonism of LY2119620 Alone

Binding studies with a radiolabeled form of LY2119620, \[^{3}H\]LY2119620, showed no detectable binding of the molecule in the absence of orthosteric ligand at the M1–M5 receptor subtypes (Schober et al., 2014). The intrinsic agonist activity of LY2119620 was also assessed by running G protein activity assays. For these GTP\(\gamma\)S experiments, membranes were incubated with LY2119620 in the absence of exogenous agonist, and the resultant concentration-response curves were normalized as a percentage of the maximal ACh response. As shown in Fig. 2C, LY2119620 showed a modest allosteric agonism of 23.2 ± 2.18% and 16.8 ± 5.01% at the M2 and M4 receptors, respectively. This agonism was less than that observed for the partial agonist control compound McN-A-343 shown in Fig. 2A. Minimal allosteric agonism (<20%) was observed for LY2119620 at the M1, M3, and M5 receptors (Supplemental Fig. 1).

Characterization of Allosteric Modulation of Orthosteric Agonists, ACh, Iperoxo, and Oxo-M by LY2119620. \[^{35}S\]GTP\(\gamma\)S-binding experiments were run to verify that LY2119620 potentiates the activity of an orthosteric agonist ligand, and to determine the degree of cooperativity between the orthosteric-allosteric sites. These experiments paired LY2119620 with agonists, ACh, Oxo-M, or iperoxo, and were run at all muscarinic receptor subtypes (M1–M5). The M2/M3/M5 receptor subtypes showed minimal differences in EC\(_{50}\) values for all agonists tested (data for iperoxo and Oxo-M not shown; data for ACh shown in Supplemental Fig. 1). Figure 3 shows positive cooperativity between LY2119620 and all agonists, with larger potentiation generally noted at M4 than M2. These changes were quantified using the allosteric ternary complex model (Christopoulos and Kenakin, 2002). The variable \(K_B\) estimates the affinity of the orthosteric ligand for the receptor.
of LY2119620 for the allosteric binding site on the unoccupied receptor and was found to be consistently about 1.9–3.4 μM (Fig. 3, insets). The cooperativity factor α, which quantifies the affinity change of one ligand by the other ligand when both are bound to the receptor simultaneously, varied widely depending on the agonist-LY2119620 pairing. LY2119620 and ACh binding led cooperativity factors of 19.5 and 79.4 for the M2 receptor and the M4 receptor, respectively (Fig. 3, A and B). The cooperativity factor of 79.4 at the M4 receptor suggests that a higher degree of positive cooperativity exists between LY2119620 and ACh than the previously described allosteric M4 > M2 receptor modulator, LY2033298 (Chan et al., 2008; Nawaratne et al., 2010). The Oxo-M and LY2119620 pairing showed the highest degree of cooperativity with an α factor of 50.1 and 282 for the M2 receptor and M4 receptor, respectively (Fig. 3, C and D). The iperoxo and LY2119620 pairing showed the most modest cooperativity with an α factor of 14.5 at the M2 receptor and 3.9 at the M4 receptor (Fig. 3, E and F). Additionally, unlike the other allosteric-orthosteric pairings (ACh and Oxo-M), LY2119620 showed a higher degree of cooperativity with iperoxo at the M2 receptor rather than the M4 receptor.

Table 1 summarizes the agonist EC50 values for each concentration of LY2119620 tested.

### Ligand-Binding Studies of LY2119620 to the M2 and M4 Receptors

#### Saturation-Binding Studies Using Both Allosteric and Orthosteric Radioligands Reveal Probe Dependence

The functional GTPγS-binding experiments above establish that LY2119620 positively modulates the G protein–coupled response of the M2/M4 receptor subtypes in a probe-dependent manner. To further elucidate this probe dependence, we set up a series of saturation-binding experiments to view these effects from both the allosteric and orthosteric ligand-binding perspective. The [3H]LY2119620 saturation binding allows assessment of probe dependence from the allosteric site perspective to all three orthosteric ligands under identical experimental conditions. Detailed characterization of the LY2119620 allosteric radioligand, [3H]LY2119620, is presented elsewhere (Schober et al., 2014). The [3H]LY2119620 saturation binding presented in Fig. 4, A and B, shows that the Bmax values for the M2 and M4 receptors were highly agonist-dependent, whereas the Kd values displayed little variance. The Bmax values at the M2 receptor were over 10-fold higher for iperoxo (2640 ± 203 fmol/mg) and Oxo-M (2540 ± 353 fmol/mg) compared with ACh (160 ± 34.0 fmol/mg) (Fig. 4A); the Kd values ranged from 11.2 ± 1.17 nM for iperoxo to 16.9 ± 0.700 nM for Oxo-M. Similarly, at the M4 receptor, the Bmax for Oxo-M was 1110 ± 157 fmol/mg, which was significantly greater than ACh (456 ± 55.0 fmol/mg) or iperoxo (291 ± 19.9 fmol/mg) (Fig. 4B); the Kd values ranged from 2.35 ± 0.237 nM for ACh to 2.93 ± 0.205 nM for iperoxo. Control experiments at varying concentrations of orthosteric agonist (0, 10, 100, 1000 μM) were performed and showed that no further cooperativity (increase in the Bmax) was observed between 10 and 100 μM. This indicated that a lack of orthosteric binding site saturation was not the primary cause for the observed Bmax differences. These results led us to hypothesize that the [3H]LY2119620 binding was only reporting the receptor G protein–coupled state (RG) population; thus, changes in the receptor G protein coupling, in this study arising from differences in the efficiency of the orthosteric ligand to couple G protein, manifest as an apparent change in Bmax. To test whether similar receptor G protein–coupled effects could also be observed from the orthosteric site, experiments were repeated with the orthosteric radioligand, [3H]Oxo-M.

Saturation-binding experiments using [3H]Oxo-M were performed in the presence of varying concentrations of the allosteric-site ligand LY2119620. Figure 4, C and D, shows a Bmax increase at the M3 receptor from 793 ± 1.95 fmol/mg to 2850 ± 162 fmol/mg upon addition of 10 μM LY2119620 (Fig. 4C), and about a 5-fold increase in Bmax at the M4 receptor, 284 ± 18.3 fmol/mg to 1340 ± 42.2 fmol/mg (Fig. 4D). The response was fully saturated at 10 μM LY2119620. The Kd values for the M3 receptor are approximately 2 nM, and for the M4 receptor decreases slightly from 3.27 ± 0.722 to 1.17 ± 0.185 nM upon LY2119620 addition (Table 2). First, these Oxo-M studies verify that allosteric ligand binding modulates the orthosteric site binding. Second, these observations are consistent with the [3H]LY2119620-binding data in which Bmax predominantly changes upon ligand site occupation without a cocommitment change in ligand affinity (Kd).

To test the hypothesis that the change in Bmax being observed may reflect a G protein–coupled active-state receptor pool, the [3H]Oxo-M saturation–binding studies were repeated in the presence of the nonhydrolyzable guanine nucleotide GppNHP. Performing [3H]Oxo-M experiments in the presence of GppNHP reduced the number of receptors in the active RG state by uncoupling the G protein. Figure 4, E and F, shows that [3H]Oxo-M–binding curve has an increase in Bmax from 721 ± 114 nM to 1890 ± 271 nM when 1 μM LY2119620 was added and that this increase was significantly reversed, Bmax of 995 ± 164 nM, by addition of

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**Table 1**

<table>
<thead>
<tr>
<th>Ligand</th>
<th>EC50 (nM)</th>
<th>Agonist Alone</th>
<th>LY2119620 (1 μM)</th>
<th>LY2119620 (10 μM)</th>
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<tr>
<td>ACh</td>
<td>34.3 ± 8.62</td>
<td>7.38 ± 2.03</td>
<td>10.1 ± 3.36</td>
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<tr>
<td>ACh</td>
<td>219 ± 42.8</td>
<td>10.0 ± 2.06</td>
<td>13.5 ± 5.03</td>
<td>31.6 ± 22.3</td>
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<tr>
<td>Oxo-M</td>
<td>22.2 ± 3.51</td>
<td>2.04 ± 0.561</td>
<td>0.956 ± 0.104</td>
<td>1.57 ± 0.270</td>
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<tr>
<td>Oxo-M</td>
<td>123 ± 19.4</td>
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<td>2.45 ± 0.0758</td>
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<tr>
<td>Iperoxo</td>
<td>0.738 ± 0.192</td>
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<tr>
<td>Iperoxo</td>
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<td>0.104 ± 0.00607</td>
<td>0.0925 ± 0.00529</td>
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Changes in the agonist EC50 upon addition of LY2119620

The change in the EC50 value of agonists ACh, Oxo-M, and iperoxo upon addition of the allosteric ligand, LY2119620, was measured using a G protein–coupled functional assay. Values presented represent the means ± S.E.M. of three independent experiments conducted in duplicate.
GppNHp at the M2 receptor (Fig. 4E). Similar trends were observed at the M4 receptor; however, the reversal was not seen to be as complete under the conditions tested (Fig. 4F).

Overall, these saturation studies support the hypothesis that LY2119620 probe dependence reflects an allosteric enhancement of the proportion of RG or G protein-coupled active-state receptors.

**[3H]NMS Competitive Binding Studies in the Presence of LY2119620.** As the equilibrium saturation studies failed to show an observable change in the $K_d$ for a series of competitive binding experiments using $[^{3}H]$NMS were performed. This tested whether the ability of the agonist iperoxo to compete with $[^{3}H]$NMS would change upon addition of LY2119620. Figure 5 shows that, upon addition of the allosteric ligand, the dissociation rate of $[^{3}H]$NMS was retarded (Fig. 5), although no overall changes, in the affinity of NMS for either the M2 or M4 receptors upon binding of the allosteric ligand LY2119620, were observed (Supplemental Fig. 2). The magnitude of this dissociation rate affect varied with receptor subtype. The dissociation rate of $[^{3}H]$NMS from the M2 receptor in the presence of iperoxo was 0.347 ± 0.0203 minute$^{-1}$ and, upon addition of LY2119620, slowed to 0.016 ± 0.00790 minute$^{-1}$ (Fig. 5A); this relative change was very similar at the M4 receptor ($k_{off}$ reduced from...
At the M4 receptor the trends remained consistent with that observed at the M2 receptor, although the potency of LY2119620 was greater (Fig. 5, A and B); Table 3 summarizes all \( t_{1/2} \) and \( k_{\text{off}} \) rates for both the M2 and M4 receptors. Overall, these results are consistent with a model in which LY2119620 is highly cooperative with orthosteric agonist binding, being more positively cooperative at the M4 relative to M2 receptor.

Additional [\(^3H\)NMS] displacement studies were performed to provide evidence that allosteric binding by LY2119620 could be observed to alter the binding affinity of the orthosteric ligand, reflected as a change in \( K_i \) of the agonist. Figure 5, C and D, shows the competitive IC50 value for iperoxo in the absence or presence of LY2119620 and shows that the IC50 values for agonist competition of [\(^3H\)NMS] binding at both the M2 and M4 receptors increased significantly upon addition of LY2119620. Specifically, the \( K_i \) value of iperoxo reduced from 19.6 ± 11.3 nM to 0.732 ± 0.287 nM at the M2 receptor; and from 15.1 ± 3.40 nM to 0.359 ± 0.0366 nM at the M4 receptor (Fig. 5, C and D; for the \( K_d \) of [\(^3H\)NMS] in the presence of LY2119620, see Supplemental Fig. 2). These [\(^3H\)NMS] data thus demonstrate that cooperativity between LY2119620 and orthosteric ligand sites can also be observed at the receptor-binding level (LY2119620 acts as a PAM).

**Discussion**

This study provides insight into the molecular pharmacology of the small molecule, LY2119620, which proves to be...
a M₂/M₄ receptor–selective PAM. Three major impacts of this work are as follows: the identification of a novel M₂/M₄ receptor allosteric pharmacological tool that can be used to inform the development of treatments for neurologic disorders such as schizophrenia; the exploration of probe dependence and the molecular mechanisms by which allosteric molecules may act upon a GPCR and its signaling properties; and the characterization of the potent muscarinic-agonist iperoxo at the muscarinic M₂ and M₄ receptor and its modulation by an allosteric ligand.

The GTPγS-binding assays performed with LY2119620 demonstrate that LY2119620 displays modest allosteric agonism (Fig. 2C) and positively modulates the functional G protein–signaling ability of an agonist at the M₂/M₄ receptor subtypes (Fig. 3). The degree of cooperativity between the orthosteric-allosteric ligands was found to vary depending on the agonist pairings, indicating probe dependence. Probe dependence for allosteric ligands has been reported before (Gregory et al., 2010; Leach et al., 2010; Luttrell and Kenakin, 2011; Suratman et al., 2011; Valant et al., 2012; see also the recent review by Wootten et al., 2013) and proves a significant challenge when trying to validate the suitability of a drug candidate. As the pharmacology can be dramatically impacted by this probe dependence, LY2119620 was evaluated with its natural agonist ACh, as well as full agonists Oxo-M and iperoxo. The results of the functional studies showed that LY2119620 enhanced the potency of all three agonists at the M₂ receptor: ACh 5-fold (34.3 ± 8.62 nM) to 7.38 ± 2.03 nM; Oxo-M 10-fold (22.2 ± 3.51 nM) to 2.04 ± 0.561 nM) and iperoxo 11-fold to a subnanomolar EC₅₀ of 71.1 ± 11.5 pM. At the M₄ receptor, the increase in potency was 22-fold for ACh (219 ± 42.8 nM to 10.0 ± 2.06 nM) and 47-fold for Oxo-M (123 ± 19.4 nM to 2.65 ± 1.05 nM); again the potency of iperoxo dropped to subnanomolar (EC₅₀ = 0.104 nM). Further exploration of whether these orthosteric-allosteric pairing effects seen in G protein signaling also manifest in other downstream signaling pathways such as extracellular regulated kinase 1/2 phosphorylation, glycogen synthase kinase 3β phosphorylation, and receptor internalization is being pursued in subsequent studies.

The radioligand saturation-binding studies presented in Fig. 4 were performed to explore whether probe dependence was also observed at the receptor-ligand–binding level. The tritiated ligand, [³H]LY2119620, allowed the binding of ACh, Oxo-M, and iperoxo under identical experimental conditions. The results of these [³H]LY2119620 studies showed that the number of allosteric receptor sites differed in an agonist-dependent manner and that this change in Bₘₐₓ was not accompanied by a readily measurable change in Kᵢ (Fig. 4, A and B). The lack of two-site binding curves indicated that LY2119620 was not binding to low- and high-affinity receptor site populations. Hypothesizing that LY2119620 binding was only able to monitor the coupled active-state receptors (RG), the differences in Bₘₐₓ perhaps arose because the various orthosteric ligands had differential abilities to promote the receptors into the RG state. Switching the radioligand to the orthosteric site, saturation-binding studies were conducted with [³H]Oxo-M in the presence of various concentrations of LY2119620 (Fig. 4, C–F). The [³H]Oxo-M results suggest that LY2119620 binding to the allosteric site could also increase the number of orthosteric binding sites available (Fig. 4, C and D). Furthermore, Fig. 4, E and F, showed that these allosteric-induced Oxo-M sites could be blocked by decoupling the G protein from the receptor (incubating the receptors with GppNHp) and shifting the receptor to a low-activity state. To summarize, the [³H]Oxo-M saturation studies suggested that the allosteric ligand LY2119620 binds preferential to coupled active-state receptors and can promote the number of receptors in this RG state. A similar redistribution of high- and low-affinity receptor sites was observed in a LY2033298, a congener M₄ receptor PAM, study (Leach et al., 2010). Overall, the allosteric and orthosteric radioligand studies suggest that both Oxo-M and LY2119620 can place the M₂ and M₄ receptors into an active G protein–bound state. Deeper insight into the complex interplay between the orthosteric, allosteric, and G protein–coupled sites is now possible with the availability of tool compounds such as [³H]LY2119620.

Additional radioligand studies were also performed to demonstrate that LY2119620 modulated the binding of agonists at the orthosteric site. Specifically, competitive binding experiments utilizing the [³H]NMS radioligand demonstrated that LY2119620 binding at the allosteric site slowed the dissociation of NMS in the presence of the agonist iperoxo (Fig. 5, A and B). In addition, equilibrium [³H]NMS saturation experiments showed that LY2119620 binding altered the affinity of iperoxo for the orthosteric site; the Kᵢ for iperoxo decreased from 25.1 ± 14.5 nM to 0.878 ± 0.343 nM at the M₂ receptor (Fig. 5, C and D). To summarize, these studies present experimental evidence that LY2119620 acts as a potent PAM for the M₄ > M₂ receptor.

**TABLE 3**

Displacement of [³H]NMS by orthosteric ligands in the presence of LY2119620 at the M₂ and M₄ receptors

<table>
<thead>
<tr>
<th>Binding</th>
<th>0 μM LY2119620</th>
<th>20 μM LY2119620</th>
<th>40 μM LY2119620</th>
<th>10 μM LY2119620</th>
</tr>
</thead>
<tbody>
<tr>
<td>M₂</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iperoxo</td>
<td>2.01 ± 0.125</td>
<td>36.9 ± 14.7</td>
<td>12.2 ± 2.70</td>
<td>5.59 ± 0.466</td>
</tr>
<tr>
<td>M₄</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iperoxo</td>
<td>10.6 ± 0.257</td>
<td>73.9 ± 0.945</td>
<td>30.6 ± 5.12</td>
<td>20.0 ± 3.12</td>
</tr>
</tbody>
</table>

Displacement of [³H]NMS by orthosteric ligands in the presence of LY2119620 at the M₂ and M₄ receptors. Displacement studies were performed in duplicate. 

- **M₂**
  - Emax values: 36.9 ± 14.7, 12.2 ± 2.70, 5.59 ± 0.466
  - Emax values: 73.9 ± 0.945, 30.6 ± 5.12, 20.0 ± 3.12

- **M₄**
  - Emax values: 10.6 ± 0.257, 73.9 ± 0.945, 30.6 ± 5.12, 20.0 ± 3.12

- **Kᵢ (nM):**
  - M₂: 2.01 ± 0.125, 36.9 ± 14.7, 12.2 ± 2.70, 5.59 ± 0.466
  - M₄: 10.6 ± 0.257, 73.9 ± 0.945, 30.6 ± 5.12, 20.0 ± 3.12
subtypes, as measured by both receptor binding (Fig. 5) and G protein functional activity (Fig. 3).

Another important result of this study was the characterization of the potent agonist, iperoxo, to date described in the literature as a M2 superagonist (Kloeckner et al., 2010; Bock et al., 2012; Schrage et al., 2013), and its modulation by an allosteric ligand. Originating from screens for novel derivatives of Oxo-M (Dallanoce et al., 1999), iperoxo was found to possess both superior affinity and efficacy over ACh. Our work recapitulated previous findings that iperoxo possessed an EC50 of 2.12 ± 0.0953 nM at the M2 receptor (EC50/Ach/EC50(iperoxo) = 56-fold; Fig. 2A), and then expanded the pharmacological characterization to the M2 receptor subtype and found similar nanomolar potency (EC50 = 8.47 ± 3.00 nM, and 61-fold enhancement in the EC50/Ach/EC50(iperoxo); Fig. 2B). Additionally, the saturation-binding and functional GTPγS studies showed that this high efficacy agonist was able to be further modulated by binding of an allosteric compound (Fig. 3; Fig. 5).

This study of LY2119620 has explored the molecular mechanism of this M2/M4 receptor subtype--selective PAM mainly in a recombinant cellular system. Moreover, these studies corroborate and extend the initial characterization of the functional cooperativity observed between LY2119620 and iperoxo in 3[H]NMS binding, 32P(GTP)-S binding, and extracellular regulated kinase 1/2 experiments (Kruse et al., 2013). The availability of a radiolabeled form of LY2119620, NMS, and Oxo-M ligands enabled the discovery that LY2119620 binding to the allosteric site increases the proportion of receptors in the G protein--coupled active state. This insight may have implications in light of the emerging structural studies, such as the recent active-state M2 receptor crystal structure with iperoxo and LY2119620 docked in their respective binding sites (Kruse et al., 2013). Although the crystal structure suggests that it is the entry of the orthosteric ligand that is critical for receptor movements leading to the appropriate unfolding of the intracellular domain region for G protein coupling, it is clear that the allosteric vestibule can also contribute to this switch to a permissive G protein--binding state. Ultimately, our work in the development of the M2/M4 receptor--selective PAM, LY2119620, supports these crystalization efforts, which provide the first atomic level understanding of such activation and allosteric modulation mechanisms of the muscarinic receptor family.

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Authorship Contributions

Conducted experiments: Croy, Schober, Xiao.
Contributed new reagents or analytic tools: Christopoulos.
Performed data analysis: Croy, Christopoulos, Schober, Felder.
Wrote or contributed to the writing of the manuscript: Croy, Felder, Quots, Schober, Christopoulos.

References


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