Development of a Radioligand, $[^{3}H]LY2119620$, to Probe the Human M$_2$ and M$_4$ Muscarinic Receptor Allosteric Binding Sites

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ABSTRACT

We have characterized a muscarinic acetylcholine receptor (mAChR) potentiator, LY2119620 (3-amino-5-chloro-6-methoxy-4-methyl-thieno(2,3-b)pyridine-2-carboxylic acid cyclopropylamid) and a derivative of this chemical scaffold, VU152100 (3-amino-N-(4-methoxybenzyl)-4,6-dimethylthieno[2,3-b]pyridine carboxamide), for its binding properties toward homologous human mAChR subtypes, M$_2$ and M$_4$. Binding studies using $[^{3}H]NMS$ further support that LY2119620 binds allosterically to the M$_2$ and M$_4$ mAChRs and was positively cooperative with muscarinic orthosteric agonists. To probe directly the allosteric sites on M$_2$ and M$_4$, we radiolabeled LY2119620. Cooperativity binding of $[^{3}H]LY2119620$ with mAChR orthosteric agonists detects significant changes in $B_{max}$ values with little change in $K_{d}$, suggesting a G protein–dependent process. Furthermore, $[^{3}H]LY2119620$ was displaced by compounds of similar chemical structure but not by previously described mAChR allosteric compounds such as gallamine or WIN 62,577 (17-$b$-hydroxy-17-$a$-ethyl-5-$b$-androstano[3,2-$b$]pyrimido[1,2-$a$]benzimidazole). Our results therefore demonstrate the development of a radioligand, $[^{3}H]LY2119620$ to probe specifically the human M$_2$ and M$_4$ muscarinic receptor allosteric binding sites.

Introduction

Acetylcholine activates two families of receptors: the nicotinic ligand-gated ion channel receptors (nAChRs) and the G protein–coupled muscarinic receptors (mAChRs), classified initially based on their differential activation by nicotine (Lindstrom, 1997) and muscarine (Wess, 1996), respectively. The wide distribution of mAChRs in the central nervous system (CNS) and periphery support their involvement in physiologic processes such as arousal, cognition, pain, exocrine gland secretion, smooth muscle, and vascular contraction (Wess et al., 2003). Muscarinic acetylcholine receptors have long been viewed as viable targets for developing therapeutic agents to treat Alzheimer’s disease and other CNS disorders. The muscarinic agonist xanomeline, for example, was developed to treat Alzheimer’s disease (Bodick et al., 1994), but it was also found to induce improvements in positive, negative, and cognitive symptoms associated with schizophrenia (Shekhar et al., 2008). However, the lack of selectivity of xanomeline led to peripheral side effects that prohibited it from advancing in the clinic. Because of the highly conserved sequence within the acetylcholine binding domain (Heinrich et al., 2009), targeting the orthosteric site for small molecule development resulted in a number of muscarinic agonist compounds with poor selectivity.

However, the discovery of allosteric sites on G protein–coupled receptors (GPCRs) is allowing more selective small molecule modulators to emerge, offering a unique approach to treating CNS diseases (Christopoulos, 2002; May et al., 2007). Allosteric modulators bind to novel sites that are distinct from the natural transmitter orthosteric binding site. Positive
allosteric modulators enhance the efficacy and/or efficacy of the endogenous ligand and have a number of therapeutic advantages compared with direct-acting agonists such as xanomeline. Besides the observation of improved receptor selectivity, positive allosteric modulators offer physiologically relevant spatial and temporal signaling that may limit undesirable side effects compared with direct-acting agonists that can lead to desensitization and long-term changes in receptor densities (Christopoulos, 2002).

Several in vitro pharmacologic methods can be used to assess allosteric binding and functional signaling modulation. Typically, muscarinic allosteric agents have been identified by the effect they induce on radioligand competition experiments at the orthosteric site (Birdsall et al., 1997). For example, allosteric modulators can be revealed by their inability to block fully radiolabeled orthosteric probes such as [3H]NMS ([3H]N-methylscopolamine). Positive allosteric modulation is typically assessed by functional signal transduction studies using various assay formats, including measurement of cAMP generation and [35S]GTPγS [5'-O-(3)-[35S]thio-triphosphate] binding. Because previous methods for studying allosteric binding mechanisms have been limited to indirect measurements using orthosteric binding, we describe here the development of a radiotracer from LY2119620 (3-amino-5-chloro-N-cyclopentyloxy-1-ethyl-6-[(4-methylpiperazin-1-y1)-2-oxoethoxy] thieno[2,3-b]pyridine-2-carboxamide) that allows direct labeling of the muscarinic allosteric site. In addition, we discuss the possibility of being able to discern muscarinic allosteric binding sites in native tissue. Our data support the hypothesis that allosteric selectivity between M2 and M4 mAChR subtypes with LY2119620 is a result of differences in cooperativity and not affinity of the orthosteric agonist, similar to what has been previously described for thiocholine (Lazareno et al., 2004) and LY2033298 (3-amino-5-chloro-6-methoxy-4-methyl-thienn[2,3-b]pyridine-2-carboxylic acid cyclopropylamide) (Leach et al., 2010) at the M4 mAChR.

Materials and Methods

Chinese hamster ovary cell lines stably expressing human M1 (B\text{max} = 4.4 pmol/mg membrane), M2 (B\text{max} = 11.0 pmol/mg membrane), M3 (B\text{max} = 7.64 pmol/mg membrane), M4 (B\text{max} = 4.2 pmol/mg membrane), or M5 (B\text{max} = 3.5 pmol/mg membrane) were purchased from PerkinElmer (Waltham, MA). Chemicals and ligands were purchased from the following sources: oxotremorine-M, VU152100 (3-amino-N-[4-methoxybenzyl]-4,6-dim ethylthieno[2,3-b]pyridine carboxamide), and VU10010 (3-amino-N-(4-chlorophenyl)methyl-4,6-dimethylthieno[2,3-b]pyridine-2-carboxamide) from Torceis (Bristol, UK); acetylcholine, WIN 62,577 (17β-hydroxy-17α-ethyl-5-[4-androstano[3,2-b]pyrimido[1,2-a]benzimidazole], and gallamine triethiodide (Sigma-Aldrich, St. Louis, MO); [3H]NMS (GE Healthcare, Piscataway, NJ); [3H]LY2119620, [3H]GTPγS (PerkinElmer), LY2119620, and LY2033298 (Lilly, Indianapolis, IN).

[3H]NMS Binding Assays. [3H]NMS binding assays were performed in HEPES buffer [20 mM HEPES, 100 mM sodium chloride (NaCl), 10 mM magnesium chloride (MgCl2), pH 7.4] as described previously (Chan et al., 2008) with the following modifications. Briefly, frozen membrane preparations were thawed and resuspended in assay buffer, and approximately 25 μg protein was added to each well and incubated with [3H]NMS for 2 hours at room temperature in a total volume of 200 μl in polypropylene 96-well plates. Nonspecific binding was determined using 10 μM atropine. Membranes were collected by rapid filtration using a Tomtec cell harvester (Tomtec, Inc., Hamden, CT) through GF/A filters that had been presoaked in 0.3% polyethyleneimine. The filters were washed with 5 ml of ice-cold 50 mM Tris buffer (pH 7.4) and air-dried overnight. The dried filters were treated with MultiLex A melt-on scintillator sheets, and the radioactivity retained on the filters was counted using a Wallac 1205 Betaplate scintillation counter (PerkinElmer). Displacement experiments for [3H]NMS were carried out in the presence of various concentrations of compounds for all five human muscarinic receptor subtypes. More specifically, in the potentiation experiments with [3H]NMS, various concentrations of orthosteric agonists were used to displace the radioligand but in the presence of 10.0, 1.0, 0.1, or 0 μM LY2119620. The dissociation kinetic binding assays were performed using a reverse-time protocol. For these experiments, P1 membrane preparations of a Chinese hamster ovary cell line stably expressing either the human M3 or M5 muscarinic mAChR were used. Membranes were added to approximately 1.0 nM [3H]NMS in the presence or absence of LY2119620 and allowed to equilibrate for 2 hours at room temperature. Once equilibrated, 100 μM oxotremorine-M was added in a time-staggered approach to allow 1- to 60-minute time point collection. In the statistical analyses, Kd values were determined from the Cheng-Prusoff relationship:

\[ K_d = \frac{IC_{50}}{1 + [\text{ligand}]/K_a} \]

where IC_{50} is determined from a four-parameter fit of displacement curves, [ligand] = 1 nM [3H]NMS, and Kd is the equilibrium dissociation constant of [3H]NMS for each mAChR subtype determined by saturation binding experiments carried out by the membrane supplier.

[3H]LY2119620 Binding Assays. [3H]LY2119620 saturation binding assays were performed in HEPES buffer (20 mM HEPES, 100 mM NaCl, 10 mM MgCl2, pH 7.4). Saturation binding was initiated by incubating 15 μg of muscarinic-containing membranes (hM1-hM4; PerkinElmer), orthosteric ligand (100 μM unless otherwise noted; oxotremorine-M or acetylcholine), and various concentrations of hot-ligand [3H]LY2119620 (0.2-60.0 nM) for 1 hour at room temperature, although equilibrium was achieved within 15 minutes (data not shown). [3H]LY2119620 displacement assays were performed in HEPES buffer as described previously. Muscarinic-containing membranes M2 or M4 receptors were incubated with 100 μM oxotremorine-M and [3H]LY2119620 at approximately the Kd concentration of the receptor, and varying concentrations (0.1 nM–10 μM) of allosteric ligands gallamine, VU152100, VU10010, WIN 62,577, LY2033298, and LY2119620. Incubations were carried out for 1 hour at room temperature. All reactions were stopped by rapid filtration on a Tomtec 96-well cell harvester. Nonspecific binding was determined using 10 μM LY2033298. Radioactivity retained on the filters was counted on a Wallac 1205 Betaplate. In the statistical analyses, the specific binding versus time data were fit to a one-site specific binding model using GraphPad Prism 6.7 and the Cheng-Prusoff relationship for [3H]LY2119620 displacement studies.

Autoradiographic Studies Using [3H]LY2119620. Male cynomolgous monkey brains were supplied from Covance (Greenfield, IN). Brains were rapidly removed, placed in ice-cold phosphate-buffered saline (PBS) for 5 minutes, frozen on dry ice, and then stored at –80°C. The brains were mounted onto chucks and sectioned at 12 μm using a cryostat (Zeiss, Thornwood, NY). Sagittal sections were thaw-mounted onto gelatin-coated slides and stored at –80°C until being assayed. Sections were initially preincubated for 10 minutes in PBS at room temperature. The sections were then placed into polypropylene containers with approximately 5.0 nM [3H]LY2119620 and either 100 μM acetylcholine or oxotremorine-M. Some near-adjacent sections were also incubated with 10 μM LY2033298 to define nonspecific binding. After a 1-hour incubation, the sections were rinsed with fresh ice-cold PBS on ice for 10 minutes each and dried rapidly. The labeled sections were exposed to FujiFilm Imaging Plate for 15 days. The...
Results

Previous studies have revealed that the mAChRs possess at least one allosteric site located extracellularly to the orthosteric site (Wess, 2005). This pocket is referred to as the “common” allosteric site because prototypical modulators, such as gallamine, alcuronium, and C7/3-phth, interact with all five mAChR subtypes, albeit with different degrees of affinity or selectivity (Christopoulos et al., 1999). In this study, we describe the identification of a novel positive allosteric modulator, LY2119620, for common allosteric site on the human M2 and M4 mAChR. For comparison’s sake, we also evaluated other muscarinic allosteric compounds, including LY2033298, VU10010, VU152100, gallamine, and WIN 62,577 (Fig. 1).

Binding Analysis of LY2119620 Using [3H]NMS. Shown in Fig. 2, LY2119620 displays little to no binding affinity for all five human mAChRs to the orthosteric pocket when interacting with the nonselective antagonist radio-ligand [3H]NMS. In contrast, the orthosteric nonselective antagonist atropine caused a concentration-dependent inhibition in the binding of [3H]NMS for all five mAChRs (Fig. 2). The competition for atropine and LY2119620 for M1–M5 mAChRs is summarized in Table 1. To address whether LY2119620 interacts with the human mAChRs in an allosteric manner, radioligand dissociation experiments were performed to determine whether coincubating LY2119620 with the nonselective muscarinic agonist, oxotremorine-M, changed the off-rate (t_{1/2}) of [3H]NMS because altered dissociation rates can be indicative of an allosteric interaction.

![Fig. 1. Structures of key pharmacologic tools.]( MolPharm_1821612_F1.png)
Binding Analysis of $[^3\text{H}]$LY2119620 as a Radioligand for Human Muscarinic Acetylcholine Receptors. To address whether $[^3\text{H}]$LY2119620 bound specifically to any of the five mAChRs, we performed saturation binding studies with membranes stably expressing the human M1–M5 mAChRs. A summary of these results can be found in Table 2. Confirming unlabeled binding studies, $[^3\text{H}]$LY2119620 did not bind to the M1, M3, or M5 mAChRs. However, $[^3\text{H}]$LY2119620 bound to the human M2 and M4 mAChRs with relatively high affinity. Depending on the orthosteric agonist used, both mAChRs bound with similar affinity ($K_d$) but very different $B_{\text{max}}$ values (number of binding sites). In the absence of orthosteric agonists, no specific binding of $[^3\text{H}]$LY2119620 was detected further, indicating a robust cooperativity between the orthosteric and allosteric sites (data not shown). The $K_d$ values for $[^3\text{H}]$LY2119620 at the human M2 were not significantly different ($P = 0.89, n = 3$, Student’s $t$ test): $12.9 \pm 3.24$ nM and $14.4 \pm 2.7$ nM in the presence of 100 $\mu$M acetylcholine or oxotremorine-M, respectively. The $B_{\text{max}}$ value for $[^3\text{H}]$LY2119620 binding to the human M2 was $160 \pm 34$ fmol/mg protein in the presence of 100 $\mu$M acetylcholine. However, in the presence of 100 oxotremorine-M, the number of binding sites increased 17-fold ($2700 \pm 383$ fmol/mg of protein). The M4 mAChR was similar to M2 in that the $K_d$ values for 100 $\mu$M acetylcholine and oxotremorine-M were not significantly different ($P = 0.57, n = 3$, Student’s $t$ test): $2.54 \pm 0.39$ nM and $2.73 \pm 0.08$ nM, respectively. The $B_{\text{max}}$ values for the M4 mAChR were significantly higher in the presence of 100 $\mu$M oxotremorine-M ($1110 \pm 157$ fmol/mg of protein) compared with acetylcholine ($468 \pm 54$ fmol/mg of protein) ($P < 0.02, n = 3$, Student’s $t$ test). The concentrations of acetylcholine and oxotremorine-M were titrated from 0–1000 $\mu$M, and 100 $\mu$M yielded a maximal response for both M2 and M4 mAChRs (data not shown). Displacement studies were conducted with $[^3\text{H}]$LY2119620 at the human M2 or M4 mAChRs to elucidate whether this compound bound to a unique allosteric site on these receptors or to previously published sites using key allosteric tool compounds. Figure 5 illustrates that $[^3\text{H}]$LY2119620 binding in the presence of 100 $\mu$M oxotremorine-M was potently displaced by unlabeled LY2119620 at both the M2 ($K_i = 15.3 \pm 1.36$ nM) and M4 ($K_i = 1.03 \pm 0.08$ nM) mAChRs. A structurally similar compound, LY2033298, displaced $[^3\text{H}]$LY2119620 binding to the human M2 mAChR with a $K_i$ of $87.1 \pm 20.9$ nM.
and M4 with a $K_i$ of $2.14 \pm 0.28$ nM (Fig. 5). We also investigated whether LY2119620 bound to either the putative strychnine or staurosporine allosteric sites by displacing $[^3H]LY2119620$ with gallamine and WIN 62,577, respectively. Neither gallamine nor WIN 62,577 displaced $[^3H]LY2119620$ from the human M2 or M4 mAChRs under these assay conditions (Table 3). Other M4 allosteric modulators similar to LY2033298, VU152100, and VU10010 were also able to displace $[^3H]LY2119620$ from the human M4 mAChR but not M2. This result was expected based on previous work that has demonstrated that these compounds are selective for the M4 receptor (Brady et al., 2008; Shirey et al., 2008).

**TABLE 1**

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>Atropine IC$_{50}$</th>
<th>LY2119620 IC$_{50}$ (nM)</th>
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<tr>
<td>M1</td>
<td>1.78 ± 0.13</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>M2</td>
<td>4.51 ± 0.22</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>M3</td>
<td>1.78 ± 0.13</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>M4</td>
<td>5.01 ± 0.79</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>M5</td>
<td>5.02 ± 1.01</td>
<td>&gt;10,000</td>
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**Fig. 3.** Dissociation rates for Chinese hamster ovary homogenates stably expressing the human mAChRs M2 (A) or M4 (B). Membranes were incubated with approximately 1.0 nM concentration of $[^4]NMS$ and various concentrations of LY2119620 for 2 hours before the addition of 100 $\mu$M oxotremorine-M. The membrane homogenates were incubated at room temperature for the duration of the experiment. A single representative graph is shown for three separate experiments. Data shown are the results of three independent experiments performed in duplicate. The symbols and error bars are expressed as the mean ± S.E.M., respectively.

**Figure Caption:** The following IC$_{50}$ values are calculated for the displacement of $[^3H]NMS$ by atropine and LY2119620 to human M1–M5 muscarinic acetylcholine receptors.

**Discussion**

The five subtypes of mAChRs are members of the superfamily of G protein–coupled receptors (Caufield and Birdsal, 1998) and are now known to have allosteric binding sites that provide significant modulation of functional signaling (Christopoulos et al., 1998; Christopoulos, 2002; Christopoulos and Kenakin, 2002). In the present study, we showed that the allosteric modulator LY2119620 exerts its modulator effects through a common site on the M4 mAChR, similar to LY2033298 (Chan et al., 2008), which has been extensively studied using mutagenesis (Leach et al., 2010). In agreement with LY2033298, our findings clearly indicate that LY2119620 is a novel allosteric compound that does not interact with the orthosteric site, similar to the properties of known allosteric compounds such as staurosporine (Lazareno et al., 2000) or strychnine (Ellis et al., 1991). To investigate whether LY2119620 bound to mAChRs in a bitopic manner (a ligand engaging both the orthosteric and allosteric sites at the same time), we used $[^3H]NMS$ displacement studies. Unlike the M2 bitopic ligand, McN-A-343 (4-[[[(3-chlorophenyl)amino]carbonyl]oxy]-N,N,N-trimethyl-2-butyn-1-aminium chloride) (Valant et al., 2008), LY2119620 does not displace the classic orthosteric pocket labeled with $[^3H]NMS$. In contrast, the orthosteric antagonist atropine readily displaces $[^3H]NMS$ from the M1–M3 mAChRs (Fig. 2). A radiolabeled allosteric modulator of mAChRs was first described for the M2 mAChR using $[^3H]dimethyl-W84$ ($N,N'$-bis[3-(1,3-dihydro-5-methyl-1,3-dioxo-2H-isindol-2-yl)propyl]-N,N,N'$-atomethyl-1,6-hexanediaminium dibromide) (Tränkle et al., 1998). Prototype muscarinic allosteric agents alcuronium and gallamine displaced in a concentration-dependent manner the high-affinity site of $[^3H]dimethyl-W84$ binding. These data led Tränkle and colleagues (1998) to conclude that this radioligand bound to the “common” allosteric site on M2.
In contrast, \[^3H\]LY2119620 was not displaced by previously described mAChR allosteric compounds such as gal- lamine or WIN 62,577. The greatest distinction between \[^3H\]dimethyl-W84 and \[^3H\]LY2119620 appears to be in how these radioligands interact with the allosteric site. \[^3H\]Dimethyl- W84 negatively modulated the M2 allosteric site, whereas \[^3H\]LY2119620 demonstrated positive cooperativity with this site.

The dissociation rate of \[^3H\]NMS was significantly reduced for both the M2 and M4 mAChRs in the presence of LY2119620 (Fig. 3). We have taken the ability of LY2119620 to slow the off-rate of \[^3H\]NMS as a measure of its allosteric effect (the binding of the allosteric ligand to the allosteric site that alters the affinity of the muscarinic orthosteric agonist to bind to the orthosteric binding pocket on the receptor). The cooperative effect was dependent on the muscarinic ligand it interacts with, which can be positive, negative, or neutral. In the \[^3H\]NMS competition binding, the interaction of LY2119620 with either acetylcholine or oxotremorine-M was positively cooperative for both M2 and M4 mAChRs (Fig. 4). Interestingly, the affinity (\(K_d\)) of \[^3H\]LY2119620 for the mAChR was similar, whereas the \(B_{\text{max}}\) varied considerably whether acetylcholine or oxotremorine-M was used. This finding of probe dependence (the interaction between allosteric and orthosteric sites changing, depending on the orthosteric ligand used) was evident in these studies because we used saturating concentrations of acetylcholine or oxotremorine-M. Probe dependence was also shown for the structurally similar

![Fig. 4. Concentration-dependent effects of LY2119620 on \[^3H\]NMS displacement binding to the M2 and M4 mAChRs in the presence of various concentrations of either oxotremorine-M (A and B) or acetylcholine (C and D). M2 and M4 Chinese hamster ovary membrane homogenates were incubated with \(\sim 1.0 \text{ nM} \[^3H\]NMS\) for 2 hours at room temperature. Data shown are the results of three independent experiments performed in duplicate and expressed as percent of control. The symbols and error bars are expressed as the mean \(\pm\) S.E.M., respectively.](image)

| Table 2 | Saturation binding of \[^3H\]LY2119620 to human M1–M5 muscarinic acetylcholine receptors in the presence of 100 \(\mu\text{M}\) of the orthosteric agonists acetylcholine or oxotremorine-M |
|-------------------|-------------------|-------------------|-------------------|-------------------|
| Receptor Subtype | Acetylcholine (100 \(\mu\text{M}\)) | Oxotremorine (100 \(\mu\text{M}\)) |
| \(B_{\text{max}}\) fmol/mg | \(K_d\) nM | \(B_{\text{max}}\) fmol/mg | \(K_d\) nM |
| M1 | BDL | BDL | BDL | BDL |
| M2 | 160 ± 34.0 | 12.9 ± 3.24 | 2700 ± 383 | 14.4 ± 2.70 |
| M3 | BDL | BDL | BDL | BDL |
| M4 | 468 ± 53.8 | 2.54 ± 0.389 | 1110 ± 157 | 2.73 ± 0.0774 |
| M5 | BDL | BDL | BDL | BDL |

BDL, below detectable limits.
compounds at the human mAChRs M2 (A) and M4 (B). Membranes were incubated with approximately 5.0 nM concentrations of [3H]LY2119620 for 1 hour. The addition of 10 μM LY2033298 was used to define nonspecific binding. Data shown are the results of three independent experiments performed in duplicate and expressed as percent of control. The symbols and error bars are expressed as the mean ± S.E.M., respectively.

### TABLE 3

<table>
<thead>
<tr>
<th>Receptor Subtype</th>
<th>LY2119620</th>
<th>LY2033298</th>
<th>VU10010</th>
<th>VU152100</th>
<th>Gallamine</th>
<th>WIN 62,577</th>
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<tr>
<td>M2</td>
<td>15.3 ± 1.36</td>
<td>87.1 ± 20.9</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
</tr>
<tr>
<td>M4</td>
<td>1.03 ± 0.0776</td>
<td>2.14 ± 0.279</td>
<td>42.6 ± 16.4</td>
<td>48.3 ± 10.7</td>
<td>&gt;10,000</td>
<td>&gt;10,000</td>
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Defining the allosteric binding site by displacing [3H]LY2119620 with other allosteric compounds in the presence of 100 μM oxotremorine-M. The K_i values shown are the result of three independent experiments performed in duplicate. Values are expressed as the averages ± S.E.M.

In summary, we have identified LY2119620 as an allosteric modulator of the human M2 and M4 mAChRs. Our data this somehow reflects the coupling of the GPCR to the G protein(s) to promote the active state (Christopoulos and El-Fakahany, 1999). Not yet tested was whether the functional positive allosteric modulation by either LY2033298 or LY2119620 can be driven by increasing the cooperativity between orthosteric ligand and G protein binding, thus increasing the number of G protein–bound mAChRs, thereby increasing the functional output of the signaling being measured. This interaction could be inferred because functional [35S]GTPγS binding was positively modulated by LY2119620 (Croy et al., 2014). Furthermore, emerging crystal structure studies with allosteric compounds might shed some light on probe dependence. Recently, the M2 mAChR was crystalized in the active state with iperoxo docked in the orthosteric binding pocket in the presence of LY2119620 (Kruse et al., 2013). The M2 crystal structure revealed that LY2119620 induces additional, albeit subtle, structural changes compared with those seen with just the orthosteric agonist.

Like its predecessor LY2033298, LY2119620 is also subject to species variability. In the initial characterization of LY2033298, it was noted that this compound had reduced in vitro potency as a modulator in the rat compared with the human M4 mAChR (Chan et al., 2008) and later for the mouse (Suratman et al., 2011). However, the affinity for LY2033298 across species has been shown to be very similar (Leach et al., 2010; Suratman et al., 2011; Valant et al., 2012) in several assay formats. In contrast, any attempt to get [3H]LY2119620 to bind to either recombinantly expressed rodent muscarinic or native tissue in the presence of any orthosteric agonist was not successful. However, it should be pointed out that the concentrations of radioligand used were limited as a result of reagent costs as well as increasing nonspecific binding at higher concentrations. These direct-labeling experiments with a radiolabeled allosteric probe contradict previous reports using various functional assays that these allosteric modulators have similar affinity across species (Leach et al., 2010; Suratman et al., 2011; Valant et al., 2012). In addition, we used [3H]LY2119620 to probe the distribution of these allosteric sites in the brain. We used nonhuman primates as the gene sequences between them and humans are nearly identical. We found the distribution of [3H]LY2119620 to be similar to the distribution of M2 and M4 mAChRs using [3H]AF-DX 384 ([N-2-[2-[(dipropylamino)methyl]-1-piperidinyl]ethyl]-5,6-dihydro-6-oxo-11H-pyrido[2,3-b][1,4]benzodiazepine-11-carboxamide), a selective M2 and M4 antagonist of the muscarinic acetylcholine receptors (Quirion et al., 1993). [3H]AF-DX 384 binds preferentially to the striatum, cortex, thalamus, and cerebellum.

In summary, we have identified LY2119620 as an allosteric modulator of the human M2 and M4 mAChRs. Our data
likewise, we report in this study that the affinity of tively with orthosteric agonist binding. 
allosteric binding governs G protein recruitment coopera-


Lazareno S, Popham A, and Birdsell NJM (2000) Allosteric interactions of staur-


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