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Allosteric Regulation of the Binding of [^{3}H]Acetylcholine to m2 Muscarinic Receptors

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ABSTRACT. Muscarinic receptors of the m2 subtype expressed in Chinese hamster ovary cells were labeled with [*methyl*-^{3}H]acetylcholine([^{3}H]ACh), and the rate of dissociation in the presence and absence of several compounds known to exert allosteric effects on labeled antagonist binding was observed. At 25°, [^{3}H]ACh bound to the receptors with a K_d of 1.2 nM and dissociated with a half-time of 1.6 min. This binding was sensitive to appropriate concentrations of guanine nucleotide and the muscarinic antagonist *N*-methylscopolamine (NMS). Gallamine, tetrahydroaminoacridine, physostigmine, obidoxime, and 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester (TMB-8) all inhibited the binding of [^{3}H]ACh and all slowed the rate of dissociation of [^{3}H]ACh in a concentration-dependent manner. However, the nature of some of the allosteric effects differed from previous studies that used other labeled ligands. In particular, TMB-8, which is very effective in slowing the dissociation of the antagonist [^{3}H]NMS, had much weaker effects on the dissociation of [^{3}H]ACh. Furthermore, TMB-8 was able to partially reverse the stronger effects of gallamine on the dissociation of [^{3}H]ACh, consistent with the possibility that TMB-8 and gallamine share a common site on the receptor. In summary, the binding of ACh to muscarinic receptors is subject to allosteric regulation, and assays using [^{3}H]ACh may be especially useful in the evaluation of potential allosteric regulators of muscarinic systems. BIOCHEM PHARMACOL 52;11:1767–1775, 1996.

KEY WORDS. muscarinic receptors; allosteric regulation; acetylcholine

Muscarinic cholinergic receptors belong to the class of G protein-coupled seven transmembrane receptors and are expressed in the central nervous system, smooth and cardiac muscle, and glands. Although these receptors are found in many different cells exerting distinct effects, they all respond to the endogenous agonist ACh.§ Earlier pharmacological evidence for heterogeneity in these receptors culminated in the demonstration by Hammer *et al.* [1] that pirenzepine binding was best explained by the existence of muscarinic receptor subtypes. Five subtypes (m1–m5) have been identified by molecular cloning [2–5] and found to be differentially distributed in peripheral tissues. For example, while all subtypes have been detected in the brain [6], only the m2 muscarinic subtype is expressed in cardiac tissue [7]. Although the five subtypes share similar affinities to antagonists such as atropine and NMS, other antagonists can differentiate between certain subtypes. Much effort has been made to identify highly selective pharmacological agents that would be able to identify each muscarinic subtype from a mixture of subtypes, but that goal remains elusive [8]. Nonetheless, when data from many somewhat selective antagonists are pooled, good correlation exists between a given cloned receptor subtype and its corresponding pharmacologically defined subtype [8]. There are other distinguishing features, as well. For example, NMS displays fairly constant affinity across the subtypes, but its rate of dissociation varies in a characteristic manner [9, 10].

Characterization of these receptors with regard to their binding kinetics, anatomical distribution, and pharmacology has been carried out largely with competitive antagonists such as NMS and QNB. These radiolabeled antagonists are technically convenient to use because they are stable, have high affinity, label all receptors regardless of state, and have low non-specific binding. In addition to compounds that compete with ACh, there are other substances that bind at a separate, allosteric site on the receptor [11]. It had been known for many years that gallamine affects muscarinic responses in a way that is not consistent with competitive interactions [12]. However, it was not until recently that this effect of gallamine was studied in more detail, facilitated in part by the ability to express individually cloned or mutated receptor subtypes. It has been shown that all five subtypes are subject to allosteric

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§ *Abbreviations:* ACH, acetylcholine; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate; [^{3}H]ACh, [*methyl*-^{3}H]acetylcholine; TMB-8, 3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester; AChE, acetylcholinesterase (EC 3.1.1.7); THA, 9-amino-1,2,3,4-tetrahydroacridine; CHO-K1 cells, Chinese hamster ovary cells; hm2, human muscarinic receptor subtype 2; and PB, sodium-potassium phosphate buffer.

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regulation [9], and that gallamine and other allosteric modulators do, in fact, act at a well-defined site [13, 14]. Other studies have begun to define the regions involved in muscarinic allosteric regulation [15–18].

The affinity of a ligand at a single site on a receptor is simply dependent upon receptor and ligand; generalizations, therefore, can be made between various ligands that interact competitively at this site. Allosteric parameters, on the other hand, are more complex in that the degree of cooperativity is necessarily unique for each pair of allosteric ligands that can bind simultaneously to the receptor. However, most studies on allosteric regulation of muscarinic receptors have utilized labeled QNB or NMS for binding to the primary site. Because of the uniqueness of cooperativity factors and because ACh is the endogenous ligand, we chose to investigate interactions between [^{3}H]ACh and a representative group of previously characterized allosteric modulators. We began this work with the m2 subtype because it has the highest affinity for muscarinic agonists and also for most allosteric modulators [8, 9, 19]. We found that the binding of ACh is subject to allosteric modulation and that, in general, modulators exerted effects similar to those previously reported in studies that used the antagonist NMS. Nonetheless, the discrepancies that we observed emphasize the point that parameters established using another muscarinic ligand, whether antagonist or agonist, cannot be assumed to hold true for ACh.

MATERIALS AND METHODS

Materials

[^{3}H]ACh and [^{14}C]ACh, specific radioactivity 60 Ci/mmol and 57.4 mCi/mmol, were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO) and New England Nuclear (Boston, MA), respectively. Atropine sulfate, gallamine triethiodide, physostigmine hemisulfate (eserine), TMB-8, AChE Type V-S from electric eel, and GDP were obtained from Sigma (St. Louis, MO), obidoxime chloride was purchased from Schweizerhall (South Plainfield, NJ), and THA was obtained from Research Biochemicals, Inc. (Wayland, MA).

Cell Culture

CHO-K1 cells transfected with the hm2 muscarinic receptor subtype [20] were used throughout this study. Cells were grown in F-12 nutrient medium (HAM, with L-glutamine) from Sigma supplemented with 5% fetal bovine serum (GIBCO, Grand Island, NY), 100 U/mL penicillin G, and 100 μg/mL streptomycin (Sigma) in a humidified incubator at 37° and 5% CO_2.

Membrane Preparation

Cells were grown to 70–90% confluency in 15-cm plates (Falcon), washed with Dulbecco's phosphate-buffered saline (GIBCO) containing no divalent cations, scraped into cold 5 mM PB (4.2 MM sodium phosphate and 0.8 mM potassium phosphate, pH 7.2), and homogenized three times for 15 sec on ice with a Bio-homogenizer (Biospec Products, Inc., Bartlesville, OK). Membranes were pelleted at 30,000 *g*, resuspended so that the protein concentration was 0.5 to 1.0 mg/mL, and stored frozen at −80° in PB. Protein was determined using the method of Lowry *et al.* [21].

Binding Assays

To measure the binding of [^{3}H]ACh, membranes (15–60 μg protein), drugs, and radioligand were made up in 5 mM PB and incubated for 18 min at 25° in a total volume of 1 mL. Samples were filtered on glass fiber filters GF/B (Whatman) presoaked in 0.2% polyethyleneimine, 40 mM PB for 30 min prior to filtration. Each sample was diluted with 5 mL of 40 mM PB at 4°, filtered immediately, and rinsed twice with an additional 5 mL PB. Filters were dried and counted by liquid scintillation. Specific binding was defined as that displaced by 3 μM atropine; non-specific binding was <10% of total binding.

Equilibrium Inhibition Studies

To measure inhibition of binding under equilibrium conditions, membranes were incubated for 18 min at 25° in the presence of 1.5 nM [^{3}H]ACh and increasing concentrations of unlabeled NMS, GDP, or various drugs. Contrary to previous studies with NMS, the approach to equilibrium was *not* affected markedly by the addition of allosteric modulators. For example, equilibrium binding of [^{3}H]ACh was achieved within 2 min both in the absence and presence of the concentration of gallamine that inhibited 50% of [^{3}H]ACh binding. Samples were filtered and counted as described above, and data from these studies were fitted to the four-parameter logistic equation: $y = R + (L - R)/[1 + (X/C)^S]$ where L is an estimate of the left plateau, R the right plateau, X the concentration of inhibitor, C the concentration at the midpoint, and S the slope factor at the midpoint [22].

Dissociation Studies

For studies of the modulation of the rate of dissociation of [^{3}H]ACh, membranes were first equilibrated with [^{3}H]ACh in a volume of 1 mL. Appropriate concentrations of allosteric modulators were then added along with 3 μM atropine. Samples were filtered at specific times after addition of the allosteric modulator, and binding was quantified as described above. Data from dissociation assays were analyzed in the following way: The apparent rate constant for the dissociation of [^{3}H]ACh was determined in the presence of each concentration of allosteric modulator and divided by the true rate constant (k_{-1}), determined in the presence of 3 μM atropine alone. The resulting value was the fold-shift in the off-rate, such that a number greater

than 1.0 would indicate dissociation faster than that observed with atropine alone while a slower dissociation rate would have a value less than 1.0. The change in rate of dissociation would be proportional to the occupancy of the allosteric site. Therefore, the data were fitted to the following equation [14]: $FS = 1 - mL/(L + K)$, where FS is the fold-shift, described above, L is the concentration of allosteric ligand, m relates to the maximal effect of that ligand on the off-rate of ACh (for example, when m is 0.8, the off-rate is reduced by 80%, or to 20% of the control value), and K is the apparent equilibrium dissociation constant for that ligand. Curve-fitting was carried out with the MINSQ program, MicroMath, Salt Lake City, UT.

AChE Assay

AChE was assayed using the method described by Younkin *et al.* [23], in 5 mM sodium phosphate buffer, pH 7.0. The reaction was initiated by the addition of 50 μL of [^{14}C]ACh such that the final concentration was 134 nM and incubated at 25° (total volume of 250 μL). Hydrolysis of [^{14}C]ACh was stopped after 15 min by the addition of 1 mL of 0.25 M glycine, 1.0 M NaCl, pH 2.5. Four milliliters of 10% isoamyl alcohol in Econofluor-2 (NEN) was added, and samples were shaken to extract [^{14}C]acetate into the non-aqueous phase. To determine the relationship between [^{14}C] acetate present and disintegrations per minute measured, a triplicate set of samples was run with 0.002 U of AChE, an amount capable of complete hydrolysis of [^{14}C]ACh under these conditions.

RESULTS

Acetylcholinesterase Activity in Membranes

In preliminary studies of interactions between ACh and allosteric modulators, we included physostigmine to ensure that the [^{3}H]ACh ligand would not be hydrolyzed by any endogenous AChE that might be in the membranes containing the receptors. However, this resulted in variable binding of ACh that correlated with the concentrations of physostigmine in the assays. This prompted us to assay esterase activity, since AChE is expressed to varying degrees in a wide range of cells. Results in Table 1 show that membranes from transfected cells hydrolyzed less than 1% of the total ACh available for hydrolysis under our typical binding assay conditions. Since esterase levels were low, and physostigmine affects the binding of ACh to muscarinic receptors (see below), AChE inhibitors were omitted from all assays described in the present report, unless otherwise noted.

[^{3}H]ACh Binding

Specific binding of [^{3}H]ACh in membranes containing the hm2 receptor reached equilibrium within 2 min (not shown) and was saturable over a concentration range of 250 pM to 16 nM. Data averaged from five experiments showed the maximal binding capacity (B_{max}) to be 0.692 ± 0.021 pmol/mg protein and the equilibrium dissociation constant (K_d) to be 1.17 ± 0.29 nM. A representative experiment is shown in Fig. 1. [^{3}H]NMS binding in these membranes averaged 1.4 ± 0.05 pmol/mg protein (average of four experiments, data not shown). The $T_{1/2}$ for dissociation of [^{3}H]ACh from the receptor was 1.6 ± 0.2 min; in the representative experiment shown in Fig. 2, it can be seen that dissociation in the presence and absence of gallamine followed a monoexponential time course.

TABLE 1. Acetylcholinesterase activity in CHO wild-type and CHOm2 cell membranes

	ACh hydrolyzed (fmol/min)
hm2 cell membranes	
30 μg	7
52.5 μg	13
135 μg	25
CHO parental	
30 μg	6
52.5 μg	25
135 μg	37

AChE activity was measured under conditions similar to those in the binding assays, pH 7.0, 25°, and 5 mM sodium phosphate buffer. Triplicate disintegrations per minute measurements were averaged, corrected for nonenzymatic hydrolysis, converted to femtomoles of [^{14}C]acetate generated during the assay, and expressed as femtomoles of acetylcholine hydrolyzed per minute. (Under these conditions, 2 mU of AChE completely hydrolyzed the [^{14}C]ACh.) The data shown are from one of two similar experiments.

Verification of Binding to the Muscarinic Receptor

Specific [^{3}H]ACh binding was undetectable in membranes from CHO cells that had not been transfected with the receptor (results not shown). Specific [^{3}H]NMS binding was also undetectable in these membranes, as reported previously by Bonner *et al.* [4]. In membrane preparations with the expressed receptor, binding of [^{3}H]ACh was sensitive to guanine nucleotides (Fig. 3A). GDP reduced the specific binding of [^{3}H]ACh with an IC_{50} of 0.7 μM. In Fig. 3B, NMS displaced the binding of 1.7 nM [^{3}H]ACh with a K_d of 0.104 nM.

Allosteric Modulation of [^{3}H]ACh Binding

In equilibrium studies, membranes were incubated for 18 min in the presence of [^{3}H]ACh and increasing concentrations of the following drugs: gallamine, obidoxime, THA, physostigmine, and TMB-8. In all cases, the allosteric modulators decreased the specific binding of [^{3}H]ACh. The modulators inhibited the binding of [^{3}H]ACh with the following order of potency: gallamine > TMB-8 > THA > obidoxime > physostigmine (Fig. 4). Data from these experiments were fitted to a parametric function to derive best estimates of the IC_{50} and slope factor. In Table 2, these parameters are presented as means ± SEM from three experiments. The allosteric modulators shown in Fig. 5 all

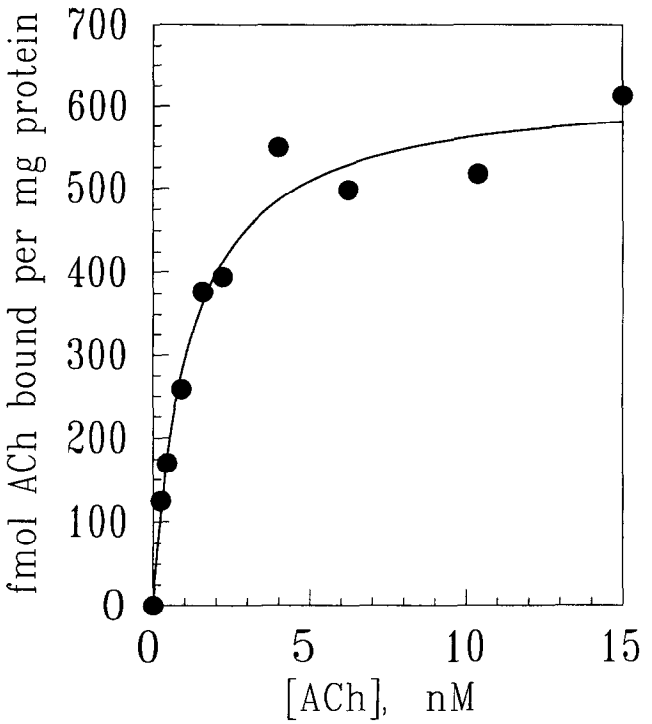

FIG. 1. Binding of [^{3}H]ACh to the m2 muscarinic receptor subtype under equilibrium conditions. Membranes (31 μg) from CHO cells transfected with the hm2 receptor were incubated with increasing concentrations of [^{3}H]ACh for 18 min at 25° and then filtered as described in Materials and Methods. The equilibrium dissociation constant, K_d, and maximal binding capacity, B_{max}, were derived using the following equation: $B = B_{max}L/(L + K_d)$ where B is the specifically bound [^{3}H]ACh, B_{max} is the maximal binding capacity, K_d is the dissociation constant for [^{3}H]ACh, and L is the concentration of [^{3}H]ACh. Data from this experiment (representative of five) yielded a Kd of 1.14 nM and a B_{max} of 0.625 pmol/mg protein.

slowed the dissociation rate of ACh, with the following order of potency: gallamine > TMB-8 > THA > obidoxime and physostigmine. THA slowed the dissociation rate of ACh by 80% at maximally effective concentrations. Gallamine and physostigmine were intermediate, slowing the rate by 70%, whereas obidoxime slowed the rate by only 54%. The effects of these modulators on the dissociation of [^{3}H]ACh are comparable to their effects in previous studies with [^{3}H]NMS (see Discussion). However, the effects of TMB-8 on the dissociation of these two ligands were quite different (Fig. 6). That is, TMB-8 has been found to be considerably more potent in assays with [^{3}H]NMS [14] and to exert a greater maximal effect, relative to [^{3}H]ACh assays (present study). Analyses of the dissociation assays are summarized in Table 3.

Reversal of the Allosteric Effect of Gallamine by TMB-8

As in the above experiments, both gallamine (1 μM) and TMB-8 (100 μM) decelerated the dissociation of [^{3}H]ACh from the receptors. Gallamine slowed the rate of dissociation by about 70%, whereas TMB-8 slowed it by only about 28%. Most interestingly, the addition of both agents did not produce an additive effect, but rather slowed the dissociation by 42%, a significantly smaller effect than that produced by gallamine alone (Fig. 7). That is, the presence of TMB-8 accelerated the dissociation when used in combination with gallamine.

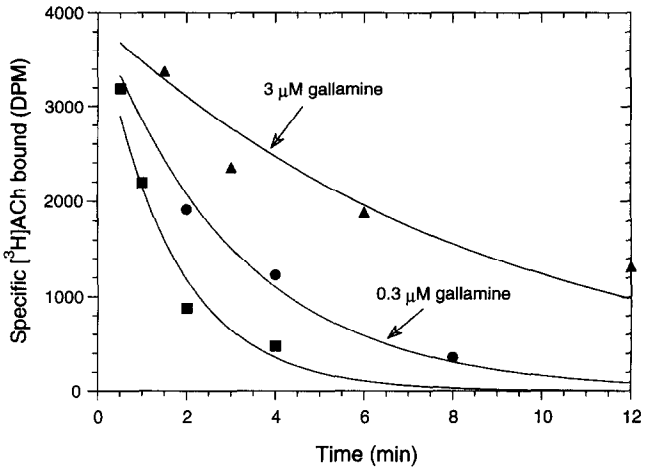

FIG. 2. Rate of dissociation of [^{3}H]ACh from m2 muscarinic receptors. Membranes (50 μg in 5 mM phosphate buffer) were incubated with 1.5 nM [^{3}H]ACh for 15 min at 25°. A final concentration of 3 μM atropine was added (with gallamine, where indicted) and, at indicated time points, samples were filtered as described in Materials and Methods. Points were fit to the exponential equation: $B = B_0e^{-kt}$ where k is the rate constant for the dissociation. In this experiment (representative of three), B_0 was 3900 dpm for all three curves; k was 0.60 min^{-1} in the absence of gallamine, corresponding to a half-time of 1.2 min; in the presence of 0.3 and 3 μM gallamine, the half-times were 2.2 and 6.0 min, respectively.

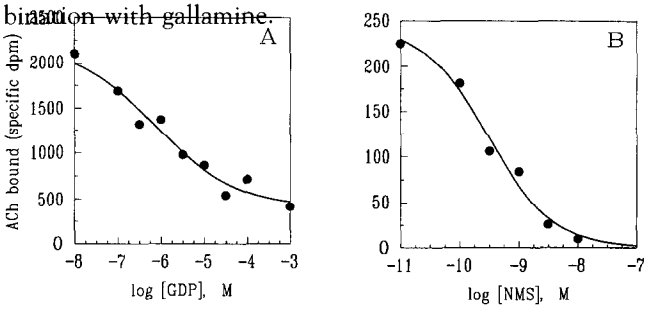

FIG. 3. Inhibition of specific [^{3}H]ACh binding to m2 receptors. (A) Membranes (56 μg in 5 mM phosphate buffer) were incubated with 4.9 nM [^{3}H]ACh and indicted concentrations of GDP for 18 min at 25°. Samples were then filtered as described. Specific binding was defined as that displaced by 3 μM atropine. Points are means of duplicate values fit to the four-parameter sigmoidal equation described in Materials and Methods. GDP reduced the binding of [^{3}H]ACh with an IC_{50} of 0.7 μM. (B) Membranes (36 μg in 5 mM phosphate buffer) were incubated with 1.73 nM [^{3}H]ACh and indicated concentrations of NMS for 18 min at 25° and then filtered as described. Points are means of triplicate determinations fit to the four-parameter equation described in Materials and Methods. The IC_{50} for NMS displacement of [^{3}H]ACh was 0.26 nM so that the K_d, corrected for the concentration of labeled ligand, was 0.104 nM.

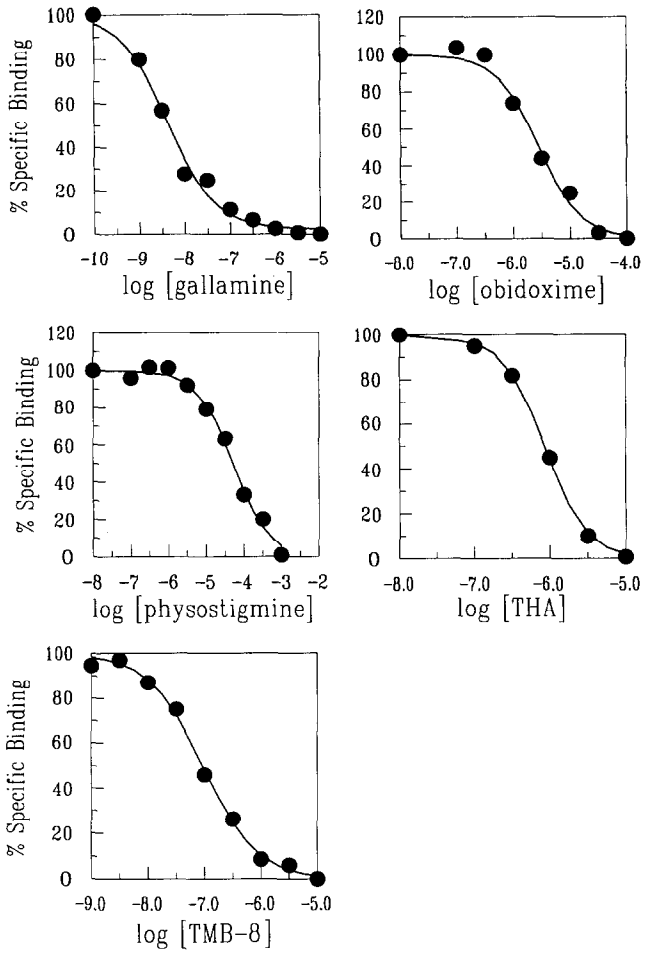

FIG. 4. Effect of allosteric modulators on the binding of [^{3}H]ACh to m2 muscarinic receptors at equilibrium. Membranes were added to [^{3}H]ACh (1.5 nM final concentration) and the indicated concentrations of allosteric modulators, and then allowed to come to equilibrium over 18 min at 25°. Specific binding in the absence of inhibitor was approximately 2500 dpm. Each point is the average of duplicate determinations from three experiments. Data were fit to the four-parameter equation described in Materials and Methods. The IC_{50} values were as follows: gallamine, 3.3 nM; obidoxime, 2.6 μM; physostigmine, 51 μM; THA, 0.85 μM; and TMB-8, 92 nM. The average IC_{50} and S values from three experiments are listed in Table 2.

DISCUSSION

Characteristics of [^{3}H]ACh Binding to Cloned Muscarinic Receptors

Reversible high affinity binding of [^{3}H]ACh to membrane preparations of CHO cells transfected with the hm2 receptor was observed. The following evidence demonstrates the muscarinic nature of this binding. First, in untransfected control cells, no specific binding was detectable. Second, the K_d and B_{max} values that were obtained for [^{3}H]ACh were consistent with competition studies utilizing [^{3}H]NMS and unlabeled ACh. That is, in the present study, approximately 50% of the sites labeled by [^{3}H]NMS could be labeled by [^{3}H]ACh, with an affinity of about 1 nM (see

TABLE 2. IC_{50} and S values for allosteric modulators on the binding of [^{3}H]ACh to m2 muscarinic receptors at equilibrium

	IC_{50} (nM)	S (slope factor)
Gallamine	5.5 ± 1.3	0.65 ± 0.07
Obidoxime	2,900 ± 600	1.17 ± 0.11
Physostigmine	55,000 ± 1,200	0.97 ± 0.07
THA	820 ± 260	1.54 ± 0.18
TMB-8	110 ± 20	0.91 ± 0.11

In each case, the parameters are given as the means ± SEM from three independent experiments, analyzed individually as described in Materials and Methods.

Results); in the competition studies, unlabeled ACh inhibited 50% of the binding of [^{3}H]NMS at low nanomolar concentrations (results not shown). Third, NMS displaced [^{3}H]ACh binding with a K_d of 0.1 nM, in good agreement with literature values for the affinity of NMS. Finally, binding of agonists to muscarinic receptors has been shown repeatedly to be modulated by guanine nucleotides [24–29]. The conversion of high- to low-affinity states induced by guanyl nucleotides is only partial for muscarinic receptors, although other receptors, including β-adrenergic receptors

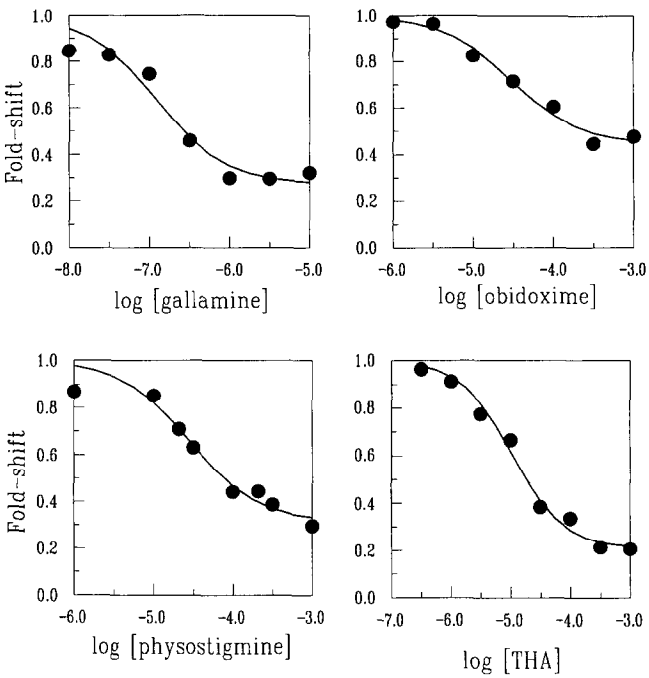

FIG. 5. Effect of allosteric modulators on dissociation of [^{3}H]ACh from m2 muscarinic receptors. The rate of dissociation of [^{3}H]ACh was determined in the presence and absence of indicated concentrations of gallamine, obidoxime, physostigmine, or THA. The effect of each modulator is expressed as a fold-shift in the dissociation rate constant of [^{3}H]ACh. Each point is the average of duplicate determinations from three experiments. Data were fit to the equation described in Materials and Methods. Best fit values were: gallamine, K_{app} = 0.11 μM, m = 0.73; obidoxime K_{app} = 26 μM, m = 0.55; physostigmine, K_{app} = 29 μM, m = 0.69; THA, K_{app} = 11 μM, m = 0.79. The average values from three experiments are shown in Table 3.

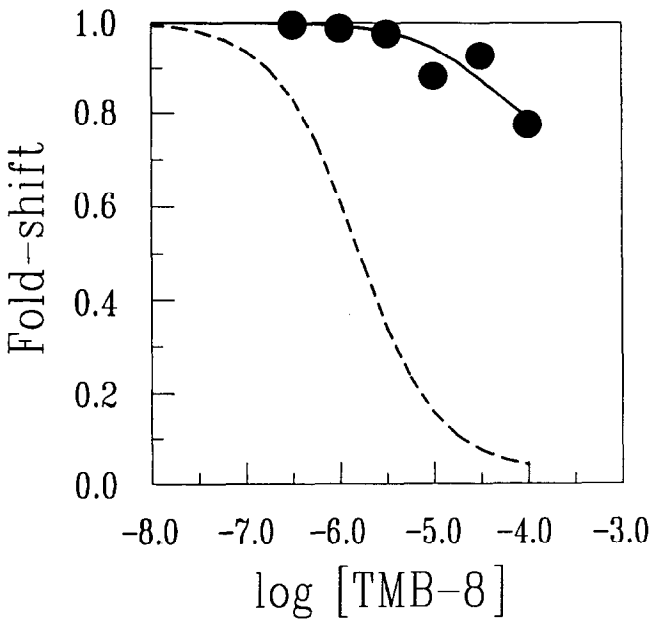

FIG. 6. Effect of TMB-8 on dissociation of [^{3}H]ACh and [^{3}H]NMS from m2 muscarinic receptors. The rate of dissociation of [^{3}H]ACh was determined in the presence and absence of the indicated concentrations of TMB-8. The effect of TMB-8 is expressed as the fold-shift in the dissociation rate constant of [^{3}H]ACh from m2 muscarinic receptors as described in Materials and Methods. Data points are averages of duplicate determinations from three different experiments (±SEM) and fit to the same equation as in Fig. 5. The curve shown represents the best fit to the aggregated data; the values were K_{app} = 47 μM, m = 0.31. The average values for K_{app} and m from the analysis of individual experiments are shown in Table 3. For comparison, the dashed line represents the effect of TMB-8 on the rate of dissociation of [^{3}H]NMS (taken from Ref. 14).

exhibit complete conversion [25]. The hm2 receptors in this study were sensitive to guanyl nucleotides; GDP reduced high-affinity [^{3}H]ACh binding by more than 80%, with an IC_{50} of 0.71 μM. Gurwitz *et al.* [28] and Kellar *et al.* [27] also reported partial conversion of muscarinic receptors from high- to low-affinity states by similar concentrations of guanyl nucleotides in rat heart atrium and in tissues rich in M2 receptors, respectively. Therefore, the kinetics and regulation by guanyl nucleotides of these cloned receptors indicate that the [^{3}H]ACh binding sites are the endogenous ligand recognition sites of high-affinity muscarinic receptors.

Acetylcholinesterase Activity in Membranes

The muscarinic acetylcholine receptors and acetylcholinesterase are structurally unrelated proteins that share a common property: interaction with acetylcholine. Muscarinic receptor activation by ACh binding results in a variety of responses due to interaction with second messengers, while AChE hydrolyzes ACh. Since this enzyme is found in virtually all tissues where ACh receptors are expressed, studies that rely on tissues as a receptor source must utilize an esterase inhibitor to prevent hydrolysis of the ligand and determine whether the inhibitor itself affects binding of the ligand. In fact, four of the five allosteric modulators used in this study are known to affect the activity of AChE. Early attempts to measure muscarinic binding sites with [^{3}H]ACh were compromised by high non-specific binding, low total binding and low specific radioactivity of the available ligand. These problems have been ameliorated by the commercial availability of ACh with high specific activity and soaking filters in polyethyleneimine, which minimizes nonspecific binding. However, the residual problem of endogenous AChE activity still requires consideration. One approach is to include AChE inhibitors in the assay, but at concentrations effective in inhibiting the esterase, the specific binding of [^{3}H]ACh may also be inhibited. Alternatively, in many tissue studies, irreversible inhibitors of the esterase have been used in a preincubation; residual free drug is then washed away prior to the assay. Of course, if the tissue lacks esterase activity, inhibitors can be avoided altogether. Since we were using cloned receptors in CHO cells, we measured the AChE activity to determine whether inclusion of inhibitors was necessary. Under conditions used in our assays, the total amount of [^{14}C]ACh hydrolyzed by the membranes containing receptors was less than 1% (Table 1). If significant AChE was present in membrane preparations, the addition of low concentrations of physostigmine (or any other inhibitor) would be expected to cause an *increase* in [^{3}H]ACh binding. Our results in Fig. 4 confirm the lack of esterase activity since neither physostigmine nor THA increased the binding of [^{3}H]ACh at any concentration.

Interaction of Allosteric Modulators with [^{3}H]ACh and Comparison with Modulation of [^{3}H]NMS

All of the modulators that we tested inhibited the binding of [^{3}H]ACh under equilibrium conditions (Fig. 4), implying that they were negatively cooperative toward ACh. We and others have commonly employed the paradigm of modulation of the rate of dissociation, because it is a purely allosteric assay. Our analysis of these experiments yields two parameters: the affinity of the modulator for the liganded receptor (K_{app}) and the maximal effect of the modulator on the rate of dissociation of the labeled ligand (m). These data are summarized in Table 3, along with similarly determined parameters obtained using [^{3}H]NMS as the dissociating ligand. Except for TMB-8, the results obtained with [^{3}H]ACh and [^{3}H]NMS were comparable. That is, the apparent affinities were similar and showed the same rank order (gallamine > THA > obidoxime). Interestingly, although the "m" values also showed the same rank order (THA > gallamine > obidoxime), they were all smaller for ACh. The greater bulk of the NMS molecule may contribute to its slower kinetics when the allosteric ligands are bound (probably restricting access to the binding site). Slow kinetics have been a common finding and a consistent problem in studies of those allosteric effects [13, 30, 31].

TABLE 3. Apparent dissociation constants of allosteric modulators at muscarinic m2 receptors labeled with [^{3}H]ACh or [^{3}H]NMS

	[^{3}H]ACh		[^{3}H]NMS	
	K_{app} (μM)	m Value	K_{app} (μM)	m Value
Gallamine	0.068 ± 0.023	0.70 ± 0.01	0.29	0.92
Obidoxime	27 ± 12	0.46 ± 0.11	53	0.58
Physostigmine	33 ± 11	0.70 ± 0.07	ND*	
THA	12 ± 2.5	0.80 ± 0.06	6.2	0.99
TMB-8	18 ± 7	0.42 ± 0.02	1.5	0.97

For studies with [^{3}H]ACh, the parameters are given as means ± SEM from three independent experiments, analyzed individually as described in Materials and Methods. For comparison, the K_{app} and m values determined from [^{3}H]NMS binding (taken from Ref. 14) are also shown. K_{app} is the affinity with which the allosteric ligand alters the rate of dissociation of the labeled ligand, while m is the maximal effect of the allosteric ligand (see Materials and Methods).

* Not determined.

Because of the moderate effects on the rate of dissociation of [^{3}H]ACh, equilibrium is achieved relatively rapidly even in the presence of these allosteric agents (see Materials and Methods).

In contrast to the above modulators, the allosteric effects of TMB-8 were markedly dependent on the nature of the labeled ligand (Table 3). While the K_{app} for TMB-8 was 1.5 μM with [^{3}H]NMS as the primary ligand, it was more than 10-fold higher with [^{3}H]ACh, at 18 μM. If the ratio of K_{app} to K_A (the affinity of TMB-8 for the free receptor) reflects

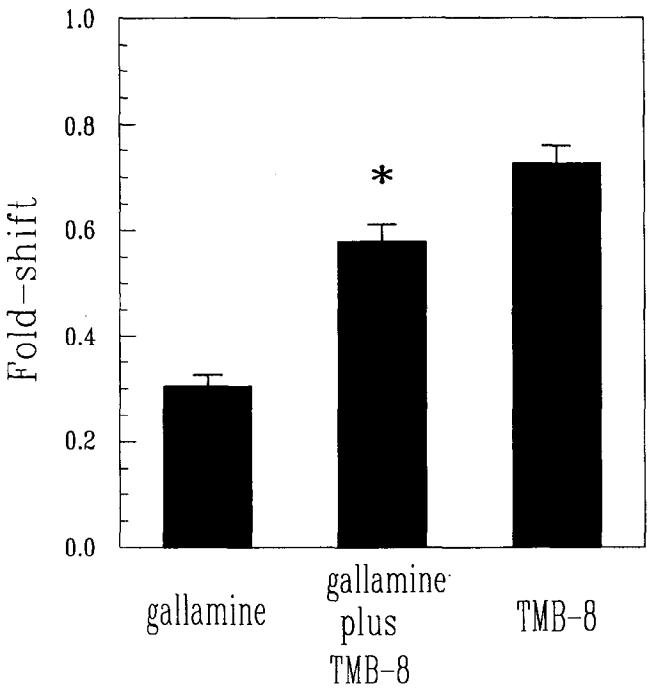

FIG. 7. Reversal by TMB-8 of gallamine's deceleration of the offrate of [^{3}H]ACh. The rate of dissociation of [^{3}H]ACh from m2 muscarinic receptors was determined in the presence of 1 μM gallamine, 100 μM TMB-8, or both. Data are the means ± SEM from four separate experiments. * The combination of gallamine plus TMB-8 slowed the dissociation significantly less than did gallamine alone ($P < 0.01$, t-test).

the cooperativity factor as expected [32], then this difference would indicate a greater degree of negative cooperativity between TMB-8 and [^{3}H]ACh than between TMB-8 and [^{3}H]NMS. In addition, the off-rate of [^{3}H]NMS could be slowed almost completely by TMB-8 (m = 0.974), but the offrate of [^{3}H]ACh was slowed only by about 40%. Unfortunately, the data for the effects of TMB-8 on the dissociation of [^{3}H]ACh are somewhat imprecise; for example, note the difference between the analysis of averaged experiments (Fig. 6) and the averages of the analyses of individual experiments (Table 3).

Thus, although it is clear in Fig. 6 that the effects of TMB-8 differ between NMS and ACh, it might be argued that the apparent affinity is, in fact, much lower than our estimate and that the maximal effect is greater, more in line with that of gallamine or THA; that is, the curve might come down further at sufficiently high concentrations. There is another approach to this question. If TMB-8 does exert a lesser maximal effect than another allosteric modulator *and* if they interact at the same site, then TMB-8 should be able to compete for the site and partially reverse the effect of the other agent (exactly the way partial agonists can reverse the effect of a full agonist under the appropriate conditions). Our results (Fig. 7) are fully consistent with the interpretation that TMB-8 exerts a fairly small effect on the dissociation of [^{3}H]ACh, even at saturating concentrations of TMB-8. Proska and Tucek [31] have suggested that muscarinic allosteric ligands absolutely prevent the dissociation of primary muscarinic ligands by "capping" the binding site. However, if gallamine and TMB-8 both acted this way, their effects would necessarily be additive. The capping model simply does not allow for the possibility that the addition of an allosteric ligand can accelerate the dissociation of the labeled primary ligand under any conditions. This effect is not isolated to the biding of [^{3}H]ACh, as we have shown previously that the allosteric effects of gallamine, THA, and TMB-8 on the rate of dissociation of [^{3}H]NMS from muscarinic receptors can be reversed by obidoxime [14].

It is tempting to attribute the dramatic ligand-dependent

differences in the allosteric effects of TMB-8 to the fact that one ligand (NMS) is an antagonist, while the other is the endogenous agonist. Clearly, agonists are sensitive to the conformational change associated with high- and low-affinity agonist states of the receptor, while antagonists are either insensitive or show a reverse sensitivity. Nonetheless, there are several reasons to resist this temptation with regard to the present allosteric effects. There are a number of cases in which an allosteric modulator exerts markedly different effects on different labeled antagonists. For example, we have found that gallamine accelerates the dissociation of [^{3}H]QNB at the same concentrations that retard the dissociation of [^{3}H]NMS [33]. Also, Tucek *et al.* [34] have shown that the neuromuscular blocker alcuronium exerts positive cooperativity on the binding of [^{3}H]NMS, but not on that of [^{3}H]QNB. Furthermore, agonists may also show unique effects. Potter *et al.* [35] found that THA did not affect the dissociation of [^{3}H]oxotremorine-M from either hippocampal or cardiac (m2) membranes and concluded that THA probably did not recognize the agonist-bound form of the receptor; however, the present study demonstrated that THA does recognize the ACh-bound receptor. Thus, until these allosteric interactions are better understood, it seems premature to draw conclusions based on the category of ligand (i.e., agonist vs antagonist). It should be noted that some of the results in the present study are not consistent with a simple allosteric mechanism. If ACh binds only to a single site, and another ligand binds only to one (allosteric) site, the slope factors (S) for the experiments shown in Fig. 4 and Table 2 should be unity. For THA, the slope factor was significantly greater than one, a characteristic that has been observed in other studies [14, 35]. On the other hand, this parameter for gallamine was significantly less than one, although this is not true for the gallamine-[^{3}H]NMS interaction (not shown). Finally, the similarity between the apparent affinities of physostigmine for the liganded (Fig. 5) and unliganded (Fig. 4) receptors would suggest that the cooperativity between [^{3}H]ACh and physostigmine is weak (see discussion of TMB-8 regarding K_{app} and K_A, above); however, the ability of physostigmine to inhibit the binding of [^{3}H]ACh in Fig. 4 suggests a strong negative cooperativity. These discrepancies may be due to the presence of more than one allosteric site or to complex binding properties of ACh. These observations are not without precedent, as a number of studies have found that muscarinic agonists can exhibit complex binding profiles [29, 36, 37]. The purpose of the present study was to determine whether the binding of [^{3}H]ACh is regulated by the same allosteric ligands that are known to regulate the binding of other muscarinic ligands. Elucidation of detailed mechanisms responsible for these allosteric interactions will require further studies.

In summary, binding of [^{3}H]ACh to recombinant m2 muscarinic receptors expressed in CHO cells could be readily measured and showed the expected pharmacological properties. In general, this binding was sensitive to the same allosteric modulators that have been identified in studies with other ligands, although there are sufficient discrepancies to necessitate testing ACh itself. Kinetic complications have often confounded studies of muscarinic allosteric interactions, and it is encouraging that the binding of [^{3}H]ACh was not found to be subject to the dramatic slowing of equilibrium that can be associated with muscarinic allosteric regulation. Thus, [^{3}H]ACh binding may prove to be especially useful in the evaluation of potential allosteric regulators of muscarinic systems.

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References

1. Hammer R, Berrie CP, Birdsall NMJ, Burgen ASB and Hulme EC, Pirenzepine distinguishes between different subclasses of muscarinic receptors. *Nature* **283:** 90–92, 1980.
2. Kubo T, Fukuda K, Mikami A, Maeda A, Takahashi H, Mishina M, Haga T, Haga K, Ichiyama A, Kangawa K, Kojima M, Matsuo H, Hirose T and Numa S, Cloning, sequencing, and expression of complementary DNA encoding the muscarinic acetylcholine receptors. *Nature* **323:** 411–416, 1986.
3. Kubo T, Maeda A, Sugimoto K, Akiba I, Mikami A, Takahashi H, Haga T, Ichiyama A, Kangawa K, Matsuo H, Hirose T and Numa S, Primary structure of porcine cardiac muscarinic acetylcholine receptor deduced from the cDNA sequence. *FEBS Lett* **209:** 367–372, 1986.
4. Bonner TI, Buckley NJ, Young AC and Brann MR, Identification of a family of muscarinic acetylcholine receptor genes. *Science* **237:** 527–532, 1987.
5. Bonner TI, Young AC, Brann MR and Buckley NJ, Cloning and expression of the human and rat m5 muscarinic acetylcholine receptor genes. *Neuron* **1:** 403–410, 1988.
6. Levey AI, Kitt CA, Simonds WF, Price DL and Brann MR, Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. *J Neurosci* **11:** 3218–3226, 1991.
7. Maeda A, Kubo T, Mishina M and Numa S, Tissue distribution of mRNAs encoding muscarinic acetylcholine receptor subtypes. *FEBS Lett* **329:** 339–342, 1988.
8. Jones SVP, Weiner DM, Levey AI, Ellis J, Novotny E, Yu S-H, Dorje SF, Wess J and Brann MR, Muscarinic acetylcholine receptors. In: *Molecular Biology of G-Protein-Coupled Receptors* (Ed. Brann MR), pp. 170–197. Birkhauser, Boston, 1992.
9. Ellis J, Huyler J, and Brann MR, Allosteric regulation of cloned m1–m5 muscarinic receptor subtypes. *Biochem Pharmacol* **42:** 1927–1932, 1991.
10. Waelbroeck M, Tastenoy M, Camus J and Christophe J, Binding of selective antagonists to four muscarinic receptors (M_1 to M_4) in rat forebrain *Mol Pharmacol* **38:** 267–273, 1990.
11. Lee NH and El-Fakahany EE, Allosteric antagonists of the muscarinic acetylcholine receptor. *Biochem Pharmacol* **42:** 199–205, 1991.
12. Clark AL and Mitchelson F, The inhibitory effect of gallamine on muscarinic receptors. *Br J Pharmacol* **58:** 323–331, 1976.
13. Waelbroeck M, Identification of drugs competing with *d*-tubocurarine for an allosteric site on cardiac muscarinic receptors. *Mol Pharmacol* **46:** 685–692, 1994.
14. Ellis J and Seidenberg M, Two allosteric modulators interact

at a common site on cardiac muscarinic receptors. *Mol Pharmacol* **42:** 638–641, 1992.
15. Lee NH, Jingru H and El-Fakahany EE, Modulation by certain conserved aspartate residues of the allosteric interaction of gallamine at the m1 muscarinic receptor. *J Pharmacol Exp Ther* **262:** 312–316, 1992.
16. Ellis J, Seidenberg M and Brann MR, Use of chimeric muscarinic receptors to investigate epitopes involved in allosteric interactions. *Mol Pharmacol* **44:** 583–588, 1993.
17. Leppik RA, Miller RC, Eck M and Paquet J-L, Role of acidic amino acids in the allosteric modulation by gallamine of antagonist biding at the m2 muscarinic acetylcholine receptor. *Mol Pharmacol* **45:** 983–990, 1994.
18. Matsui H, Lazareno S and Birdsall NJM, Probing of the location of the allosteric site on m1 muscarinic receptors by site-directed mutagenesis. *Mol Pharmacol* **47:** 88–98, 1995.
19. Lee NH and El-Fakahany EE, Allosteric interactions at the m1, m2, and m3 muscarinic receptor subtypes. *J Pharmacol Exp Ther* **256:** 468–479, 1991.
20. Buckley NJ, Bonner TI, Buckley CM and Brann MR, Antagonist binding properties of five cloned muscarinic receptors expressed in CHO-K1 cells. *Mol Pharmacol* **35:** 469–476, 1989.
21. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193:** 265–275, 1951.
22. Lennox RH, Hendley D and Ellis J, Desensitization of muscarinic receptor-coupled phosphoinositide hydrolysis in rat hippocampus: Comparisons with the α_1-adrenergic response. *J Neurochem* **50:** 558–564, 1988.
23. Younkin SG, Rosenstein C, Collins PL and Rosenberry TL, Cellular localization of the molecular forms of acetylcholinesterase in rat diaphragm. *J Biol Chem* **257:** 13630–13637, 1982.
24. Berrie CP, Birdsall NJM, Burgen ASV and Hulme EC, Guanine nucleotides modulate muscarinic receptor binding in the heart. *Biochem Biophys Res Commun* **87:** 1000–1006, 1979.
25. Harden TK, Scheer AG and Smith MM, Differential modification of the interaction of cardiac muscarinic cholinergic and *beta*-adrenergic receptors with a guanine nucleotide binding component(s). *Mol Pharmacol* **21:** 570–580, 1982.
26. Kurose H, Katada T, Amano T and Ui M, Specific uncoupling by islet activating protein, pertussis toxin, of negative signal transduction via α-adrenergic, cholinergic, and opiate receptors in neuroblastoma and glioma hybrid cells. *J Biol Chem* **258:** 4870–4875, 1983.
27. Kellar KJ, Martino AM, Hall DP, Schwartz RD and Taylor RL, High-affinity binding of [^{3}H]acetylcholine to muscarinic cholinergic receptors. *J Neurosci* **5:** 1577–1582, 1984.
28. Gurwitz D, Kloog Y and Sokolovsky M, High affinity binding of [^{3}H]acetylcholine to muscarinic receptors. Regional distribution and modulation by guanine nucleotides. *Mol Pharmacol* **28:** 297–305, 1985.
29. Mattera R, Pitts BJR, Entman ML and Birnbaumer L, Guanine nucleotide regulation of a mammalian myocardial muscarinic receptor system. *J Biol Chem* **260:** 7410–7421, 1985.
30. Stockton JM, Birdsall NJM, Burgen ASV and Hulme EC, Modification of the binding properties of muscarinic receptors by gallamine. *Mol Pharmacol* **23:** 551–557, 1983.
31. Proska J and Tucek S, Mechanisms of steric and cooperative actions of alcuronium on cardiac muscarinic acetylcholine receptors. *Mol Pharmacol* **45:** 709–717, 1994.
32. Lazareno S and Birdsall NJM, Detection, quantitation, and verification of allosteric interactions of agents with labeled and unlabeled ligands at G protein-coupled receptors: Interactions of strychnine and acetylcholine at muscarinic receptors. *Mol Pharmacol* **48:** 362–378, 1995.
33. Ellis J and Seidenberg M, Gallamine exerts biphasic allosteric effects at muscarinic receptors. *Mol Pharmacol* **35:** 173–176, 1989.
34. Tucek SL, Musilkova J, Hedoma J, Proska J, Shelkovnikov S and Vorlicek J, Positive cooperativity in the binding of alcuronium and *N*-methylscopolamine to muscarinic acetylcholine receptors. *Mol Pharmacol* **38:** 674–680, 1990.
35. Potter LT, Ferrendelli CA, Hanchett HE, Hollifield MA and Lorenzi MV, Tetrahydroaminoacridine and other allosteric antagonists of hippocampal M1 muscarinic receptors. *Mol Pharmacol* **35:** 652–660, 1989.
36. Hirschberg BT and Schimerlik MI, A kinetic model for oxotremorine-M binding to recombinant porcine m2 muscarinic receptors expressed in Chinese hamster ovary cells. *J Biol Chem* **269:** 26127–26135, 1994.
37. Wreggett KA and Wells JW, Cooperativity manifest in the binding properties of purified cardiac muscarinic receptors. *J Biol Chem* **270:** 22488–22499, 1995.