

# Binding of Orthosteric Ligands to the Allosteric Site of the M<sub>2</sub> Muscarinic Cholinergic Receptor

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## ABSTRACT

The M<sub>2</sub> muscarinic receptor has two topographically distinct sites: the orthosteric site and an allosteric site recognized by compounds such as gallamine. It also can exhibit cooperative effects in the binding of orthosteric ligands, presumably to the orthosteric sites within an oligomer. Such effects would be difficult to interpret, however, if those ligands also bound to the allosteric site. Monomers of the hemagglutinin (HA)- and FLAG-tagged human M<sub>2</sub> receptor therefore have been purified from coinfecting Sf9 cells and examined for any effect of the antagonist *N*-methyl scopolamine or the agonist oxotremorine-M on the rate at which *N*-[<sup>3</sup>H]methyl scopolamine dissociates from the orthosteric site (*k*<sub>obsd</sub>). The predominantly monomeric status was confirmed by coimmunoprecipitation and by cross-

linking with bis(sulfosuccinimidyl)suberate. Both *N*-methyl scopolamine and oxotremorine-M acted in a cooperative manner to decrease *k*<sub>obsd</sub> by 4.5- and 9.1-fold, respectively; the corresponding estimates of affinity (log *K*<sub>L</sub>) are  $-2.55 \pm 0.13$  and  $-2.29 \pm 0.14$ . Gallamine and the allosteric ligand obidoxime decreased *k*<sub>obsd</sub> by more than 100-fold (log *K*<sub>L</sub> =  $-4.12 \pm 0.04$ ) and by only 1.1-fold (log *K*<sub>L</sub> =  $-1.73 \pm 0.91$ ), respectively. Obidoxime reversed the effect of *N*-methyl scopolamine, oxotremorine-M, and gallamine in a manner that could be described by a model in which all four ligands compete for a common allosteric site. Ligands generally assumed to be exclusively orthosteric therefore can act at the allosteric site of the M<sub>2</sub> receptor, albeit at comparatively high concentrations.

The M<sub>2</sub> muscarinic acetylcholine receptor is a member of the rhodopsin-like family of G protein-coupled receptors (GPCRs) and contains at least two ligand-binding sites: the orthosteric site and a topographically distinct allosteric site. The former is located within a pocket formed by the cluster of transmembrane helices (Curtis et al., 1989; Hulme et al., 1990), and binding involves amino acid residues that are well conserved among the five muscarinic subtypes (Curtis et al., 1989; Hulme et al., 2003). The allosteric site seems to be located at the entrance to the orthosteric pocket (Tuček and Proška, 1995), and it is thought to involve residues from the second and third extracellular loops (Ellis et al., 1993; Ellis and Seidenberg, 2000).

Muscarinic receptors are known to form oligomers (Wreg-

gett and Wells, 1995; Zeng and Wess, 1999; Park and Wells, 2004; Goin and Nathanson, 2006; Ma et al., 2007), a property that is common to many and perhaps all GPCRs (e.g., Gomes et al., 2001; Milligan, 2001; Angers et al., 2002; Park et al., 2004). Oligomerization results in two or more orthosteric and allosteric sites per multimer, and such an arrangement could allow for several types of cooperative interaction. Heterotropic cooperativity between allosteric and orthosteric sites could be either intra- or intermolecular with respect to individual molecules of the receptor. Homotropic cooperativity requires oligomers and could occur between orthosteric sites on the one hand or allosteric sites on the other.<sup>1</sup>

Heterotropic cooperativity between allosteric modulators such as gallamine and orthosteric ligands such as *N*-methyl scopolamine is evident in the effect of the former on the rate

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**ABBREVIATIONS:** HA, hemagglutinin; BS<sup>3</sup>, bis(sulfosuccinimidyl)suberate; Chin3/6, 9hexane-1,6-bis[dimethyl-3'-(4-oxo-2-phenyl-3,4-dihydro-2*H*-quinazolin-1-yl)propylammonium bromide]; NMS, *N*-methyl scopolamine; Obi, obidoxime; Oxo-M, oxotremorine-M; W84, hexane-1,6-bis[dimethyl-3'-phthalimidopropylammonium bromide]; UH-AH 37, 6-chloro-5,10-dihydro-5-((1-methyl-4-piperidyl)acetyl)-11*H*-dibenzo(*b,e*)(1,4)-diazepine-11-one; AF-DX 116, otenzepad; AF-DX 384, 5,11-dihydro-11-(((2-((dipropylamino)methyl)-1-piperidiny)ethyl)amino)carbonyl)-6*H*-pyrido(2,3-*b*)(1,4)-benzodiazepin-6-one methanesulfonate.

of dissociation of the latter (Stockton et al., 1983; Lee and el-Fakahany, 1988; Waelbroeck et al., 1988). Homotropic cooperativity in the binding of orthosteric ligands has been inferred from noncompetitive effects between the antagonists *N*-methyl scopolamine and quinuclidinyl benzilate in studies at thermodynamic equilibrium. A quantitative description of the data requires four interacting sites, presumably within a tetramer (Wreggett and Wells, 1995; Park et al., 2002; Ma et al., 2007). When the receptor is purified as a monomer, the same antagonists bind in a competitive manner (Park and Wells, 2003; Ma et al., 2007). Homotropic cooperativity among four orthosteric sites also can account for the guanyl nucleotide-sensitive binding patterns revealed by muscarinic agonists, which raises the possibility that cooperativity has a role in the signaling process (Wreggett and Wells, 1995; Chidiac et al., 1997; Ma et al., 2007).

The notion that noncompetitive effects denote cooperativity within an oligomer presupposes that muscarinic ligands such as *N*-methyl scopolamine, quinuclidinyl benzilate, and various agonists bind exclusively to the orthosteric site. Also, it generally is assumed that the effect of an allosteric modulator such as gallamine on the dissociation of a supposed orthosteric ligand such as *N*-methyl scopolamine is strictly intramolecular. The interpretation of such effects is less clear, however, if the ligands are less than wholly selective for one site or the other. Although gallamine seems to recognize only the allosteric site (Waelbroeck, 1994; Ellis and Seidenberg, 1999), the affinity of that site for orthosteric ligands and the consequences of any such interaction are unknown.

The present investigation was prompted by preliminary evidence that the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine from the orthosteric site of the M<sub>2</sub> receptor was slowed by oxotremorine-M in a manner similar to that of gallamine. The effect was mechanistically ambiguous, however, owing to the presence of oligomers. Two experimental tools therefore have been used to examine whether supposed orthosteric ligands affect dissociation of the radioligand via the allosteric site or a neighboring orthosteric site. First, the effect has been characterized in a preparation of monomeric M<sub>2</sub> receptors purified from *Sf9* cells (Park and Wells, 2003). Second, the pharmacological specificity associated with the effect has been examined by means of the allosteric ligand obidoxime, which binds to the same site as gallamine but has little effect on the dissociation of *N*-methyl scopolamine from the orthosteric site (Ellis and Seidenberg, 1992). The results indicate that compounds commonly viewed as orthosteric ligands can bind to the allosteric site and mimic gallamine in their effect on the binding of *N*-methyl scopolamine.

## Materials and Methods

**Ligands, Antibodies, and Other Materials.** *N*-[<sup>3</sup>H]Methyl scopolamine was purchased from GE Healthcare (80 Ci/mmol; Chalfont St. Giles, Buckinghamshire, UK). Unlabeled *N*-methyl scopolamine hydrobromide, oxotremorine-M, gallamine triethiodide, carbamoylcholine chloride, and aminobenzotropine were purchased from Sigma-Aldrich (St. Louis, MO). Obidoxime chloride was from Toronto Research Chemicals, Inc. (Toronto, ON, Canada).

Digitonin used for solubilization and purification of the receptor was purchased from Wako Bioproducts (Richmond, VA) at purity near 100%. Digitonin used for buffers to pre-equilibrate and elute Sephadex G-50 columns in binding assays was purchased from Cal-

biochem (San Diego, CA). Cholic acid was from Sigma-Aldrich. Sephadex G-50 Fine was purchased from Sigma-Aldrich, and Fast-Flow DEAE-Sepharose was from GE Healthcare. Other chemicals were obtained as follows: magnesium chloride and sodium chloride (BDH, Inc., Poole, Dorset, UK), dithiothreitol (Bioshop Canada, Burlington, ON, Canada), glycerol (Caledon Laboratories Ltd., Georgetown, ON, Canada), methanol, SDS, and potassium chloride (EMD Chemicals, Inc., San Diego, CA), BS<sup>3</sup> (Pierce Biotechnology, Inc., Rockford, IL), HEPES (Roche Diagnostics, Indianapolis, IN), and EDTA, glycine, magnesium sulfate, phenylmethylsulfonyl fluoride, Tris (Trizma base), and Tween 20 (Sigma-Aldrich).

Econo-Pacs and Econo-Columns were purchased from Bio-Rad Laboratories (Mississauga, ON, Canada), and receptor was concentrated using Centricon and Centriprep concentrators (Amicon) purchased from Millipore Corporation (Billerica, MA). Total protein concentration was estimated by means of bicinchoninic acid using the BCA Protein Assay Kit and bovine serum albumin, taken as the standard, purchased from Pierce.

Anti-HA antibody conjugated to horseradish peroxidase (goat) was purchased from Roche Diagnostics. Agarose-conjugated anti-FLAG antibody used for immunoprecipitation was from Sigma-Aldrich (M<sub>2</sub>, mouse).

**Muscarinic Receptor.** Human M<sub>2</sub> muscarinic receptor bearing the hemagglutinin (HA; YPYDVPDYA) or FLAG (DYKDDDDA) epitope at the amino terminus was expressed in *Sf9* cells and purified as described previously (Wreggett and Wells, 1995; Park et al., 2001; Ma et al., 2007). *Sf9* cells were cultured at 27°C in Ex-Cell 400 insect media (JRH Biosciences, Lenexa, KS) containing 2% fetal bovine serum, 1% Fungizone (amphotericin B), and 0.01% gentamicin (all from Invitrogen Canada, Burlington, ON, Canada). Cells growing at a density of  $2 \times 10^6$  cells/ml were infected with one or both baculoviruses and harvested 48 h later. The total multiplicity of infection was five. The membranes were solubilized in digitonin-cholate (0.86% digitonin, 0.17% cholate), and the receptor was purified via successive passage on DEAE-Sepharose, 3-(2'-aminobenzhydryloxy)tropane-Sepharose, and hydroxyapatite. The final concentrations of digitonin and cholate were 0.1 and 0.02%, respectively. Purified receptor was stored at -75°C.

**Cross-Linking.** Samples of the unprocessed extract or the purified receptor were supplemented with an aliquot of the cross-linking reagent BS<sup>3</sup> in deionized water (20 mM) to yield a final concentration of 2 mM. The mixture was incubated for 30 min at room temperature, and the reaction was terminated by the addition of Tris-HCl (1 M, pH 8.00) to a final concentration of 20 mM. After further incubation for 15 min at room temperature, the sample was placed on ice pending examination by electrophoresis as described below. Samples lacking BS<sup>3</sup> but treated in a parallel and otherwise identical manner were used as controls.

**Immunoprecipitation, Electrophoresis, and Western Blotting.** Those procedures were carried out essentially as described previously (Park et al., 2001; Park and Wells, 2003). To test for coimmunoprecipitation, aliquots of tagged receptor (500 μl) were supplemented with a 50% slurry of the agarose-conjugated anti-FLAG antibody (20 μl) and shaken overnight at 4°C. Immunoabsorbed receptor was collected by centrifugation for 5 min at 4°C and 1000g, and the precipitated beads were washed four times by resuspension in 1 ml of buffer A (9.1 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.7 mM NaH<sub>2</sub>PO<sub>4</sub>, and 150 mM NaCl, adjusted to pH 7.40 with NaOH) and subsequent centrifugation.

Samples for electrophoresis were heated at 65°C for 5 min before loading on precast polyacrylamide gels from Bio-Rad Laboratories (Ready Gel Tris-HCl, 10%). It has been shown previously that these conditions do not induce aggregation of the M<sub>2</sub> muscarinic receptor from *Sf9* cells (Park and Wells, 2003). Resolved proteins were transferred onto nitrocellulose membranes (Bio-Rad Laboratories, 0.45 μm) in a Mini Trans-Blot Transfer Cell (Bio-Rad Laboratories). The membranes were then treated with the horseradish peroxidase-conjugated, anti-HA antibody for 2 h at a dilution of 1:1000. Proteins

were visualized by chemiluminescence using reagents and film purchased from GE Healthcare (ECL, Hyperfilm MP). Densitometry was performed on images that were digitized at a resolution of 200 dots per inch, and the intensities of the bands were quantified using ImageJ (<http://rsb.info.nih.gov/ij>).

**Binding Assays.** For binding at equilibrium, *N*-[<sup>3</sup>H]methyl scopolamine and any unlabeled ligands were dissolved in buffer B (250 mM HEPES, 0.8 mM EDTA, 10 mM MgCl<sub>2</sub>, and 0.1 mM phenylmethylsulfonyl fluoride, adjusted to pH 7.40 with NaOH) supplemented with 0.1% digitonin and 0.02% cholate. An aliquot (50 μl) was added to a sample of the purified receptor (3 μl) in a polypropylene microcentrifuge tube, and the reaction mixture was incubated at 30°C for 45 min. Bound radioligand then was separated by applying an aliquot (50 μl) to a column of Sephadex G-50 Fine (0.8 × 6.5 cm) pre-equilibrated with buffer C (20 mM HEPES, 20 mM NaCl, 5 mM MgSO<sub>4</sub>, and 1 mM EDTA, adjusted to pH 7.40 with NaOH) supplemented with 0.017% digitonin. The assays were performed in triplicate. Nonspecific binding was estimated in the presence of 1 mM unlabeled *N*-methyl scopolamine.

To measure the time course of dissociation, a solution of *N*-[<sup>3</sup>H]methyl scopolamine in buffer B (1509–1651 μl) was added to a sample of the purified receptor (91–99 μl) in polypropylene microcentrifuge tubes. The final concentration of the radioligand was 6 to 10 nM. The reaction mixture was incubated at 30°C for 45 min. Two aliquots (50 μl) then were removed and applied to Sephadex G-50 as described above to obtain an estimate of initial binding (i.e.,  $t = 0$ ). Net dissociation of *N*-[<sup>3</sup>H]methyl scopolamine was initiated by the addition of unlabeled *N*-methyl scopolamine either alone or together with a second ligand being examined for its allosteric effect on binding of the radioligand (i.e., oxotremorine-M, *N*-methyl scopolamine, obidoxime, or gallamine). The final concentration of unlabeled *N*-methyl scopolamine was 10 μM except in those assays when it was being examined for its allosteric effect. Incubation was continued at 30°C, and duplicate aliquots (50 μl) were removed at different times and applied to Sephadex G-50 as described above. All concentrations were corrected as required to accommodate an increase of 1 to 13% in the volume of the reaction mixture upon the addition of the allosteric modulator. Nonspecific binding was measured in parallel assays performed at equilibrium as described above. The final concentration of the radioligand was essentially the same as that in the time course, and any difference was accommodated by a linear correction to the estimate of nonspecific binding.

**Analysis of Data.** Concentrations of receptor and ligand refer throughout to the concentration in the binding assay. Data were analyzed empirically in terms of the Hill equation, formulated as eq. 1 or eq. 2.

$$B_{\text{obsd}} = B_{\text{max}} \frac{([P]_t - B_{\text{sp}})^{n_H}}{K^{n_H} + ([P]_t - B_{\text{sp}})^{n_H}} + \text{NS}([P]_t - B_{\text{sp}}) \quad (1)$$

The variable  $B_{\text{obsd}}$  in eq. 1 represents the total binding of *N*-[<sup>3</sup>H]methyl scopolamine (P) at the total concentration  $[P]_t$ , and  $B_{\text{sp}}$  is the corresponding value of specific binding. The parameter  $B_{\text{max}}$  represents maximal specific binding,  $n_H$  is the Hill coefficient, and  $K$  is the concentration of unbound radioligand that yields half-maximal occupancy of the receptor. The parameter NS represents the fraction of unbound radioligand that appears as nonspecific binding, which was approximately 0.012% for *N*-[<sup>3</sup>H]methyl scopolamine over the course of the investigation (i.e.,  $\text{NS} = 0.00012 \pm 0.00001$ ,  $n = 30$ ).

$$\frac{k_{\text{obsd}}}{k_0} = (Y_{[L]=0} - Y_{[L] \rightarrow \infty}) \frac{\text{EC}_{50}^{n_H}}{\text{EC}_{50}^{n_H} + [L]^{n_H}} + Y_{[L] \rightarrow \infty} \quad (2)$$

In eq. 2,  $k_{\text{obsd}}$  and  $k_0$  represent rate constants for the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine. Further details are described below. The parameters  $Y_{[L]=0}$  and  $Y_{[L] \rightarrow \infty}$  represent the asymptotic values of  $k_{\text{obsd}}/k_0$ , and  $\text{EC}_{50}$  is the concentration at which ligand L achieves a half-maximal reduction in  $k_{\text{obsd}}/k_0$ .

Net dissociation of *N*-[<sup>3</sup>H]methyl scopolamine was analyzed in

terms of a single exponential, as shown in eq. 3. The fits were not improved by the addition of a second exponential. The variable  $B_{\text{obsd}}$  represents total binding at time  $t$  after the addition of the allosteric modulator to the pre-equilibrated mixture of receptor and radioligand. The parameters  $B_{t=0}$  and  $B_{t \rightarrow \infty}$  represent binding immediately before the addition of the modulator and at infinite time, respectively, and  $k_{\text{obsd}}$  is the apparent rate constant. The value of  $B_{t \rightarrow \infty}$  was fixed throughout at the value obtained for nonspecific binding at equilibrium in the presence of 1 mM unlabeled *N*-methyl scopolamine, corrected as required for any difference in the concentration of the radioligand. This restraint generally was without appreciable effect on the goodness of fit, as indicated by the weighted sum of squares, and it was required when the asymptote was not defined by the data.

$$B_{\text{obsd}} = (B_{t=0} - B_{t \rightarrow \infty})e^{-k_{\text{obsd}}t} + B_{t \rightarrow \infty} \quad (3)$$

Dose-dependent allosteric effects on the rate of dissociation of *N*-[<sup>3</sup>H]methyl scopolamine were assessed in a manner similar to that described previously (Ellis and Seidenberg, 1992). The analyses were based on Scheme 1, in which three ligands (A, B, and C) compete for the allosteric site of a receptor (R) occupied at the orthosteric site by a radiolabeled probe (P). If the allosteric ligands equilibrate rapidly with the receptor on the time scale of the binding assay, the observed rate constant for dissociation of the probe is given by

$$k_{\text{obsd}} = k_P \frac{[RP]}{[RP]_t} + k_A \frac{[ARP]}{[RP]_t} + k_B \frac{[BRP]}{[RP]_t} + k_C \frac{[CRP]}{[RP]_t} \quad (4)$$

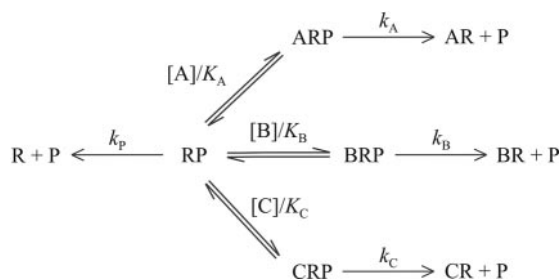
The parameter  $k_P$  in eq. 4 is the first-order rate constant for dissociation of the probe when the allosteric site is vacant, and the parameters  $k_A$ ,  $k_B$ , and  $k_C$  are the corresponding constants when the allosteric site is occupied by ligand A, B, or C, respectively. The quantity  $[RP]_t$  is defined by the equation of state for receptor bound with probe at the orthosteric site; that is,

$$[RP]_t = [RP] + [ARP] + [BRP] + [CRP] \quad (5)$$

The ratio  $[RP]/[RP]_t$  therefore can be expressed as

$$\frac{[RP]}{[RP]_t} = \frac{1}{1 + \frac{[A]}{K_A} + \frac{[B]}{K_B} + \frac{[C]}{K_C}} \quad (6)$$

where  $[A]$ ,  $[B]$ , and  $[C]$  represent the free concentrations of the allosteric ligands, and the parameters  $K_A$ ,  $K_B$ , and  $K_C$  represent the equilibrium dissociation constants for the interaction of each ligand with the allosteric site (e.g.,  $[A][RP]/[ARP] = K_A$ ). The difference between free and total concentration was negligible at the concentrations used in the assays. Likewise, the ratio  $[ARP]/[RP]_t$  can be expressed as



**Scheme 1.** Dissociation of a radioligand from the orthosteric site of a receptor in the presence of three ligands that compete for a common allosteric site. The radioligand was *N*-[<sup>3</sup>H]methyl scopolamine (P). The allosteric ligands included various combinations of gallamine or oxotremorine-M (A), obidoxime (B), and unlabeled *N*-methyl scopolamine (C). Further details are described under *Analysis of Data*.



$$\frac{[\text{ARP}]}{[\text{RP}]_t} = \frac{\frac{[\text{A}]}{K_A}}{1 + \frac{[\text{A}]}{K_A} + \frac{[\text{B}]}{K_B} + \frac{[\text{C}]}{K_C}}, \quad (7)$$

and analogous equations can be written for  $[\text{BRP}]/[\text{RP}]_t$  and  $[\text{CRP}]/[\text{RP}]_t$ .

If the rate constant for dissociation in the presence of an allosteric ligand is expressed as a fraction of that in its absence (e.g.,  $k_A = f_A k_P$ ), eq. 4 can be combined with eqs. 6 and 7 to obtain

$$k_{\text{obsd}} = k_P \left( \frac{1 + f_A \frac{[\text{A}]}{K_A} + f_B \frac{[\text{B}]}{K_B} + f_C \frac{[\text{C}]}{K_C}}{1 + \frac{[\text{A}]}{K_A} + \frac{[\text{B}]}{K_B} + \frac{[\text{C}]}{K_C}} \right). \quad (8)$$

If the effect of the allosteric ligand is expressed as the change in  $k_P$  relative to the unmodulated value (e.g.,  $m_A = 1 - f_A$ ), eq. 8 can be rewritten as

$$k_{\text{obsd}} = k_P \left( \frac{1 + (1 - m_A) \frac{[\text{A}]}{K_A} + (1 - m_B) \frac{[\text{B}]}{K_B} + (1 - m_C) \frac{[\text{C}]}{K_C}}{1 + \frac{[\text{A}]}{K_A} + \frac{[\text{B}]}{K_B} + \frac{[\text{C}]}{K_C}} \right). \quad (9)$$

The value of  $k_{\text{obsd}}$  was estimated throughout in the presence 10  $\mu\text{M}$  unlabeled *N*-methyl scopolamine, either alone or together with gallamine, obidoxime, oxotremorine-M, or additional *N*-methyl scopolamine. Each experiment included up to four kinetic traces: one with unlabeled *N*-methyl scopolamine alone at a concentration of 10  $\mu\text{M}$ , and those in the presence of an additional ligand or ligands. The value of  $k_{\text{obsd}}$  from the former trace has been designated  $k_0$  and was used to normalize those from all other traces. The resulting values of  $k_{\text{obsd}}/k_0$  from several experiments then were pooled and analyzed in terms of eq. 10 to obtain fitted estimates of  $k_P/k_0$ ,  $m_L$ , and  $K_L$  ( $L \equiv A, B$ , or  $C$ ).

$$\frac{k_{\text{obsd}}}{k_0} = \frac{k_P}{k_0} \left( \frac{1 + (1 - m_A) \frac{[\text{A}]}{K_A} + (1 - m_B) \frac{[\text{B}]}{K_B} + (1 - m_C) \frac{[\text{C}]}{K_C}}{1 + \frac{[\text{A}]}{K_A} + \frac{[\text{B}]}{K_B} + \frac{[\text{C}]}{K_C}} \right) \quad (10)$$

Unlabeled *N*-methyl scopolamine was designated throughout as ligand C, and the concentration was entered as either 10  $\mu\text{M}$  or a higher value as appropriate. Ligand B was obidoxime, and ligand A was either oxotremorine-M or gallamine.

**Statistical Procedures.** All parameters were estimated by nonlinear regression. The data were weighted according to the standard error ( $B_{\text{obsd}}$ , eq. 1) or the error as estimated from the diagonal element of the covariance matrix ( $k_{\text{obsd}}$ , eq. 2 and 10). Means are presented together with the standard error. Parametric values derived from a single analysis of one or more sets of data are presented together with the errors as estimated from the covariance matrix. In all figures, values plotted on the y-axis were normalized in the context of the fitted model as described previously [i.e., eq. 6 in Chidiac et al. (1997)]. Further details regarding the statistical procedures have been described previously (Chidiac et al., 1997; Ma et al., 2007; and references therein).

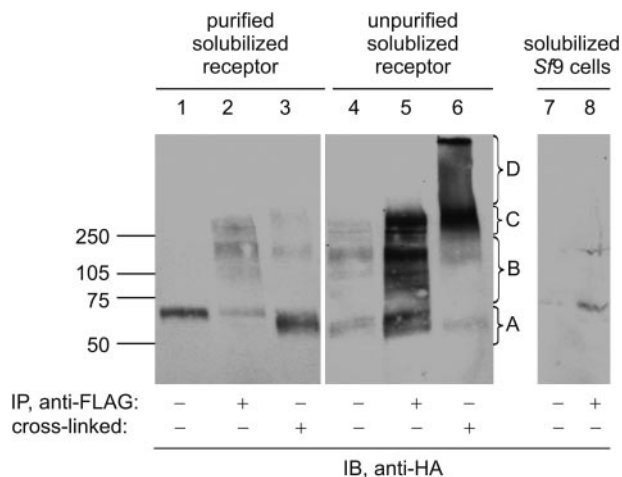
## Results

**Monomers of the Purified Human M<sub>2</sub> Muscarinic Receptor.** HA- and FLAG-tagged M<sub>2</sub> muscarinic receptors were coexpressed in *S*/9 cells and purified by successive passage on DEAE-Sepharose, 3-(2'-aminobenzhydryloxy)-tropine-Sepharose, and hydroxyapatite. The receptor was

recovered primarily as a monomer, in accord with previous reports (Park and Wells, 2003; Ma et al., 2007). Densitometric analyses indicated that most of the immunoreactive material identified on Western blots migrated as a single band with a relative molecular mass of  $58,000 \pm 3000$  ( $n = 7$ ). Comparatively faint bands corresponding to dimers and larger oligomers accounted for the balance (Fig. 1, lane 1; Table 1). The largely monomeric status of the purified receptor was confirmed by the small effect of BS<sup>3</sup>, which slowed only a minor fraction of the immunoreactive material (Fig. 1, compare lanes 1 and 3; Table 1), and by the negligible coimmunoprecipitation of receptors tagged with the HA and FLAG epitopes (Fig. 1, lane 2).

The M<sub>2</sub> receptor is at least partly oligomeric in unprocessed extracts of *S*/9 cells (Park and Wells, 2003), which therefore can serve as a positive control for cross-linking and coimmunoprecipitation. Receptors in the extract migrated as a mixture of forms, and the relative molecular masses of the major bands were as follows:  $52,000 \pm 2000$  ( $n = 5$ ),  $108,000 \pm 5000$  ( $n = 4$ ),  $143,000 \pm 5000$  ( $n = 4$ ), and  $277,000 \pm 31,000$  ( $n = 5$ ) (Fig. 1, lane 4). Only 22% of the immunoreactive material was monomeric, and that was reduced to 1% after treatment with BS<sup>3</sup> (Table 1). Approximately 90% of the cross-linked receptor migrated as if it were a tetramer or larger oligomer (Fig. 1, lane 6; Table 1).

The complement of monomers in the purified preparation was 88 and 71% before and after cross-linking, respectively (Table 1), and only 9% of the cross-linked, purified receptor



**Fig. 1.** Detection of monomers and apparent oligomers in solubilized and purified preparations of the M<sub>2</sub> muscarinic receptor. The gel was loaded with samples of the unprocessed extract (lanes 4–6) and the purified receptor (lanes 1–3). The samples were taken without further treatment (lanes 1 and 4), after immunoprecipitation with the agarose-coupled anti-FLAG antibody (lanes 2 and 5), and after cross-linking with BS<sup>3</sup> (lanes 3 and 6). Extract from uninfected cells was used as a control (lanes 7 and 8). All lanes are from the same gel. The same amount of receptor was applied to lanes 1, 3, 4, and 6 (0.07 pmol), as estimated from the specific binding of [<sup>3</sup>H]QNB at a concentration of 40 nM. To obtain the immunoprecipitate applied to lanes 2 and 5, the antibody was mixed with a volume of sample that was 20-fold larger than that applied in the other lanes. The area under the densitometric trace was determined in four segments, as shown in the figure (A002D–D), and the relative contribution of each segment to the total intensity is listed in Table 1. The efficiency of coimmunoprecipitation was estimated as the total area under the trace obtained for the precipitated sample (lanes 2 and 5) divided by that obtained for the corresponding untreated sample (lanes 1 and 4) and adjusted for the 20-fold difference in the amount of receptor. The mean values are as follows: unprocessed extract,  $47 \pm 12\%$  ( $n = 4$ ); purified receptor,  $4 \pm 1\%$  ( $n = 4$ ).

was tetrameric or larger (Fig. 1, lane 3; Table 1). To the extent that the densitometric signal may have been nonlinear at higher levels of exposure, the fraction corresponding to monomers is underestimated by the values listed in Table 1. In unprocessed extracts, approximately 47% of the HA-tagged receptor was precipitated by the anti-FLAG antibody (Fig. 1, lane 5); the corresponding value after purification was only 4% (Fig. 1, lane 2).

**Binding of  $N$ -[ $^3\text{H}$ ]Methyl Scopolamine and Its Modulation via the Allosteric Site.** Binding to the allosteric site required concentrations up to 100 mM with the ligands used in the present investigation. All assays therefore were conducted at a comparatively high concentration of HEPES. Under those conditions,  $N$ -[ $^3\text{H}$ ]methyl scopolamine bound to an apparently homogeneous population of sites ( $n_{\text{H}} \approx 1$ ) with an equilibrium dissociation constant of approximately 10 nM ( $\log K = -8.02 \pm 0.03$ ; Fig. 2). The affinity was 2- to 3-fold weaker than that measured previously at lower ionic strength (Park and Wells, 2003). Approximately 50% of the sites were occupied by  $N$ -[ $^3\text{H}$ ]methyl scopolamine at the concentration used to monitor its dissociation from the receptor.

The net dissociation of  $N$ -[ $^3\text{H}$ ]methyl scopolamine was well described by a single exponential under all conditions (Fig. 3). Reassociation of the radioligand was prevented by the inclusion of unlabeled  $N$ -methyl scopolamine at a concentration of 10  $\mu\text{M}$ , which was sufficient to block the orthosteric site (Fig. 2) without binding to the allosteric site (see below). Thus, the value of  $k_{\text{obsd}}$  estimated in the presence of 10  $\mu\text{M}$   $N$ -methyl scopolamine alone (i.e.,  $k_0$ ) is expected to approximate the rate constant for dissociation of the radioligand from an otherwise vacant receptor (i.e.,  $k_{\text{P}}$ ). Each experiment included one such trace, and the mean value of  $k_0$  from different estimates obtained over the course of the investigation is  $0.036 \pm 0.001 \text{ min}^{-1}$  ( $n = 28$ ). Individual values of  $k_0$  were in good agreement within the same experiment but varied by up to 1.5-fold between different experiments ( $0.029$ – $0.043 \text{ min}^{-1}$ ). All values of  $k_{\text{obsd}}$  therefore were normalized to the corresponding value of  $k_0$  for subsequent analyses.

Four ligands were examined for their effect on the rate of dissociation of  $N$ -[ $^3\text{H}$ ]methyl scopolamine: the known allosteric ligands gallamine and obidoxime, the muscarinic agonist oxotremorine-M, and the antagonist  $N$ -methyl scopolamine.

TABLE 1

Distribution of the  $M_2$  receptor among the monomeric and various oligomeric states identified on Western blots

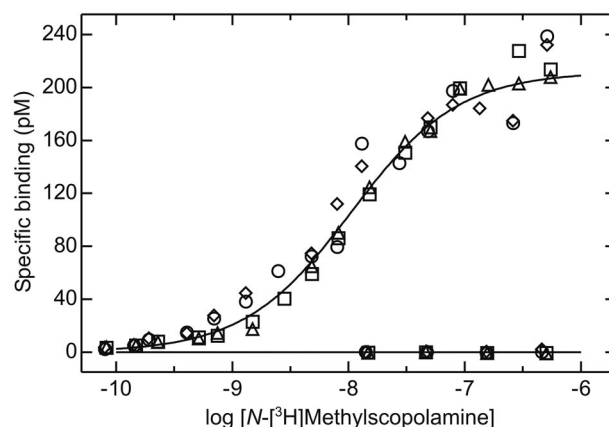
The area under the densitometric trace from each lane of Western blots such as that illustrated in Fig. 1 was estimated in four segments corresponding roughly to monomers (A), dimers and trimers (B), and larger aggregates (C and D), as shown in the figure. Each value was expressed as a percentage of the total area for that lane, and the values from different blots were averaged to obtain the means ( $\pm$  S.E.M.) listed in the table. The number of blots is shown in parentheses. Cross-linking was carried out in the presence of 2 mM  $\text{BS}^3$ . Further details are described in the legend to Fig. 1.

Preparation	Intensity			
	A	B	C	D
	%			
Solubilized receptor				
Untreated with $\text{BS}^3$ (6)	$22 \pm 7$	$44 \pm 4$	$31 \pm 10$	$2 \pm 2$
Cross-linked (6)	$1 \pm 1$	$7 \pm 2$	$47 \pm 4$	$45 \pm 5$
Purified receptor				
Untreated with $\text{BS}^3$ (7)	$88 \pm 7$	$5 \pm 2$	$6 \pm 3$	$1 \pm 1$
Cross-linked (6)	$71 \pm 4$	$20 \pm 4$	$8 \pm 4$	$1 \pm 1$

The dissociation was slowed appreciably by gallamine (Fig. 3E), oxotremorine-M (Fig. 3C), and  $N$ -methyl scopolamine (Fig. 3A). At the highest concentrations used in the experiments, both  $N$ -methyl scopolamine (10 mM) and oxotremorine-M (32 mM) decreased  $k_{\text{obsd}}$  by 3- to 4-fold: that is, from  $0.038 \text{ min}^{-1}$  to  $0.013 \text{ min}^{-1}$  (Fig. 3A), and from  $0.038 \text{ min}^{-1}$  to  $0.011 \text{ min}^{-1}$  (Fig. 3C), respectively. Gallamine effected a 20-fold decrease at a concentration of 1 mM (i.e., from  $0.040 \text{ min}^{-1}$  to  $0.0018 \text{ min}^{-1}$ ; Fig. 3E). Obidoxime caused a much smaller, 1.5-fold decrease at a concentration of 100 mM (i.e., from  $0.038 \text{ min}^{-1}$  to  $0.026 \text{ min}^{-1}$ ; Fig. 4) but reversed the effects of the other three ligands (Fig. 3, B, D, and F).

Data from a series of experiments such as those illustrated in Figs. 3 and 4 were analyzed together in terms of Scheme 1, in which all ligands compete for a single allosteric site. The fit of the model is illustrated in Fig. 5, and the parametric values are listed in Table 2. Mechanistic consistency was enforced for all parameters except the affinity of obidoxime. In that case, separate values of  $K_{\text{L}}$  were assigned to data acquired with obidoxime as the only unlabeled ligand in addition to 10  $\mu\text{M}$   $N$ -methyl scopolamine and to data acquired with obidoxime plus gallamine, oxotremorine-M, or higher concentrations of  $N$ -methyl scopolamine (i.e.,  $>10 \mu\text{M}$ ). The analysis therefore gave four estimates of  $K_{\text{L}}$  for obidoxime: one from the effect of obidoxime alone on the dissociation of  $N$ -[ $^3\text{H}$ ]methyl scopolamine, and three inferred from the effect of obidoxime in the presence of another ligand. The latter values of  $K_{\text{L}}$  are identified as  $K_{\text{Obl}}$  in Table 2. If binding is strictly competitive, as postulated in Scheme 1, the affinity of obidoxime is expected to be the same irrespective of the nature of the assay.

The model affords a good description of the data, as illustrated by the fitted curves in Fig. 5. Among the 36 estimates of  $k_{\text{obsd}}/k_0$ , 31 were within 5% of 1; of those, 17 were within 2% of 1. All ligands affected the dissociation of  $N$ -[ $^3\text{H}$ ]methyl scopolamine, although the affinities of  $N$ -methyl scopolamine, oxotremorine-M, and obidoxime were comparatively

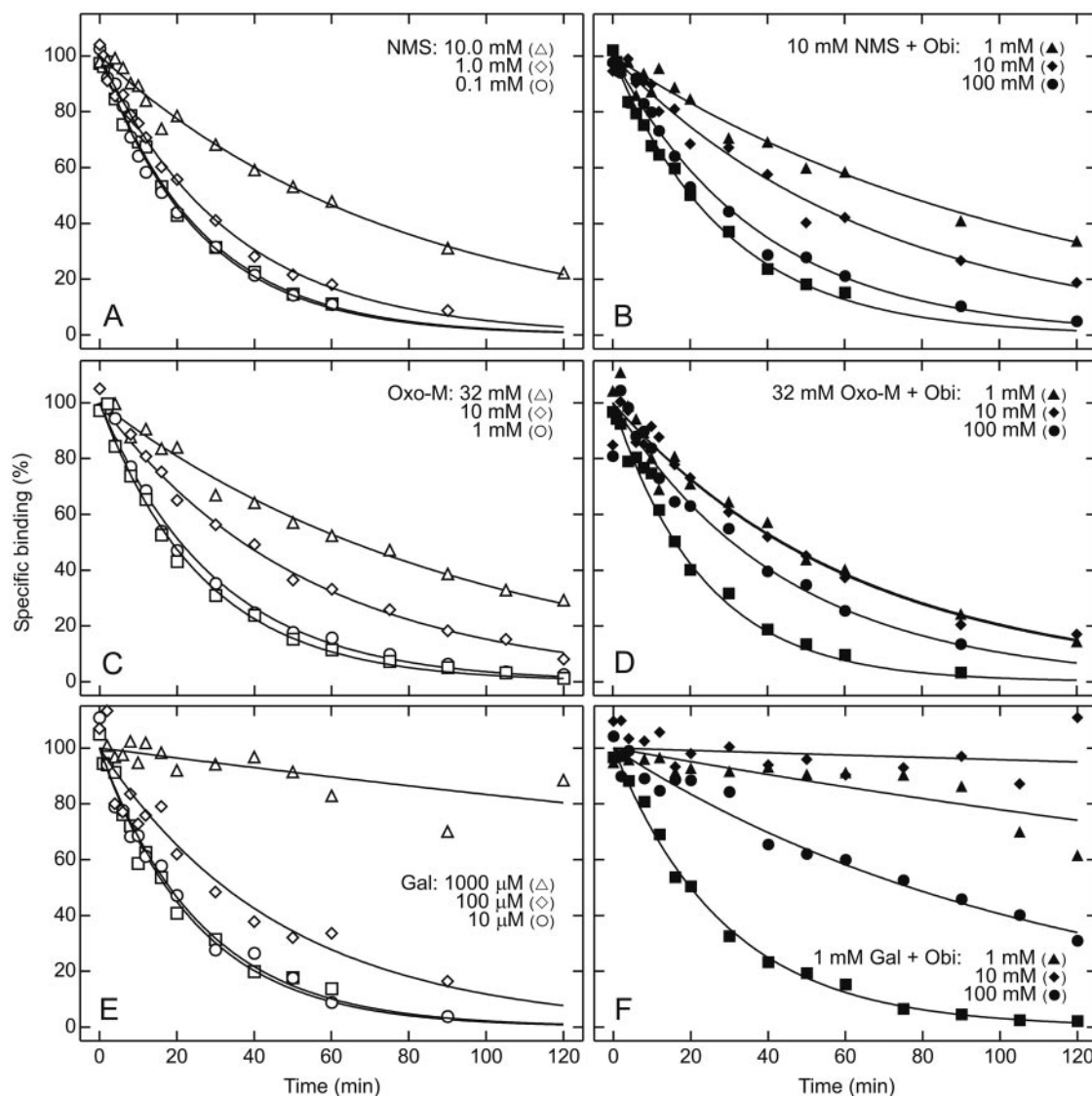


**Fig. 2.** Binding of  $N$ -[ $^3\text{H}$ ]methyl scopolamine to purified  $M_2$  receptor. Total binding was measured at graded concentrations of [ $^3\text{H}$ ]NMS alone (upper curves) or in the presence of 1 mM unlabeled NMS (baseline). The line represents the best fit of eq. 1 to the pooled data from four independent experiments identified by different symbols ( $\square$ ,  $\triangle$ ,  $\diamond$ ,  $\circ$ ). Single values of  $\log K$  and  $n_{\text{H}}$  were common to all of the data, whereas separate values of  $B_{\text{max}}$  and  $\text{NS}$  were assigned to the data from each experiment. The fitted estimates of  $\log K$  and  $n_{\text{H}}$  are  $-7.98 \pm 0.05$  and  $0.94 \pm 0.05$ , respectively. Individual estimates of  $B_{\text{obsd}}$  were adjusted to the mean value of  $B_{\text{max}}$  (i.e.,  $212 \pm 13 \text{ pM}$ ,  $n = 4$ ), as described under *Materials and Methods*. The sum of squares was not increased appreciably when  $n_{\text{H}}$  was fixed at 1 ( $p = 0.22$ ), and the corresponding value of  $\log K$  is  $-8.02 \pm 0.03$ .

weak. The values of  $K_L$  indicate that gallamine was at least 30-fold more potent than the other three compounds (Table 2). Saturating concentrations of gallamine decreased  $k_{\text{obsd}}$  by at least 100-fold ( $m_L \approx 1$ ), whereas the effects of *N*-methyl scopolamine and oxotremorine-M were 9.1-fold ( $m_L = 0.89$ ) and 4.5-fold ( $m_L = 0.78$ ), respectively (Table 2). In contrast, the estimated decrease in  $k_{\text{obsd}}$  at saturating concentrations of obidoxime was only 1.1-fold ( $m_L = 0.11$ ). The latter value is somewhat smaller than the 2.5- to 5.4-fold decrease reported previously for the muscarinic receptor in myocardial membranes (Ellis and Seidenberg, 1992; Tränkle and Mohr, 1997).

The affinities of all four compounds exceeded 70  $\mu\text{M}$  ( $\log K_L \geq -4.12$ , Table 2). Both gallamine and obidoxime

bound more weakly than in previous studies, as discussed below. The reaction mixture contained 10 mM magnesium, which has been shown to compete with the compounds W84 and Chin3/6 for the allosteric site of the M<sub>2</sub> receptor in porcine ventricular membranes and to slow the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine by 60% (Burgmer et al., 1998). The concentration dependence of the effect was biphasic, with inflections at 0.40 mM and 1.6 mM  $\text{Mg}^{2+}$ . Neither value is sufficiently low to account for the weak affinities of gallamine and obidoxime at the concentration of magnesium used in the present investigation. In addition, the effect of gallamine on  $k_{\text{obsd}}$  was characterized for its dose dependence in a modified buffer B that lacked magnesium. Under those conditions, the affinity of gal-



**Fig. 3.** Allosteric effects of *N*-methyl scopolamine, oxotremorine-M, and gallamine on the dissociation *N*-[<sup>3</sup>H]methyl scopolamine and their reversal by obidoxime. Purified M<sub>2</sub> receptor was equilibrated with [<sup>3</sup>H]NMS, and total binding was measured at different times after the addition of unlabeled NMS (10  $\mu\text{M}$ ) either alone (□, ■) or together with additional ligands as shown in the figure and described below. The mean concentration of [<sup>3</sup>H]NMS was  $7.74 \pm 0.22$  nM ( $n = 30$ ). The left-hand panels depict traces measured at three different concentrations of unlabeled NMS (A), oxotremorine-M (B), or gallamine (C). The right-hand panels depict traces measured at a single concentration of unlabeled NMS (B, 10 mM), oxotremorine-M (D, 32 mM), or gallamine (F, 1 mM) and three different concentrations of obidoxime (▲, 1 mM; ◆, 10 mM; ●, 100 mM). The lines represent the best fits of eq. 3 to the data in each trace taken separately, optimizing the values of  $k_{\text{obsd}}$  and  $B_{t=0}$ . To obtain the values plotted on the y-axis, the measured estimates of  $B_{\text{obsd}}$  were normalized to the fitted value of  $B_{t=0}$  taken as 100 and the value of  $B_{t=\infty}$  taken as zero. Further details are described under *Materials and Methods*. In each panel, all data shown were obtained in parallel and are representative of three to five experiments at any given concentrations of modulator or modulators.

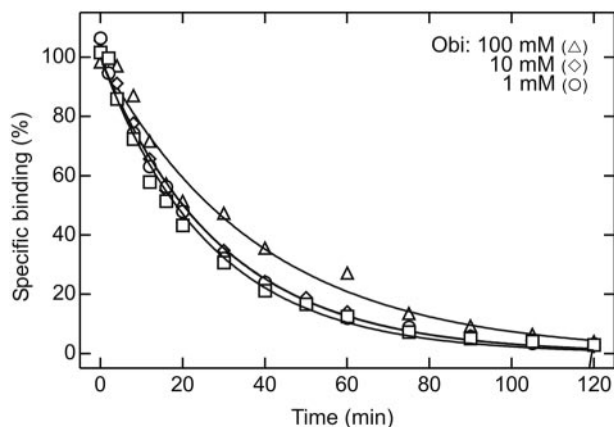


lamine for the allosteric site was unchanged from that listed in Table 2 ( $\log K_L = -4.24 \pm 0.15$ ; eq. 10).

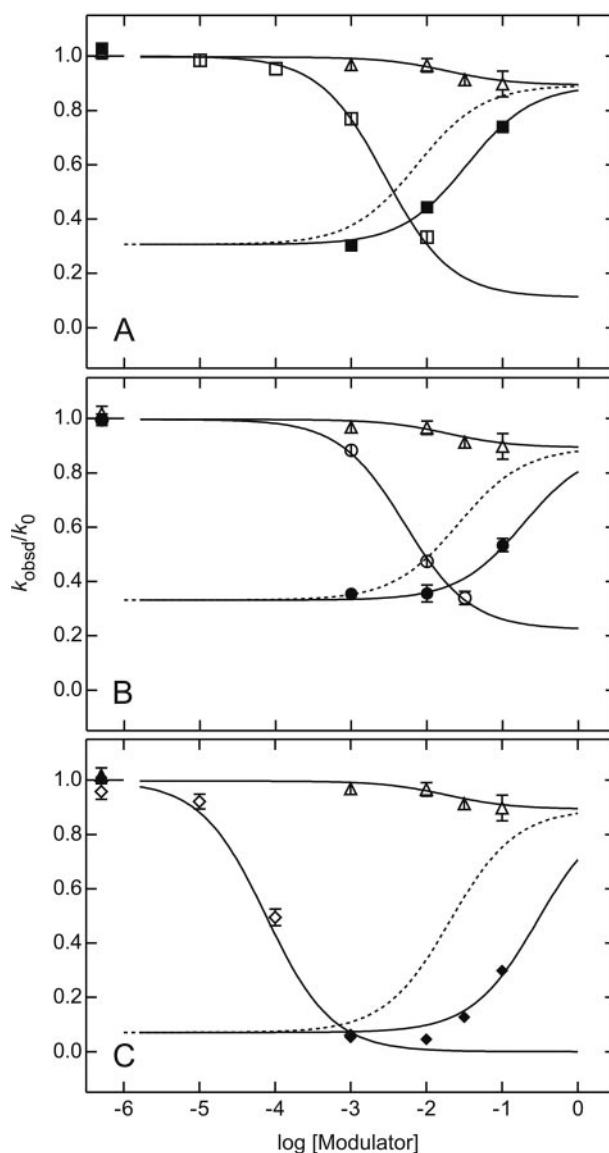
Owing to the differences in  $m_L$ , sufficient concentrations of obidoxime are expected to reverse the effect of *N*-methyl scopolamine, oxotremorine-M, or gallamine if binding is competitive (Ellis and Seidenberg, 1992). Saturating concentrations are expected to return  $k_{\text{obsd}}$  to the value that would be observed with obidoxime alone. Although saturation was precluded by the weak affinity (i.e.,  $\log K_L = -1.73$ , Table 2), it was approached in the case of obidoxime plus 10 mM *N*-methyl scopolamine. As illustrated in Fig. 5A, approximately 77% of the total effect expected of obidoxime was achieved at the highest concentration used in the experiment (i.e., 100 mM). The reversal is smaller in the presence of 32 mM oxotremorine-M (Fig. 5B) or 1 mM gallamine (Fig. 5C), but the available data are well described by the model.

The competitive nature of the reversal also is supported by good agreement between the affinity obtained for obidoxime alone ( $\log K_L = -1.73 \pm 0.91$ , Table 2) and that inferred from its effect in the presence of an additional ligand (i.e.,  $K_{\text{Obi}}$  in Table 2). Values differ by 3.5-fold or less between pairs that include obidoxime plus 10 mM *N*-methyl scopolamine, and by 1.4-fold or less with other combinations. The weighted sum of squares is not increased significantly if the analysis represented in Table 2 is repeated with one value of  $K_L$  rather than four ( $p = 0.089$ ). Although binding was comparatively weak for all ligands except gallamine, the estimates of  $K_{\text{Obi}}$  are defined to within a narrow tolerance by the substantial effects of *N*-methyl scopolamine, oxotremorine-M, and gallamine on the potency of obidoxime. The magnitude of those effects is illustrated by the differences between the dashed and corresponding solid lines in Fig. 5.

The values listed in Table 2 were obtained with  $m_L$  for gallamine fixed at 1. That constraint was imposed to avoid a fitted value greater than 1 (i.e.,  $m_L = 1.07 \pm 0.02$ ), which has no physical correlate. When analyzed in terms of eq. 2, the dose dependence of  $k_{\text{obsd}}/k_0$  on gallamine yields a Hill coefficient of  $1.23 \pm 0.26$  and a lower asymptote indistinguishable from 1 (i.e.,  $Y_{[L] \rightarrow \infty} = 1.00 \pm 0.05$ ). The decrease in  $k_{\text{obsd}}/k_0$  therefore is somewhat steeper than can be accommodated by eq. 10 at realistic values of  $m_L$ . The constraint on  $m_L$  is



**Fig. 4.** Allosteric effect of obidoxime on the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine. Purified  $M_2$  receptor was equilibrated with 8 nM [<sup>3</sup>H]NMS, and total binding was measured at different times after the addition of unlabeled NMS (10  $\mu$ M) either alone ( $\square$ ) or together with obidoxime at the concentrations shown in the figure. The data are representative of 4 such experiments. Further details are described in the legend to Fig. 3.



**Fig. 5.** Competition among *N*-methyl scopolamine, oxotremorine-M, gallamine, and obidoxime for the allosteric site affecting the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine. Values of  $k_{\text{obsd}}/k_0$  were estimated from a total of 115 kinetic traces obtained in 36 different experiments such as those illustrated in Figs. 3 and 4. Each experiment represented by the open symbols included a control trace in the presence of 10  $\mu$ M unlabeled NMS alone ( $k_0$ ) and traces at one or more different concentrations of NMS (A,  $\square$ ), oxotremorine-M (B,  $\circ$ ), gallamine (C,  $\diamond$ ), or obidoxime (A–C,  $\triangle$ ). The data shown for obidoxime are the same in each panel. Each experiment represented by the closed symbols included a control ( $k_0$ ) and traces at different concentrations of obidoxime in the presence of 10 mM NMS (A,  $\blacksquare$ ), 32 mM oxotremorine-M (B,  $\bullet$ ), or 1 mM gallamine (C,  $\blacklozenge$ ). In C, the points at 1 mM gallamine ( $\diamond$ ) and at 1 mM obidoxime in the presence of 1 mM gallamine ( $\blacklozenge$ ) are superimposable. The solid lines represent the best fit of Scheme 1 to the data taken together, and the parametric values are listed in Table 2. Individual estimates of  $k_{\text{obsd}}/k_0$  were normalized to the value of  $k_P/k_0$  taken as 1, and the adjusted values of  $k_{\text{obsd}}/k_0$  at the same concentration of modulator then were averaged to obtain the mean ( $\pm$  S.E.M.,  $n = 3$ –5) plotted on the y-axis. When not shown, the range of error lies within the diameter of the symbol. The dashed lines were calculated according to the equation  $Y = (Y_{L \rightarrow \infty} - Y_{L=0})[L]/(K + [L]) + Y_{L \rightarrow \infty}$ , with the values of  $Y_{L \rightarrow \infty}$  and  $Y_{L=0}$  taken as the asymptotic values of the fitted curves for obidoxime in the presence of NMS (A,  $\blacksquare$ ), oxotremorine-M (B,  $\bullet$ ), or gallamine (C,  $\blacklozenge$ ); the value of  $K$  was taken as the corresponding, fitted value of  $K_L$  for obidoxime (i.e.,  $K_{\text{Obi}}$  in Table 2). The difference between the dashed line and the accompanying fitted curve illustrates the magnitude of the change effected by NMS, oxotremorine-M, or gallamine in the potency of obidoxime. Further details regarding the analysis are described in the legend to Table 2.

accompanied by a significant increase in the sum of squares ( $p = 0.00019$ ). Virtually all of the increase derives from the data for gallamine in the absence of obidoxime, which lie above the values predicted by the model (Fig. 5C). The fitted curves otherwise are superimposable with those shown in Fig. 5, and the values of other parameters are essentially the same as those listed in Table 2. The reason for this anomaly in the effect of gallamine is unclear.

## Discussion

M<sub>2</sub> muscarinic receptors from porcine atria and baculoviral-infected Sf9 cells exhibit noncompetitive effects in the binding of orthosteric ligands (Wreggett and Wells, 1995; Park et al., 2002; Ma et al., 2007). The data can be described in terms of cooperative interactions among at least four sites, implying that the receptor is tetrameric or larger. In the same vein, a complex containing the HA-, c-Myc-, and FLAG-tagged forms of the M<sub>2</sub> receptor could be purified from Sf9 cells coinfecting with the three baculoviruses (Park and Wells, 2004), suggesting that the receptor is at least trimeric. In addition, the M<sub>2</sub> receptor has been shown by cross-linking to form tetramers when purified as a monomer from Sf9 cells and reconstituted in phospholipid vesicles (Ma et al., 2007).

As inferred from cooperativity, the notion of a tetramer is based on the assumption that ligands such as *N*-methyl scopolamine and oxotremorine-M bind only to the single orthosteric site within the cluster of transmembrane helices (cf. Hulme et al., 2003; Rasmussen et al., 2007). This disregards the possibility of an interaction with the allosteric site recognized by compounds such as gallamine, but the preference of ligands for the two sites is not necessarily absolute. Allosteric modulators of the M<sub>2</sub> receptor are structurally diverse, and compounds such as UH-AH 37 (Ellis and Seidenberg, 1999), methoctramine (Waelbroeck, 1994; Boselli and Grana, 1995), and AF-DX 116 (Lee and el-Fakahany, 1991; Waelbroeck, 1994) also may bind to the orthosteric site. Conversely, M<sub>2</sub>-selective antagonists, such as AF-DX 384, that bind to the orthosteric site (Miller et al., 1991) also may bind to the allosteric site (Tränkle et al., 1998).

A tetramer of the M<sub>2</sub> receptor would contain four orthosteric and four allosteric sites, with considerable potential for homo- and heterotropic cooperativity. Noncompetitive effects between supposed orthosteric ligands would be difficult to

interpret if those ligands also bound to the allosteric site and mimicked the effects of gallamine. That possibility therefore has been examined using a preparation of monomers, which are expected to exhibit only heterotropic effects. HA- and FLAG-tagged M<sub>2</sub> receptors extracted from coinfecting Sf9 cells were predominantly oligomeric, as indicated by their degree of coimmunoprecipitation and their electrophoretic mobility before and after cross-linking with BS<sup>3</sup>. In contrast, and in agreement with previous results (Park and Wells, 2003; Ma et al., 2007), the affinity-purified receptor was predominantly monomeric. There was little coimmunoprecipitation of the tagged adducts, and most of the receptor migrated as a monomer after treatment with BS<sup>3</sup>.

Both oxotremorine-M and *N*-methyl scopolamine slowed the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine, which bound only to the orthosteric site at the concentration of 6 to 10 nM used in the assays. Whereas the affinity of the radioligand for the orthosteric site was 10 nM, that estimated for the allosteric site was at least 3 orders of magnitude weaker, as discussed below. Inasmuch as monomers are not expected to exhibit intermolecular interactions between orthosteric sites, the effect of either compound on the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine was heterotropic in nature.

*N*-Methyl scopolamine, oxotremorine-M, gallamine, and obidoxime all seem to affect the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine via a common allosteric site. Scheme 1 provides a good description of the data, which yield four estimates for the affinity of obidoxime: one estimate from the dependence of  $k_{\text{obsd}}$  on the concentration of obidoxime alone ( $K_L$ ) and three from the potency of obidoxime in the presence of *N*-methyl scopolamine, oxotremorine-M, or gallamine ( $K_{\text{Obi}}$ ). The fitted values of  $K_L$  and  $K_{\text{Obi}}$  are indistinguishable, and obidoxime therefore was competitive with respect to the other three ligands. In the case of obidoxime and gallamine, the present results are consistent with the results of an earlier study in which the interaction between those two ligands also was found to be competitive (Ellis and Seidenberg, 1992). It follows that *N*-methyl scopolamine and oxotremorine-M bound to the same site as gallamine and obidoxime.

The affinities of both obidoxime and gallamine for the allosteric site of the radioligand-occupied receptor were comparatively weak. The present values of 19 mM and 76  $\mu$ M obtained for  $K_L$  are at least 260-fold greater than those reported for the effect of obidoxime and gallamine, respectively, on the dissociation of *N*-[<sup>3</sup>H]methyl scopolamine from the M<sub>2</sub> receptor in rat myocardial membranes (Ellis and Seidenberg, 1992). In a more recent study on porcine myocardial membranes, the effect of obidoxime was found to be biphasic, with inflections at 23  $\mu$ M and 0.96 mM (Tränkle and Mohr, 1997). In addition, the maximal decrease effected by obidoxime in  $k_{\text{obsd}}$  was only 11% in the present investigation; in contrast, a decrease of at least 50% has been found in studies on the receptor in myocardial membranes (Ellis and Seidenberg, 1992; Tränkle and Mohr, 1997).

The origin of such differences in the properties of obidoxime and gallamine remains unclear. They apparently are not caused by magnesium, and the reported effects of ionic strength on the affinity of gallamine are comparatively small (Pedder et al., 1991). They may be related to the state of aggregation of the receptor, which can exist as oligomers in whole cells (Goin and Nathanson, 2006), in detergent-solubi-

TABLE 2  
Affinities of ligands for the allosteric site, estimated in terms of Scheme 1

The data represented in Fig. 5 were analyzed simultaneously in terms of Scheme 1 to obtain the parametric values listed in the table. Values of  $K_L$  and  $m_L$  (L = A, B, or C in eq. 10) were assigned to each ligand as follows: in the case of NMS, oxotremorine-M, and gallamine, single values of each parameter were common to all of the relevant data; in the case of obidoxime, a single value of  $m_L$  was common to all of the data, and separate values of  $K_L$  were assigned to data acquired with obidoxime taken alone and in the presence of 10 mM NMS, 32 mM oxotremorine-M, or 1 mM gallamine. The latter values are listed in the table under  $K_{\text{Obi}}$ , where the value of  $K_L$  for obidoxime alone is reprinted in brackets for the purpose of comparison. A separate value of  $k_p/k_0$  was assigned to the data from each experiment, and the mean is  $1.01 \pm 0.01$  ( $n = 36$ ). The value of  $m_L$  for gallamine was fixed at 1 during the fitting process, as described in the text. The weighted sum of squares was not increased appreciably when the affinity of obidoxime was estimated as 1 parameter rather than 4 ( $p = 0.089$ ), and the resulting value of  $K_L$  is  $-1.73 \pm 0.08$ .

Ligand	log $K_L$	log $K_{\text{Obi}}$	$m_L$
<i>N</i> -Methyl scopolamine	$-2.55 \pm 0.13$	$-2.13 \pm 0.18$	$0.89 \pm 0.06$
Oxotremorine-M	$-2.29 \pm 0.14$	$-1.58 \pm 0.17$	$0.78 \pm 0.04$
Gallamine	$-4.12 \pm 0.04$	$-1.68 \pm 0.10$	1.0
Obidoxime	$-1.73 \pm 0.91$	$[-1.73 \pm 0.91]$	$0.11 \pm 0.05$



lized preparations (Park et al., 2001; Park and Wells, 2004), and after reconstitution (Ma et al., 2007). Such aggregation seems to have functional consequences, which may extend to the binding and effects of allosteric ligands.

Under some conditions, the detergent-solubilized  $M_2$  receptor exhibits a pattern of noncompetitive behavior wherein the binding of [ $^3$ H]quinuclidinyl benzilate is inhibited by *N*-methyl scopolamine at sites that are inaccessible to the latter (Park et al., 2002; Colozo et al., 2007). The effect can be described quantitatively in terms of cooperativity among four or more interacting and presumably orthosteric sites (Park et al., 2002). Such effects are absent from preparations of purified monomers (Park and Wells, 2003; Ma et al., 2007), but they are recovered upon reconstitution of the receptor in phospholipid vesicles (Ma et al., 2007). The parametric values reported here for gallamine and obidoxime pertain to the allosteric site of a monomer. If the receptor in myocardial membranes is predominantly oligomeric, the values of  $K_L$  and  $m_L$  in those preparations may be affected by modes of cooperativity and other factors that are absent from a monomer.

The allosteric effects of *N*-methyl scopolamine and oxotremorine-M raise the possibility that such interactions contribute to events heretofore assumed to occur at the orthosteric site. The affinity of *N*-methyl scopolamine for the allosteric site of the ligand-occupied monomer was 2.8 mM ( $K_L$ , Table 2). Its affinity for the allosteric site of the vacant monomer is unknown, but a cooperativity factor ( $\alpha$ ) of 46 has been estimated for the degree of negative heterotropic cooperativity between gallamine and *N*-[ $^3$ H]methyl scopolamine at the cardiac muscarinic receptor (Tränkle et al., 1999). Gallamine is one of the more efficacious allosteric modulators of *N*-[ $^3$ H]methyl scopolamine (Mohr et al., 2003); accordingly, the value of  $\alpha$  for the heterotropic interaction between *N*-methyl scopolamine and its radiolabeled analog seems unlikely to exceed 46. It follows that the equilibrium dissociation constant for the binding of *N*-methyl scopolamine to the allosteric site of the vacant monomer is likely to exceed 0.06 mM (i.e.,  $K_L/\alpha$ ).

Based on the lower limit estimated above, the affinity of *N*-methyl scopolamine for the allosteric site is at least 2800-fold weaker than its affinity for the orthosteric site [10 nM, Fig. 2;  $\leq$  22 nM, Ma et al. (2007)]. It is at least 1000-fold weaker than the affinity that emerges from the noncompetitive inhibition of [ $^3$ H]quinuclidinyl benzilate by *N*-methyl scopolamine at the  $M_2$  receptor solubilized in cholate-NaCl [46 nM, Park et al. (2002)] or reconstituted in phospholipid vesicles [27 nM, Ma et al. (2007)]. These considerations suggest that the noncompetitive effect of *N*-methyl scopolamine at concentrations below 10  $\mu$ M derives entirely from interactions among orthosteric sites. Such a system is potentially complex, however, and the combined effect of several cooperativity factors on the affinity of *N*-methyl scopolamine for the allosteric sites is unknown.

In the case of oxotremorine-M, the affinity for the allosteric site of the ligand-occupied monomer was approximately 5 mM ( $K_L$ , Table 2). If the value of  $\alpha$  for the heterotropic interaction with *N*-[ $^3$ H]methyl scopolamine is assumed once again to be 46 or less, the equilibrium dissociation constant for oxotremorine-M at the allosteric site of the vacant receptor is 0.11 mM or more. That is approximately 170-fold greater than the dissociation constant of oxotremorine-M for

the low-affinity form of the muscarinic receptor in myocardial membranes, as determined at equilibrium in the presence of *N*-[ $^3$ H]methyl scopolamine (0.66  $\mu$ M; Ehlert, 1988), and it approximates the corresponding value obtained for the reconstituted  $M_2$  receptor in the presence of [ $^3$ H]quinuclidinyl benzilate (0.35 mM; Ma et al., 2007). Oxotremorine-M therefore reveals comparatively little if any difference between its estimated affinity for the allosteric site of the monomer and its affinity for at least some of the presumed orthosteric sites. It follows that the inhibitory effect of oxotremorine-M on the binding of an antagonist such as *N*-[ $^3$ H]methyl scopolamine may derive in part from an interaction at the allosteric site. Such an effect would have implications for our understanding of the GTP-sensitive heterogeneity revealed by agonists at the muscarinic and perhaps other G protein-coupled receptors.

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