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The Binding of Antagonists to Brain Muscarinic Receptors

E. C. HULME, N. J. M. BIRDSALL, A. S. V. BURGEN, AND P. MEHTA

Division of Molecular Pharmacology, National Institute for Medical Research, Mill Hill, London NW7 1AA

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SUMMARY

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The equilibrium binding of muscarinic antagonists to a crude synaptosome fraction from rat cerebral cortex obeys the simple Langmuir isotherm. The affinity constants measured range from 10^5 to 10^{10} M^{-1} and in all cases agree within a factor of 3 with the corresponding values derived from measurements of anticholinergic potency on smooth muscle. The methodology which must be applied to obtain valid measurements of affinity constants from *in vitro* studies using radiolabelled ligands is discussed.

INTRODUCTION

A number of studies have demonstrated the presence of high affinity binding sites for radiolabelled muscarinic antagonists in subcellular fractions from brain and smooth muscle, and in intact tissues [for review, see (1)]. A major component of such binding satisfies criteria which suggest that it occurs specifically to muscarinic receptors. These criteria are (1) saturability—a component of the binding saturates with increasing concentration of the radiolabelled ligands; (2) specificity—pharmacologically active concentrations of muscarinic drugs inhibit the saturable component of binding, while drugs whose pharmacological properties suggest a different primary site of action do not inhibit binding, at least at those concentrations at which they exert their predominant pharmacological effects. These criteria are fulfilled both by reversible muscarinic antagonists (2-5) and by irreversible antagonists based on benzilylcholine mustard (6-12). The assumption that antagonists and agonists undergo competitive and mutually exclusive binding to a uniform receptor population has been crucial to the interpretation of the whole tissue pharmacology of muscarinic drugs. Preliminary reports from this laboratory suggest that while muscarinic antagonists appear to bind to a single uniform population of binding sites in brain subcellular fractions, the binding of agonists may not be so described (2, 3, 12). The purpose of this paper is to examine the above hypothesis critically in the case of binding of antagonists, and in addition to examine the conformity of *in vitro* antagonist binding studies to a more rigorous version of the second of the criteria for receptor-specific binding stated above, namely that the affinity constants describing the specific binding of a wide range of antagonists to muscarinic receptors in a subcellular fraction *in vitro* should agree quantitatively with values estimated from antagonism of muscarinic physiological responses of whole tissue.

MATERIALS AND METHODS

Radiolabelled Antagonists

N-([^{3}H]methyl)-atropinium iodide ([^{3}H]MeAtr)[1]. Atropine free base (1.16 mg) in acetone (0.5 ml) was methylated with [^{3}H]methyl iodide (9 mCi, 2.2 Ci/mmole) at

[1] Abbreviations: MeAtr, N-methylatropine; PrBCh, propylbenzilylcholine; Atr, atropine; QNB, quinuclidinylbenzilate.

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room temperature in the dark for 36 hr. The solution was evaporated to dryness under N_2 and the [^{3}H]MeAtr purified by preparative thin-layer chromatography (silica gel G, chloroform/methanol/acetic acid/water (65:25:5:5; Rf.0.4). The product (specific activity 2.2 Ci/mmole, radiochemical purity 98%) had the same Rf as N-methylatropine bromide (Sigma) in the above solvent system and in butan-1-ol/acetic acid/water (5:3:3). [^{3}H]MeAtr was stored in solution in absolute ethanol at −20°C.

N-(2',3'[$^3H_2$]propyl)-N,N-dimethyl-2-aminoethylbenzilate ([^{3}H]PrBCh). N-Allyl-N-dimethyl-2-aminoethylbenzilate bromide (70 mg, mp 160-1) in methanol (0.5 ml) was reduced by tritium gas (15 Ci) in the presence of 5% Pd/C (20 mg) and potassium benzilate (10 mg). After filtration of the crude tritiation product through Hyflo, labile tritium was removed by repeated evaporation of methanol. The product (37 mg, 3.5 Ci) was recrystallized from methanol/ethyl acetate (1.5 ml, 1:10) and stored in absolute ethanol at −20°C. The product was further purified by preparative thin-layer chromatography (silica gel, butan-1-ol/acetic acid/water 5:3:3, Rf = 0.7) and the [^{3}H]PrBCh (98% radiochemical purity, specific activity 40 Ci/mmole) stored in absolute ethanol at −20°C. The product had the same Rf as an authentic sample of PrBCh [mp 187.5–188.5, literature mp 186-7 (28)] on thin layer chromatography on silica gel in the solvent systems (butanol/acetic acid/water 4:1:1 and chloroform/methanol/acetic acid/water 75:25:5:5).

N-([^{3}H]methyl) - scopolamine. Scopolamine free base was methylated with [^{3}H]methyl iodide (3.3 Ci/mmole) using the same method as for the preparation of N-([^{3}H]methyl)-atropine. The product was purified by thin-layer chromatography (silica gel G, butan-1-ol/acetic acid/water 5:3:3, Rf scopolamine 0.55, N-methyl-scopolamine 0.35). The purified material had the same Rf as N-methyl-scopolamine bromide (Sigma) in the above solvent system and in chloroform/methanol/acetic acid/water (65:25:5:5) and was >98% radiochemically pure. It was stored in solution in absolute ethanol at −20°.

[^{3}H]atropine (0.32 Ci/mmole) and [^{3}H]-quinuclidinylbenzilate (13 Ci/mmole) were purchased from the Radiochemical Centre, Amersham, Bucks., U. K.

Binding assays. A crude synaptosome fraction was prepared from rat cerebral cortex as follows: cerebral cortices from 6–10 rats (male Wistars, 180–200 g body weight) were pooled and homogenized in 9 volumes of ice cold 0.32 M sucrose using a Potter-Elvehjem homogenizer with a rotating teflon pestle (520 rpm, 6 up and down strokes). Subsequent steps were carried out at 2–4°C. The homogenate was centrifuged at 1000 × *g* for 10 min. The pellet was discarded, and the supernatant centrifuged at 10,000 × *g* for 20 min. The resulting pellet, designated the crude synaptosome fraction, was resuspended to a protein concentration of 10 mg/ml in Krebs-Henseleit solution (pH 7.4) and kept at 0°C. Binding studies were performed as soon as possible, usually within 2–4 hr of preparation of the crude synaptosome fraction. However, the crude synaptosome pellet can be frozen and stored at −70°C for prolonged periods (weeks) without loss of binding activity.

For binding assays, the crude synaptosome fraction was diluted to a protein concentration of 1 mg/ml or 0.2 mg/ml as appropriate in Krebs-Henseleit solution, and preincubated at 30°C for 15 min. One milliliter aliquots of the suspension were then pipetted into plastic microcentrifuge tubes (1.5 ml, Eppendorf, Hamburg, West Germany) containing the [^{3}H]antagonist together with a competing drug if appropriate, giving a final incubation volume of 1.02 ml. Over the concentration range used, there was no detectable binding of [^{3}H]ligands to microcentrifuge tubes. Assays were carried out in quadruplicate. Tubes were mixed and incubation continued at 30°C for 15 min. The incubation was terminated by centrifugation at 14,000 × *g* for 30 sec (1 mg/ml) or 60 sec (0.2 mg/ml) at room temperature using a microcentrifuge (Quickfit, model 320). Pellet formation was essentially complete within 15 sec. After decanting the supernatant, the pellets were rapidly and superficially washed with 3 × 1.5 ml of Krebs Henseleit solution, to remove radioactivity adhering to the sides of

the tube, and allowed to drain. The tips of the microcentrifuge tubes containing the pellets were then cut off and placed in 10 ml of liquid scintillation medium (toluene/triton-X-100/water, 8:4:1, butyl PBD 4 g/liter). The pellets dissolved completely after agitation, and bound radioactivity was measured by liquid scintillation spectrometry at a counting efficiency of 30%.

Protein concentrations were determined by the method of Lowry *et al.* (13).

DATA ANALYSIS

Theoretical binding curves were fitted to experimental data points by nonlinear least-squares regression analysis, using a computer program described by Batchelor (14). Individual data points were the mean of four observations, and were weighted according to the inverse of the standard error of the mean. The fitting program provided estimates of the best-fit parameter values, together with estimates of the SEM and confidence limits for each parameter. The latter estimates were based on the assumption that each parameter makes an independent contribution to the residual sum of squares around the optimum solution; since this is not necessarily the case when nonlinear models are under consideration, such estimates must be regarded as conservative.

The possible existence of systematic deviations of experimental data from theoretical binding curves was examined by the analysis of residuals, using a statistical procedure suggested by Reich *et al.* (15). The residuals e_i defined as the difference between the ith measured and calculated values are ranked in order of increasing ligand concentration, and a correlation coefficient calculated for the set of pairs of values (e_i, e_{i+1}), $i = 1, n-1$, where n is the number of points. This parameter, designated the nearest-neighbor residual correlation coefficient (NNRC) has a low or negative value if the experimental points are randomly scattered around the best fit curve, but takes on a positive value approaching unity as deviations from the theoretical curve become systematic, so that large positive and large negative residuals tend to be grouped. The statistic

$$t_2 = \text{NNRC}\sqrt{\left(\frac{n-3}{1\text{-NNRC}^2}\right)}$$

has a t-distribution, with n-3 degrees of freedom (15) enabling one to test the significance of the NNRC.

RESULTS

Specific and nonspecific binding. Substantial binding of the three radiolabelled antagonists to crude synaptosome preparations could be observed within the concentration range 10^{-10}–10^{-7} M. Such binding equilibrated rapidly, reaching a steady value within 1–2 min in the case of [^{3}H]-PrBCh, and 5 min in the cases of [^{3}H]Atr and [^{3}H]MeAtr.

A large percentage of the binding of the [^{3}H]antagonists was inhibited by incubation with a second, unlabelled muscarinic drug. This inhibition was concentration-dependent, and attained a limiting value at a sufficiently high concentration of the competing ligand; Table 1 shows that the maximum inhibition of the binding of 10^{-10} M [^{3}H]PrBCh by a series of muscarinic antagonists, agonists, and partial agonists was equal, within the experimental error to that given by 10^{-6} M atropine on the same crude synaptosome preparation. Exactly analogous results were obtained when displacement of the binding of [^{3}H]Atr and [^{3}H]-MeAtr was studied. The proportion of the binding inhibited is independent of the exact nature of the competing ligand. This can be accounted for by positing mutually exclusive binding of muscarinic ligands to a common set of binding sites; such binding is hereafter referred to as *specific* binding, and the residual nondisplaceable binding designated *nonspecific* binding. Both specific and nonspecific binding of the [^{3}H]-antagonists were found to be directly proportional to the concentration of membrane protein in the incubation mixture, over the entire range used in these studies (0.2–2.0 mg/ml) provided that the free (i.e., unbound) concentration of the [^{3}H]ligand was maintained at a constant value.

Dependence of specific and nonspecific binding on the concentration of [^{3}H]antagonist. In confirmation of previous reports (1–5) specific binding of [^{3}H]antago-

TABLE 1

Displacement of [^{3}H]PrBCh binding by muscarinic ligands at saturation: comparison with displacement produced by 10^{-6} M atropine

Crude synaptosome preparations (1 mg/ml) were incubated with ^{3}H-PrBCh (10^{-10} M), in the presence and absence of the indicated concentrations of the unlabelled competing ligand for 15 min at 30°C. Bound radioactivity was measured as described in Materials and Methods. The values tabulated represent the percentage inhibition of [^{3}H]PrBCh binding given by each of the competing ligands. Each pair of values was derived from a separate experiment, using a different crude synaptosome preparation.

Ligand	concn	%I ± SEM	%I, 10^{-6} M atropine ± SEM	Δ ± SEM[a]
	M			
Pilocarpine	10^{-3}	83.6 ± 0.5	83.5 ± 0.2	+0.1 ± 0.54
Acetylcholine	10^{-3}	84.7 ± 0.6	85.2 ± 0.6	−0.5 ± 0.83
Oxotremorine	10^{-4}	86.5 ± 1.3	87.6 ± 0.3	−1.1 ± 1.33
C12-TMA	10^{-4}	89.4 ± 0.2	88.3 ± 0.2	+1.1 ± 0.28
N-dimethyl-4-piperidinyl-phenylacetate	10^{-4}	80.2 ± 0.2	80.8 ± 0.2	−0.6 ± 0.28
PrBCh	10^{-6}	84.4 ± 0.4	85.7 ± 0.6	−1.3 ± 0.72
Lachesine	10^{-7}	87.3 ± 0.6	88.1 ± 0.6	−0.8 ± 0.83

[a] %I (ligand) − %I (10^{-6} M atropine)

nists was found to be a saturable function, and nonspecific binding a linear function of free [^{3}H]antagonist concentration, over a wide concentration range. This situation is exemplified in Fig. 1, which shows binding of [^{3}H]PrBCh; specific binding is defined as that fraction of the binding inhibited by simultaneous incubation with 10^{-6} M atropine.

Specific binding of [^{3}H]PrBCh was adequately described by a simple bimolecular association model

$$R + L \overset{K}{\rightleftharpoons} RL$$

where R represents free binding sites, L is the ligand, RL is the complex and K is the affinity constant for binding. Free ligand concentration was taken to be the difference between the total and the bound concentrations, and amounted to 85% of the total concentration at 10^{-10} M ligand. Application of the law of Mass Action to the above model yields the simple Langmuir isotherm

$$[RL] = [R_t]\cdot K\cdot[L]/(1 + K[L]) \quad (1)$$

where $[R_t]$ is the total concentration of specific binding sites, $[L]$ is the concentration of free ligand, and $[RL]$ the concentration of complex. The conformity of the [^{3}H]PrBCh binding curve to Eq. (1) was investigated by curve-fitting; (see MATERIALS AND METHODS). The best fit curve, represented by a full line in Fig. 1b, is derived from Eq. (1) with $[R_t] = 1.384 \pm 0.029 \times 10^{-9}$ M, and $K = 8.3 \pm 0.5 \times 10^7$ M^{-1}.

The possibility of the existence of systematic deviations of the binding curve from Eq. (1) was examined in two ways: first, by computation of the nearest neighbor correlation coefficient (15) (MATERIALS AND METHODS)—this gave a value of 0.1, ($t = 0.28$, 8 degrees of freedom; $p > 0.05$) indicating that the deviations were random; second, the Hill coefficient of the binding curve was computed in two ways, (a) by fitting the Hill equation

$$[RL] = [R_t]\cdot(K\cdot[L])^n/(1 + (K\cdot[L])^n) \quad (2)$$

to the binding curve, providing simultaneous estimates of $[R_t]$, K and the Hill coefficient, n, and (b) by estimation of n from the linearized form of Eq. (2), the Hill plot

$$\log [RL]/([R_t] - [RL]) = n\log[L] + n\log(K) \quad (3)$$

using the value of $[R_t]$ derived from fitting Eq. (1). Both methods gave estimates of n which are very close to 1.0, namely 0.99 ± 0.06, and 0.98 ± 0.02, respectively (see Fig. 3b). When $n = 1.0$, Eq. (2) reduces to the

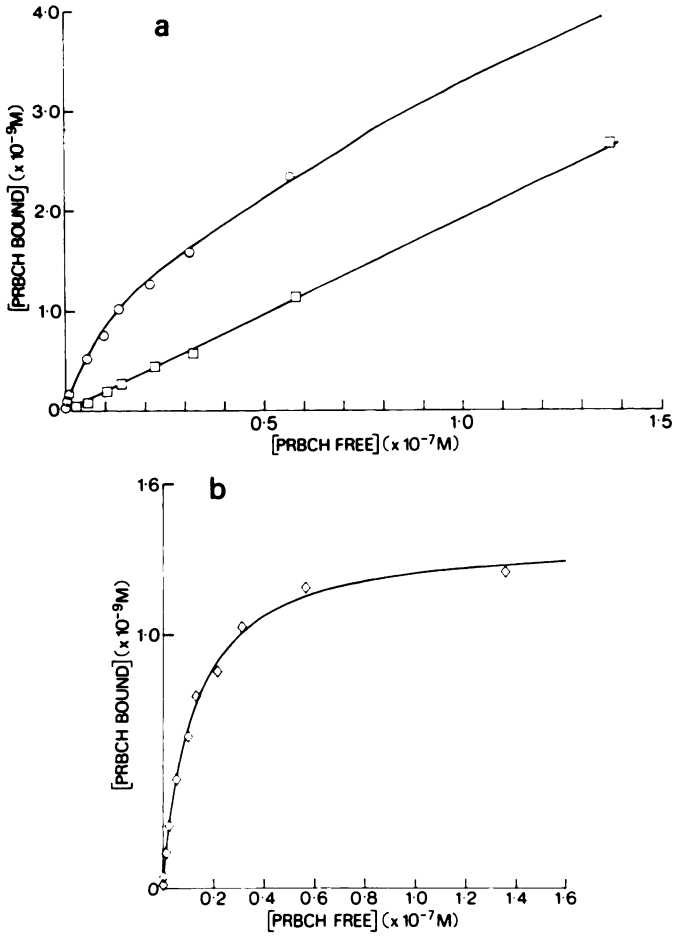

FIG. 1. *Total, specific and non-specific binding of [^{3}H]PrBCh*

(a) (○)—binding measured in the absence of atropine; the full line represents the best fit to the equation

$$B_t = [R_t]\cdot K\cdot[L]/(1 + K\cdot[L]) + C\cdot[L]$$

where $[L]$ is the concentration of unbound [^{3}H]PrBCh and $[R_t] = 1.46 \times 10^{-9}$ M, $K = 7.6 \times 10^7$ M^{-1} and $C = 0.019$. [□]—binding measured in the presence of 10^{-6} M atropine; the full line is given by

$$B_{\text{non-specific}} = 0.019\cdot[L]$$

(b) Specific binding of [^{3}H]PrBCh; the full line represents the best fit to the equation

$$[RL] = [R_t]\cdot K\cdot[L]/(1 + K\cdot[L])$$

with $[R_t] = 1.38 \times 10^{-9}$ *M* and $K = 8.3 \times 10^7$ M^{-1}

simple mass-action isotherm described by Eq. (1), which therefore provides an adequate fit to the data.

Similar analysis demonstrated the precisely additive nature of specific and non-specific binding; thus the following equation

$$B_t = [R_t]\cdot K\cdot[L]/(1 + K\cdot[L]) + C\cdot[L] \quad (4)$$

where B_t is the total measured binding, and C is a constant, provides a good fit to the experimentally-measured values of [^{3}H]-PrBCh binding in the absence of atropine as a function of free [^{3}H]PrBCh concentration (Fig. 1a). The values of $[R_t]$ and K derived from fitting Eq. (4) to this curve were 1.46×10^{-9} M, and 7.6×10^7 M^{-1}, in good agreement with the values obtained from application of Eq. (1) to the specific

binding curve. The value of C obtained was 0.019, which provides an excellent fit to values of [^{3}H]PrBCh binding measured in the presence of 10^{-6} M atropine (Fig. 1a).

Exactly analogous methods have been applied to the analysis of binding curves for [^{3}H]MeAtr and [^{3}H]Atr. In both cases it was assumed that only the more pharmacologically active (−)-isomers of these drugs[16] undergo significant specific binding. This assumption receives direct support from the results of the experiment summarised in Fig. 2; here a low, fixed concentration (10^{-9} M) of [^{3}H]MeAtr was titrated with a series of increasing concentrations of crude synaptosomal fraction. Depletion of the free concentration of the [^{3}H]antagonist, as a result of binding, led to continuous curvature of the binding/concentration relationship. The curve eventually reached an asymptote at a protein concentration of about 3 mg/ml, at which point 47% of the available radioactivity was specifically bound. This presumably corresponds to the pharmacologically active (−)-isomer.

Depletion of the active isomer of the [^{3}H]ligand at half-saturation of the binding sites exceeds 50% when the product $K \cdot [R_t]$ exceeds 2, where K is the affinity constant for the active isomer. Under such conditions, estimation of the free ligand concentration becomes difficult, leading to unacceptable errors in the final value of K. This problem can be countered by lowering the concentration of binding sites, but the extent to which this is possible is limited by the specific radioactivity of the [^{3}H]ligand. In the case of [^{3}H]MeAtr, measurements of binding were conducted at the lowest protein concentration consistent with good recovery in the microcentrifugation assay, namely 0.2 mg/ml, corresponding to a binding site concentration of $2–4 \times 10^{-10}$ M. One would expect to be able to measure affinity constants of up to about 5×10^9 M^{-1} with an acceptable degree of precision at this binding site concentration, using the difference between total and bound ligand concentration as a measure of the concentration of free ligand. In the case of [^{3}H]atropine, however, the low specific radioactivity of the [^{3}H]antagonist made it impracticable to use concentrations of binding sites below

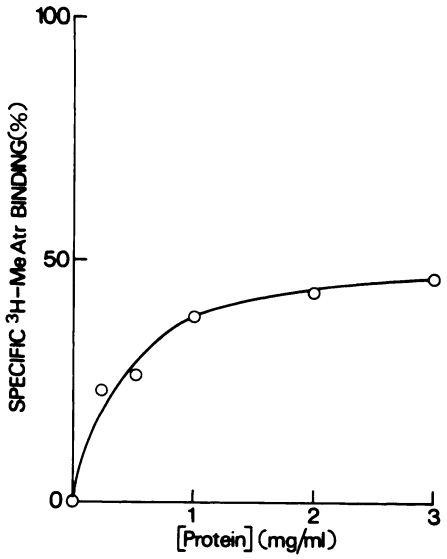

FIG. 2. *Titration of [^{3}H]MeAtr with a crude synaptosome preparation*

10^{-9} M [^{3}H]MeAtr was incubated with a series of increasing concentrations of a crude synaptosome preparation for 15 min at 30°C. Specifically bound radioactivity was expressed as a percentage of the total radioactivity present in the incubation medium.

10^{-9} M. Typical occupancy/concentration curves for the [^{3}H]antagonists, demonstrating the best-fit mass-action curves are shown in Fig. 3a; Hill plots of these curves are presented in Fig. 3b, and the values of the Hill coefficients and the affinity constants for the pharmacologically active (−)-isomers are summarised in Table 2.

The Hill coefficients of both MeAtr and Atr binding curves are somewhat less than 1.0, assessed both by means of the Hill plot and by fitting the Hill equation. In the case of MeAtr, neither value was significantly different from 1.0 ($p > 0.05$). In agreement with this, calculation of the nearest neighbor residual correlation coefficient of the curve gave a value of 0.51 ($t = 1.68$, 8 degrees of freedom) a value not significantly different from 0. According to these criteria the MeAtr binding curve is adequately described by Eq. (1). In the case of the atropine binding curve, the estimate of the Hill coefficient derived from fitting the Hill equation was 0.87, the lowest value encountered in the entire study. This value appeared to be marginally different from

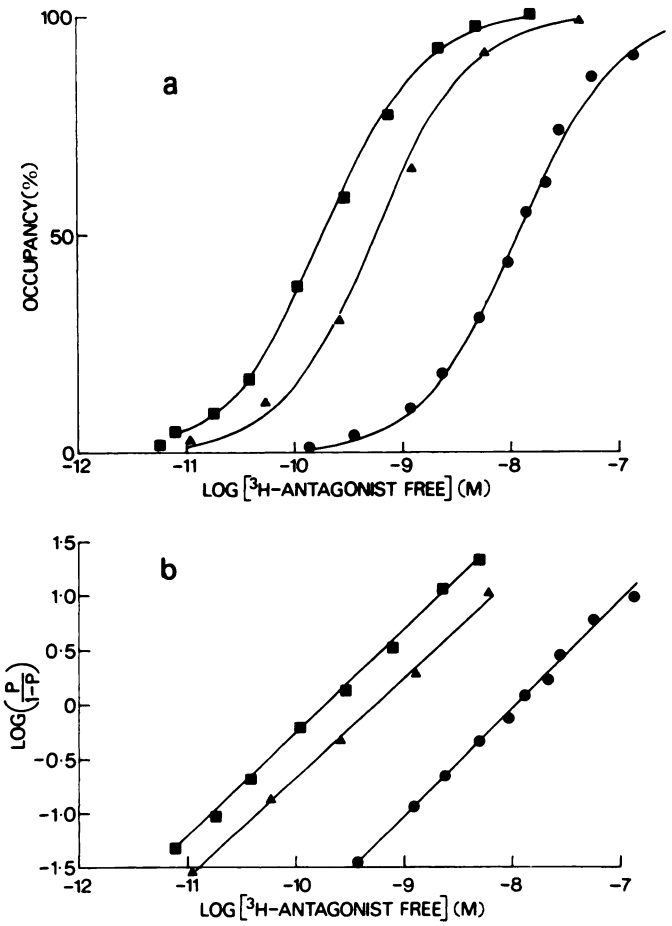

FIG. 3. *Binding of [^{3}H]antagonists*

(a) Occupancy as a function of free ligand concentration for (−)-[^{3}H]MeAtr [■], (−)-[^{3}H]atropine (▲) and [^{3}H]PrBCh (●). The full lines represent the best fit mass action curves derived by fitting the equation

$$[RL] = [R_t] \cdot K \cdot [L]/(1 + K \cdot [L])$$

to the binding data; values of $[RL]$ have been normalized by division by the appropriate value of $[R_t]$ to yield estimates of occupancy (P).

(b) Hill plots of the data given in Fig. 3(a), showing the best-fit straight lines. The slopes of these lines (the Hill coefficient) are 0.96 ± 0.013 ([^{3}H]MeAtr); 0.92 ± 0.03 ([^{3}H]atropine) and 0.98 ± 0.02 ([^{3}H]PrBCh).

1.0 ($p < 0.05$). However, the existence of any systematic deviation of the binding curve from Eq. (1) was not supported by calculation of the nearest neighbor correlation coefficient, which gave 0.4 ($t = 0.8$, 4 d.f.) a value not significantly different from 0. Significant deviations have not been found in other experiments. On balance, therefore, there seems to be insufficient evidence to maintain that the atropine binding curve deviates from Eq. (1). If such deviations exist, they are too small to detect reliably by equilibrium binding measurements using our present techniques. Measurements of the kinetics of binding should give more accurate information.

Binding capacities for [^{3}H]antagonists. Mean values of the binding capacities for [^{3}H]PrBCh, [^{3}H]Atr and [^{3}H]MeAtr determined from complete binding curves measured using a number of different crude synaptosome preparations were similar, amounting to 1.7 ± 0.2 nmoles/g protein for PrBCh (6 determinations), 1.6 ± 0.4

TABLE 2

Affinity constants, and values of the Hill coefficient for binding of [^{3}H]antagonists

Antagonist	K_A	n	
		Hill plot	Hill equation
	M^{-1}		
[^{3}H]PrBCh	$8.3 \pm 0.5 \times 10^7$	0.98 (0.93–1.03)[a]	0.99 (0.87–1.11)
(−)-[^{3}H]atropine	$1.6 \pm 0.16 \times 10^9$	0.92 (0.81–1.03)	0.87 (0.77–0.97)
(−)-[^{3}H]N-methylatropine	$5.7 \pm 0.3 \times 10^9$	0.96 (0.93–1.00)	0.93 (0.87–1.00)

[a] 95% confidence limits given in parentheses.

nmoles/g protein for atropine (3 determinations) and 1.7 ± 0.2 nmoles/g protein for MeAtr (4 determinations). The identity of the binding capacities for several different [^{3}H]antagonists was established as the result of a number of experiments in which the levels of specific binding at saturation were measured on the same subcellular preparation and found to be equal within the experimental error. The results of one such experiment are summarised in Table 3, which shows equality of the binding capacities for [^{3}H]PrBCh, [^{3}H]Atr, [^{3}H]-MeAtr, [^{3}H]N-methylscopolamine, and [^{3}H]quinuclidinylbenzilate. This identity suggests that the antagonists bind to a common set of sites.

Nonspecific binding of [^{3}H]antagonists. Table 3 also contains estimates of the nonspecific binding coefficients [see Eq. (4)] of the [^{3}H]antagonists, expressed as % ligand bound/mg protein/ml. These values tend to be about 2% for the quaternary amines, but are much larger for the tertiary antagonists [^{3}H]atropine (6%) and [^{3}H]quinuclidinyl-benzilate (17%). This presumably reflects penetration of the tertiary amines in uncharged form into lipophilic sites from which the quaternary ligands are excluded by their charges.

The ratio of specific to nonspecific binding at any given level of occupancy P of the binding site is given by

$$[RL]/(B_t - [RL]) = K \cdot [R_t] \cdot (1 - P)/(a \cdot C) \quad (5)$$

where $[R_t]$ and C are given in terms of binding/unit protein concentration, and a is 1 for optically pure ligands but 2 for ligands composed of equal portions of an active and an inactive isomer. At 95% saturation this ratio is 39 for (−)-N-methylscopolamine, 14.3 for (+/−)-MeAtr, 1.48 for (+/−)-QNB, 1.13 for (+/−)-Atr, and 0.34 for PrBCh. There is thus no doubt of the superiority of the potent quaternary antagonists over the tertiary ligands for making accurate measurements of the concentration of binding sites present in any given sample of tissue homogenate.

TABLE 3

Binding capacities for [^{3}H]antagonists

Binding capacities for atropine, N-methylscopolamine, N-methylatropine and QNB were calculated from quadruplicate measurements of specific binding conducted at single, high concentration of the [^{3}H] ligand, using 10^{-6} M unlabelled QNB to define nonspecific binding. Free [^{3}H]ligand concentrations were: 9.5×10^{-8} M ((−)-atropine), 8.2×10^{-9} M ((−)-N-methylscopolamine), 9.04×10^{-9} M ((−)-N-methylatropine), 5×10^{-10} M ((−)-QNB). In the case of QNB, the measured binding was corrected for incomplete saturation, using an affinity constant of 7.1×10^9 M^{-1} for the (−)-isomer (see Table 4). The binding capacity for [^{3}H]PrBCh was obtained from a complete binding curve, using concentrations of 10^{-9}–10^{-7} M [^{3}H]-PrBCh; this gave an affinity constant of 9×10^7 M^{-1}.

Antagonist	Specific binding capacity	Non specific binding (C[a])
	nmoles/g protein	*% bound/mg protein/ml*
[^{3}H]QNB	1.95 ± 0.07	17.2
[^{3}H]N-methyl-scopolamine	2.08 ± 0.03	2.2
[^{3}H]N-methyl-atropine	2.14 ± 0.02	1.7
[^{3}H]atropine	2.16 ± 0.10	6.0
[^{3}H]PrBCh	2.04 ± 0.02	2.5

[a] C is the coefficient of nonspecific binding (see text).

Competitive determination of antagonist affinity constants. When binding of a ligand L is determined in the presence of a second ligand L' which binds to the same site with an affinity constant K', Eq. (1) becomes

$$[RL] = [R_t]\cdot K\cdot[L]/ \cdot(1 + K\cdot[L] + K'[L']) \quad (6)$$

If $[RL]$ is determined for a set of values of $[L]$ and $[L']$ then the affinity constants for binding of the two ligands can be estimated by fitting Eq. (6) to the resultant data. Usually such an experiment takes the following form: L is the radiolabelled ligand; inhibition of formation of the complex RL by a series of increasing concentrations of L' is monitored at a concentration of L which is, as nearly as possible, constant. In this case Eq. (6) simplifies to

$$[RL] = [RL^0]/(1 + K'\text{app}\cdot[L']) \quad (7)$$

where $[RL^0]$ is the specific binding of L measured in the absence of L' and K'app $= K'/(1 + K\cdot[L])$. K'app is readily determined by graphical analysis, or by fitting Eq. (7) to the displacement curve; K' then can be calculated provided that K and L are known.

The use of Eqs. (6) and (7) is predicated on the accurate knowledge of the free ligand concentrations $[L]$ and $[L']$. Where these cannot be measured or estimated accurately the displacement equation takes the more complex form

$$[RL](1 + 1/(K\cdot([L_t] - [RL])) + K'\cdot[L_t']/(K\cdot([L_t] - [RL]) + K'\cdot[RL])) = [R_t] \quad (8)$$

This equation can be solved numerically for the concentration of the receptor-radioactive ligand complex, $[RL]$, in terms of the total ligand concentration $[L_t]$ and $[L_t']$ and the concentration of binding sites, $[R_t]$ and applied directly to the measured displacement curve using the curve fitting program; the values quoted in Table 4 for the affinity constants of lachesine, atropine, and MeAtr measured by competition with [^{3}H]PrBCh were estimated by direct fitting of Eq. (8) after substitution of the value of the affinity constant for [^{3}H]PrBCh given in Table 2.

Use of Eq. (8) in the competitive estimation of affinity constants is complex from a computational point of view. However, conditions can be identified under which Eq. (8) reduces to the form of Eq. (7) leading to the design of experiments which minimize the distortion of competition curves caused by depletion of radioactive and nonradioactive ligands. These conditions and the accompanying experiments are discussed below. The pitfalls of measurements of this type have been extensively discussed by Cuatrecasas and Hollenberg (30).

Competition with [^{3}H]PrBCh. Experimental conditions for these measurements involved the use of $1\text{–}2 \times 10^{-9}$ M binding sites, and 10^{-10} or 10^{-9} M [^{3}H]PrBCh. Since the affinity constant for [^{3}H]PrBCh is 10^8 M^{-1} (Table 2), these concentrations only produce fractional occupancy of the binding sites, i.e., $K\cdot[L_t] \ll 1$.

Under these conditions, the [^{3}H]antagonist can be used as a virtually nonperturbing probe to indicate the concentration of binding sites unoccupied by a competing ligand. To obtain maximum accuracy in the measurement of the competition curve it is, however, necessary to correct a small distortion attributable to depletion of the [^{3}H] ligand. Thus the concentration of bound [^{3}H]ligand is given by

$$[RL] = K\cdot[R]\cdot[L_t]/(1 + K\cdot[R])$$

where $[R]$ is the concentration of free binding sites. The denominator term in the above expression is contributed by depletion of the radioactive ligand, which reduces the free concentration of L from $[L_t]$ to $[L_t]/(1 + K\cdot[R])$. This effect can be corrected by multiplication of $[RL]$ by the ratio of total to free radioactive ligand concentration, yielding

$$[RL]_{corr} = [RL]\cdot[L_t]/([L_t] - [RL]) = K\cdot R\cdot[L_t] \quad (9)$$

The value of $[RL]_{corr}$ thus calculated is directly proportional to the concentration of free binding sites. Calculation shows that this simple linear correction reduces the maximum error which depletion of the [^{3}H]-ligand contributes to estimation of the occupancy of the binding site by a competing ligand to less than 0.5% for values of $K\cdot[L_t] < 0.2$. The concentration of competing ligand required to reduce $[RL]_{corr}$ to half its initial value, which gives the apparent dissociation constant of the competing ligand, is

TABLE 4

Competitive determination of antagonist affinity constants and comparison with pharmacological values

Antagonist	Competition with				Phamacological affinity constant[d] log (K_A)
	[^{3}H]PrBCh		[^{3}H]MeAtr		
	log (K_A)	n	log (K_A)	n	
(−)-3-quinuclidinylbenzilate			9.85 ± 0.04 (−0.68)	1.09 ± 0.10	9.80
(−)-N-methylatropine	9.49 ± 0.08 (−0.38)		9.65 ± 0.03 (0.3)	1.00 ± 0.01	9.65
(−)-atropine	9.25 ± 0.06 (0.47)[a]		9.26 ± 0.02 (−0.3)	0.95 ± 0.03	9.20
(−)-scopolamine			9.09 ± 0.01 (−0.17)	1.01 ± 0.02	9.35
(+)-N-methyl-3-quinuclidinylbenzilate			9.12 ± 0.04 (0.28)	0.95 ± 0.03	9.43
(−)-N-methyl-3-quinuclidinylbenzilate			9.31 ± 0.03 (−0.78)	1.02 ± 0.03	9.43
Lachesine[e]	8.85 ± 0.01 (0.18)	0.98 ± 0.06	8.92 ± 0.04 (0.12)	0.97 ± 0.04	8.75
N-dimethyl-4-piperidinyl-diphenylacetate			9.00 ± 0.03 (0.14)	0.99 ± 0.03	9.06
Tricyclamol[e]			8.77 ± 0.1 (−0.11)	0.98 ± 0.01	8.92
Benzhexol[e]	8.14 ± 0.03 (−0.21)	0.98 ± 0.02			8.27
Transergan[e]			8.01 ± 0.01 (−0.86)	1.01 ± 0.02	8.10
Secergan[e]			7.96 ± 0.02 (−0.25)	0.99 ± 0.01	8.29
PrBCh	7.98 ± 0.05[b]	1.00 ± 0.02[b]	7.86 ± 0.04 (0.37)	0.92 ± 0.04	8.03[c]
N-methyl-3-quinuclidinyl-diphenylacetate	7.91 ± 0.03 (−0.1)	1.09 ± 0.01			7.86
Caramiphen[e]	7.67 ± 0.02 (−0.6)	0.97 ± 0.03			7.62
Diphenacetylcholine	7.20 ± 0.02 (0.53)	0.91 ± 0.02	7.32 ± 0.03 (0.31)	0.93 ± 0.02	7.16
N-dimethyl-4-piperidinyl-phenylacetate	6.34 ± 0.01 (0.05)	0.95 ± 0.06			6.20
N-methyl-3-quinuclidinyl-phenylacetate	5.84 ± 0.61 (−0.21)	0.99 ± 0.02			5.50
Decyltrimethylammonium	5.65 ± 0.03	1.12 ± 0.04			5.28
N-dimethyl-3-piperidinyl-phenylacetate	5.54 ± 0.03 (0.15)	0.95 ± 0.01			5.11

[a] log (K_A) ± SEM (NNRC).

[b] Mean of 6 determinations.

[c] R. B. Barlow, personal communication.

[d] References (5, 16, 23–29); these values refer to determinations of antimuscarinic potency in the isolated guinea pig ileum.

[e] Chemical identities of these compounds are given in reference (26).

$$[L_t'] = (1 + K\cdot[L_t])/K' + [R_t]/2$$

Provided that $1/K' \gg [R_t]/2$, the displacement curve follows the simple form of Eq. (7), and to a first approximation, the form of the curve is independent of the actual values of both K and $[L_t]$ (since $K\cdot[L_t] \ll 1$). When $1/K' < [R_t]/2$, the method loses precision, due to depletion of the competing ligand, and suffers from precisely the same disadvantages incurred in this situation by direct measurements of the binding of [^{3}H]-ligands. Where the condition $1/K' \gg [R_t]/2$ was fulfilled, experimental data were fitted to the equation

$$B_{corr} = B_t\cdot[L_t]/[L] = [RL^0]/(1 + K'_{app}\cdot[L_t']) + NS \quad (10)$$

yielding the parameters $[RL^0]$, K'_{app}, and an estimate of the nonspecific binding, NS, which was identical in all cases to the binding measured in the presence of 10^{-6} M unlabelled atropine, or QNB. The values of K' were calculated from K'_{app} by multiplication by the factor $[1 + K\cdot[L_t])$. In the

case of lachesine, atropine and MeAtr, where $1/K'$ approaches $[R_t]/2$, values of [^{3}H]PrBCh binding in the presence of the competing ligand were fitted to Eq. (8), after prior subtraction of nonspecific binding, using an independent estimate of the concentration of binding sites, $[R_t]$. Values of K', and the nearest neighbor correlation coefficients of the fits for these antagonists are summarized in Table 4. For display, values have been expressed in terms of occupancy of the binding site, given by

$$P = 1 - \frac{(B_{corr} - NS)}{[RL^0]} = K'_{app} \cdot [L_t']/(1 + K'_{app} \cdot [L_t'])$$

and are plotted in Fig. 4. Hill coefficients were obtained by fitting a straight line to plots of log $P/(1 - P)$ against log$[L_t']$ [cf. Eq. (3)].

Equation (10) provides an excellent fit to the competition data over a range of K' extending from 10^5 to 10^8 M^{-1}; this is illustrated by the closeness of individual values of the Hill coefficients to 1.0, and by the small size of the nearest neighbor residual correlation coefficients. The Hill coefficients for atropine/[^{3}H]PrBCh, and MeAtr/[^{3}H]PrBCh (see Fig. 4) competition are both significantly greater than 1.0, when the value plotted on the abscissa of the graph is the total ligand concentration $[L_t']$; that neither curve should be described by Eq. (10) is expected from non-fulfillment of the condition $1/K' > [R_t]/2$; both curves are, however, well fitted by Eq. (8). The high values of n do not therefore indicate cooperativity of binding, but are a result of depletion of the competing ligand, which also leads to underestimation of the affinity constant (30).

Displacement of [^{3}H]PrBCh by all of these compounds thus accords well with the assumption of simple competitive binding to a single, essentially homogeneous set of binding sites. In agreement with this hypothesis, the affinity constants for PrBCh itself, and for atropine and MeAtr measured by competition, agree closely with the values derived from direct binding measurement using the [^{3}H]ligands.

Competition with [^{3}H]MeAtr. The diffi-

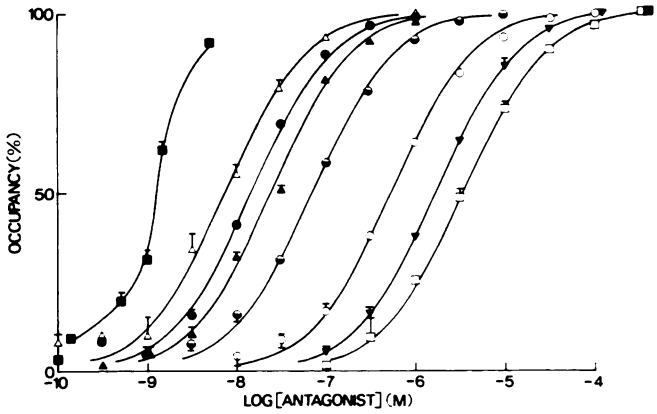

FIG. 4. *Occupancy/concentration curves for unlabelled antagonists derived by inhibition of specific binding of [^{3}H]PrBCh*

The concentration of [^{3}H]PrBCh was 10^{-10} or 10^{-9} M, and the concentration of crude synaptosome suspension was 0.5–1.0 mg protein per ml. The abscissa shows the total concentration of the competing antagonist. ■—MeAtr; △—benzhexol; ●—N-methyl-3-quinuclidinyl-diphenylacetate; ▲—caramiphen; ◒-diphenacetylcholine; ○—N-dimethyl-4-piperidinylphenylacetate; ▼—N-methyl-3-quinuclidinylphenylacetate; □—N-dimethyl-3-piperidinylphenylacetate. Except in the case of MeAtr, the fitted curves (full lines) are of the form

$$P = K'_{app} \cdot [L'_t]/(1 + K'_{app} \cdot [L'_t])$$

where $[L'_t]$ is the total concentration of competing ligand and K'_{app} is its apparent affinity constant. The inhibition curve given by MeAtr was fitted to the more complex function described in the text. Unless otherwise indicated, the standard errors of the points lie within the size of the symbol.

culties inherent in estimation of the affinity constants of high affinity ligands which undergo substantial depletion, by competition with a radiolabelled ligand of relatively low affinity, such as [^{3}H]PrBCh, can be overcome by performing competition experiments using a high concentration of a potent [^{3}H]ligand such as [^{3}H]MeAtr. The required conditions are (1) $K \cdot [L_t] \gg 1$; (2) $[L_t] \gg [R_t]$. Depletion of the radioactive ligand can then be ignored. The value of K'_{app}, defined as the concentration of competing ligand needed to reduce the concentration of the bound [^{3}H]ligand to half its initial value is then given by

$$1/K'_{app} = (1 + K \cdot [L_t])/K' + [R_t]/2$$

This equation is of exactly the same form as that encountered in displacement of the binding of low affinity [^{3}H]ligands. However, the importance of depletion of the unlabelled competing ligand is reduced as a result of the reduction in its apparent affinity constant induced by the large size of the term $(1 + K \cdot [L_t])$. A series of estimates of affinity constants have been made under these conditions, using [^{3}H]MeAtr at a concentration of 10^{-8} M, giving a concentration of the active isomer equal to 25 times its dissociation constant, and at a concentration of binding sites of $2 - 4 \times 10^{-10}$ M. Calculation shows that, under these conditions, competition curves are closely described by Eq. (10). For values of K' up to 10^{10} M^{-1} there is negligible depletion of the competing agonist, and the expected Hill coefficient of the competition curve is 0.99. The data were therefore fitted to Eq. (10) without correction for depletion of the [^{3}H]antagonist and values, together with the fitted curves, again expressed as occupancy as a function of ligand concentration, are shown in Fig. 5. The corresponding values of K', n and the nearest neighbor correlation coefficient are presented in Table 4; the value of the affinity constant for (−)[^{3}H]MeAtr given in Table 2 was used to correct apparent affinity constants for the [^{3}H]MeAtr-induced shift.

Once again these data are well described by Eq. (10). The Hill coefficients of the curves are all close to 1.0; the lowest values encountered were 0.92 (PrBCh) and 0.93 (diphenacetylcholine). Examination of the nearest neighbor residual correlation coefficients of individual competition curves, however, indicates that in no case is there any significant deviation from Eq. (10).

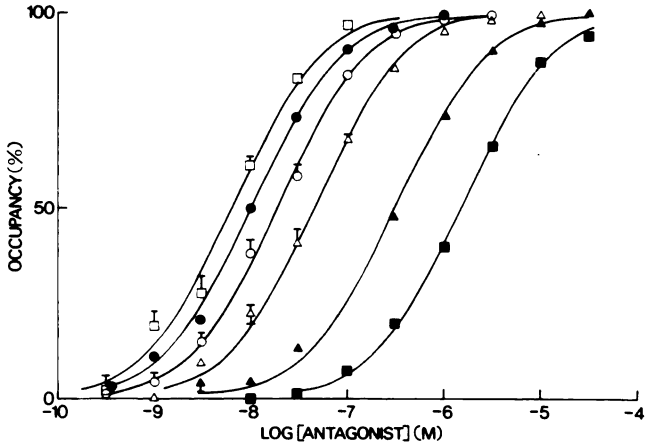

FIG. 5. *Occupancy/concentration curves for unlabelled antagonists, derived by inhibition of specific binding of [^{3}H]MeAtr*

The concentration of [(+/−)-^{3}H]MeAtr was 10^{-8} M, and the concentration of crude synaptosome fraction was 0.2 mg/ml. □—QNB; ●—MeAtr; ○—atropine; △—lachesine; ▲—PrBCh; ■—diphenacetylcholine. The fitted curves are of the form

$$P = K'_{app} \cdot [L'_t]/(1 + K'_{app} \cdot [L'_t])$$

where $[L'_t]$ is the total concentration of competing antagonist, and K'_{app} is its apparent affinity constant. Unless otherwise indicated, the standard errors of the points lie within the size of the symbols.

The affinity constants for diphenacetylcholine, lachesine, atropine and MeAtr agree closely with the corresponding values obtained by competition with [^{3}H]PrBCh (Table 4), and by direct measurement of [^{3}H]ligand binding (Table 2). The closeness of this agreement underlines the essential validity of the simple competitive binding model.

Competition with a high concentration of a high affinity radiolabelled ligand provides the best strategy for estimating K' for ligands for which $1/K' < [R_t]/2$. The major drawback of this technique is that uncertainty in the value of $K \cdot [L_t]$ used to correct K'_{app} makes a major contribution to uncertainty in the estimated value of K'.

DISCUSSION

The equilibrium binding of muscarinic antagonists to brain membrane fractions conforms closely to the simplest possible model, namely that of competitive interaction with a single, uniform set of sites. The key findings which support this conclusion are as follows:

1) Direct and competitive measurements of antagonist affinity constants are mutually consistent.

2) Binding capacities for different [^{3}H]-antagonists are equal; this contrasts with reports of unequal binding capacities for [^{3}H]Atr and [^{3}H]MeAtr in intact smooth muscle (16) and for [^{3}H]benzetimide and [^{3}H]Atr in a smooth muscle membrane preparation (17).

3) The Hill coefficients of antagonist binding curves are close, if not exactly equal, to 1.0.

The affinity constants for atropine, MeAtr and QNB derived from the present series of experiments agree well with published estimates, derived from both direct and competitive measurements of binding of these antagonists to brain and smooth muscle subcellular preparations, and in the case of [^{3}H]Atr and [^{3}H]MeAtr, to intact smooth muscle (4, 11, 16, 19).

The pharmacological relevance of the affinity constants determined by *in vitro* binding measurements is immediately established by comparison with the corresponding values estimated from pharmacological antagonism of whole-tissue muscarinic responses, such as smooth muscle contraction and ion flux (20, 21) or biochemical effects, such as activation of guanylate cyclase (22). A comparison of the antagonist affinity constants reported here with those derived by antagonism of contraction of the guinea pig ileum is included in Table 4. Agreement between the two sets of values is excellent, within a factor of two for all the compounds studied with affinities above 10^6 M^{-1}. The only sign of systematic deviation occurs for the compounds of lowest affinity, namely 3-phenylacetoxy-N-methylpiperidine, 3-phenylacetoxyquinuclidine, and decyltrimethylammonium; here, the concentrations used to produce antagonism may be high enough to cause effects additional to muscarinic blocking activity. The correlation coefficient of the logarithms of affinity constants derived from binding studies with the corresponding pharmacological values is 0.994. The slope and intercept of the regression line (pharmacological on binding data) are 1.1 ± 0.03 and −0.83, respectively; if antagonists with affinity constants less than 10^6 M^{-1} are omitted, these values become 1.03 ± 0.04 and −0.17, respectively. Over the range 10^{10}–10^6 M^{-1}, the two methods thus give virtually identical estimates of the affinity constant.

The degree of agreement which exists is perhaps particularly surprising in that the comparisons made are between different tissues (brain, with smooth muscle) and also between different species (rat with guinea pig). In relation to the latter point, it has also been shown that the antagonist and agonist binding properties of the muscarinic receptors of the frog brain are also virtually identical to those of the rat brain.[2] These results suggest that the ligand-binding subunits of central and peripheral muscarinic receptors are similar, if not identical, and that the functional properties of the receptor have been strongly conserved during the course of vertebrate evolution.

Studies of the binding of agonists (see accompanying paper) strongly suggest the existence of two major classes of muscarinic receptor in brain subcellular preparations, which differ widely (by up to 300-fold) in their affinities for potent agonists.

[2] Birdsall, Hulme, Mehta, unpublished data.

The present study enables us to put an upper limit on the extent to which these two populations of sites can be distinguished by the binding of antagonists. The lowest Hill coefficients measured for antagonist binding have been about 0.9, corresponding to a difference of no more than threefold between the relative affinities of the subpopulations of binding sites for the corresponding ligands. It seems unlikely that such small heterogeneities can be reliably established by equilibrium binding measurements. Investigation of the kinetics of association and dissociation should eventually provide much better insight into the question of whether real differences exist. If they do, they seem to be of little practical significance as the internal consistency of the binding studies and their excellent agreement with pharmacological studies demonstrate.

In summary, we have shown that studies of the specific binding of radiolabelled muscarinic antagonists to brain membrane fractions *in vitro* yield estimates of affinity constants which are almost identical to the pharmacologically estimated values. The hypothesis that the binding of these ligands to muscarinic receptors is competitive and mutually exclusive and described by the law of mass action is fulfilled to a high degree of precision. These studies have therefore established the validity and internal consistency of the *in vitro* binding technique, which is a prerequisite for its extension to the much more complex problems of agonist interactions.

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