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Two Allosteric Modulators Interact at a Common Site on Cardiac Muscarinic Receptors

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SUMMARY

The abilities of gallamine, obidoxime, tetrahydroaminoacridine (THA), and 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate (TMB-8) to alter the rate of dissociation of *N*-[^{3}H]methylscopolamine from rat cardiac muscarinic receptors were investigated. All four ligands monotonically slowed the dissociation, with the order of potency gallamine > TMB-8 > THA > obidoxime. There was a dramatic difference in the efficacy of these allosteric modulators. Gallamine, TMB-8, and THA slowed the dissociation of *N*-methylscopolamine by >90% at maximally effective concentrations, whereas obidoxime was capable of slowing it by only about 50%. In a manner analogous to the action of a partial agonist, obidoxime was able to partially reverse the effects of the other three modulators. Furthermore, the concentration-dependent effects of combinations of obidoxime and gallamine were in good agreement with the model of competitive interaction between these two ligands. These results provide the first evidence that two muscarinic allosteric modulators interact competitively at a well defined site.

Muscarinic receptors comprise a family of five receptor subtypes that possess seven transmembrane domains and are activated by the neurotransmitter acetylcholine (1). It is likely that muscarinic ligands bind within a pocket formed by the transmembrane regions, which may be large enough to accommodate more than one ligand at a time (2). Two ligands might, therefore, interact, either by virtue of steric hindrance or through induced conformational changes in the receptor protein. Such interactions can be detected as deviations from competitive behavior, by using a variety of techniques (3). We have recently shown that all five muscarinic receptor subtypes are subject to allosteric regulation (4). The flexible nature of allosteric interactions makes allosteric sites attractive for therapeutic intervention (5) but can also make it more difficult to characterize the sites in detail. Indeed, it has been recognized that, on the basis of the available data, different muscarinic allosteric ligands might act at different sites (6, 7) or might even act nonspecifically (8, 9). This is a crucial issue, because if the reported allosteric phenomena were based on nonspecific interactions the possibility for eventual therapeutic application would be nil. In the present study, we have investigated four ligands. Gallamine, TMB-8, THA, and pyridinium oximes closely related to obidoxime have previously been shown to exert allosteric effects at muscarinic receptors (3), and our data have confirmed their activities at cardiac receptors. However, they did not exert identical maximal effects, and we were able to exploit the antagonistic property of a "partial" allosteric modulator (obidoxime) to demonstrate that several allosteric modulators appear to interact with overlapping sites on cardiac muscarinic receptors. The interaction between gallamine and obidoxime was characterized in detail and found to be entirely consistent with competition for a well defined allosteric site.

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Materials and Methods

Atropine, gallamine, and TMB-8 were obtained from Sigma (St. Louis, MO). Obidoxime was obtained from Schweizerhall (So. Plainfield, NJ), THA was from Research Biochemicals Inc. (Wayland, MA), and [^{3}H]NMS (81.5 Ci/mmol) was from NEN DuPont (Boston, MA).

Cardiac membranes were obtained by mincing ventricles from male Sprague Dawley rats (150–200 g) with scissors and then homogenizing the minced ventricles with a biohomogenizer (Biospec Products, Bartlesville, OK), in 40 mM Na-K PB at 0° (two times, 30 sec each). The resulting homogenate was filtered through two layers of cheesecloth and centrifuged at 50,000 × *g* for 20 min. The pellet was resuspended in PB and stored at −70°.

Binding assays were conducted in 5 mM PB at 22°. Membranes (0.3 mg of protein in 1 ml) were prelabeled with 1 nM [^{3}H]NMS for 30 min. Dissociation of the labeled ligand was initiated by the addition of 1 μM atropine, with or without allosteric modulators, and the incubation was allowed to continue for the appropriate length of time. The incubation was terminated by filtration through S&S no. 32 glass fiber filters (Schleicher and Schuell, Keene, NH), followed by two rinses with 40 mM PB (0°). Nonspecific binding was determined by the inclusion of 1 μM atropine during the prelabeling period.

ABBREVIATIONS: TMB-8, 8-(*N*,*N*-diethylamino)octyl-3,4,5-trimethoxybenzoate; NMS, *N*-methylscopolamine; QNB, quinuclidinylbenzilate; THA, tetrahydroaminoacridine; PB, phosphate buffer.

The data from the dissociation assays were treated in the following manner. The apparent rate constant for the dissociation of [^{3}H]NMS was determined in the presence of each concentration of allosteric modulator and divided by the true rate constant (k_{off}), determined in the presence of 1 μM atropine only. The resulting number was designated the fold-shift in the off-rate, such that a value of 2 would indicate a dissociation of [^{3}H]NMS that was twice as fast as the control rate and a value of 0.5 would be slower than control. We expected the change in rate of dissociation to be proportional to the occupancy of the allosteric site. Therefore, we fitted the data from these experiments to the following equation:

$$FS = 1 - \sum_{i=1}^{n} \frac{m_i L_i}{K_i (1 + \sum_{i=1}^{n} \frac{L_i}{K_i})}$$

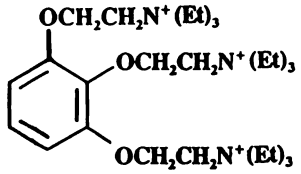
where FS is the fold-shift (described above), L_i is the i^{th} allosteric ligand, m_i is the maximal effect of that ligand on the off-rate of NMS, and K_i is the apparent equilibrium dissociation constant for that ligand (for the NMS-bound form of the receptor). Curve-fitting was carried out with the MINSQ program (MicroMath, Salt Lake City).

Results

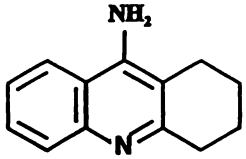
In the absence of allosteric modulators, the rate constant for the dissociation of [^{3}H]NMS from rat cardiac muscarinic receptors (k_{off}) was 0.159 min^{-1} ($t_{1/2}$ = 4.4 min). Gallamine, TMB-8, THA, and obidoxime (Fig. 1) all monotonically slowed the rate of dissociation (Fig. 2), with the following order of potency: gallamine > TMB-8 > THA > obidoxime. Whereas gallamine, TMB-8, and THA slowed the dissociation of NMS by >90% at maximally effective concentrations, obidoxime was capable of slowing it by only about 50%. The data for gallamine, TMB-8, and obidoxime were in good agreement with a binding isotherm (see Materials and Methods and Fig. 2), as would be expected if the binding of the modulator to a well defined allosteric site resulted in a conformational change in the receptor that rapidly led to a decrease in the rate of dissociation of NMS. However, the data for THA were noticeably steeper than a binding isotherm. These data were further analyzed with a sigmoidal three-parameter equation [$y = R + (1 - R)/(1 + (X/C)^B)$] that assumes a left plateau of unity and estimates the right plateau (R), midpoint (C), and slope through the midpoint (S). For THA (but not for the others), the fit to this function was significantly better when the slope was estimated (S = 1.73) than when it was fixed at the value of 1.0 that is characteristic of mass action [$F(1,21) = 23.0$].

The lesser maximal effect of obidoxime was exploited to test whether the modulators acted at a common site. In a manner analogous to the action of a partial agonist, obidoxime should reduce the effects of the other modulators at appropriate concentrations, if they compete for a common site of action. Fig. 3 indicates that obidoxime was able to partially reverse the effects of gallamine, TMB-8, and even THA. For example, compared with the allosteric effect caused by gallamine alone, the additional presence of obidoxime appeared to accelerate the rate of dissociation of NMS, toward that observed with obidoxime alone. As noted above, when used alone obidoxime only slowed the dissociation of NMS.

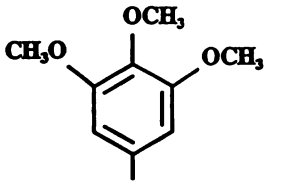
Fig. 4 displays the dose-dependent reversal of the effects of different concentrations of gallamine by obidoxime. As in Fig. 2, the model (see legend to Fig. 4) assumed a proportional coupling of occupancy to effect (see above), and the data fit the proposed model very well, upon visual inspection. Additionally,



GALLAMINE



THA



TMB-8

OBIDOXIME

Fig. 1. Allosteric modulators used in this study.

the affinity of obidoxime determined from the reversal curves did not differ significantly from that obtained from the direct obidoxime curve (Fig. 4). That is, the sum of squares obtained when these two estimates of affinity were determined independently was not significantly smaller than that obtained when they were constrained to be equal [$F(1,24) = 2.35$].

Discussion

These results provide the first experimental evidence that any two allosteric modulators interact at a common site on a muscarinic receptor subtype and, as a corollary, that there is a well defined allosteric site on the cardiac (m2) subtype. Some authors have cautioned that, especially at the high concentrations that are commonly used in studies of the muscarinic allosteric effect, nonspecific interactions may predominate. Thus, Henis *et al.* (8) have noted that hydrophobic modulators might alter general membrane properties, and Hulme *et al.* (9) suggested that high concentrations of positively charged ligands might create a "haze" of positive charge that could limit access to the binding site. However, the dramatic and qualitative

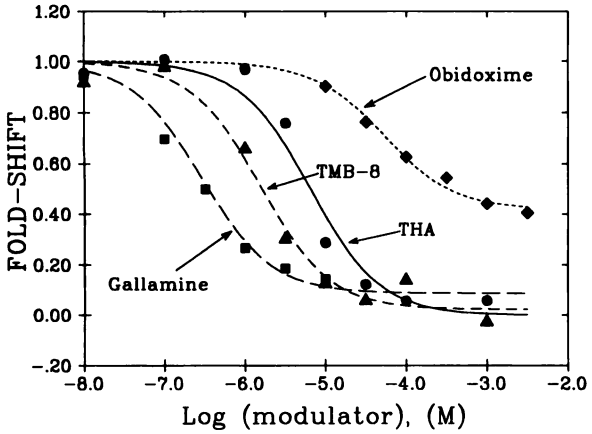


Fig. 2. Allosteric modulators slow the dissociation of [^{3}H]NMS from rat cardiac muscarinic receptors. The rate of dissociation of [^{3}H]NMS was determined in the presence and absence of the indicated concentrations of gallamine, TMB-8, THA, and obidoxime. The effect of each modulator has been expressed as the fold-shift in the dissociation rate constant of NMS (see Materials and Methods). The data points, averaged from three experiments for each curve, were fitted to the equation

$$FS = 1 - \frac{mL}{L + K}$$

where *FS* is the fold-shift (see Materials and Methods), *L* is the concentration of modulator, *m* is the maximal reduction in the rate constant that can be exerted by *L*, and *K* is the equilibrium dissociation constant for the interaction between *L* and the NMS-bound form of the receptor (see text). The best-fit values were as follows: gallamine, $K = 0.29$ μM, $m = 0.915$; TMB-8, $K = 1.5$ μM, $m = 0.974$; THA, $K = 6.2$ μM, $m = 0.992$; obidoxime, $K = 53$ μM, $m = 0.581$.

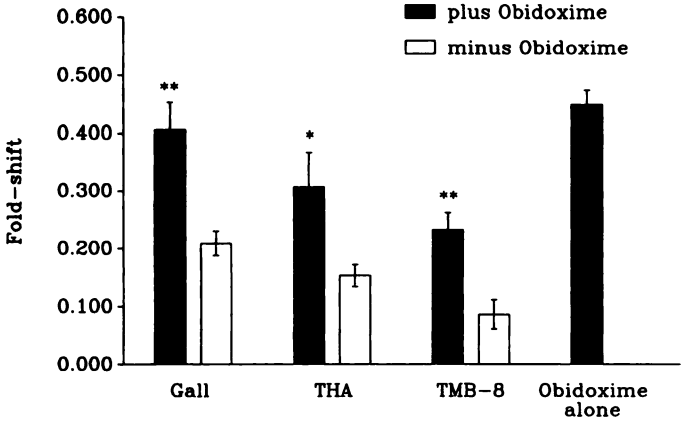


Fig. 3. Obidoxime partially reverses the allosteric effects of gallamine (*Gall*), TMB-8, and THA at rat cardiac muscarinic receptors. The off-rate of [^{3}H]NMS was determined in the presence and absence of the indicated combinations of modulators and is expressed as in Fig. 2 (obidoxime, 1 mM; gallamine, 3 μM; THA, 30 μM; TMB-8, 10 μM). Data are means ± standard errors of three experiments. For each modulator, the data in the presence of obidoxime were compared with those obtained in the absence of obidoxime, by a one-tailed *t* test. *, $p < 0.05$; **, $p < 0.01$.

reversals of the effects of the other modulators by obidoxime cannot be explained by such membrane or charge effects.

Allosteric modulators have as yet shown no strikingly common structural features (Fig. 1), and they are drawn from different pharmacological classes as well. Thus, it has been suggested that, even if muscarinic allosteric modulators did act at specific sites, they might act at different sites (10). The present study focused primarily upon the effects of gallamine and obidoxime, whose complex interactions at cardiac receptors

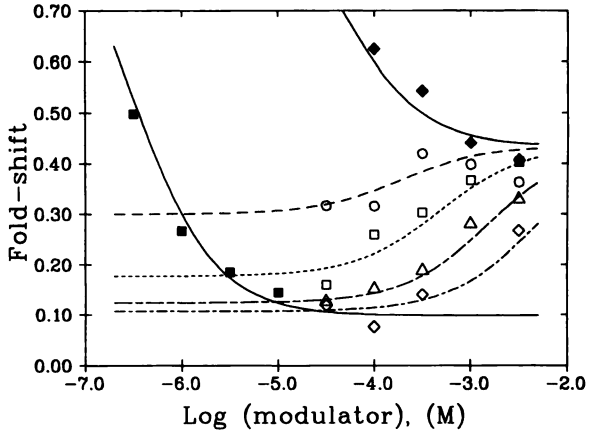


Fig. 4. Obidoxime reverses the allosteric effect of gallamine in a concentration-dependent manner. Experiments were conducted as in Fig. 3, except that multiple concentrations of each modulator were used. ■, Gallamine alone; ◆, obidoxime alone; ○, 1 μM gallamine plus obidoxime (at the indicated concentrations); □, 3 μM gallamine plus obidoxime; △, 10 μM gallamine plus obidoxime; ◇, 30 μM gallamine plus obidoxime. *Points* shown are the averages of three to six independent experiments, but the upper portions of the noninteractive curves (■, ◆) have been truncated for clarity. The *curves* represent the simultaneous best fit to the model of competitive interaction between gallamine and obidoxime. The equation used was

$$FS = 1 - \frac{m_o L_o}{L_o + K_o\left(1 + \frac{L_g}{K_g}\right)} - \frac{m_g L_g}{L_g + K_g\left(1 + \frac{L_o}{K_o}\right)}$$

where the parameters were defined as for Fig. 2 and the subscripts *o* and *g* refer to obidoxime and gallamine, respectively. The best-fit parameters were $K_g = 0.29$ μM, $m_g = 0.902$, $K_o = 42$ μM, and $m_o = 0.566$.

are neatly explained by a single common site of action (Fig. 4). Although the significant reversal of the effect of TMB-8 by obidoxime (Fig. 3) is consistent with a similar mechanism, we have not yet carried out as detailed a study with this ligand. For THA, in addition to this caveat, the steepness in the concentration-effect curve suggests a more complex interaction (see Fig. 2 and Results). Potter *et al.* (7) also observed a steep Hill coefficient for THA in the modulation of the dissociation of [^{3}H]pirenzepine from hippocampal membranes and concluded that THA must act at multiple allosteric sites. We have also suggested that there may be multiple sites of allosteric action (at m2 receptors), based on biphasic effects of gallamine and tubocurarine on the binding properties of [^{3}H]QNB; however, both modulators monotonically slowed the dissociation of [^{3}H]NMS (4). It appeared (a) that the deceleration of the dissociation of NMS and the acceleration of the dissociation of QNB were probably mediated by the same allosteric site and (b) that the dissociation of NMS was insensitive to whatever mechanism may have slowed the dissociation of QNB at higher concentrations of gallamine or tubocurarine (4). The latter effect remains poorly defined and may yet be explained by membrane or charge effects (see above). Tuček *et al.* (11) have recently reported a biphasic effect of alcuronium on the binding of NMS, but not QNB, at m2 receptors. In this case, the binding of subsaturating concentrations of NMS was dramatically increased by 10^{-7} to 10^{-5} M alcuronium but decreased at higher concentrations. The authors suggested that the latter effect could be due to a competitive interaction between alcuronium and NMS; membrane or charge effects might be considered here also.

Although a number of bisquaternary oximes have been found to modulate muscarinic receptors allosterically (8), Jepsen *et al.* (12) reported that obidoxime did not alter the rate of dissociation of NMS from guinea pig cardiac receptors. The discrepancy between their findings and ours is not likely to be due to species differences, because we have observed the same interaction between gallamine and obidoxime in rat cardiac membranes (present report) and in CHOK1 cells transfected with the human m2 subtype (data not shown). It may be due to differences in assay conditions, coupled with the low efficacy and potency of obidoxime.

We chose to use the modulation of dissociation as an index of allosteric action. It should be noted that the potencies cited reflect the affinities of the modulators for the NMS-bound form of the receptor. The experimental design did not allow for estimates of either the affinities for the free receptor or the degrees of cooperativity between NMS and the modulators. However, the values of this paradigm are that it is simple and that it ensures that only allosteric effects are reflected. Many investigators have suggested that the allosteric drugs interact with both the competitive and the allosteric sites (3, 8, 13) and even that the competitive components of some allosteric drugs may be more potent than the allosteric components (8, 14). If this is so, then the interpretation of functional studies (e.g., Refs. 15 and 16) may be greatly complicated, because the competitive aspect cannot be negated as it is in the dissociation paradigm used in the present study. On the other hand, if the competitive component is insignificant, then functional studies analogous to those presented in this paper will provide similarly useful information, if modulators with suitably different efficacies can be identified.

The same mathematical model applies to the intramolecular (17) allosteric regulation of muscarinic receptors and to the interactions between γ-aminobutyric acid and benzodiazepines at the γ-aminobutyric acid receptor-chloride channel-benzodiazepine receptor complex (5, 18). Thus, allosteric ligands may be found that will either augment or reduce muscarinic responses, in manners analogous to those of diazepam and methyl-6,7-dimethoxy-4-ethyl-β-carboline-3-carboxylate, respectively. Subtype-specific augmenting agents would appear to be good candidates for treatment of the cholinergic deficiency in Alzheimer's disease, for example (10). Of more immediate interest to pharmacologists, however, would be ligands analogous to Ro 15–1788, which exerts little effect itself but competitively antagonizes the effects of other benzodiazepines (18). It is well recognized that binding sites are best characterized in terms of the affinities of competitive antagonists (19). With the caveat that regulation of antagonist (NMS) binding is somewhat removed from regulation of agonist-induced receptor function, obidoxime may represent a significant step toward the discovery of such low-efficacy muscarinic allosteric modulators.

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