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Interactions of Alcuronium, TMB-8, and Other Allosteric Ligands with Muscarinic Acetylcholine Receptors: Studies with Chimeric Receptors

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ABSTRACT

A series of ligands that allosterically modulate the binding of classical ligands to muscarinic receptors was evaluated at wild-type and chimeric receptors. All of the ligands studied had highest affinity toward the M_2 subtype and lowest affinity toward the M_5 subtype. The chimeric receptors were mostly M_5 sequence; the amount of M_2 sequence ranged from about 6 to just under 30%. Alcuronium and TMB-8 had much higher affinity for the chimeric receptor that included the M_2 second outer loop of the receptor plus flanking regions of TM4 and TM5 than for any of the other chimeric receptors (the affinities of which remained similar to that of the M_5 subtype). However, this chimera retained the negative cooperativity between alcuronium and the classical antagonist *N*-methylscopolamine that is characteristic of M_5 (these ligands are positively cooperative at M_2). Verapamil, tetrahydroaminoacridine, and *d*-tubocurarine were also sensitive to that chimeric substitution, although verapamil and tetrahydroaminoacridine had even higher affinity for a chimera with M_2 sequence in TM7. None of these ligands shared gallamine's sensitivity to a region of the third outer loop, but studies in which obidoxime reversed the allosteric effects of gallamine and other ligands suggested that they nevertheless compete for a common site. In summary, although the present data are consistent with previous studies that have suggested that allosteric ligands bind to the outermost regions of muscarinic receptors, it appears that different allosteric ligands may derive subtype selectivity from different regions of the receptor.

The five subtypes of muscarinic receptors belong to the largest superfamily of transmembrane signaling receptors, the G protein-coupled receptors (GPCRs), and comprise the most highly conserved of the biogenic amine GPCR families. As in the superfamily as a whole, the conservation is especially great in the transmembrane regions (TM) (Jones et al., 1992). It is likely that acetylcholine and competitive agonists and antagonists bind within a pocket formed by the transmembrane regions at the level of a crucial aspartic acid residue in TM3 (Curtis et al., 1989; Fraser et al., 1989). The high degree of conservation around this binding site may be responsible for the difficulty in developing competitive muscarinic agonists and antagonists that are highly subtype-selective.

There is a second ligand-binding site on muscarinic receptors. A wide array of compounds has been identified, each of which is capable of modulating the binding of classical ligands to all five muscarinic subtypes (Lee and El-Fakahany, 1991; Ellis, 1997; Christopoulos et al., 1998). These modulators are believed to bind to more extracellular regions of the receptors than do classical ligands, and it is speculated that the lesser degree of conservation in these regions will permit correspondingly greater subtype selectivity. Allosteric modulation can also present another degree of selectivity by virtue of potential subtype-specific cooperativities (Birdsall et al., 1997). In addition to these possibilities for subtype selectivity, allosteric modulators have a number of theoretical therapeutic advantages over directly acting agonists or antagonists. First, they are capable of enhancing or inhibiting the action of the endogenous agonist to a fixed and finite degree at saturating concentrations of modulator. That is, the effect can become independent of concentration, largely avoiding the problems of activating or inhibiting the system too profoundly, which might otherwise occur because of overdosing or through kinetic variations in available drug levels (Ehlert, 1986). Second, allosteric drugs have a unique potential to preserve the physiological spatiotemporal patterning of signal input. For systemically acting hormones, this is not so important, but for the enhancement of central nervous system chemical neurotransmission it may be a crucial advantage. A modulator that merely enhances the affinity of an

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ABBREVIATIONS: GPCR, G protein-coupled receptor; TM, transmembrane region of the receptor; GABA, γ-aminobutyric acid; NMS, *N*-methylscopolamine; PB, sodium-potassium phosphate buffer, pH 7.4; BBSS, balanced buffered salt solution; QNB, quinuclidinyl benzilate; CR, chimeric receptor; THA, tetrahydroaminoacridine; k_{obs}, apparent rate constant; k_o, true rate constant.

endogenous transmitter will essentially amplify the natural signal when and where the transmitter is released. By contrast, a direct agonist will activate all receptors for as long as it is present, or until it desensitizes the receptor (which presents another problem). Drugs that prolong the duration of action of the transmitter by inhibiting re-uptake or metabolism will necessarily alter temporal patterning and may promote spatial spreading of the signal, as well. The advantages of allosterically acting drugs are well illustrated by the safety and efficacy of benzodiazepines that enhance the affinity of GABA for the $GABA_A$ (ion channel) receptor; there are no therapeutic applications for directly acting GABA agonists.

At present, acetylcholine is the only transmitter for which a complete family of GPCRs is sensitive to ligand-ligand interactions, but there is a growing number of reports of allosteric modulation at other GPCRs, including the A_1 adenosine (Bruns and Fergus, 1990), α_1 adrenergic (Waugh et al., 1999), α_2 adrenergic (Leppik et al., 1998), and D_2 dopamine (Hoare and Strange, 1996) receptors. However, the muscarinic receptor family remains by far the most intensively studied GPCR system for the allosteric interactions of small molecules, from both pharmacological and molecular perspectives. Thus, muscarinic receptors should be considered a model system for the study of allosteric modulation at GPCRs, in addition to the potential utility of such modulators at muscarinic receptors themselves. Selective muscarinic regulation could be useful in researching or treating a num-

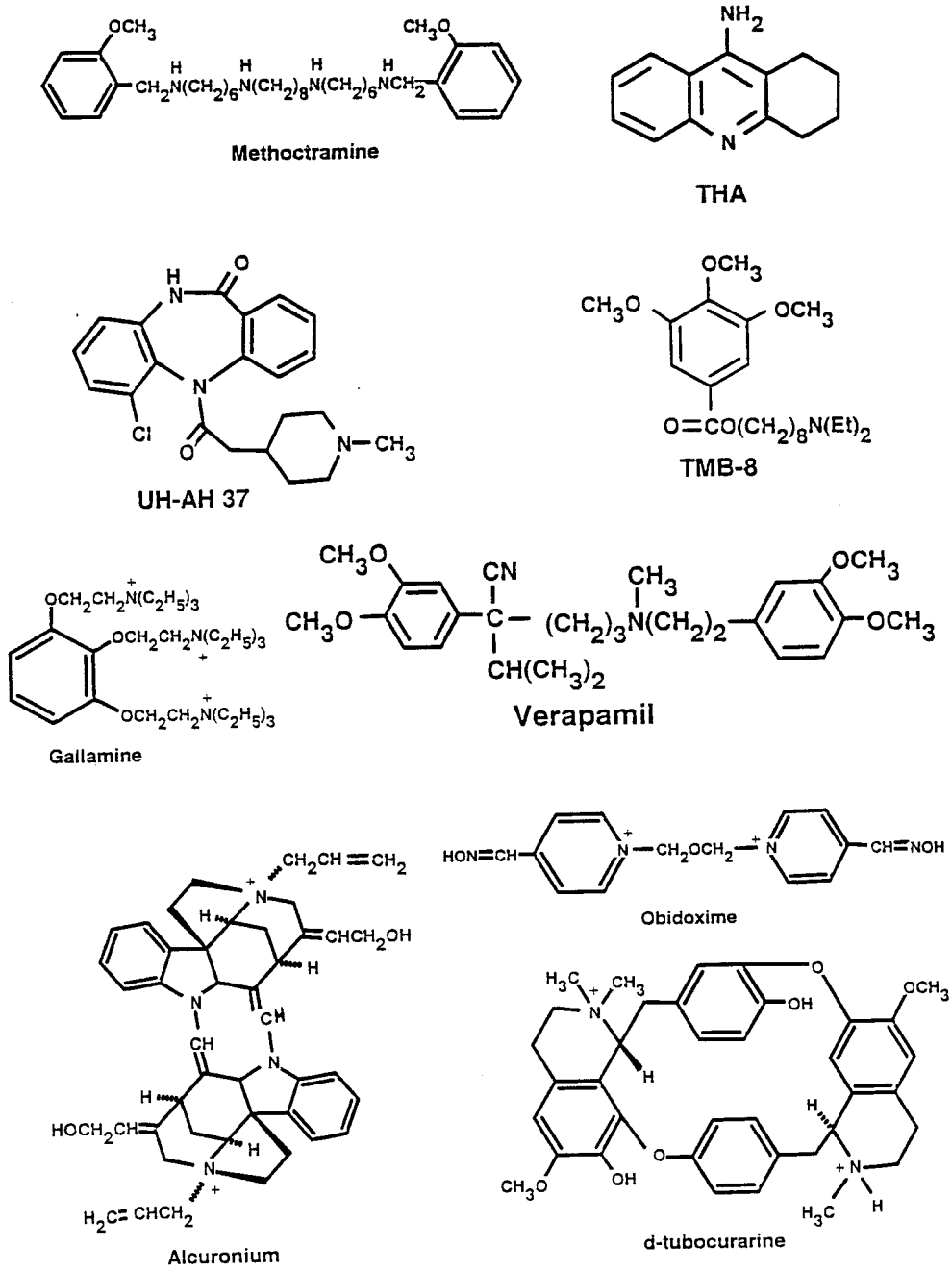

Fig. 1. Structures of muscarinic allosteric ligands investigated in this study.

ber of physiological or pathological conditions (Caulfield and Birdsall, 1998). In particular, levels of acetylcholine are dramatically reduced in Alzheimer's disease and, as mentioned above, a selective allosteric enhancer seems to be ideally suited to ameliorating transmitter depletion in the central nervous system.

Knowledge of which structural features of ligands and receptors interact with each other is fundamental to understanding how receptor function is regulated and what regulation is possible. Gallamine was the first allosteric muscarinic ligand to be described and has been evaluated in mutagenic studies of the muscarinic allosteric site (Lee et al., 1992; Ellis et al., 1993; Leppik et al., 1994; Matsui et al., 1995). We have reported that two regions of the receptor contain epitopes that contribute to gallamine's selectivity (Gnagey et al., 1999). In the present study we have investigated a variety of allosteric modulators, which present quite different structural features. One of these ligands, alcuronium, is of special interest because it exerts positive cooperativity with the antagonist NMS and because some ligands with closely related structures are positively cooperative with acetylcholine. We found that the affinities of the different compounds appear to be mainly sensitive to two regions of the receptor: 1) the second outer loop and adjacent regions of TM4 and TM5; and 2) TM7.

Experimental Procedures

Materials

Atropine, verapamil, THA, TMB-8, *d*-tubocurarine, polyethyleneimine (60,000 mol. wt., average), and gallamine were obtained from Sigma (St. Louis, MO). Methoctramine was obtained from RBI/Sigma (Natick, MA). Obidoxime was obtained from Schweizerhall (South Plainfield, NJ). Alcuronium was obtained from Roche Laboratories (Nutley, NJ). UH-AH 37 was a kind gift of Karl Thomae (Biberach, Germany). Labeled *N*-methylscopolamine chloride ([^{3}H]NMS; 84.5 Ci/mmol) and quininuclidinyl benzilate ([^{3}H]QNB; 52 Ci/mmol) were obtained from Perkin Elmer Life Sciences (Boston, MA).

Receptors

Wild-Type Receptors. Except when compared directly with chimeric receptors, human wild-type receptors were stably expressed in Chinese hamster ovary cells. Cells were grown to 80% confluency, and membranes were prepared as described below for chimeric receptors.

Chimeric Receptors. The M_2/M_5 chimeric muscarinic receptor genes used in this study have been investigated previously in allosteric studies (Ellis et al., 1993; Ellis and Seidenberg, 1999). The M_2/M_3 chimeric receptor gene was generously provided by Dr. Mark Brann. Schematic diagrams of the chimeric receptors are shown in Fig. 5. The exact sequence compositions are as follows: CR1: hM_2 1–69, hM_5 77–532; CR2: hM_5 1–76, hM_2 70–155, hM_5 163–532; CR3: hM_5 1–162, hM_2 156–300, hM_5 336–532; CR4: hM_5 1–445, hM_2 391–421, hM_5 477–532; CR5: hM_2 1–155, hM_5 163–532; CR6: hM_2 1–69, hM_5 77–445, hM_2 391–466; CR7: hM_2 1–207, rM_3 252–491, hM_2 389–466. Plasmids containing the various gene constructs were transfected into COS-7 cells by calcium phosphate precipitation. Cells were harvested 72 h after transfection by scraping into 5 mM sodium-potassium phosphate buffer, pH 7.4 (PB), homogenizing, and collecting the membranes at 50,000*g* for 20 min. The membranes were resuspended in 5 mM PB and stored as aliquots at −70°C.

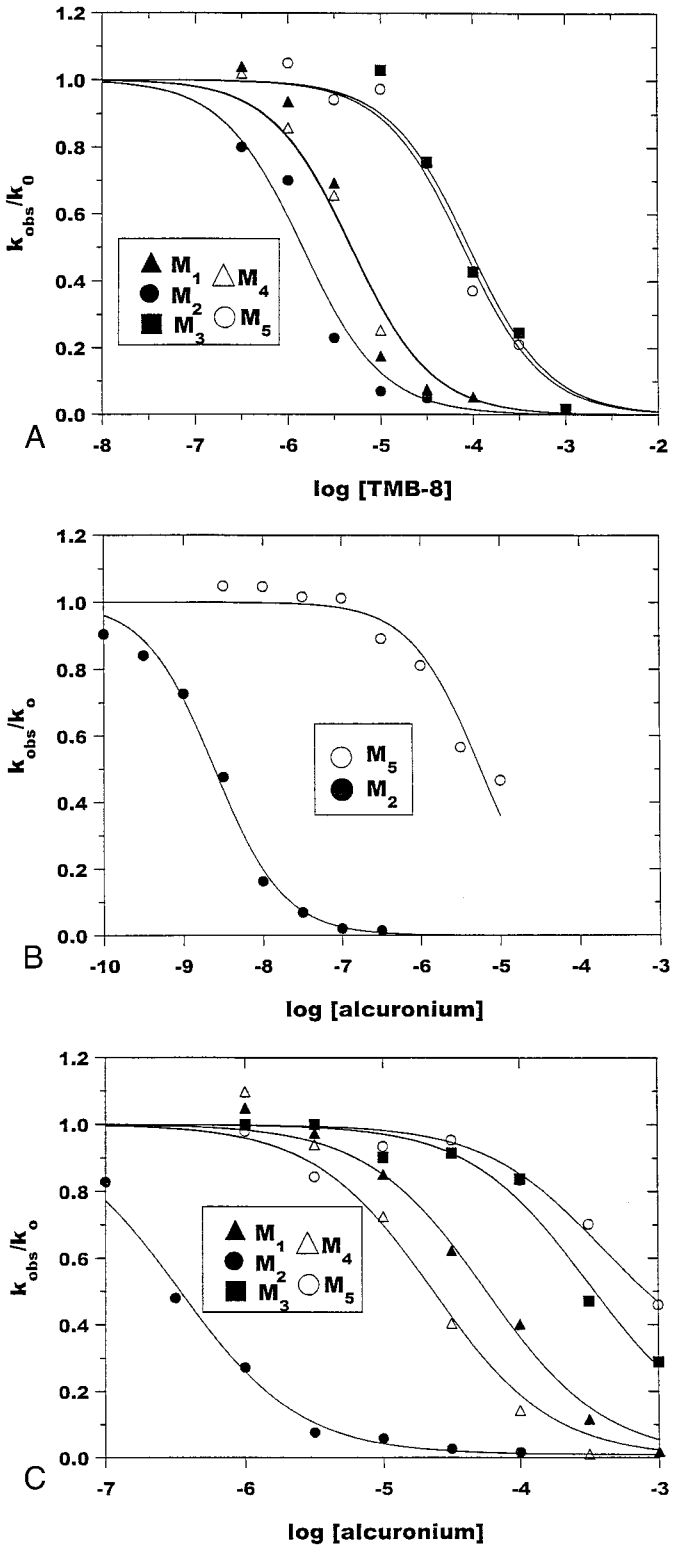

Fig. 2. Potencies with which alcuronium and TMB-8 slow the rates of dissociation of [^{3}H]NMS from wild-type muscarinic receptors. The receptors were prelabeled with [^{3}H]NMS, which was then allowed to dissociate in the absence or presence of the indicated allosteric modulator. The ratio of the rate of dissociation in the presence of each concentration of modulator (k_{obs}) to the rate of dissociation of [^{3}H]NMS alone (k_0) is plotted on the *y*-axis. The curves are the best fits to the simple allosteric model for dissociation data (see *Experimental Procedures*). Experiments in panels A and B were conducted in 5 mM PB, whereas the experiment in panel C was conducted in BBSS. The data shown are from representative experiments (summary data are presented in Fig. 3).

Binding Assays

Dissociation Assays. Assays were conducted at 25°C ([^{3}H]NMS) or 37°C ([^{3}H]QNB), in either 5 mM PB or a balanced buffered salt solution (BBSS; composition, in mM: NaCl, 136; KCl, 5; $CaCl_2$, 2; $MgS0_4$, 1; Na_2HPO_4, 1; Na-HEPES, 10; pH 7.4). Membranes (approx 30 μg of protein in 1 ml) were prelabeled with 1 nM [^{3}H]NMS or 0.2 nM [^{3}H]QNB for 30 min. Dissociation of the labeled ligand was initiated by the addition of 3 μM atropine, with or without the indicated concentration of allosteric ligand, and the incubation was allowed to continue for up to about 2 to 3 times the half-time for the dissociation of the labeled ligand from the receptor in the absence of allosteric modulator (Matsui et al., 1995; Kostenis and Mohr, 1996). The incubation was terminated by filtration through S&S 32 glass fiber filters (Schleicher and Schuell, Keene, NH) that had been pretreated with 0.1% polyethyleneimine, followed by two rinses with 40 mM PB (0°C). Nonspecific binding was determined by the inclusion of 3 μM atropine during the prelabeling period.

Dissociation assays were used extensively because they guarantee that allosteric effects are being measured. The dissociation of the specific binding of [^{3}H]NMS from each of the receptors has previously been shown to be first-order and complete in the absence of allosteric ligands (Ellis et al., 1993). It is difficult to unambiguously confirm these criteria at very high concentrations of most allosteric ligands because of the degree to which they slow the kinetics, but we have not observed any deviations with any of the ligands we have tested, in agreement with other studies (Matsui et al., 1995). The data from dissociation assays were treated in the following manner. The apparent rate constant (k_{obs}) for the dissociation of the labeled ligand was determined in the presence of each concentration of allosteric modulator and divided by the true rate constant (k_0), determined in the presence of 3 μM atropine only; thus, a resulting number of less than one indicates a slowing of the dissociation of the labeled ligand. The concentrations of modulators used in these studies are expected to lead to rapid equilibration with the allosteric site. Under these conditions, the concentration-dependent effects of an allosteric ligand on the dissociation of the labeled ligand should be proportional to the occupancy of the allosteric site, as previous studies have confirmed (Ellis and Seidenberg, 1992; Ellis, 1997). Therefore, data from these experiments were fitted to the following equation:

$$\frac{k_{obs}}{k_0} = 1 - \frac{mA}{A + K_{app}} \quad (1)$$

where A is the concentration of the allosteric modulator, m is the maximal reduction in the rate constant that can be exerted by the modulator, and K_{app} is the apparent equilibrium dissociation constant (for the interaction between the modulator and the *NMS-bound or QNB-bound* form of the receptor). When allosteric modulators were studied in combination (Figs. 6 and 9), a more general form of eq. 1 was employed (Ellis and Seidenberg, 1992):

$$\frac{k_{obs}}{k_0} = 1 - \frac{m_1A_1}{A_1 + K_1(1 + (A_2/K_2))} - \frac{m_2A_2}{A_2 + K_2(1 + A_1/K_1))} \quad (2)$$

where the parameters are as defined above, except that K_{app} has been replaced by K for clarity and the subscripts refer to the two different allosteric modulators present in the assay. Curve-fitting was carried out with the Scientist (MicroMath, Salt Lake City, UT) and MLAB (Civilized Software, Bethesda, MD) programs.

Equilibrium Assays. Membranes (approximately 30 μg of protein) were incubated with [^{3}H]NMS and the indicated concentrations of alcuronium at 25°C in BBSS for 3 h. Nonspecific binding was determined by the inclusion of 3 μM atropine in the assay. The incubation was terminated by filtration, which was conducted as described above for dissociation assays. Data from these assays were expressed as the percentage of the specific binding of [^{3}H]NMS in the absence of alcuronium and then plotted.

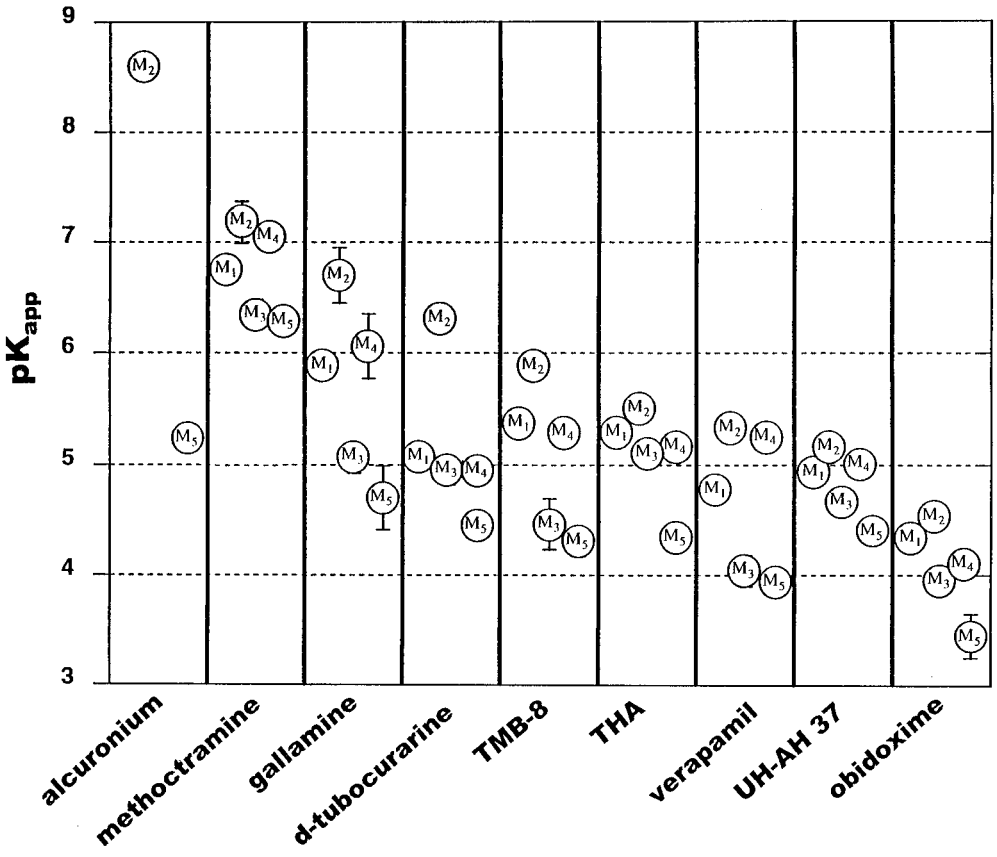

Fig. 3. Summary data for dissociation experiments carried out with wild-type receptors in Chinese hamster ovary cell membranes. Data from individual experiments carried out in 5 mM PB were fitted to the same model used in Fig. 2 (see *Experimental Procedures*) and the means ± S.E.M. of the pK_{app} values obtained from the curve-fitting are plotted for each modulator and subtype. Where error bars are not visible, they are smaller than the symbols. Data represent three to six experiments. Data for gallamine and UH-AH 37 have been reported previously (Ellis et al., 1993; Ellis and Seidenberg, 1999).

Results

The rates of dissociation of [^{3}H]NMS from the wild-type and chimeric receptors in the absence of allosteric ligands (k_0) have been reported previously (Ellis et al., 1993; Gnagey et al., 1999). These half-times range from about 5 min at M_2 to about 80 min at M_5, and from about 4 min at CR6 to about 180 min at CR2, in 5 mM PB. Dissociation was faster in BBSS by about 2-fold. The half-time for the dissociation of [^{3}H]QNB from the M_2 receptor was about 60 min, as reported previously (Ellis et al., 1991). The structures of the compounds investigated in these studies are presented in Fig. 1. Both TMB-8 and alcuronium markedly slow the dissociation of [^{3}H]NMS from all five subtypes of muscarinic receptors (Fig. 2). In hypotonic phosphate buffer, both modulators have much higher affinity for the M_2 subtype than for the M_5 subtype. At M_2, TMB-8 exerts half-maximal effects at low micromolar concentrations, whereas alcuronium exerts half-maximal effects in the low nanomolar range. The subtype selectivity of TMB-8 is $M_2 > M_1 = M_4 > M_3 = M_5$. In the physiological buffer BBSS, alcuronium displays lower affinities, but the relative order of potency is preserved ($M_2 \gg M_4 \approx M_1 > M_3 \approx M_5$; Fig. 2C).

Figure 3 presents summary data for the potencies of nine allosteric modulators in slowing the rate of dissociation of [^{3}H]NMS from the muscarinic receptor subtypes (in 5 mM PB). All nine have the highest affinity for M_2 and the lowest affinity for the M_5 subtype. The ratios of these affinities range from about 10-fold for methoctramine, UH-AH 37, and obidoxime, to more than 1000-fold for alcuronium. The patterns of intermediate affinity also vary. Gallamine's subtype selectivities are similar to those described above for TMB-8 and alcuronium: $M_2 > M_4 \approx M_1 > M_3 \approx M_5$. On the other hand, in the case of *d*-tubocurarine, the affinities of M_1, M_3, and M_4 cluster fairly close to that of M_5; in the case of THA, they cluster near the affinity of M_2.

The structural bases for the allosteric selectivities of these modulators were investigated using M_2/M_5 chimeric receptors. TMB-8 slowed the dissociation of [^{3}H]NMS from all of the chimeric constructs (Fig. 4). All but one of the chimeras exhibited similar affinities to that of the M_5 subtype. CR3, which contained a segment of M_2 sequence that includes the extracellular half of TM4, the second outer loop, all of TM5, and a portion of the third intracellular loop, had nearly as high affinity for TMB-8 as did the M_2 subtype (schematic diagrams of the chimeric receptors are presented in Fig. 5). Alcuronium exhibited a very similar pattern, with CR3 again showing dramatically higher affinity than any of the other chimeric receptors. As in the studies of wild-type receptors, the affinity of alcuronium was much greater in 5 mM PB than in BBSS, but the relative difference between CR3 and the other chimeras was not altered (Fig. 4, B and C).

Summary data for the potencies with which TMB-8, alcuronium, and six other allosteric ligands alter the rate of dissociation of [^{3}H]NMS from the chimeric receptors are shown in Fig. 5. Most of the ligands exhibit considerable sensitivity to the sequence in CR3. The two exceptions are gallamine and UH-AH 37, which have been reported on previously (Ellis et al., 1993; Ellis and Seidenberg, 1999). Alcuronium, TMB-8, and *d*-tubocurarine have highest affinity for CR3, compared with the other chimeras. On the other hand, verapamil, UH-AH 37, THA, gallamine, and methoctramine have highest affinity for CR6. Of these, verapamil and THA also show considerable preference for CR3, compared with the remaining chimeras. THA exhibited steep curves at all of the wild-type receptors, with Hill slopes ranging from 1.3 to

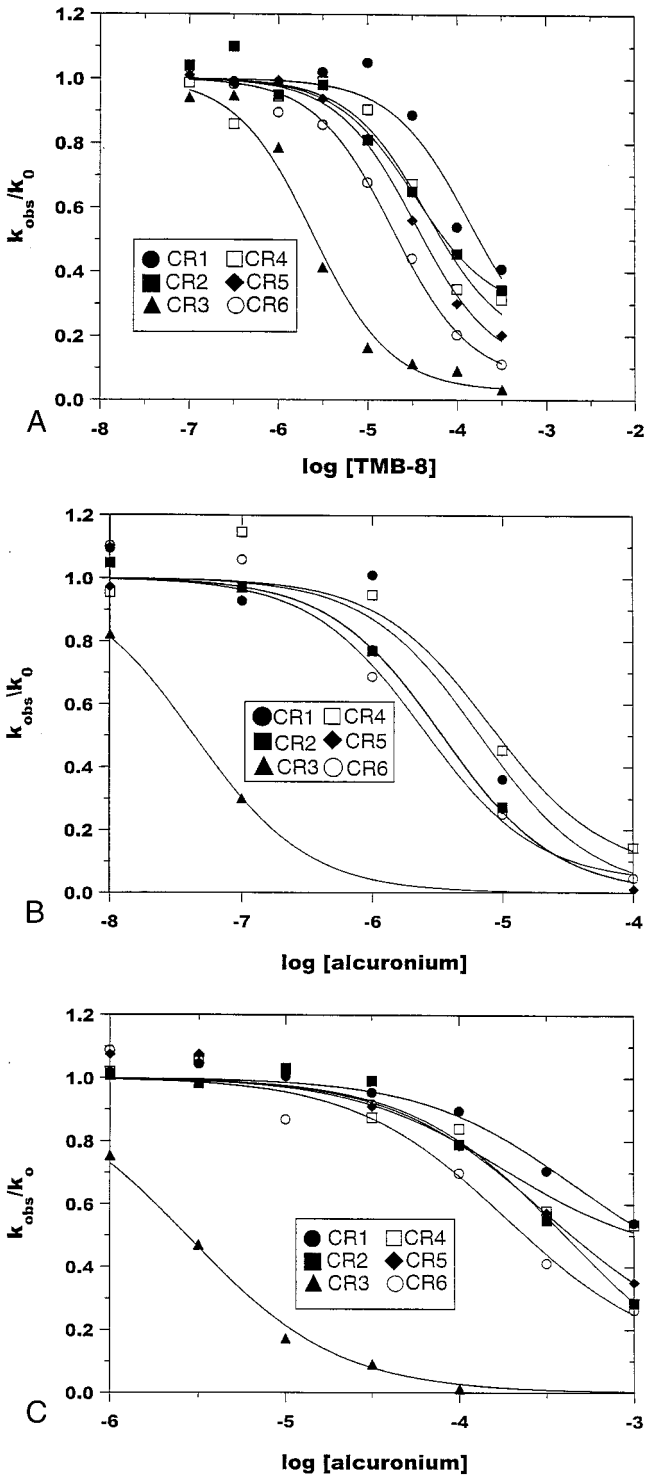

Fig. 4. Potencies with which alcuronium and TMB-8 slow the rates of dissociation of [^{3}H]NMS from chimeric muscarinic receptors. Experiments were conducted as in Fig. 2, using the indicated M_2/M_5 chimeric receptors. Schematic representations of the chimeric receptors are presented with summary data in Fig. 5. Experiments in panels A and B were conducted in 5 mM PB, whereas the experiment in panel C was conducted in BBSS.

1.7 (data not shown), in agreement with previous studies (Potter et al., 1989; Ellis and Seidenberg, 1992; Mohr and Trankle, 1994). All of the chimeric receptors also displayed steep curves, with Hill slopes in the same range.

The model with which we analyzed the dissociation assays includes the parameter *m*, which defines the maximal alteration of the off-rate of the labeled ligand that can be accomplished by very high concentrations of a given allosteric ligand (see *Experimental Procedures*). The *m* values from our analyses are summarized in Table 1. Our assays were designed to optimize the determination of K_{app} values, however, rather than *m* values, because we place much greater importance on affinities (see *Discussion*). For this reason, *m* values greater than 0.9 (meaning that the off-rate can be slowed to less than 10% of control values) are simply listed as ">0.9" in the Table.

The most unambiguous way to demonstrate that a particular ligand's *m* value is less than one is to use that ligand to reverse the effect of a more efficacious ligand. Indeed, we have previously found that obidoxime is only capable of slowing the dissociation of [^{3}H]NMS from the M_2 receptor by about a factor of 2, whereas gallamine and other allosteric ligands can slow it to a much greater extent; we were able to use this property to demonstrate that gallamine and obidoxime act at the same site (Ellis and Seidenberg, 1992). Figure 6 shows that obidoxime has essentially no effect on the rate of dissociation of [^{3}H]QNB from the M_2 receptor, whereas TMB-8 and alcuronium markedly slow that dissociation and gallamine accelerates it. Obidoxime reverses the effects of each of the other three ligands in a concentration-dependent manner. The goodness of fit between the model and the data in Fig. 6 suggests that these four ligands interact with each other competitively at a common allosteric site on the M_2 receptor.

Less detailed experiments have been carried out with the other ligands. It can be seen in fig. 7A that obidoxime also reverses the allosteric effects of *d*-tubocurarine, verapamil, and THA. Methoctramine itself only partially slows the dissociation of [^{3}H]QNB and, like obidoxime, is capable of reversing the effects of other allosteric ligands (Fig. 7B).

Alcuronium was the first allosteric ligand shown to exert positive cooperativity with a classical muscarinic ligand. In fig. 8 it can be seen that alcuronium causes a concentration-dependent increase in the binding of [^{3}H]NMS to the M_2 subtype (in BBSS at 25°C, 3 h of incubation). At the highest concentrations of alcuronium (above 3 μM), the enhancement disappears and is replaced by an apparent inhibition, due to the dramatic slowing of the association of [^{3}H]NMS, as previously demonstrated (Proska and Tucek, 1994). When similar experiments were carried out with the chimeric receptors, no positive cooperativity was observed. As in the dissociation experiments, alcuronium had dramatically

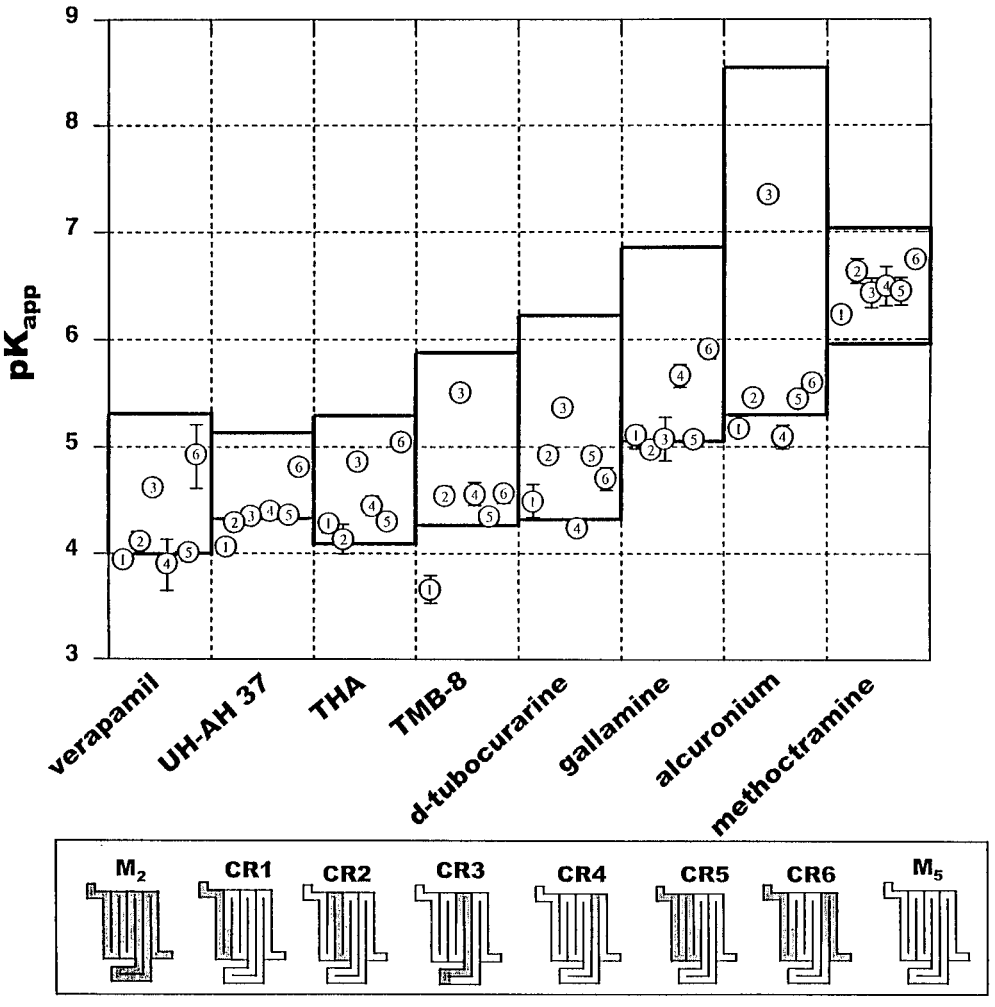

Fig. 5. Summary data for dissociation experiments carried out with chimeric receptors in COS-7 cell membranes. Data from individual experiments were fitted to the same model used in Fig. 2 (see *Experimental Procedures*) and the means ± S.E.M. of the pK_{app} values obtained from the curve-fitting are plotted for each modulator and chimeric receptor. The circled numbers represent the chimeric receptors, schematic representations of which are shown below the graph; detailed sequence information is given under *Experimental Procedures*. Where error bars are not visible, they are smaller than the symbols. In each column, the bottom and top of the bolded box represent the pK_{app} values for the wild-type M_5 and M_2 receptors (expressed in COS-7 cells), respectively. The error bars for these wild-type data have been omitted for clarity, but were always ±0.2 or less. The data for gallamine and UH-AH 37, included for comparison, have been published previously (Ellis et al., 1993; Ellis and Seidenberg, 1999). Data represent three to seven experiments.

higher affinity for the CR3 construct than for the other chimeras; however, even at the lowest concentrations, it only inhibited the binding of [^{3}H]NMS. Because there is a portion of the third intracellular loop that was not swapped in the set of M_2/M_5 chimeras, we also investigated a chimeric receptor that was composed of M_2 sequence everywhere except for the third intracellular loop, which was made up of M_3 sequence. This chimeric receptor had the same intrinsic dissociation rate for [^{3}H]NMS as did the M_2 receptor (a half-time of about 2 min in BBSS). The effects of alcuronium at this chimeric receptor were the same as at the M_2 receptor in both pseudo-equilibrium assays and dissociation assays (Fig. 9). However, the affinity of the agonist carbachol at CR7 was significantly reduced compared with M_2 (data not shown), as expected (Wess et al., 1990).

Discussion

Many compounds have been demonstrated to allosterically modulate the binding of classical ligands to muscarinic receptors; these modulators represent multiple pharmacological classes, as well. For the present study, we chose an array of modulators that span different classes and present a variety of structural features. A number of our choices are best known for other cholinergic properties, such as nicotinic receptor antagonism (alcuronium, gallamine, *d*-tubocurarine) or interaction with acetylcholinesterase (THA, obidoxime). Verapamil and TMB-8 are better known as blockers of calcium-dependent signaling. Methoctramine and UH-AH 37 were introduced as (competitive) muscarinic ligands before their allosteric properties were known. Interestingly, all of these diverse ligands display greatest allosteric affinity toward the M_2 subtype and lowest allosteric affinity toward M_5, although their absolute affinities for these subtypes and the ratios of these affinities vary widely. Thus, chimeric receptors composed of segments of M_2 and M_5 sequence are the most promising tools with which to investigate subtype-specific epitopes that affect the selectivities of these compounds.

The chimeric receptors tested were all predominantly M_5 sequence, with the amount of sequence donated by the M_2 gene ranging from about 6% (CR4) to almost 30% (CR5) of amino acid residues; of course, there is also a great deal of conservation of sequence among the muscarinic receptors (Jones et al., 1992). Most of the ligands studied were sensitive to only one or two of the chimeric substitutions. For example, alcuronium exhibited much greater affinity toward CR3 than toward any of the other chimeras; the affinities of the remaining chimeras were essentially the same as the M_5 receptor. TMB-8 was also uniquely sensitive to CR3. Gallamine has previously been found to be sensitive to the sequence highlighted in CR4; that entire sequence is also included in CR6 (Ellis et al., 1993). Subsequent studies have found that the residue in the CR4 region that is responsible for the enhancement of gallamine's affinity appears to be an asparagine in the third outer loop, at M_2^{419} (Gnagey et al., 1999). Somewhat paradoxically, the affinity of gallamine has also been shown to be sensitive to an epitope in the second outer loop, yet it is not sensitive to CR3, which includes this region (Ellis et al., 1993; Leppik et al., 1994). The explanation appears to be that both M_5 and M_2 contain the essential sequence, whereas the M_1 subtype lacks it (Gnagey et al., 1999). Thus, those ligands that display sensitivity to CR3 are likely to be sensitive to residues other than those that affect gallamine's affinity.

Verapamil, UH-AH 37, and THA were all sensitive to CR6, but not to CR1 or CR4. This suggests that the residue(s) responsible for these sensitivities lie between the top of TM7 and the carboxyl terminus of the receptor (see schematics in Fig. 5). Previous studies of UH-AH 37 have revealed that its subtype specificity depends on whether it is measured at equilibrium or in dissociation assays. The results of several independent experimental approaches suggested that UH-AH 37 interacts both competitively and allosterically with muscarinic receptors (Ellis and Seidenberg, 1999). Mutational studies have indicated that the subtype selectivity of the competitive interaction is caused by an epitope within the CR4 region, specifically a threonine residue in the middle of TM6 that is present in every mammalian subtype except M_2, which has an alanine (M_2^{401}) at this position (Ellis and Seidenberg, 2000). As noted above, the epitope responsible the allosteric subtype selectivity of UH-AH 37 lies outside of the CR4 region.

With the possible exception of methoctramine, none of the allosteric ligands were very sensitive to substitutions in the N-terminal half of the receptor. The methoctramine data is difficult to interpret, however, because of the broad sensitivity and the small magnitudes of the effects. This suggests that the C-terminal half of the receptor plays the major role in generat-

TABLE 1

Summary of *m* values obtained from analysis of dissociation assays

The upper portion of the table refers to receptors expressed in CHO cells, while the rest of the table refers to receptors expressed in COS-7 cells. The data are the means ± S.E.M. from three or more determinations, except for the UH-AH 37 data, which are from two to three determinations. See text for further discussion.

	Alcuronium (BBSS)	Alcuronium	*d*-Tubocurarine	Methoctramine	Obidoxime	THA	TMB-8	Verapamil	UH-AH 37	Gallamine
M_1	>0.9	N.D.	0.88 ± 0.04	>0.9	0.72 ± 0.03	>0.9	>0.9	>0.9	0.76 ± 0.06	>0.9
M_2	>0.9	>0.9	>0.9	>0.9	0.67 ± 0.06	>0.9	>0.9	>0.9	0.88 ± 0.04	>0.9
M_3	0.86 ± 0.09	N.D.	>0.9	>0.9	0.69 ± 0.08	>0.9	>0.9	>0.9	0.73 ± 0.08	>0.9
M_4	>0.9	N.D.	>0.9	>0.9	0.79 ± 0.02	>0.9	>0.9	>0.9	>0.9	>0.9
M_5	0.84 ± 0.10	>0.9	>0.9	0.79 ± 0.02	0.64 ± 0.03	>0.9	0.86 ± 0.09	>0.9	0.66 ± 0.12	0.82 ± 0.05
M_2	>0.9	>0.9	>0.9	>0.9	N.D.	>0.9	>0.9	>0.9	>0.9	>0.9
M_5	0.74 ± 0.11	>0.9	0.88 ± 0.06	0.67 ± 0.08	N.D.	>0.9	0.71 ± 0.10	0.81 ± 0.12	0.72 ± 0.14	0.75 ± 0.06
CR1	0.84 ± 0.09	>0.9	0.88 ± 0.06	0.64 ± 0.11	N.D.	>0.9	>0.9	0.79 ± 0.11	>0.9	>0.9
CR2	0.83 ± 0.06	>0.9	0.86 ± 0.11	0.68 ± 0.06	N.D.	0.78 ± 0.10	0.74 ± 0.05	0.79 ± 0.09	>0.9	>0.9
CR3	>0.9	>0.9	0.84 ± 0.03	0.75 ± 0.06	N.D.	>0.9	>0.9	>0.9	>0.9	>0.9
CR4	0.69 ± 0.07	>0.9	0.89 ± 0.06	0.77 ± 0.07	N.D.	>0.9	0.69 ± 0.08	0.82 ± 0.17	>0.9	>0.9
CR5	>0.9	>0.9	0.85 ± 0.02	0.76 ± 0.06	N.D.	0.83 ± 0.04	>0.9	>0.9	>0.9	0.72 ± 0.07
CR6	>0.9	>0.9	>0.9	>0.9	N.D.	>0.9	>0.9	>0.9	>0.9	>0.9

N.D., not determined.

ing subtype-selectivity and, coupled with evidence that muscarinic allosteric ligands probably bind to the most extracellular portions of the receptor (Jakubik and Tucek, 1994; Gnagey et al., 1999), focuses attention on the second outer loop and the tops of TM4 and TM5 and on the top of TM7. THA is sensitive to both of these regions. The steepness of the binding curves for THA suggests that it may interact with more than one allosteric site, including the possibility that its binding is sensitive to receptor dimerization (Potter et al., 1989).

Although there are at least some differences between the epitopes that modify the binding of gallamine and those that affect alcuronium and TMB-8, obidoxime was able to reverse the effects of all three ligands on the dissociation of [^{3}H]QNB (Fig. 6). The goodness of fit obtained (for the family of curves) with a single value of the apparent affinity of obidoxime suggests that all four of these ligands are interacting competitively at the allosteric site; presumably, like gallamine, alcuronium and TMB-8 will be found to interact with extracellular portions of the receptor. Similar but less extensive data suggest that the other small ligands also interact with the same common allosteric site.

Most of the data presented in this report were obtained from dissociation assays. The dissociation assay provides the advantage that it reflects only allosteric interactions. However, it determines the affinity of these ligands for the NMS-bound receptor. Equilibrium studies are required in order to estimate the affinity of allosteric ligands for the unliganded receptor, but they can be misleading for ligands that also interact competitively at the receptor, as we have shown for UH-AH 37 (Ellis and Seidenberg, 1999, 2000). Allosteric ligands that exhibit positive cooperativity are less likely to be confounded, and alcuronium is believed to interact purely allosterically with muscarinic receptors (Proska and Tucek, 1995). Data from equilibrium and dissociation assays both indicate that the binding of alcuronium is sensitive to an epitope in the region of the CR3 substitution, which includes part of TM4, all of the second outer loop, all of TM5, and a portion of the third inner loop. Most of the present assays

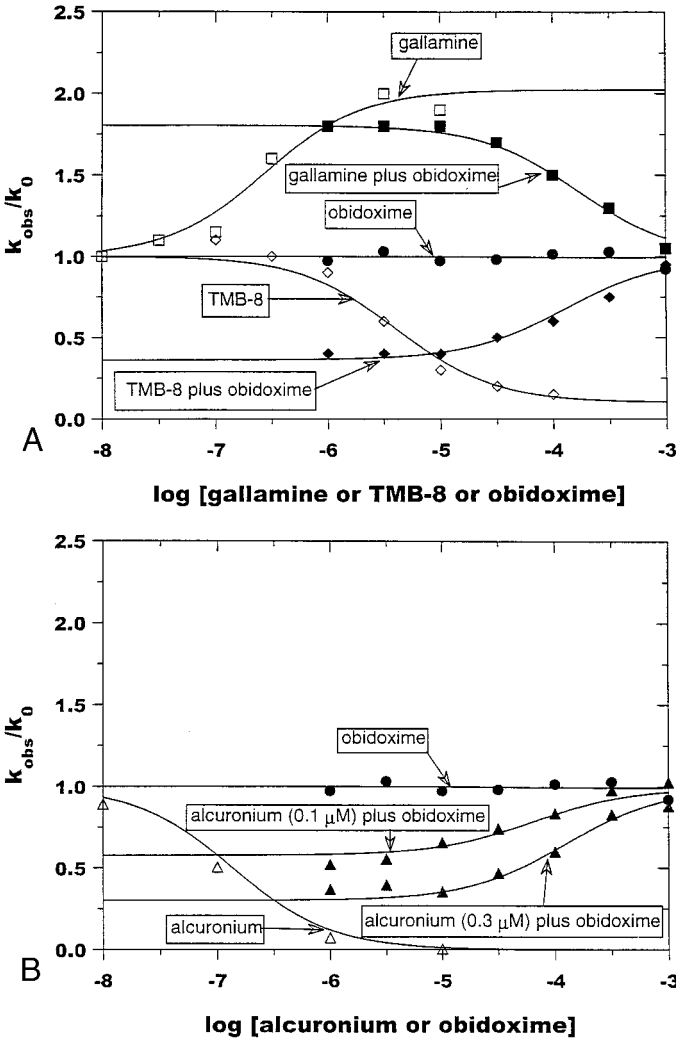

Fig. 6. Reversibility by obidoxime of the allosteric effects of alcuronium, gallamine, and TMB-8 on the rate of dissociation of [^{3}H]QNB from M_2 muscarinic receptors. Experiments were conducted in 5 mM PB as in Fig. 2, except that receptors were prelabeled with [^{3}H]QNB before the initiation of dissociation. The concentration of obidoxime was titrated in the presence of 1 μM gallamine (■), 10 μM TMB-8 (♦), 0.1 or 0.3 μM alcuronium (▲), or by itself (●). Alcuronium, gallamine, and TMB-8 were also titrated by themselves, as indicated by the open symbols. The entire set of data (both panels) was simultaneously fitted to model 2 (see *Experimental Procedures*) for the interactions of multiple modulators at a single allosteric site. The curves shown are all based on the following set of best-fit parameters: obidoxime, $m = 0.0062$, $K_{app} = 36$ μM; alcuronium, $m = 0.99$, $K_{app} = 0.13$ μM; gallamine, $m = -1.02$, $K_{app} = 0.27$ μM; TMB-8, $m = 0.90$, $K_{app} = 4.0$ μM.

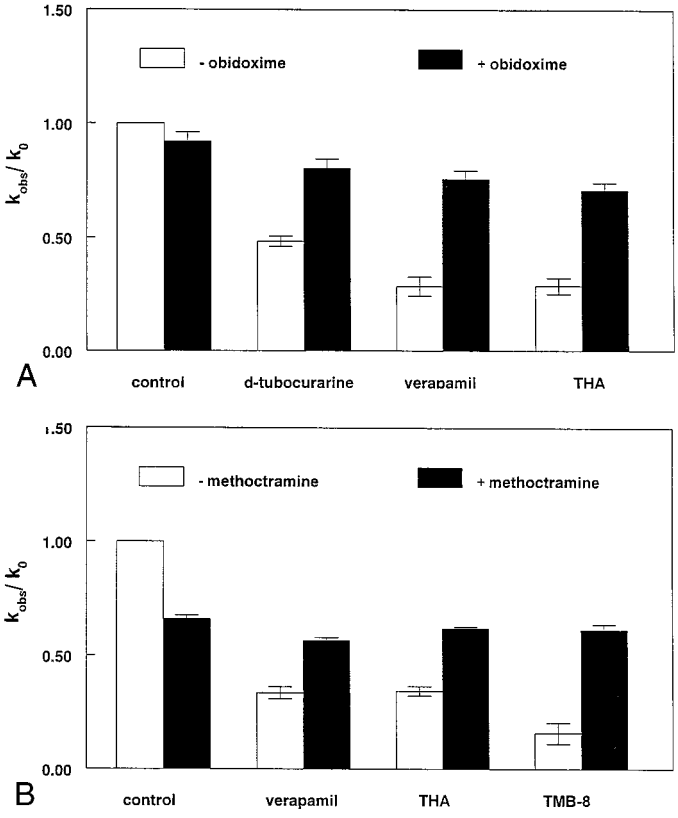

Fig. 7. Ability of obidoxime and methoctramine to reverse the effects of other allosteric ligands on the rate of dissociation of [^{3}H]QNB from M_2 muscarinic receptors. Experiments were conducted as in Fig. 6, using single concentrations of each ligand. A, 300 μM obidoxime was able to significantly reverse the effects of 300 μM *d*-tubocurarine, 100 μM verapamil, and 100 μM THA on the rate of dissociation of [^{3}H]QNB from M_2 receptors. B, 100 μM methoctramine exerts a partial effect on the off-rate of [^{3}H]QNB and is able to significantly reverse the effects of 100 μM verapamil, 100 μM THA, and 10 μM TMB-8. Bars represent the mean ± S.E.M. for two to three experiments.

were also performed in a hypotonic buffer in which the affinities of many muscarinic ligands, both allosteric and classical, are significantly greater than they are in physiological buffers (Birdsall et al., 1979; Ellis et al., 1991; Waelbroeck, 1994; Trankle et al., 1996). Nonetheless, we would expect the epitopes involved in the binding of most ligands to be independent of the ionic strength of the assay, and that expectation has been upheld for alcuronium (see Fig. 4) and gallamine (Gnagey et al., 1999).

In our studies of pseudoequilibrium binding, alcuronium interacted with [^{3}H]NMS in a positively cooperative manner at the M_2 subtype, but did not enhance [^{3}H]NMS binding at the M_3 or M_5 subtypes, in agreement with previous reports (Jakubik et al., 1995). Similar studies of the chimeric receptors found that, as in the dissociation studies, CR3 exhibited much higher affinity toward alcuronium than did the other chimeric receptors; however, alcuronium was not positively cooperative with NMS at any of the chimeric receptors. Because our M_2/M_5 chimeric receptor series covers the receptor expanse comprehensively everywhere except the third inner loop, we also examined an M_2/M_3 chimeric receptor in which the third inner loop of the M_2 receptor is replaced by the homologous sequence of M_3 (the binding of alcuronium is very similar at the M_3 and M_5 subtypes, as illustrated in Figs. 2 and 8). This M_2/M_3 chimeric receptor did not differ significantly from the M_2 receptor in equilibrium or dissociation studies, implying that the third inner loop dictates neither alcuronium's affinity nor cooperativity.

Our inability to confer positive cooperativity by chimeric substitution suggests that cooperativity may be a global property of receptor structure. On the other hand, affinity seems to be more tightly related to specific receptor epitopes. Our strategy has been fairly conservative, in that we have been attempting to confer *enhanced* affinity onto the lower affinity subtype (M_5) by replacing relatively small portions of the receptor with sequence from the higher affinity subtype (M_2). This differs from the more usual approach of mutating suspected residues that are conserved across a family; however, because affinity may be

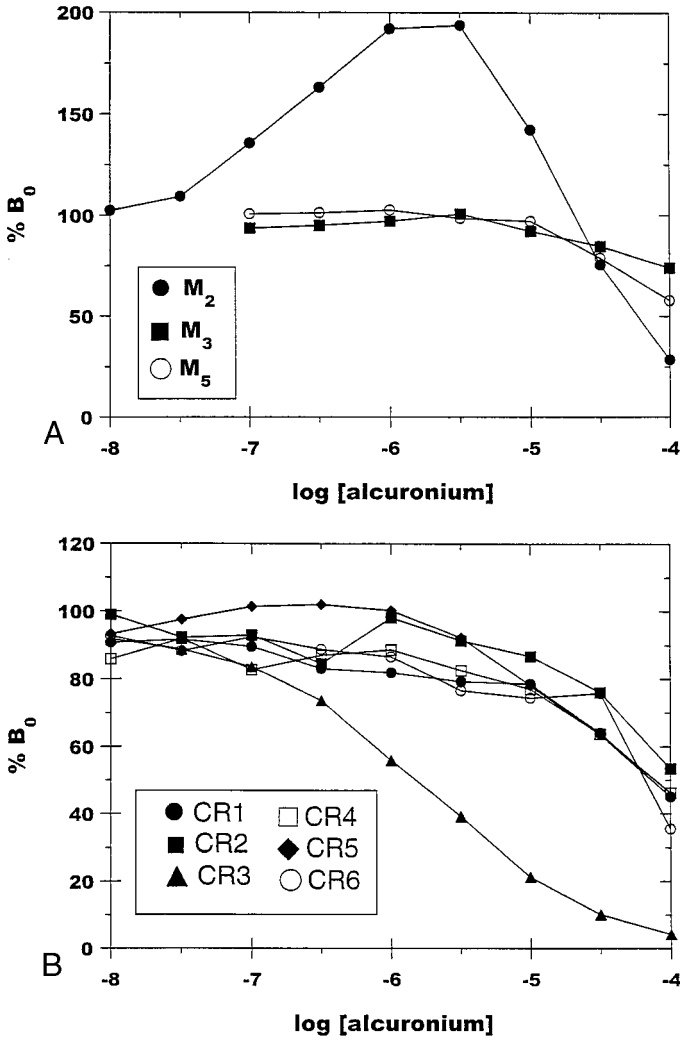

Fig. 8. Effects of alcuronium on the binding of [^{3}H]NMS to wild-type (A) and chimeric (B) receptors under pseudoequilibrium conditions. Membranes containing the indicated receptors were incubated in BBSS with 0.05 nM [^{3}H]NMS for 3 h at 25°C, in the presence or absence of the indicated concentrations of alcuronium. The specific binding determined at each concentration of alcuronium is expressed as the percentage of that found in the absence of alcuronium (B_0). The data shown are representative of two to four similar experiments. See Fig. 5 for schematic representations of the chimeric receptors.

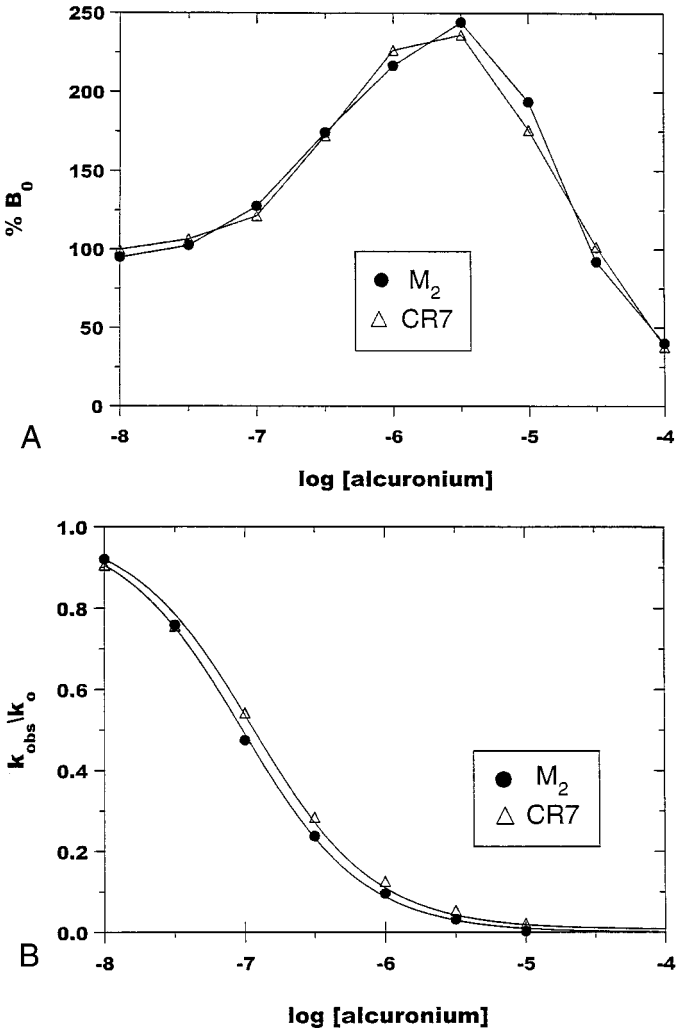

Fig. 9. Lack of importance of the nature of the third intracellular loop of the receptor on the affinity of alcuronium and the cooperativity between alcuronium and [^{3}H]NMS. Experiments were conducted in BBSS, as in Figs. 8A or 2B, using either the wild-type M_2 receptor or a chimeric receptor (CR7) composed of M_2 sequence everywhere except the third intracellular loop, which contained M_3 sequence (see *Experimental Procedures* for detailed sequence information).

disrupted for many reasons, it can be harder to interpret the results of mutations of conserved residues (Huang et al., 1994; Birdsall et al., 1995; Schwartz et al., 1997). Identification of subtype-specific residues presents the opportunity to examine reciprocal mutations at different subtypes to determine whether consistent results are observed; so far, we have found such consistent effects on the allosteric binding of gallamine (Ellis et al., 1993; Gnagey et al., 1999). We have previously discussed reasons why we do not expect rate constants (k_0) to be closely and consistently tied to particular receptor epitopes (Ellis et al., 1993). The same arguments apply to the m parameter in our modeling function because it merely serves to define another rate, mk_0. By a related argument, we are more hopeful that specific epitopes may be responsible for cooperativity, because it serves to define a new affinity (αK). Even so, because cooperativity reflects the adjustment of conformational strain between different binding sites, it is not unreasonable that multiple features of the receptor may influence this parameter, and in this case, it may be more informative to attempt to disrupt positive cooperativity than to confer it. Future studies of the interactions between alcuronium and NMS at chimeras that are predominantly M_2 sequence may be useful in this regard.

In summary, we have found that a diverse group of muscarinic allosteric modulators is selective for the M_2 subtype over the M_5 subtype. In studies with chimeric receptors, most of these modulators were sensitive to a region that includes the second outer loop of the receptor. The other region that influenced the affinities of several compounds included the seventh transmembrane domain. Based on recent findings with gallamine, it seems reasonable to expect that subsequent studies will be able to attribute the subtype selectivities of some of these ligands to specific residues in these regions.

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