Vol. 81, No. 3, 1978 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1978 Pages 710-717

MUSCARINIC BINDING TO MOUSE BRAIN RECEPTOR SITES

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Received February 10, 1978

In mouse brain the binding of [^{3}H]-Atropine to the muscarinic receptor seems to be a simple mass-action determined process as gauged both by approach to equilibrium kinetics and binding at equilibrium. In contrast, using isotopic dilution technique, dissociation measurements indicate the existence of two receptor-ligand complexes. It would appear that association and dissociation rates of binding of the muscarinic antagonists atropine, scopolamine, N-methyl-4-piperidyl benzilate (4NMPB) and 3-quinuclidinyl benzilate (QNB) decrease with increasing affinity based on comparisons of kinetic binding data. The differences between the association rate constants are small whereas those between the dissociation rate constants differ markedly. This kinetic behavior is similar to the well-known time profile of antimuscarinic activity in isolated tissues. These phenomena are discussed in terms of possible isomerization of the receptor-ligand complex, as has been proposed recently for [^{3}H]-scopolamine and [^{3}H]-4NMPB binding.

Atropine generally behaves as a competitive antagonist of acetylcholine at muscarinic receptors; its onset and offset of antagonism are slow. It has been suggested that the rates of onset and offset of atropine blockade are determined by the rates of binding to and dissociation from the muscarinic receptor (1-3). This communication describes studies on the binding properties of the muscarinic receptors of mouse brain using [^{3}H]-atropine. The main features of the kinetic behavior of the system are reported here and compared to those of its direct analogue scopolamine and the highly potent antagonist N-methyl-4-piperidyl benzilate (4NMPB)(4-6).

EXPERIMENTAL

[^{3}H]-atropine 2.6 Ci/mmole, [^{3}H]-scopolamine 1.7 Ci/mmole and [^{3}H]-N-methyl-piperidyl benzilate (4NMPB) 6 Ci/mmole were prepared by a catalytic tritium exchange as described elsewhere (7). The chemical and radiochemical purity was determined by analytical thin-layer chromatography (Merck Silica 60 plates, 0.25 mm thickness) in two solvent systems: n-butanol, acetic acid, water (4:1:1), and chloroform, acetone, diethylamine (5:4:1). [^{3}H] drugs moved as a single peak, identical to the authentic, unlabeled compound in these two systems. The purity was [^{3}H]-atropine and [^{3}H]-scopolamine > 99%, and [^{3}H]-4NMPB > 97%.

Male ICR mice (20-25 gr) were decapitated, brains were removed (within 2 minutes) and homogenized in ice cold 0.32M sucrose using a motor-driven

0006-291X/78/0813-0710$01.00/0

teflon pestle (950 rpm) in a glass homogenizer to yield a 10% homogenate (w/v). The entire homogenate was centrifuged for 10 min. at 1000xg (Sorvall RC2-B), the pellet discarded and the resultant supernatant fluid (S_1) was used for assays of binding as described elsewhere (4, 5). 50 µl of S_1 fraction were incubated at 25° in 2 ml modified Krebs Henselite solution (25 mM Tris HCl instead of bicarbonate), pH 7.4, containing the labeled ligand. After various times of incubation ice cold Krebs solution (3 ml) was added and the contents were passed rapidly through a glass filter (GF/C, Whatman 25 mm diameter) by suction. The filters were washed 3 times (3 ml of ice cold Krebs solution). All procedures were completed within less than 10 sec. and all binding experiments were performed in triplicate together with triplicate samples containing $5x10^{-5}M$ of unlabeled ligand. The filters were placed in vials containing 5 ml of scintillation liquid (33 ml Triton X-100, 66 ml toluene, 5.5 gr PPO (Packard) and 0.1 gr dimethyl-POPOP (Merck), maintained at 25° for 30 min. and the radioactivity then assayed by liquid scintillation spectrometry (Packard Tricarb model 2002, 31% efficiency).

Specific binding is defined as the total minus the non-specific binding i.e., binding in the presence of $5x10^{-5}M$ of unlabeled ligand.

Approach to equilibrium kinetics were analyzed by assuming a simple bimolecular reaction. A best fit calculation method was employed and curve-fitting was carried out with a CDC 6400 computer using program D506 (MINUIT) from the CERN computer 6000 series, program library I. The observed time course of dissociation was fitted to the sum of two first order exponentials, pertaining to fast (k_{-2}) and slow (k_{-1}) steps using a best fit calculation method (5).

RESULTS

The binding of [^{3}H]-atropine at 25° was studied as described recently for [^{3}H]-4NMPB and [^{3}H]-scopolamine (5) using S_1 fraction of mouse brain homogenate. The binding capacity of the preparation for these drugs is very similar: 1.55, 1.68 and 1.62 pmole/mg protein for [^{3}H]-atropine, [^{3}H]-4NMPB and [^{3}H]-scopolamine respectively when calculated at the present conditions.*

Equilibrium binding studies were performed at a range of concentration from 0.25 to 20 nM, shown in Fig. 1 for specific and non-specific binding of [^{3}H]-atropine. Specific ligand binding (Fig. 1, upper) exhibits the hyperbolic shape typical for saturation phenomena (half saturation at 1.3 nM ligand). Non-specific binding is much lower and varies linearly with ligand concentration (Fig. 1, lower). The binding data when replotted in a double reciprocal form gave a straight line (Fig. 1, insert), therefore [^{3}H]-atropine binding reflects a single population of binding sites at the concentration range investigated. Binding was investigated further kinetically. Fig. 2 shows the time course of association of [^{3}H]-atropine at three ligand concentrations, corrected for non-specific binding. The solid lines represent curve fitting, assuming a simple bimolecular reaction scheme. The same kinetic parameters $k_1=2.9x10^8M^{-1}$ min^{-1} and $k_{-1}=0.45$ min^{-1} accomodate the association data at all [^{3}H]-atropine concentrations investigated. The dissociation

* In our previous work we determined the binding capacity to be 0.62 and 0.68 pmole/mg protein for [^{3}H]-4NMPB and [^{3}H]-scopolamine respectively for those experiments.

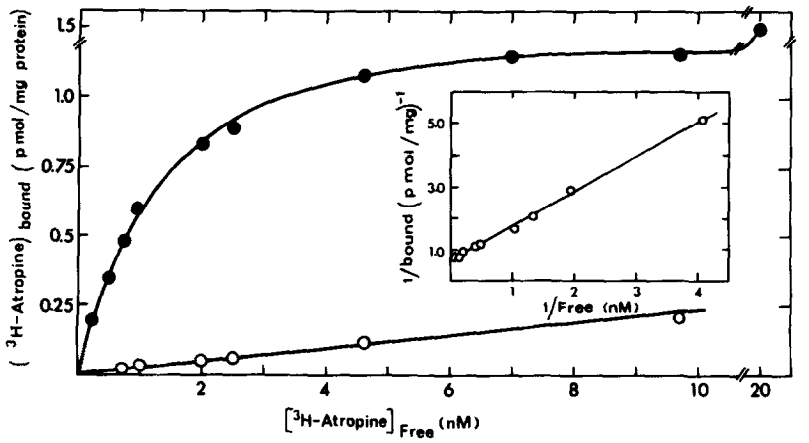

Fig. 1. Binding of [^{3}H]-atropine at 25° as a function of concentration. 0.05 ml S_1 samples were incubated with varying concentrations of [^{3}H]-atropine for 10 min. at 25° in 2 ml Krebs solution (pH 7.4). Lower curve: nonspecific binding (in the presence of $5x10^{-5}$M atropine). Upper curve: specific binding. Each point is the mean of triplicate samples whose standard error was less than 5%. Insert: Double reciprocal plot.

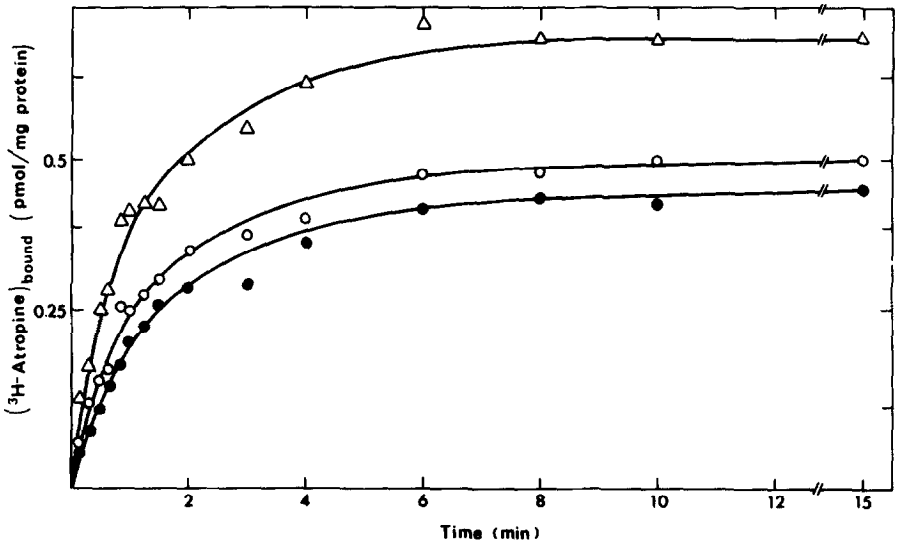

Fig. 2. Time course of [^{3}H]-atropine binding at 25°. S_1 samples (0.05 ml) were incubated in 2 ml modified Krebs solution with [^{3}H]-atropine for various periods of time at 25°. Specific binding was determined as described in "Methods". Each experimental point is the mean of triplicate samples whose standard error was less than 5%. The kinetics were measured with [^{3}H]-atropine at the following concentrations: (●-●)0.75 nM, (o-o)1.0 nM, (Δ-Δ)1.5 nM.

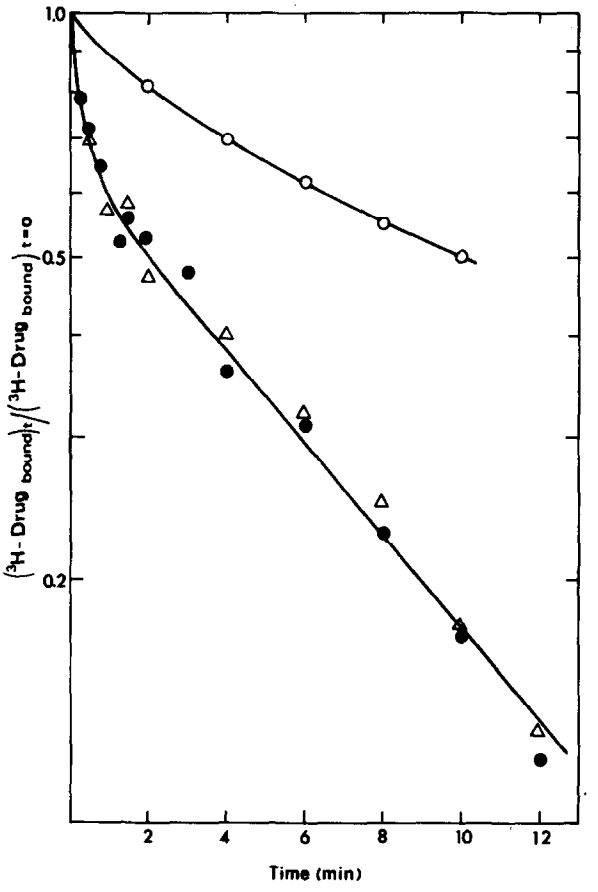

Fig. 3. Dissociation of [^{3}H]-atropine and [^{3}H]-scopolamine at 25°. S_1 samples (0.05 ml) were incubated with 6.0 nM(●-●), 0.6 nM(Δ-Δ) [^{3}H]-atropine for 10 min. and 3.7 nM [^{3}H]-scopolamine (o-o) for 30 min. in 2 ml modified Krebs solution. $5x10^{-5}$M unlabeled ligand was then added and the samples were filtered immediately (zero times), and at the times indicated. Specific [^{3}H]-drug binding was determined as described in text. Each experimental point is the mean of triplicate samples whose standard error was less than 5%.

constant deduced from the ratio k_{-1}/k_1 is very similar to the Kd value as determined at equilibrium (1.5 nM and 1.3 nM respectively).

An isotopic dilution technique is suitable to follow the course of dissociation of ligand-receptor complex. Fig. 3 demonstrates the first order plot for the dissociation of [^{3}H]-atropine and the same plot for dissociation of [^{3}H]-scopolamine is also given for comparison. As in the case of [^{3}H]-scopolamine and [^{3}H]-4NMPB at 25° (5) the first order plot deviates from linearity. The data can, however, be fitted with the sum of two first

order exponential terms, viz: $\alpha_1 \exp(-k_{-1}t)+\alpha_2 \exp(-k_{-2}t)$, where the first order rate constants for atropin are $k_{-1}=2.87\ min^{-1}$, $k_{-2}=0.13\ min^{-1}$ with the corresponding fractions of binding sites $\alpha_1=0.35$ and $\alpha_2=0.65$.

To avoid possible artifacts due to the presence of "closed structures" control experiments were performed. The dissociation reaction was terminated with double distilled water rather than buffer; this results in osmotic shock (as confirmed by the [^{3}H]-GABA uptake system (8)). In these experiments the dissociation of ligand-receptor complex was similar to that measured in the original experiments; thus it seems that the biphasic dissociation cannot be attributed to trapped, labeled ligand.

DISCUSSION

The binding of [^{3}H]-atropine measured by approach to equilibrium and at equilibrium can be described by a simple, mass-action determined process. At first sight these results are not in keeping with the dissociation measurements showing the existence of two different populations of binding sites (Fig. 3). Indeed, the measurements of association kinetics appear to contradict the possibility of two preexisting binding sites. Moreover, if there is a heterogeneiety of binding sites, then the rate of dissociation of ligand-receptor complex should change with the degree of occupancy. As shown in Fig. 3, the dissociation rate of [^{3}H]-atropine-receptor complex after incubation with 0.6 nM [^{3}H]-atropine or 6.0 nM are similar, thus eliminating the possibility of heterogeneiety. The biphasic dissociation of receptor-ligand complex observed using an isotopic dilution technique seems to contradict the possibility of negative cooperativity per se. Under the conditions used here the receptor sites are fully occupied by ligand; hence, based solely on negative cooperativity, dissociation should be monophasic.

The minimal reaction sequence $R+L \rightleftarrows RL \rightleftarrows R^*L$ can account for the combined data. Here it is assumed tacitly that the binding of [^{3}H]-atropine (L) to its receptor (R) involves an isomerization step ($RL \rightleftarrows R^*L$) and that this is much slower than the association-dissociation reactions. Thus, the association step would depend only on the reactions leading to the formation of the RL complex (where $k_1=2.9\times10^8 M^{-1}\ min^{-1}$ and $k_{-1}=0.45\ min^{-1}$). On the other hand, the rates of dissociation, measured after equilibrium between the RL and R*L forms had been reached, would reflect the backward reactions of both RL and R*L. It is worth adding here that estimation of the entrophy from the association and dissociation reactions of e.g. 4NMPB yielded $\Delta S= +40$ eu. Such a positive entrophy value is frequently associated with conformational changes in macromolecules. Compared to the model proposed recently for the binding of [^{3}H]-4NMPB and [^{3}H]-scopolamine (4, 5) the present one is

simplified. In the former scheme we had to assume rapid dissociation of R*L to R* and L and irreversible transformation of R* to R, to result in a non-Michaelis-Menten type binding curve. Since there are only slight structural differences between the molecules of atropine and scopolamine, the possibility suggests itself that this structural difference (the epoxy group) might account for their different binding features perhaps including the formation of a hydrogen bond and concomitant conformational change.

It should be noticed that the kinetic binding pattern of all of these antimuscarinic drugs is similar, i.e. association seems to be a simple bimolecular reaction, whereas dissociation reactions are biphasic. This behavior is consistent with either models. It should be emphasized that at the moment a combination of allosteric interactions and isomerization cannot be ruled out. Moran and Triggle (9) working on BHM ($Ph_2CHO\ CH_2CH_2NMe\ CH_2CH_2Cl$) on rat jejunum observed that the inactivation of response by BHM is reversible and that the two distinct rates of recovery of the response observed correlate quite well with the rate of loss of ligand from the tissue. They suggest that BHM has two sites of action, one being the acetylcholine receptor proper and the other an allosteric or regulatory site.

The binding constant value for atropine reported here is in excellent agreement with equilibrium binding studies reported prveiously (10-16). Most of the labeled muscarinic antagonists studied in subcellular preparations bind to a single high-affinity site whereas most of the agonists bind to multiple sites (15,16). Our kinetic experiments seem to support the conclusion that antagonists bind to a homogeneous population of binding sites. The biphasic dissociation of atropine, scopolamine and 4NMPB on the other hand cannot be explained by a simple mass-action process. Biphasic dissociation rates have been described for various ligand-receptor interactions (17-20). The slow isomerization of the muscarinic-receptor-ligand complex may thus be a common feature of membrane receptor-ligand interactions.

The binding of labeled muscarinic antagonists to subcellular preparations enables the direct study of their rate of association to and dissociation from the receptor sites with minimal, if any, limitations of access. This obviates many of the difficulties encountered when working with isolated tissues and measuring rates of antagonism (9 and references therein). The kinetic binding studies of QNB, 4NMPB, scopolamine and atropine (Table 1) lead to the conclusion that 1. kinetic constants measured by direct binding of labeled antagonists are much greater than those of antagonism, where the antagonist is applied to an organ bath, (2, 6). 2. Increasing affinity correlates with decrasing dissociation and association rates (Table 1) e.g. the time required to reach equilibrium at 0.6nM ligand is 40 min. for QNB

TABLE I. KINETIC PARAMETERS FOR BINDING OF MUSCARINIC LIGANDS

ligand	association experiments			dissociation experiments
	$k_1 (M^{-1} min^{-1})$	$k_{-1} (min^{-1})$	k_{-1}/k_1 (nM)	$t_{1/2}$ (min)
QNB [a]	2.0×10^8	0.012	0.06	57
4NMPB	2.3×10^8	0.08	0.34	12
Scopolamine	2.2×10^8	0.15	0.68	10
Atropine	2.9×10^8	0.45	1.55	2

a Data taken from Ref. 14 measured at 35° in rat brain preparation

(at 35°), 23 min. for scopolamine (25°) and about 9 min. for atropine (25°). Such correlation was also established for muscarinic antagonism (1, 6, 21). These data suggest that although there are factors other than association to and dissociation from the receptors which determine the rates of onset and offset of antagonism, the events at the receptor sites are dominant. Similar conclusions have been reached recently by Bolton (22) by a different route of experimentation.

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