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Tetrahydroaminoacridine and Other Allosteric Antagonists of Hippocampal M1 Muscarine Receptors

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SUMMARY

Tetrahydroaminoacridine (THA) and a variety of other nonclassical antagonists of muscarine receptors were studied for their ability to bind to primary and allosteric sites on muscarine receptors in rabbit hippocampal membranes. Competition curves between 13 antagonists and 1 nM [^{3}H]pirenzepine (K_d = 3 nM) were simple mass action curves, but THA produced steeper curves, indicating positive cooperativity. Nonetheless, THA inhibited the binding of low concentrations of [^{3}H]pirenzepine, [^{3}H]*N*-methylscopolamine, and [^{3}H]oxotremorine-M to M1 receptors with similar IC_{50} values, indicating competition for primary sites. Antagonists were also compared for their ability to bind to allosteric sites and to slow the dissociation of [^{3}H]pirenzepine from primary sites. THA was 6–8-fold more potent than verapamil, *d*-tubocurare, quinidine, and secoverine, the next most effective allosteric agents, and THA was more effective. McN-A-343, gallamine, pancuronium, and pirenzepine showed weaker allosteric effects. The large size and considerable rigidity of these compounds suggest large allosteric sites. The Hill coefficient for the allosteric effects of THA was 1.7, indicating more than one allosteric site. Solubilization of receptors did not alter steep inhibition curves between THA and [^{3}H]quinuclidinyl benzilate or THA-induced slowing of the dissociation of this ligand. Hence, cooperative allosteric effects of THA are probably exerted on receptor monomers. Inhibition curves between THA and [^{3}H]oxotremorine-M were not steep, and THA had no (allosteric) effect on the dissociation of this ligand from M1 or M2 receptors. Thus, the high affinity agonist conformation of muscarine receptors, once formed, may not bind THA readily. The present results indicate that compounds that can act allosterically may compete with acetylcholine for primary receptor sites but that allosteric effects of these drugs on muscarine receptors are not likely to be important clinically.

The term "allosteric" was introduced into the biochemical literature in 1963 by Monod *et al.* (1) to explain the nature of feedback inhibition of enzymes at branch points in metabolic pathways. They indicated that end-product inhibition of the first enzyme in a sequence was due to the binding of the product to an allosteric site on the enzyme that was distinct and distant from the site that bound the usual enzyme substrate. An example is the inhibition of threonine deaminase by isoleucine. In their 1968 review of the catalytic and regulatory properties of enzymes, Koshland and Neet (2) called this kind of allosteric site a "regulatory site" to distinguish it from other kinds of allosteric sites. In almost all the known cases of this kind of regulatory allosterism, the allosteric ligand is inhibitory and the stated assumption is made that this ligand produces a conformational change in the enzyme, which alters the affinity of the "active site" for its normal substrate. This is one kind of negative cooperativity. A few cases of positive regulatory effects are known; for example, norleucine prevents the inhibitory action of isoleucine (1).

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In 1965, Monod *et al.* (3) complicated our understanding of the term allosteric by using it to describe a different kind of distant-site phenomenon, namely the cooperative effects of the binding of ligands to two or more molecules ("subunits or protomers") of an oligomeric enzyme or other protein. In almost all the known cases of this kind of allosterism, the same kind of substrate or ligand is capable of binding to two or more different active sites, and the specific assumption is made that changes in the conformation of one protomer modify the binding of the substrate or other ligand to other protomers. The cooperative binding of oxygen to hemoglobin is the best known example. In some cases, different ligands can bind to separate protomers and produce either positive or negative cooperativity (1–3).

In the pharmacological literature the term allosteric has been used most commonly to describe the action of an inhibitor ("allosteric antagonist") on a receptor. Here we will use the term "primary site," rather than "active site" for the site that binds agonists and classical antagonists, because receptors are not enzymes. A well known example concerns the binding of

ABBREVIATIONS: PBCM, propylbenzilylcholine mustard; THA, tetrahydroaminoacridine; QNB, quinuclidinylbenzilate; NMS, *N*-methylscopolamine; NEM, *N*-ethylmaleimide; Gpp(NH)p, guanyl-5′-yl-imidodiphosphate.

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gallamine to muscarine receptors. Gallamine (10–1000 μM) can interact noncompetitively with both M1 and M2 muscarine receptors, as evidenced by incomplete inhibition of the binding of classical antagonists, nonlinear Schild plots with slopes below 1, decreased rates of radioligand dissociation, and lack of protection of primary sites from alkylation by PBCM (4–11). The allosteric nature of the effects of gallamine is most clearly evident from its ability to slow radioligand dissociation from primary sites, even in the presence of a large excess of classical antagonists like atropine or QNB, which prevent the action of gallamine on unoccupied primary sites. Tests of slowed radioligand dissociation have also been used to demonstrate the allosteric actions of other nonclassical antagonists, including pancuronium (5), alcuronium (10), bisquaternary pyridinium oximes (12), 4-aminopyridine (13), quinidine (14), verapamil (15–17), secoverine (18), and THA (19).

It may be noted that allosteric sites on muscarine receptors differ in an important way from the regulatory allosteric sites first described by Monod *et al.* (1). There is no presumption that the sites on receptors are specific for endogenous ligands or that the sites were selected during evolution for any key regulatory function. Indeed, from the variety of sizes and solubilities of allosteric muscarine antagonists, one might suppose that allosteric sites are nonspecific and quite possibly not identical. A further point is that the nature of the primary ligand affects the effectiveness of the allosteric ligand. For example, 1 mM gallamine fully blocks the dissociation of [^{3}H]NMS from cortical muscarine receptors but has far less effect on the dissociation of [^{3}H]pirenzepine (8). Both in theory (20) and in practice (6), the stated assumption is that the allosteric ligand exerts negative cooperativity on the binding of the primary ligand and the primary ligand exerts negative cooperativity on the binding of the allosteric ligand. Gallamine is also known to interact with primary sites, inasmuch as low concentrations (0.01–1 μM) can protect primary sites from alkylation by PBCM (7). Under such conditions gallamine shows the same K_i for inhibiting a variety of different radiolabeled classical antagonists (11). Because gallamine can interact at both primary and allosteric sites, it is probable that its binding to one site inhibits its own binding to the other. This is presumably the explanation for why concentrations of gallamine that affect allosteric sites can still leave primary sites open for alkylation by PBCM.

Yet a fourth kind of cooperativity has been considered, concerning the binding of two molecules of acetylcholine (or other agonist) to two subunits of nicotine receptor channel molecules. In this special case it is clear that there are two genetically identical protomers with primary sites, but it is probable that these are not contiguous (21), as is the case for the allosterically interacting protomers considered by Monod *et al.* (3). Effectively simultaneous saturation of both protomers with acetylcholine is believed to occur physiologically and is clearly necessary for normal channel opening (22). Although binding studies *in vitro* have suggested allosteric interactions between primary sites, the available physiological evidence obtained before receptor desensitization does not suggest that cooperative binding phenomena play a substantial role in nicotine receptor function (22).

Our interest in further studies of the antagonistic effects of THA and other muscarine antagonists was prompted by four kinds of observations. First, we found that 3 μM THA significantly inhibited (by 25%) the binding of 10 nM [^{3}H]pirenzepine to M1 muscarine receptors in rabbit hippocampal membranes suspended in artificial cerebrospinal fluid at 37° (19). Because this concentration of THA is only 10-fold higher than the plasma concentration of THA found in patients given the drug as an esterase inhibitor for the treatment of Alzheimer's disease (23) and because THA is concentrated 10-fold in the brain (24), it appeared that the inhibitory effects of THA on receptors might be significant clinically. Because physiologists are now reporting the ability of 1–1000 μM THA to block potassium channels at cholinergic synapses (e.g., Ref. 25), the action of THA as a receptor antagonist also appeared important for further physiological experiments. Moreover, 100 μM THA was highly effective for inhibiting the dissociation of [^{3}H]pirenzepine, suggesting that THA might be exceptionally effective as an allosteric agent. We wanted, therefore, to find out how effective THA is in inhibiting the binding of an agonist, as well as antagonists. In addition, because THA does not resemble acetylcholine to the same extent as is the case for other allosteric antagonists, we wanted to find out whether THA interacted with primary sites at all. Because we are interested in any tools that may help dissect the structure and function of muscarine receptors, we also wished to determine the relative effectiveness of THA and other antagonists as allosteric agents, so as to choose the one for further studies that is most effective. This has not been accomplished previously. Here we have compared the ability of 14 compounds to inhibit the binding of [^{3}H]pirenzepine to M1 receptors, when both primary and allosteric sites are available, and we have compared the ability of 13 of these agents to slow the dissociation of [^{3}H]pirenzepine, as a measure only of allosteric effects. Direct measurements have been made of the ability of THA to inhibit the binding of the agonist, [^{3}H]oxotremorine-M. The results show that THA acts on both primary and allosteric sites and that THA is the most potent and effective allosteric agent yet tested. However, it appears to compete with an agonist only at primary sites.

Our second observation was that inhibition curves between THA and either [^{3}H]pirenzepine or [^{3}H]QNB were steeper than mass action curves, indicating a form of allosteric antagonism having features of positive as well as negative cooperativity. Third, there is now evidence that muscarine receptors may be paired. Specifically, we have shown that only half of the total M1 muscarine receptors in the hippocampus can form a guanine nucleotide-sensitive high affinity complex with oxotremorine-M (26) or six other highly effective M1 agonists (27), and Mattera *et al.* (28) have shown that guanine nucleotides reveal the presence of cooperative binding of QNB to cardiac M2 receptors. Because pure M1 (29) and M2 (30) receptors bind only 1 mol of antagonist/mol of receptor protein, these observations indicate the possibility of receptor-receptor interactions. Fourth, we found that THA did not inhibit the dissociation of [^{3}H]oxotremorine-M from brainstem M2 receptors (19), under conditions in which there might be positive cooperativity between agonist-liganded receptors. These three kinds of observations raised the question of whether THA exerted any of its effects via receptor-receptor interactions. Here we have carried out further experiments with THA using M1 receptors liganded with agonists or antagonists and have studied the allosteric effects of THA on QNB-saturated receptors in solution, under conditions strongly favoring receptor monomers. The results further substantiate the lack of allosteric effects of

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THA on the high affinity agonist conformation of muscarine receptors, demonstrate that solubilization has no effect on the allosteric effects of THA, and indicate that THA binds to more than one allosteric site cooperatively.

Methods

Drugs were obtained from Sigma Chemical Co. (St. Louis, MO) except as follows: clomiphene (Serono Labs Inc., Randolph, MA), McN-A-343 and (±)-QNB (Research Biochemicals Inc., Natick, MA), pancuronium (Organon Inc., West Orange, NJ), pirenzepine (Boehringer Ingelheim Indianapolis, IN), secoverine (Duphat, Amsterdam), and THA (Aldrich Chemical Co., Milwaukee, WI). Radioisotopes were purchased from the New England Nuclear Division of Dupont (Boston, MA).

Membranes were prepared from fresh rabbit hippocampi as described by Flynn and Potter (31) and Potter *et al.* (26). In brief, hippocampi were homogenized in ice-cold 50 mM sodium phosphate buffer containing 10 mM Na_3EDTA and 0.1 mM phenylmethylsulfonyl fluoride, pH 7.4. Membranes were collected by centrifugation for 10 min at 38,000 × g_{max}, and resuspended in assay medium, using sufficient buffer to achieve 20 ml of suspension/g of original tissue. Each assay was performed with 0.1 ml of suspension, thus utilizing unselected membranes from 5 mg of tissue. The same procedure was used to prepare cardiac membranes from fresh rabbit atria.

To assess the ability of THA and other drugs to inhibit the binding of [^{3}H]pirenzepine, freshly prepared membranes were incubated with various concentrations of the drugs at 25° for 45–60 min in 1.0 ml of 20 mM Tris·HCl buffer containing 1 mM $MnCl_2$, pH 7.4 (Tris-Mn buffer) and 1.0 nM [*N-methyl*-^{3}H]pirenzepine (87 Ci/mmol). Samples without and with 1 μM (±)-QNB served to establish total and nonspecific binding and the difference was taken as specific binding to receptors. After incubation, 9 ml of ice-cold buffer were added to each tube, and membranes with bound [^{3}H]pirenzepine were collected by filtration on 2.4-cm Reeve-Angel 934 AH glass-fiber filters. Filters were rinsed twice with 4 ml of buffer, placed in counting vials, dried in an oven at 60°, covered with 4 ml of Liquiscint (National Diagnostics Co., Somerville, NJ), and counted at an efficiency of about 37%. Under the conditions of these assays, [^{3}H]pirenzepine associates with, dissociates from, and comes to equilibrium with only a single population of binding sites having a K_d (determined by self-competition) of 3 nM (26). To assess the ability of THA to inhibit the binding of [^{3}H]oxotremorine-M to cardiac M2 receptors, the same assay conditions were used except that the ligand was 0.5 nM [*methyl*-^{3}H]oxotremorine-M (87 Ci/mmol). The K_d, determined by self-competition, was about 0.5 nM. The same conditions were also used to measure the effects of THA on the binding of the agonist to hippocampal M1 receptors, except that the concentration of [^{3}H]oxotremorine-M was 5 nM, and 0.1 mM NEM was included in the incubation buffer to prevent the binding of the agonist to the highest affinity state of the few M2 receptors in the hippocampus (31). The K_d at M1 receptors, determined by competition between oxotremorine-M and 1 nM [^{3}H]pirenzepine, is about 8 nM (32). Tris-Mn buffer was used for these assays because low ionic strength helps to stabilize receptor complexes with pirenzepine (26) and oxotremorine-M (26, 27) and because the binding of oxotremorine-M to the highest affinity states of muscarine receptors is strongly facilitated by the transition metal ion Mn (Ref. 26 and references therein). The same buffer was used to examine the ability of THA to inhibit the binding of [*methyl*-^{3}H]NMS (80 Ci/mmol). For these assays, incubations were in 10 ml of 0.1 nM ligand. The K_d for NMS, determined by self-competition, was about 0.1 nM.

To study the ability of THA and other drugs to inhibit the dissociation of [^{3}H]pirenzepine and [^{3}H]oxotremorine-M, membranes were first equilibrated with these ligands as described above. The membranes were then diluted 10-fold with incubation buffer containing 1 μM (±)-QNB and the drug under study. For incubations involving [^{3}H]NMS, (±)-QNB and the drug under study were added after receptor labeling, in 100 μl of buffer, to achieve the same final concentrations as used for pirenzepine and oxotremorine-M.

To study the effects of solubilization on the allosteric actions of THA, hippocampal receptors were incubated with [^{3}H]QNB at 37° in phosphate-EDTA buffer. These conditions facilitate full saturation of primary sites and assays in digitonin (31, 33), results that are difficult to achieve with [^{3}H]pirenzepine. K_d values for [^{3}H]QNB in membranes and in solution under these conditions are about 4 and 6 pM, respectively (31). Membranes from 5 mg of tissue were incubated with or without THA for 45 min with 10 ml of 1 nM (−)-[*benzilic*-4,4′-^{3}H]QNB (43 Ci/mmol) in 50 mM sodium phosphate buffer containing 1 mM EDTA, pH 7.4 (33). For dissociation rate experiments, membranes were first labeled with QNB and dissociation was then initiated by the addition of 20 μl of buffer containing 100 μM (±)-QNB plus THA. Incubation was then continued and samples were taken at various time points. For studies in solution, rabbit hippocampal membranes were dissolved in assay buffer containing 1% digitonin, and insoluble material was removed by centrifugation (31). Membrane proteins from 10 mg of tissue were incubated with or without THA for 45 min, in 2 ml of 1 nM [^{3}H]QNB in phosphate-EDTA buffer, and receptors with bound QNB were then precipitated with polyethylene glycol and protamine and collected by filtration (31). For dissociation experiments, receptors were first labeled and dissociation was then initiated by the addition of 20 μl of buffer containing 100 μM (±)-QNB plus THA. Incubation was then continued while samples were taken. For each of these assays, 1 μM (±)-QNB was used during incubation to assess nonspecific binding.

All of the assays noted were carried out in triplicate and each experiment was repeated at least once.

Results

Potential interactions of nonclassical antagonists with primary receptor sites. The ability of various compounds to inhibit the binding of [^{3}H]pirenzepine, [^{3}H]NMS, and [^{3}H] oxotremorine-M was explored using radioligand concentrations at or below their K_d values. When the radioligand concentration is low, the counterligand concentration can be kept relatively low, minimizing negative cooperativity between ligands at primary and allosteric sites and maximizing the chances of measuring the affinities of allosteric counterligands for primary sites. For an allosteric agent like gallamine, which shows higher affinity for primary than allosteric sites (7, 11), the concentration required to inhibit half the radioligand (IC_{50} value) reflects the affinity of gallamine for primary sites. For a purely allosteric compound, binding curves can theoretically be identical to those for a purely primary site ligand (20), and IC_{50} values would then pertain only to allosteric sites. When radioligand concentrations are at or below their K_d values, IC_{50} values lie within a factor of 2 of the (higher affinity) K_i value, whether the sites involved are primary, allosteric, or both (6, 20). This is important in the case of THA, because steep binding curves do not yield data appropriate for the correction of IC_{50} values to K_i values.

Fig. 1 shows inhibition curves between 1 nM [^{3}H]pirenzepine (K_d = 3 nM) and four antagonists at hippocampal M1 receptors. Each of the 14 drugs tested in this manner (see Table 1) was capable of full inhibition of the binding of pirenzepine and, with the exception of THA, each binding curve closely approximated a mass action curve. For THA, it is apparent that the curve is steeper than a mass action curve, indicating some kind of positive cooperativity. IC_{50} values for different antagonists varied from 0.0025 to 50 μM (Table 1).

The ability of THA to antagonize the binding of 0.1 nM [^{3}H]

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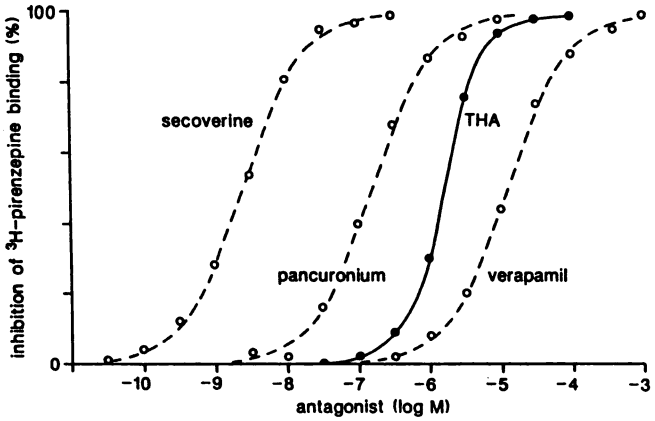

Fig. 1. Competition between four nonclassical muscarine antagonists and 1 nM [^{3}H]pirenzepine for M1 muscarine receptors in rabbit hippocampal membranes. *Points* are mean values from 2–4 experiments (each carried out in triplicate). *Dashed lines* are mass action curves. IC_{50} values for these 4 and 10 other antagonists are given in Table 1. The specific binding of [^{3}H]pirenzepine alone averaged 7800 ± 830 cpm (mean ± SE) in this series of experiments. Note the steepness of the curve with THA.

TABLE 1

Relative affinities of various compounds for hippocampal M1 muscarine receptors

The following mean values were obtained from full inhibition curves between the drugs and 1 nM [^{3}H]pirenzepine. See Fig. 1 for details. Two to eight experiments were performed for each antagonist; results varied less than 12% between experiments. Except for the value for THA, these data can be converted to K_i values using the Cheng-Prusoff equation, by dividing by 1.33.

Compound	IC_{50}
	μM
Secoverine	0.0025
Pirenzepine	0.004
Pancuronium	0.16
Gallamine	0.6
McN-A-343	1.3
THA	1.7
Quinidine	6.0
Decamethonium	6.3
Clomiphene	6.0
d-Tubocurare	8.0
Verapamil	13
4-Aminopyridine	32
Succinylcholine	40
Hexamethonium	50

NMS to hippocampal muscarine receptors ($K_d \approx 0.1$ nM) is shown in Fig. 2. As for pirenzepine, the inhibition curve for [^{3}H]NMS was steeper than a mass action curve, and the IC_{50} value (2.5 μM) was close to that found for [^{3}H]pirenzepine (1.7 μM).

The ability of THA to inhibit the binding of 5 nM oxotremorine-M to hippocampal M1 receptors ($K_d \approx 8$ nM) is shown in Fig. 3. The component of binding studied was Gpp(NH)p-sensitive binding in the presence of 0.1 mM NEM, because this concentration of NEM fully dissociates the Gpp(NH)p-sensitive highest affinity state of the few M2 receptors in the hippocampus (31) but does not affect the binding of oxotremorine-M to the Gpp(NH)p-sensitive high affinity state of M1 receptors (26, 32). The IC_{50} value for THA versus [^{3}H]oxotremorine-M (1.8 μM) was very similar to that found in experiments with [^{3}H]pirenzepine and [^{3}H]NMS. However, the THA inhibition curve in Fig. 3 is a mass action curve, unlike those in Fig. 1 and 2.

We considered the possibility that the unusual shape of the

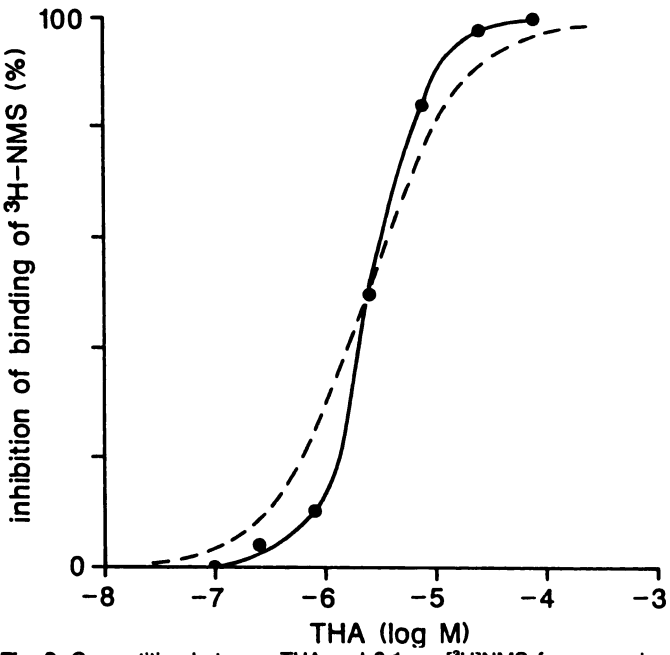

Fig. 2. Competition between THA and 0.1 nM [^{3}H]NMS for muscarine receptors in rabbit hippocampal membranes. *Points* are mean values from two experiments. The *dashed line* is a mass action curve drawn through the IC_{50} value (2.5 μM). The specific binding of [^{3}H]NMS alone averaged 17,200 cpm. This competition curve is as steep as that shown for THA versus [^{3}H]pirenzepine in Fig. 1.

THA versus [^{3}H]pirenzepine curve might be due to nonequilibrium binding, because allosteric antagonists must slow radioligand association more than dissociation, in order to decrease the overall affinity of radioligands for primary sites. Measurements of the rates of association of [^{3}H]pirenzepine in the presence and absence of 3 μM THA are shown in Fig. 4. This concentration of THA prevented the binding of [^{3}H]pirenzepine to four fifths of the total M1 receptors. Nonetheless, [^{3}H]pirenzepine bound to and progressively decreased the number of unoccupied receptors at the same rate in the presence and absence of THA. This observation and our prior evidence that inhibition curves between THA and 10 nM [^{3}H]pirenzepine at 37° are steep (19) show that nonequilibrium conditions are not a significant factor in the shape of the inhibition curve.

Interactions of nonclassical antagonists with allosteric sites. In most of the following experiments, primary receptor sites were first labeled with [^{3}H]pirenzepine or [^{3}H]NMS and various antagonists were then tested for their relative potency and/or effectiveness in slowing the dissociation of the radioligand used in the presence of an excess of (±)-QNB. Under these conditions, nonclassical antagonists act only on allosteric sites.

Fig. 5 shows the effects of various concentrations of THA on the rates of dissociation of [^{3}H]pirenzepine from hippocampal M1 receptors. Dissociation slowed progressively with increasing concentrations of THA and stopped completely in 300 μM THA. The presence of 0.2 mM Gpp(NH)p had no effect on the ability of THA to decelerate dissociation (Fig. 5). NEM (0.1 mM) was also without effect (data not shown), in keeping with prior evidence that NEM does not affect the ability of gallamine to slow the dissociation of [^{3}H]NMS (9). Only four of the other compounds tested were capable of slowing the dissociation of [^{3}H]pirenzepine by 50% when tested in this manner; verapamil,

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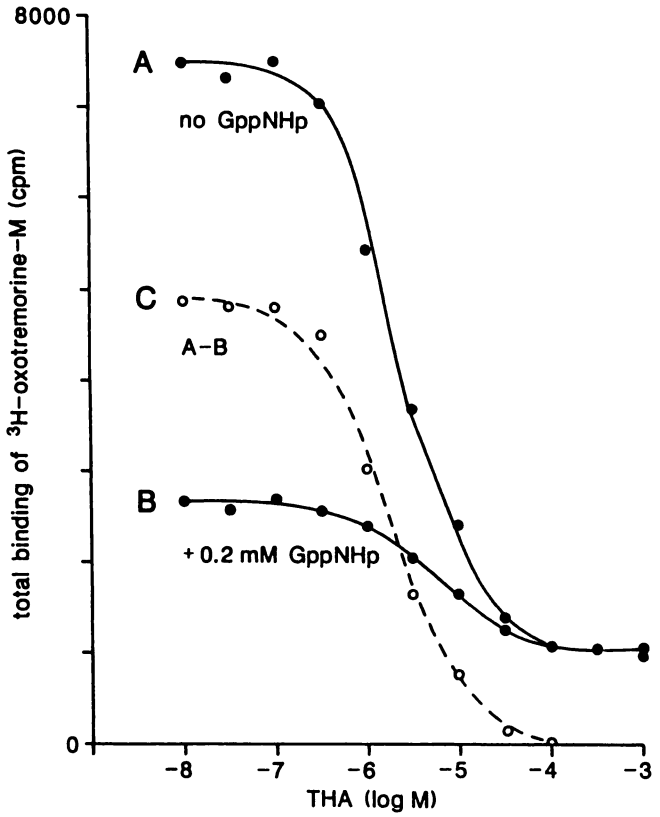

Fig. 3. Competition between THA and 5 nM [^{3}H]oxotremorine-M for M1 muscarine receptors in rabbit hippocampal membranes, in the presence of 0.1 mM NEM and with or without 0.2 mM Gpp(NH)p. *Solid points* are mean values from two experiments. The *dashed line* is a mass action curve fit to the calculated Gpp(NH)p-sensitive but NEM-insensitive component of agonist-binding to M1 receptors. The IC_{50} value for this component is 1.8 μM.

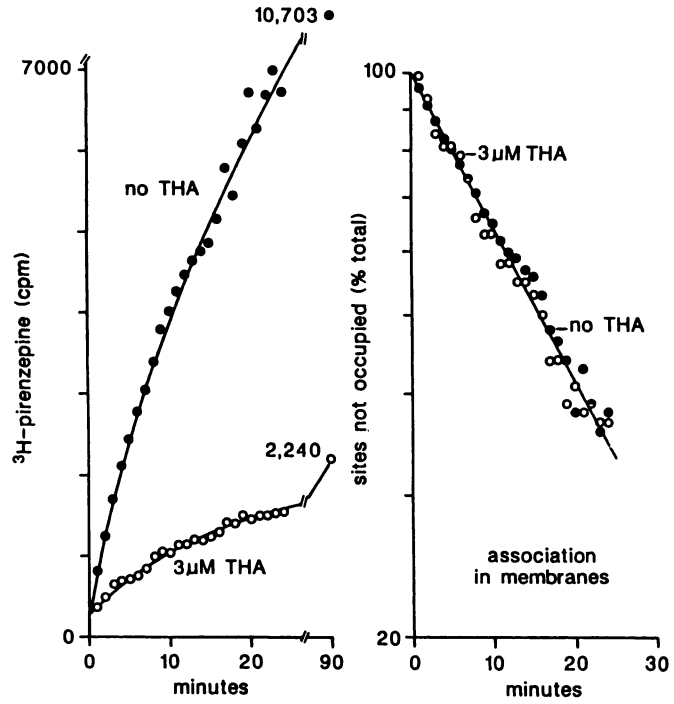

Fig. 4. Rate of association of [^{3}H]pirenzepine (1 nM) with M1 muscarine receptors in rabbit hippocampal membranes in the absence and presence of 3 μM THA. In the *left panel*, *points* are single values for total binding from one of three experiments. The *right panel* shows the rate of decline of free receptors in this typical experiment. The calculated association rate without THA was 2.57×10^7 M^{-1} min^{-1}.

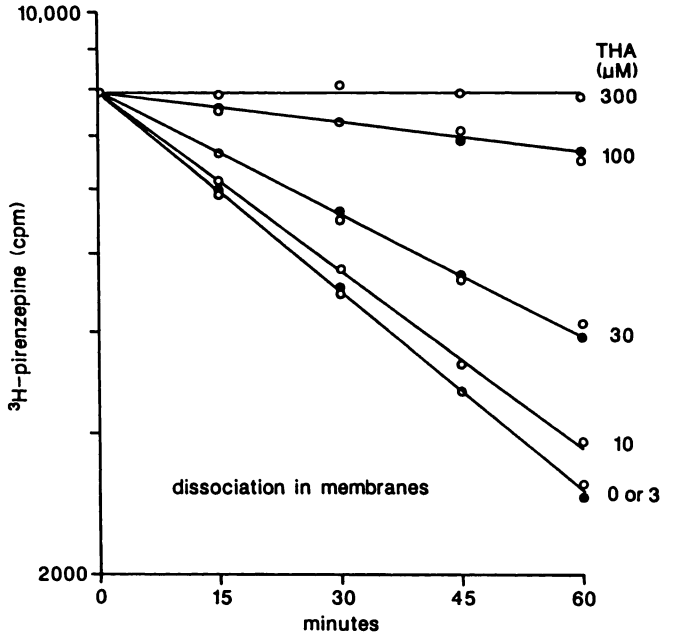

Fig. 5. Effect of THA on the dissociation of [^{3}H]pirenzepine from M1 receptors in rabbit hippocampal membranes. Receptors were labeled with 1 nM [^{3}H]pirenzepine. Dissociation was initiated with 1 μM (±)-QNB with or without THA as shown. *Points* are mean values for specific binding from three experiments; *solid points* indicate the presence of 0.2 mM Gpp(NH)p. *Lines* are linear regressions. The calculated dissociation rate without THA was 1.78×10^{-2} min^{-1}.

d-tubocurare, quinidine, and secoverine. Fig. 6 shows a plot of the relationship between the degree of slowing of dissociation and the concentration of THA and these four other antagonists. Two conclusions may be drawn from this figure. First, it is evident that THA was the most potent and effective allosteric antagonist tested. Second, the inhibition curve for THA at allosteric sites alone is still steeper than a mass action curve (the Hill coefficient is 1.7), demonstrating positive cooperativity at allosteric sites. The concentration of THA required to slow dissociation by 50% was about 40 μM. The corresponding value for verapamil and for *d*-tubocurare was about 250 μM, and the value for quinidine and secoverine was about 330 μM (Fig. 6).

The relative effectiveness of all but one of the compounds listed in Table 1, to slow the dissociation of [^{3}H]pirenzepine, was compared using 1 mM levels of each drug (Table 2). Clomiphene (34) could not be tested because of its limited solubility in aqueous media. It is evident from Table 2 that McN-A-343, gallamine, pancuronium, and pirenzepine had measurable allosteric effects on M1 receptors, whereas 4-aminopyridine, hexamethonium, decamethonium, and succinylcholine showed no significant allosteric effects by this technique. These data represent the first comparison of the relative allosteric effectiveness of different compounds and the first demonstration of the allosteric effects of *d*-tubocurare and pirenzepine. It has been suggested previously that pirenzepine might act allosterically, on the basis that its binding to receptors in

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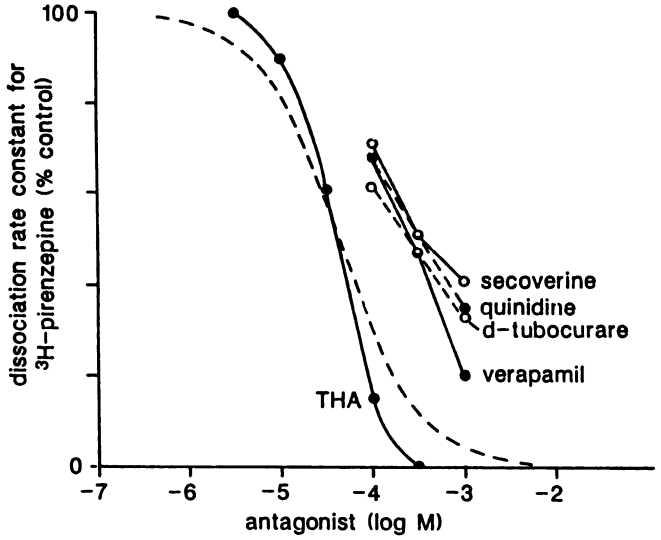

Fig. 6. The ability of five antagonists to slow the rate of dissociation of [^{3}H]pirenzepine from M1 receptors in rabbit hippocampal membranes, in the presence of 1 μM (±)-QNB. The data for THA are taken from Fig. 5 and are compared with similar mean data (two experiments) for each of the other antagonists. A *dashed mass action curve* is drawn through the IC_{50} value for THA (40 μM for this allosteric effect) to show the steepness of the binding curve. The Hill coefficient for this curve is 1.7. The other IC_{50} values are given in the text.

TABLE 2

Relative effectiveness of various compounds for allosterically inhibiting the dissociation of [^{3}H]pirenzepine from hippocampal M1 receptors in the presence of 1 μM (±)-QNB

The following mean data were obtained from dissociation measurements like those shown in Fig. 5. Two to nine experiments were performed for each drug; results varied less than 9% between different experiments.

Compound (1 mM)	Dissociation rate constant	Slowing
	× 100 min	%
THA	0	100
Verapamil	0.36	80
d-Tubocurare	0.59	67
Quinidine	0.62	65
Secoverine	0.73	59
McN-A-343	1.28	28
Gallamine	1.36	23
Pancuronium	1.50	16
Pirenzepine	1.55	13
4-Aminopyridine	1.72	3
Decamethonium	1.78	None
Hexamethonium	1.78	None
Succinylcholine	1.78	None
No drug	1.78	Control

solution is unstable compared with that of QNB (35). The M1 agonist McN-A-343 has been shown previously to cause incomplete inhibition of the binding of [^{3}H]NMS (36) but not to slow radioligand dissociation. Its effectiveness compared with that of gallamine is impressive.

Whereas 3 μM THA had no detectable effect on the rate of association of [^{3}H]pirenzepine (2.57×10^7 M^{-1} min^{-1}; Fig. 4) or its rate of dissociation (1.78×10^{-2} min^{-1}; Fig. 5), measurements of competition between pirenzepine and [^{3}H]pirenzepine (Fig. 7) in the presence of THA showed that 3 μM THA increased the K_d for pirenzepine from about 3 to 13 nM. These rate measurements do not yield the same K_d as these equilibrium measurements because receptors with bound pirenzepine isomerize (37).

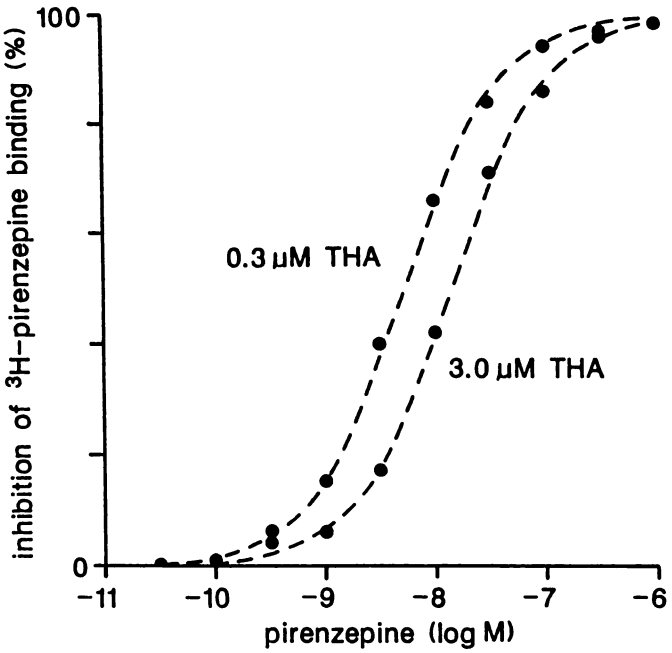

Fig. 7. Self-competition between pirenzepine and 1 nM [^{3}H]pirenzepine for M1 muscarine receptors in rabbit hippocampal membranes, in the presence of 0.3 and 3.0 μM THA. *Points* are mean values for specific binding from two experiments; *lines* are mass action curves. Uninhibited specific binding was 6430 and 2460 cpm. The calculated K_d values are 4 and 13 nM; without THA, the K_d is 3.0 nM (20).

Fig. 8 shows the effect of various concentrations of THA on the rates of dissociation of [^{3}H]NMS from hippocampal muscarine receptors. Dissociation slowed progressively with increasing THA. A plot of the dissociation rate constants versus the concentration of THA (data not shown) was steeper than a mass action curve; the Hill coefficient was 1.7. The concentration of THA required to slow the dissociation of [^{3}H]NMS by 50% was 50 μM, a value unexpectedly close to that found for THA versus [^{3}H]pirenzepine.

Fig. 9 shows that THA had no effect on the dissociation of [^{3}H]oxotremorine-M from hippocampal or cardiac membranes labeled under conditions that yield binding of the radioligand only to the highest affinity states of M1 and M2 receptors. Thus, THA had no observable allosteric effects on receptors after agonist binding. It may be noted that quinidine, verapamil, and secoverine have been reported to slow the dissociation of [^{3}H]oxotremorine-M slightly from cardiac M2 receptors (14, 15, 18). However, the conditions of these prior experiments were such that intermediate affinity states of M2 receptors could have been labeled. Hence, only the Gpp(NH)p-sensitive highest affinity states of muscarine receptors may be insensitive to allosteric ligands.

Effect of solubilization of receptors on the allosteric action of THA. For these studies, membranes were dissolved in phosphate buffer containing 1% digitonin and 1 mM EDTA. These conditions fully dissociate the Gpp(NH)p-sensitive highest affinity states of M1 and M2 receptors (31) and are likely to yield receptor monomers.

The ability of THA to inhibit the binding (Fig. 10) and dissociation (Fig. 11) of [^{3}H]QNB to hippocampal muscarine receptors was compared in membranes and in solution. Inhibition curves were unaffected by receptor solubilization and were steeper than mass action curves (Fig. 10). The allosteric

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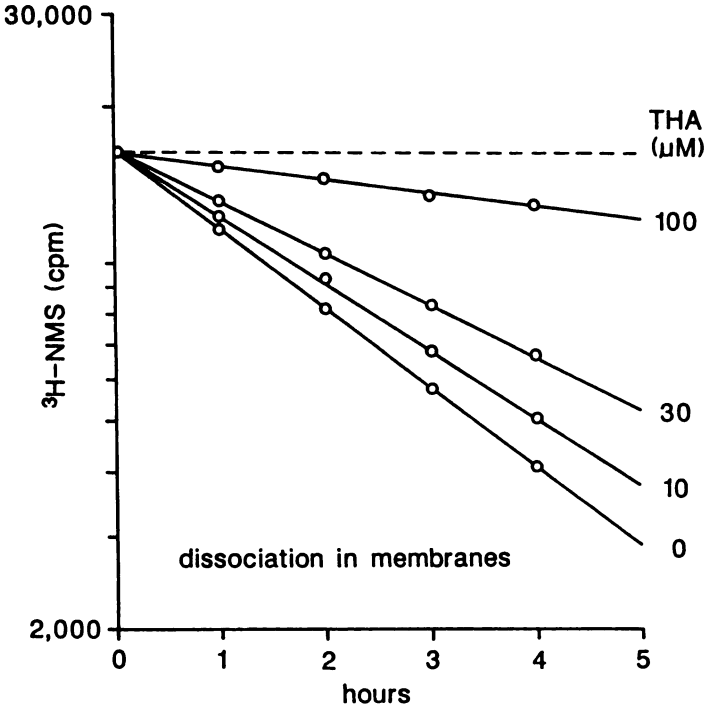

Fig. 8. Effect of THA on the dissociation of [^{3}H]NMS from muscarine receptors in rabbit hippocampal membranes. Receptors were labeled with 0.1 nM [^{3}H]NMS, and dissociation was initiated with 1 μM (±)-QNB with or without THA as shown. *Points* are mean values for specific binding from two experiments; *solid lines* are linear regressions. The *dashed line* is horizontal for comparison with the data in Fig. 5. The dissociation rate without THA was 5.7×10^{-3} min^{-1}. Analysis of these data (not shown) in the manner shown for THA in Fig. 6 indicates an IC_{50} value of 50 μM for the allosteric effect of THA on [^{3}H]NMS and a Hill coefficient of 1.7.

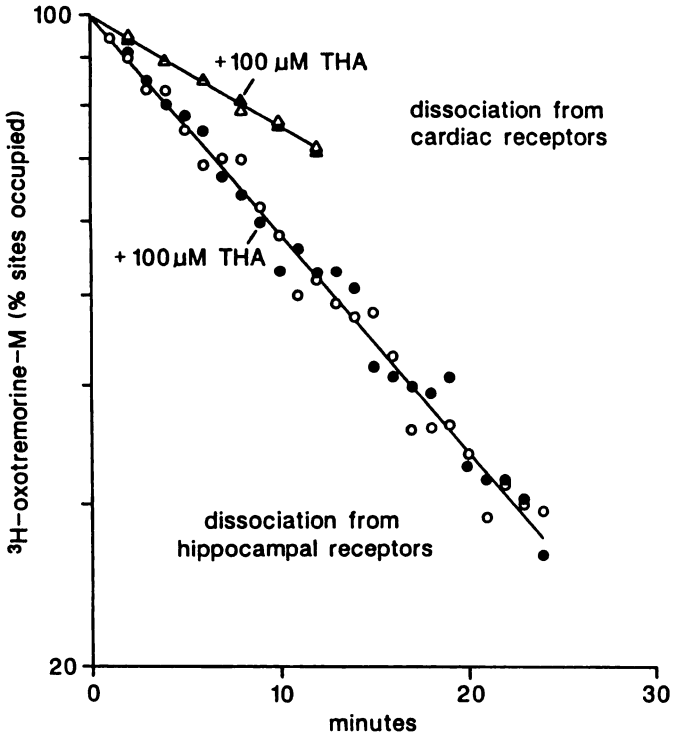

Fig. 9. Lack of effect of THA on the rate of dissociation of [^{3}H]oxotremorine-M from muscarine receptors. M1 receptors in rabbit hippocampal membranes were labeled with 5 nM radioligand in the presence of 0.1 mM NEM; initial specific binding was 4400 cpm. M2 receptors in rabbit atria were labeled with 0.5 nM radioligand without NEM; initial specific binding was 1450 cpm. *Points* are single values; *lines* are linear regressions. *Hollow points* show results without THA. Note that 100 μM THA was without effect on dissociation, although this concentration fully blocked the equilibrium binding of oxotremorine-M to M1 receptors (Fig. 3).

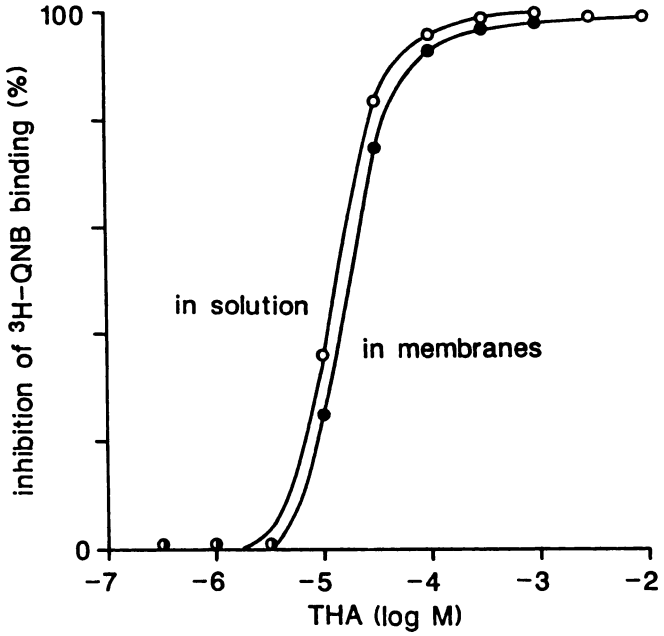

Fig. 10. Comparison of the ability of THA to inhibit the binding of 1 nM [^{3}H]QNB to rabbit hippocampal muscarine receptors in membranes and in solution. These experiments were carried out in phosphate-EDTA buffer at 37°, before and after dissolution of membrane proteins in 1% digitonin. Specific binding of [^{3}H]QNB alone to membranes from 5 mg of tissue averaged 20,100 cpm, whereas soluble receptors from 10 mg of tissue bound an average of 11,050 cpm.

effects of 200 μM THA on the dissociation of [^{3}H]QNB were also similar before and after dissolving receptors. These results and the lack of any effect of Gpp(NH)p on the ability of THA to slow the dissociation of [^{3}H]pirenzepine (Fig. 5) provide the best available evidence that the effects of an allosteric antagonist are exerted directly on receptor molecules and not via associated molecules. Unpublished work with gallamine has yielded the same conclusion (6).

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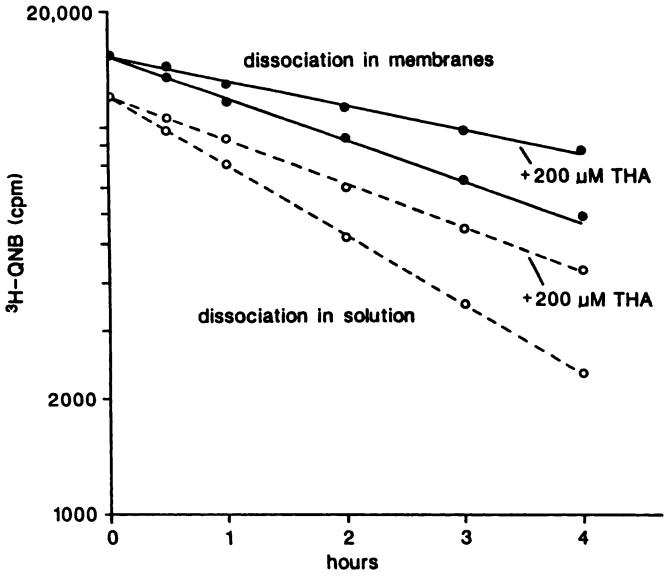

Fig. 11. Comparison of the ability of THA to slow the dissociation of [^{3}H]QNB from rabbit hippocampal muscarine receptors in membranes and in solution. Receptors were labeled with 1 nM [^{3}H]QNB under the conditions noted for Fig. 10. Dissociation was initiated with 1 μM (±)-QNB with or without THA as shown. *Lines* are linear regressions. Dissociation is slightly more rapid from receptors in solution (31). The allosteric effect of THA clearly remains after solubilization.

Discussion

We found that low concentrations of THA were able to inhibit the binding of low concentrations of [^{3}H]pirenzepine, [^{3}H]NMS, and [^{3}H]oxotremorine-M to hippocampal muscarine receptors with approximately the same IC_{50} value (1.7–2.5 μM). In contrast, the concentrations of THA required to allosterically inhibit the dissociation of [^{3}H]pirenzepine and [^{3}H]NMS by half were 40 and 50 μM, respectively, and 100 μM THA had no effect on the dissociation of [^{3}H]oxotremorine-M. We interpret these results as evidence that low concentrations of THA can compete with other ligands for primary receptor sites, presumably including the ligand acetylcholine. Under more physiological conditions (artificial cerebrospinal fluid at 37°) than those used in the present experiments, the IC_{50} value for THA versus 10 nM[^{3}H]pirenzepine ($K_d \approx 35$ nM) was about 6 μM. Because plasma levels of THA in humans can reach 0.3 μM (23) and THA is concentrated 10-fold in the brain (24), probably because of its high lipid solubility, the levels of THA used for esterase inhibition *in vivo* may begin to inhibit the binding of acetylcholine to muscarine receptors. The more potent of the other compounds listed in Table 1 may also produce clinically relevant competition with acetylcholine at primary sites. In contrast, the lack of any allosteric effect of THA on the binding of oxotremorine-M makes it very unlikely that THA or any of the other compounds tested produces any clinically relevant allosteric effects on the binding of acetylcholine.

The data in Fig. 6 and Table 2 demonstrate that THA is the most potent and the most effective allosteric agent yet tested on muscarine receptors. It is, therefore, a logical choice of an allosteric drug for further mechanistic studies of these receptors. A point of interest for further work is the striking lack of effect of THA on the conformations of M1 and M2 receptors which show the highest affinity for agonists. The reason for this lack of effect is not clear. The most likely possibility is that the formation of complexes between agonist, receptor, and GTP-binding protein, quite possibly with paired receptors (26, 28), produces a receptor conformation that does not bind THA readily. Alternatively, THA may bind but produce little negative cooperativity from allosteric → primary sites. In any case, THA may prove to be useful for identifying distinct receptor conformations.

Each of the nine allosterically active compounds listed in Table 2 has a large ring-bearing structure and most of these drugs are quite rigid, especially *d*-tubocurare. This suggests that allosteric sites represent a relatively large pocket or surface on receptors, compared with primary sites. Because *d*-tubocurare, pancuronium, gallamine, and McN-A-343 are all quaternary amines, at least some portion of allosteric interactions must be on the extracellular part of muscarine receptors. On the other hand, the high lipid solubility of THA, verapamil, and quinidine may permit their access to additional sites on receptors, which could help explain their high allosteric activity. Our sense, after looking at the structures of these compounds, is that allosteric sites are likely to be nonspecific. If this is correct, then the information that might be gained from carrying out competition experiments between one labeled and several unlabeled allosteric agents, for allosteric sites, may be limited.

The unique steepness of THA inhibition curves is intriguing. Because this phenomenon occurs in solution, in the presence of EDTA, and in the presence of Gpp(NH)p, it is probable that THA is interacting directly with receptor molecules. Because the purely allosteric effect of THA on the dissociation of [^{3}H]pirenzepine or [^{3}H]NMS still has a Hill coefficient of 1.7, it is probable that more than one molecule of THA binds to each receptor molecule. The most general model for interactions between allosteric and primary sites assumes that the occupation of one site will modify the binding of ligands to the other site, with no assumptions as to interdependent couples or the ratio of the numbers of both kinds of sites (20). We have been unable to computer-fit steep curves for allosteric interactions with any set of assumptions for this model (including negative cooperativity from allosteric → primary sites plus positive cooperativity from primary → allosteric sites) so long as the assumption is made that interactions between one ligand with a certain fraction of one kind of site leads to a conformational change of the *same* fraction of the other kind of site. We are left with the reasonable idea that THA either binds cooperatively or that the binding of more than one THA molecule produces a nonproportionally large allosteric effect on primary sites.

An unresolved question is whether there are any interactions between THA-liganded and THA-free receptors. If receptors are paired, it remains possible that the binding of THA to primary or allosteric sites on one receptor modifies the binding properties of the sites on the other member of the receptor pair.

The present results add to knowledge concerning the binding properties of M1 receptor subtypes. Bonner and his colleagues (38, 39) have demonstrated that the rat hippocampus expresses all three of the "M1" receptors that bind pirenzepine with high affinity, in the proportions m1 > m3 ≫ m4. It is, therefore likely that the rabbit hippocampus also has substantial numbers of more than one M1 receptor subtype. [^{3}H]pirenzepine

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associates with, dissociates from, and comes to equilibrium with only a single population of receptors in rabbit hippocampal membranes under the conditions used for the present study (26), and only a single affinity state is seen for each of 16 agonists in competing with [^{3}H]pirenzepine in the presence of Gpp(NH)p (27). In the present study, [^{3}H]pirenzepine still associated with (Fig. 4), dissociated from (Fig. 5), and came to equilibrium with (Fig. 7) only one population of receptors after a large fraction of the total M1 receptors were inhibited by THA. Hence, the major subtypes of M1 receptors in fresh rabbit hippocampal membranes have equal or nearly equal affinity for pirenzepine and for each agonist. THA has equal inhibitory effects on hippocampal M1 and brainstem M2 receptors (19) and is not likely to be selective for different M1 receptors. Inhibition curves between 12 other antagonists and [^{3}H]pirenzepine were mass action curves, indicating that none of these nonclassical antagonists could distinguish major M1 receptor subtypes in the rabbit hippocampus.

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