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The Binding of Agonists to Brain Muscarinic Receptors

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SUMMARY

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The interaction of agonists with muscarinic receptors has been investigated by measuring the binding of [^{3}H]muscarinic agonists to membrane preparations from the rat cortex and by means of [^{3}H]agonist/agonist and [^{3}H]antagonist/agonist competition experiments. The binding data can be explained by the presence of two major populations of agonist binding sites which do not interconvert during the binding experiments and have the same affinity constants for antagonists. The ratio of the affinity constants of an agonist for the two sites can vary from 1 to about 275. There is quantitative agreement between the agonist affinity constants for the two sites and parameters derived from the action of muscarinic agonists on smooth muscle. A third (minor) population of "super-high" affinity agonist binding sites has been detected.

INTRODUCTION

We have previously presented evidence that some agonists exhibit unusual binding properties for the muscarinic receptor (1–3). This paper is concerned with the detailed analysis of the interactions of agonists, alone and in combination with antagonists, with the muscarinic receptors in the rat brain.

MATERIALS AND METHODS

Radiolabelled Agonists

[^{3}H]Oxotremorine-M. N-[4(2-Oxopyrrolidin-1′-yl)but-2-ynyl]dimethylamine was prepared by a modification of the method of Bebbington *et al.* (4); for the preparation of 4-(2-oxopyrrolidin-1′-yl) but-2-yne from pyrrolidin-2-one and propargyl bromide, sodium hydride was used as the base.

The tertiary amine (290 μg/100 μl acetone) was added to acetone (0.5 ml) containing [^{3}H]methyl iodide (5 mCi, 3.5 Ci/mM). After 16 hr at room temperature in the dark the solution was evaporated under a stream of nitrogen to a volume of about 100 μl and purified by thin layer chromatography (Merck pre-coated silica gel plates) in chloroform:methanol:acetic acid:water (65:25:5:5). The radiolabeled [^{3}H]Oxo-M[1] (R_f ~0.2 c.f. starting material ~0.6) was eluted with 3:1 methanol:water, evaporated to dryness, taken up in methanol, filtered and stored at −20°. The product (specific activity 3.5 Ci/mM, radi-

[1] The abbreviations used are: Oxo-M, Trimethyl[4-(2-oxopyrrolidin-1′-yl) but-2-ynyl]ammonium iodide; PrBCh, N,N-dimethyl-N-propyl-2-aminoethyl-benzilate; NMS, N-methylscopolamine; C_n-TMA, monoalkyltrimethylammonium salts, C_n being the number of carbon atoms in the n-alkyl chain; ACh, acetylcholine; PrBCM, N-2-chloroethyl-N-propyl-2-aminoethyl-benzilate; dilvasene, N,N,N-trimethyl-2-aminomethyl-1,3-dioxolane; d/l-cis-methyl-dilvasene, d/l-cis-4-methyl-N,N,N-trimethyl-2-aminomethyl-1,3-dioxolane; methylfurmethide, 5-methyl-N,N,N-trimethyl-2-aminomethyl-furan; furmethide, N,N,N-trimethyl-2-aminomethylfuran; K_A, affinity constant.

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ochemical purity >98%) had the same R_f as Oxo-M on silica gel G thin layer plates in the above solvent system, in chloroform:methanol:acetone:acetic acid:water (30:10:40:10:5) and in chloroform:acetone:diethylamine (50:40:10). [^{3}H]Oxo-M of higher specific activity was also synthesized using [^{3}H]CH_3I (specific activity 10 Ci/mM).

[^{3}H]Methylfurmethide. 5-Methylfurfuryldimethylamine, prepared by the method of Holdren (5), was quaternized by [^{3}H]-methyl iodide (5 mCi, 3.5 Ci/mM) under the same conditions as for the preparation of [^{3}H]Oxo-M. The product, purified as described for [^{3}H]Oxo-M, was greater than 98% radiochemically pure and had the same R_f as an authentic sample of methylfurmethide, as judged by thin layer chromatography in the above three solvent systems.

The preparation of [^{3}H]propylbenzilylcholine and [^{3}H]N-methylscopolamine are described elsewhere (6).

[^{3}H]Acetylcholine (10 Ci/mM) and [^{3}H]methyl iodide (3.5 Ci/mM) were obtained from the Radiochemical Centre, Amersham; [^{3}H]pilocarpine (30 Ci/mM) and [^{3}H]methyl iodide (10 Ci/mM) were obtained from New England Nuclear.

Chemicals. The alkyltrimethylammonium compounds (C_2-C_6 TMA) were prepared by quaternization of trimethylamine with the appropriate alkyl bromide in butan-2-one. The precipitated products were filtered and recrystallized from ethanol/ether. Oxotremorine-M, furmethide and methylfurmethide were synthesized using minor modifications of published procedures (4, 5, 7). The resolved optical isomers of acetyl β-methyl choline were kind gifts of Dr. J. M. Young; (d/l)cis-methyldilvasene and dilvasene were generous gifts of Professor B. Belleau.

All other materials were obtained from commercial suppliers as reagent grade or better and were used without further purification.

Binding assays. The detailed procedure is given elsewhere (6). Briefly, a crude synaptosome (P2) fraction from rat cerebral cortex was prepared and diluted to a protein concentration of 0.2–1.0 mg/ml in Krebs-Henseleit solution. Following preincubation at 30° for 15 min, 1 ml aliquots were pipetted into plastic microcentrifuge tubes containing the [^{3}H]ligand together with a competing drug if appropriate, giving a final volume of 1.02 ml. Assays were always carried out in quadruplicate. The tubes were incubated at 30° for 15 min. This temperature was originally chosen because of the relative instability of the muscarinic receptor from smooth muscle. It provides a satisfactory compromise between the long-term stability of the receptor and a rapid approach to equilibrium. The nature of the results described in this paper are not changed by incubation at 0° or 37°. After incubating, the tubes were centrifuged at 14,000 × g for 30 sec (1 mg protein/ml) or 60 sec (0.2 mg/ml), the pellets rapidly washed three times with Krebs-Henseleit solution and dissolved in scintillant medium (Toluene:Triton X-100; water, 8:4:1, butyl PBD, 4g/liter) and bound radioactivity measured by liquid scintillation spectrometry at 30% counting efficiency. Some experiments using [^{3}H]ACh as a radioligand necessitated enrichment of the receptor above that present in a P2 pellet (1.3–2.0 nmole/g protein). For these experiments a purified lysed synaptosome fraction was prepared. Non-specific binding was estimated by carrying out incubations in the presence of 10^{-6} M atropine or N-methyl atropine.

Rat cortices were homogenized in 0.3 M sucrose/1 mM $MgCl_2$/50 μM $CaCl_2$ (10 vol) in a Potter Elvehjem homogenizer. The homogenate was spun at 900 × g for 10 min, the pellet rehomogenized and spun again at 900 × g for 10 min. The pooled supernatants were then centrifuged at 10,000 × g for 20 min, and the pellet resuspended in 10 μM $CaCl_2$ for 15 min at 4° (1 mg protein/ml). The lysed membranes were layered on a 3-step sucrose gradient (0.6 M/0.85 M/1.2 M) containing 50 μM $CaCl_2$ and centrifuged at 100,000 × g for 2 hr. The membrane fraction at the 0.85/1.2 M sucrose interface was diluted 1:1 with 50 μM $CaCl_2$, centrifuged at 100,000 × g for 20 min and the pellets frozen rapidly, stored at −70° and used generally within one week of preparation. The above procedure resulted in a 2–5 fold enrichment in the concentration of muscarinic receptors.

In experiments using [^{3}H]PrBCh and [^{3}H]NMS the standard errors of the mean of the quadruplicate determinations of bound radioactivity were generally in the range 0.5–2.0% and only on rare occasions exceeded 3%. Experiments using [^{3}H]Oxo-M or [^{3}H]ACh had larger errors in the estimates of the bound radioactivity, the SEM values being in the range 1–4% and rarely exceeding 6%. Preliminary experiments using the tritiated ligands indicated that, at the concentrations used, binding reached an equilibrium in less than 2 min and remained at that level for over 1 hr.

In all experiments involving choline esters, susceptible to cholinesterases, the membrane suspension was treated with 10^{-5} M neostigmine during the last five minutes of preincubation. This procedure inhibited >99% of the cholinesterase activity without markedly affecting the agonist or antagonist binding properties. Protein concentrations were determined by the method of Lowry *et al.* (8).

Data analysis. Theoretical binding curves were fitted to the experimental data points by non-linear least squares regression analysis using a computer program described by Batchelor (9). Individual data points were the mean of four observations and were weighted according to the inverse of the standard error of the mean. The program provides estimates of the best-fit parameter values together with estimates of the SEM and confidence limits for each parameter. The latter estimates assume that each parameter makes an independent contribution to the residual sum of squares about the optimum solution. This is not necessarily the case for non-linear models and such estimates must be regarded as conservative.

RESULTS

Agonist-[^{3}H]antagonist competition studies. We have so far examined in detail 21 agonists and all of them, at sufficiently high concentrations, completely inhibit the specific binding of [^{3}H]antagonists. The binding of the agonists can thus be deduced from an analysis of this competitive behavior. When the [^{3}H]antagonist has a relatively low binding constant ($K_A < 1/10R_o$ M^{-1}, where R_o is the concentration of receptor sites in the binding assay) and a sufficiently high specific activity so that it can be employed at concentrations where only a small fraction of the receptors are occupied, the analysis is particularly simple, the depression of specific [^{3}H]antagonist binding being a direct measure of the receptor sites occupied by the agonist. [^{3}H]Propylbenzilylcholine ([^{3}H]PrBCh, $K_A = 1 \times 10^8$ M^{-1}; specific activity 40 Ci/mM) has been the radioligand of choice in these experiments. At 10^{-10}–10^{-9} M concentrations, the receptor occupancy is 1–10%. An example of an agonist-[^{3}H]PrBCh competition curve is shown in Fig. 1. The d/l-cis methyldilvasene-[^{3}H]PrBCh inhibition curve does not correspond to a simple mass action curve, as may be seen by comparison with a theoretical mass action curve, calculated from its I_{50} value (6.5×10^{-6} M). The experimental curve is flatter than the mass action curve; the deviation may be quantified by comparison of the ratio of the concentrations of agonist for 9% and 91% inhibition of ~7,000, compared with the value of 100 expected for mass action binding. The deviations from mass action behavior are especially marked at low agonist concentrations.

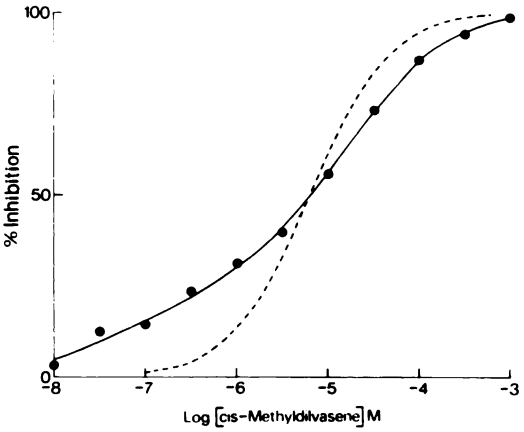

FIG. 1. *Occupancy concentration curve for (d/l) cis methyldilvasene derived by inhibition of the specific binding of [^{3}H]PrBCh*

The concentration of [^{3}H]PrBCh was 10^{-10} M and that of the crude synaptosome preparation 1.0 mg/ml. The dotted line shows a mass action curve centered on the I_{50} value for displacement of [^{3}H]PrBCh.

A Hill plot [log $(I/(100 - I))$ vs. log C where I is the percent inhibition of specific binding] of the data provides another way of assessing the deviations from a simple mass action relationship (Fig. 2). This shows that the slope is not constant but is everywhere less than 1 and reaches a minimum of 0.31 in the region I ~30%. Curves of this general form have been obtained for most agonists as can be seen from the listing of Hill coefficients in Table 1. The lowest value was found for the oxotremorine analogue, oxotremorine-M and the values range up to unity for the lowest members of the table, all of which are partial agonists.

Flat binding curves have also been observed for agonists in competition with [^{3}H]atropine, [^{3}H]N-methylatropine and [^{3}H]N-methylscopolamine. However, the analysis of such experiments is, in general, complicated because it is difficult to avoid either relatively high occupancies by these [^{3}H]antagonists (because of the high affinity constants) or changes in the free concentration of the [^{3}H]antagonist during the agonist-[^{3}H]antagonist competition experiment. The problems raised in analyzing such curves for antagonists have been discussed elsewhere (6); artifacts which can arise from a simple approach to the analysis of inhibition curves of potent [^{3}H]antagonists by agonists will be the subject of a separate paper.[2] If the concentration of the [^{3}H]antagonist is much greater than the concentration of receptors, the inhibition curves will be displaced by a factor $[(1 + K_I [I])$, where K_I is the affinity constant of the [^{3}H]antagonist at a concentration, I] from the intrinsic inhibition curve. An example is shown in Fig. 3 for the methylfurmethide/[^{3}H]N-methylscopolamine inhibition curve. The same values of the Hill coefficient and for the 91%/9% inhibitory concentration ratio are observed. In addition, the above correction of the inhibition curve gives an 'intrinsic' curve which is very close to that for methylfurmethide-[^{3}H]PrBCh competition. This establishes that the low Hill slopes are a function of agonist binding and not of an agonist-antagonist interac-

TABLE 1

I_{50} Values and Hill coefficients for agonist competition with [^{3}H]PrBCh. n_H values are minimum measurable values ([^{3}H]PrBCh = 10^{-10} M)

The values are, in general, means of at least three separate determinations.

Agonist	n_H	I_{50}
		M
Oxotremorine-M	0.25	1.6×10^{-6}
(d/l)-cis-methyldilvasene	0.31	6.5×10^{-6}
carbachol	0.33	1.5×10^{-5}
acetylcholine	0.45	3.3×10^{-6}
methylfurmethide	0.48	3.0×10^{-6}
furmethide	0.52	1.3×10^{-5}
(+)-acetyl-β-methylcholine	0.52	1.0×10^{-5}
(+)-muscarine	0.62	8.3×10^{-6}
dilvasene	0.70	4.5×10^{-5}
arecoline	0.75	5.0×10^{-6}
C_4-TMA	0.75	6.3×10^{-5}
oxotremorine	0.80	4.6×10^{-7}
tetramethylammonium	0.80	5.0×10^{-4}
C_5-TMA	0.82	2.0×10^{-5}
(−)-acetyl-β-methylcholine	0.84	6.4×10^{-4}
propionylcholine	0.89	1.7×10^{-4}
C_2-TMA	0.93	2.3×10^{-4}
C_3-TMA	0.93	2.3×10^{-4}
pilocarpine	0.95	1.7×10^{-5}
C_6-TMA	1.00	6.3×10^{-6}
butyrylcholine	1.05	1.6×10^{-4}

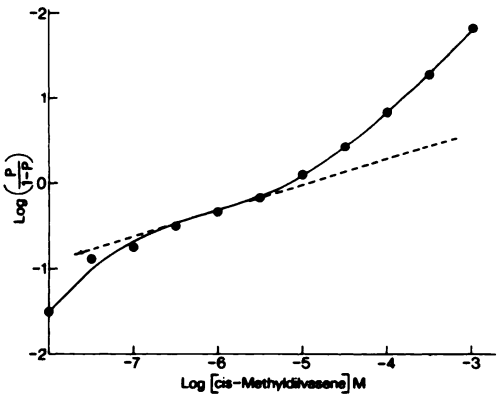

FIG. 2. *Hill Plot of (d/l) cis methyldilvasene-[^{3}H]PrBCh competition curve*

The data used to construct the Hill plot are given in Fig. 1. The solid line shows the multiphasic nature of the plot, with the gradient approaching unity at the extremities and having a minimum value of 0.31 (indicated by the dotted line) at ~30% occupancy.

[2] Wells, Birdsall, Burgen, Hulme, manuscript in preparation.

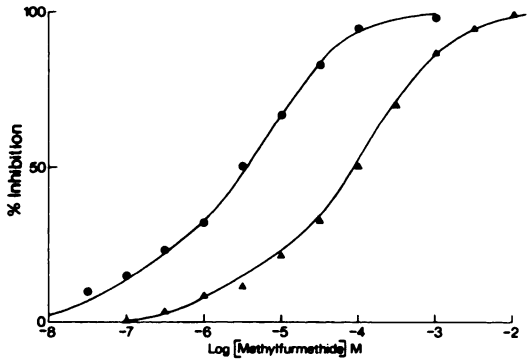

FIG. 3. *Inhibition by methylfurmethide of the specific binding of (a) [^{3}H]Propylbenzilylcholine and (b) [^{3}H]N-methylscopolamine*

(a) The concentration of [^{3}H]PrBCh was 10^{-10} M and the membrane protein concentration, 1.0 mg/ml. The line through these data points (●) (discussed later in RESULTS) is a theoretical curve for 25% of the sites having a high affinity (K_A = 1.3 × 10^7 M^{-1}), the remaining sites having a K_A of 1.3 × 10^5 M^{-1}.

(b) The concentration of [^{3}H]N-methylscopolamine (3 × 10^{-9} M) was considerably higher than the concentration of muscarinic receptors (0.3 × 10^{-9} M, 0.2 mg/ml protein) and the dissociation constant for binding of [^{3}H]N-methylscopolamine to muscarinic receptors in the rat cortex (1.4 × 10^{-10} M, Birdsall and Hulme, unpublished results). The curve through the data points for [^{3}H]N-methylscopolamine/methylfurmethide (▲) is parallel to the [^{3}H]PrBCh/methylfurmethide inhibition curve. The actual parallel shift of a factor of 24 agrees with the value of 22 predicted by simple competition [$(1 + K_{NMS}[NMS])$ where K_{NMS} is the affinity constant for binding of [^{3}H]N-methylscopolamine].

tion. Furthermore, the fact that the antagonist can be totally displaced from the receptor under these conditions of high occupancy (~96%) at least places severe restrictions on the possibility of ternary agonist-antagonist receptor complexes while not totally excluding it.

Several explanations for the low Hill coefficient have been examined. First, the possibility of receptor desensitization may be considered. In muscarinic responses in smooth muscle, apparent desensitization is concentration and time-dependent, the time course being on the scale of seconds to minutes. However, exposure of membrane preparations to concentrations of carbachol (10^{-7}–10^{-4} M) for periods of 15–1000 sec did not result in any time-dependent change in the inhibition of [^{3}H]-PrBCh binding by carbachol (Fig. 4).

A second possibility is that there are negatively cooperative interactions between agonist binding sites. This possibility can be examined by irreversibly blocking some of the receptors with propylbenzilylcholine mustard (PrBCM) (10). The principle of this test is as follows: Suppose that the receptors are organized as a negatively cooperative dimer. If random alkylation of the receptors occurs then when 50% are inactivated only 33% of the residual receptors will be paired and the agonist inhibition curve should be steepened. If 90% of the receptors are inactivated, only 5.3% of the residual receptors will be paired and the agonist inhibition curve should be indistinguishable from a mass action curve. The experimental results (Table 2) show that there is no detectable change in the carbachol-[^{3}H]PrBCh curve even when more than 90% of the receptors have been inactivated. This excludes the possibility of simple pairwise cooperative interactions; if one considers tetrameric or hexameric cooperative groups then at 95% inactivation of the receptors, the residual multimeric arrangements will be only 7.9% and 13.2% of the total receptor groups, respectively, so that again a substantial steepening of the curves would be expected. It is therefore possible to rule out that the low slope is due to a stable oligomer of binding sites.

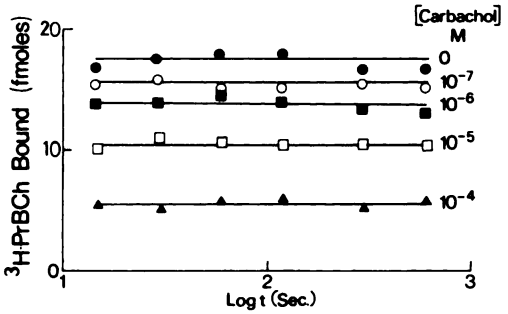

FIG. 4. *Time course of inhibition of specific [^{3}H]-PrBCh binding by carbachol*

Crude synaptosomal membranes (1.0 mg/ml) were incubated with [^{3}H]PrBCh (10^{-10} M) and appropriate concentrations of carbachol (10^{-7}–10^{-4} M) at 30° for differing lengths of time. The binding process was terminated by rapid microcentrifugation (centrifugation was essentially complete within 10–15 sec) and superficial washing of the pellets.

TABLE 2

Effect of PrBCM pretreatment of membranes on the carbachol-[^{3}H]PrBCh competition curve

Resuspended P2 pellets (0.3 mg/ml) were preincubated with PrBCM for 15 min at 30°. The suspension was centrifuged (10,000 × *g*, 20 min), resuspended in Krebs-Henseleit solution to 0.7 mg/ml and the carbachol-[^{3}H]PrBCh (1.0×10^{-9} M) inhibition curve determined for the remaining unalkylated receptors. The occlusion of the binding sites was determined using [^{3}H]PrBCh, preliminary experiments having shown that treatment of the membranes with PrBCM did not affect the affinity constant of [^{3}H]PrBCh for the unalkylated receptors. At the higher levels of receptor occlusion, the results of the carbachol-[^{3}H]PrBCh curves are considerably less precise than those measured when lower concentrations of PrBCM are used.

[Carbachol]	[Mustard] nM					
	0	0.2	0.5	1.0	4.0	10.0
M	*% inhibition of specific [^{3}H]PrBCh binding*					
10^{-7}	8	4	15	10	14	1
10^{-6}	26	27	27	31	32	23
10^{-5}	50	49	49	50	57	—
10^{-4}	72	71	69	68	80	61
10^{-3}	93	93	91	86	98	90
% occlusion of binding sites	0	17	38	67	80	92

There is, however, also the possibility that the binding sites can reversibly form associates as occurs in solution with monomer-dimer and dimer-tetramer equilibria in enzymes. If the ability to form associates resides in unalkylated binding sites, then the cooperative interactions would be maintained and the carbachol-[^{3}H]PrBCh curve at the unalkylated sites unchanged. The experiment illustrated in Fig. 5 argues against this explanation. PrBCM was reacted with the membrane preparation in the presence of a concentration of carbachol sufficient to occupy ~50% of the binding sites. After removing the carbachol, a carbachol/[^{3}H]PrBCh competition curve was carried out. The binding properties of the residual (unalkylated) receptors are clearly changed by this procedure. There is both an increase in the apparent affinity for carbachol as well as some increase in the steepness of the curve.

Undoubtedly the simplest explanation of the flat agonist/[^{3}H]antagonist competition curves is that there is some heterogeneity in the population of agonist binding sites. The simplest case to examine is that there are just two classes of binding sites which are not interconvertible (at least within the time scale and under the conditions of the experiments). An example of the fit to this model is seen in the control carbachol/[^{3}H]PrBCh curve in figure 5. The best fit, obtained by a non-linear least squares pro-

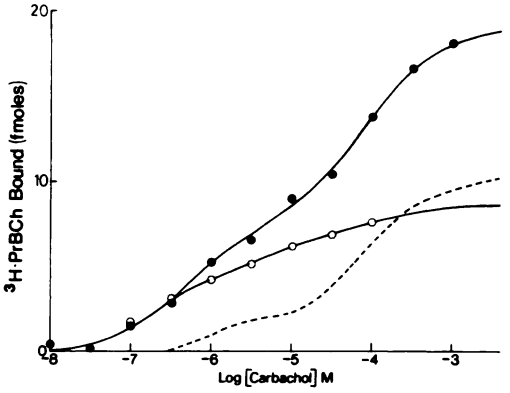

FIG. 5. *Partial protection of PrBCM binding by carbachol*

A crude synaptosomal membrane preparation (0.3 mg/ml) was incubated at 30° in the presence of carbachol (10^{-5} M) with PrBCM (4 nM) which had been cyclized to the aziridinium ion as described by Young *et al.* (10). After 15 min, the membranes were centrifuged twice. This procedure resulted in alkylation of 53% of the antagonist binding sites (measured by [^{3}H]N-methylscopolamine) whereas there was 87% alkylation of the sites in a control experiment in which there was no carbachol. The occupancy concentration curves for inhibition of the specific binding of [^{3}H]PrBCh (10^{-10} M) by carbachol in membrane preparations treated (○) or untreated (●) with PrBCM are shown. The control (●) curve is the non-linear least squares fit of the experimental data to the two-site model and corresponds to 40% of a high affinity site (K_A 2.2×10^6 M^{-1}) with the low affinity site having an affinity constant of 1.1×10^4 M^{-1}. The difference between the control and treated curve is indicated by the dotted line.

TABLE 3

Estimates of high and low affinity agonist binding constants, and of percentage of high affinity sites, from agonist/[^{3}H]PrBCh competition curves

Agonist	Log(K_H) ± SEM	Log(K_L) ± SEM	Ratio K_H/K_L	% High affinity site ± SEM
Oxotremorine-M	7.58 ± 0.10	5.14 ± 0.08	275	37 ± 3
(d/l)-cis-methyldilvasene	7.20 ± 0.15	4.80 ± 0.10	250	29 ± 4
oxotremorine	7.18 ± 0.30	6.08 ± 0.10	11	19 ± 14
acetylcholine	7.08 ± 0.14	5.08 ± 0.06	100	26 ± 4
methylfurmethide	7.04 ± 0.19	5.23 ± 0.09	65	30 ± 6
arecoline	6.92 ± 0.23	4.98 ± 0.10	87	24 ± 6
(+)-acetyl-β-methylcholine	6.40 ± 0.10	4.50 ± 0.07	80	35 ± 3
carbachol	6.33 ± 0.10	4.05 ± 0.10	195	40 ± 3
furmethide	6.34 ± 0.12	4.46 ± 0.06	75	30 ± 4
muscarine	6.28 ± 0.24	4.40 ± 0.20	75	37 ± 11
C_5-TMA	5.52	4.52	10	(32)
dilvasene	5.34 ± 0.14	3.82 ± 0.02	33	37 ± 7
C_4-TMA	5.17	3.80	23	(32)
C_6-TMA	5.20		1	
pilocarpine	5.22		1	
propionylcholine	4.23 ± 0.17	3.30 ± 0.20	8	49 ± 19
tetramethylammonium	4.15	3.08	12	(32)
(−)-acetyl-β-methylcholine	4.03	3.00	11	(32)
C_2-TMA	4.00	3.48	3	(32)
C_3-TMA	4.00	3.48	3	(32)
butyrylcholine	3.80		1	

gram, gives values of the affinity constant for the high affinity site (K_H) of (1.7 ± 0.3) × 10^6 M^{-1} and for the low affinity site (K_L) of (1.2 ± 0.2) × 10^4 M^{-1} with 42 ± 3% of the sites having high affinity and 58% low affinity. The closeness of the fit of the two site model to the agonist/[^{3}H]antagonist curves is also evident in Figs. 3 and 7. Such an analysis has been applied successfully to all the agonists studied (Table 3). It can be seen that in all cases this analysis appears to demonstrate that the low affinity site is the predominant site; the estimates of the proportion of high affinity sites show variation in the range 19–49% with a mean of 32 ± 8% (SD). For the agonists with low Hill slopes the ratio of the affinity constants (K_H/K_L) ranged from 275 to 10. For agonists whose Hill coefficients were greater than 0.8, the fitting algorithm did not converge satisfactorily with three variable parameters. In these cases, it was assumed that 32% of the sites were of high affinity and after insertion of this fixed value the resulting affinity constants obtained using the same program. For the three partial agonists, pilocarpine, butyrylcholine and hexyltrimethylammonium, a single affinity constant provided an excellent fit to the experimental data.

For fitting the two site binding curves, a less precise method that obviates the use of the iterative procedure is to use the relationships:

$$P(\text{min}) = \frac{\alpha + (1-\alpha)\beta}{1+\beta} \quad (1)$$

$$n_H\,(\text{min}) = \frac{2}{1 + \left\{\dfrac{\alpha + (1-\alpha)\beta}{\beta[1+\alpha(\beta-1)]}\right\}^{1/2}} \quad (2)$$

and

$$K_H \cdot I_{50} = \tfrac{1}{2}\{[(\beta-1)(1-2\alpha)^2 + 4\beta]^{1/2} - (\beta-1)(1-2\alpha)\} \quad (3)$$

From the occupancy of receptors, P(min), at which the Hill slope has a min-

TABLE 4

Binding of [^{3}H]agonists

Agonist	Concentration	Specific binding	Specific/nonspecific binding
	nM	*pmoles/g protein*	
[^{3}H]Oxotremorine-M	8–9	92 ± 31 (46)[a]	1.9 ± 0.40 (46)
[^{3}H]Methylfurmethide	8	35	0.25
[^{3}H]Acetylcholine	9	12 ± 5 (4)	0.14 ± 0.07 (4)
[^{3}H]Acetylcholine[b]	8–10	52 ± 16 (6)	0.61 ± 0.18 (6)

[a] Values are expressed as the mean ± standard deviation with the number of separate experiments given in parentheses.

[b] These experiments were carried out using a purified synaptosomal membrane preparation. The other experiments utilized a P2 membrane preparation.

imum value, n_H(min), it is possible to estimate α, the fraction of the high affinity sites and β, the ratio K_H/K_L. Using these values of α and β, K_H, and hence K_L can be calculated using equation (3). Construction of the appropriate isotachs of n_H and P(min) as functions of α and β aids this form of analysis.

The two site hypothesis also provides a simple explanation of the results presented in figure 5. Carbachol has selectively protected the high affinity sites from alkylation so that the competition curve of the residual sites is shifted to high affinity and correspondingly the lost sites, as demonstrated by the difference between the two curves for the unalkylated and the alkylated receptors, have certainly been selected from the low affinity receptor population. This is precisely the behavior expected for heterogeneous binding sites. The high affinity sites are more fully occupied by carbachol and hence are more effectively protected against alkylation by PrBCM than the poorly occupied low affinity sites.

Binding of [^{3}H]agonists. The binding of three tritiated agonists [acetylcholine, ([^{3}H]ACh), oxotremorine-M ([^{3}H]Oxo-M) and methylfurmethide] has been studied. In all cases it was possible to determine a binding component which was inhibited by atropine (10^{-6} M) or by self saturation with the non-radioactive ligand.[3] It can be seen in Table 4 that oxotremorine-M had the most favorable properties for further study and has the added advantage over acetylcholine of chemical stability and insensitivity to cholinesterases. Studies on [^{3}H]ACh binding were mostly carried out on membrane preparations enriched in muscarinic receptors by fractionation on a sucrose density gradient.

A binding curve for [^{3}H]Oxo-M is shown in Fig. 6. When a [^{3}H]Oxo-M binding curve is scaled to the antagonist capacity of the preparation and compared with the occupancy determined by competition with [^{3}H]-PrBCh, there is essentially coincidence of the results (Fig. 7). An analogous result is also given by acetylcholine (Fig. 7). Due to the relatively unfavorable ratio of specific to non-specific binding, the precision of the points becomes steadily less good as the [^{3}H]agonist concentration is increased. It has never been possible to obtain satisfactory data at occupancies greater than 60% and thus the 'low affinity' region of the binding curve is undefined by direct binding. However, the results shown in Fig. 7 demonstrate a 1:1 stoichiometry of agonist and antagonist binding sites in the high affinity region of the binding curve.

As the muscarinic receptor binding sites exhibit essentially no dispersion in their affinity for antagonists (6) it would be expected that if the binding of a tritiated agonist, at low receptor occupancy, were measured in the presence of increasing concentrations of an antagonist, the resulting inhibition curve should be indistinguishable from the direct binding curve for the antagonist. This essentially is observed (Fig. 8, Table 5), the mean Hill coefficient being 0.97 ± 0.04 but the antagonists have inhibition constants of the order of 2 times weaker than those given by direct binding measurements or by antagonist/[^{3}H]antagonist competition.

[3] No specific binding of [^{3}H]pilocarpine could be detected.

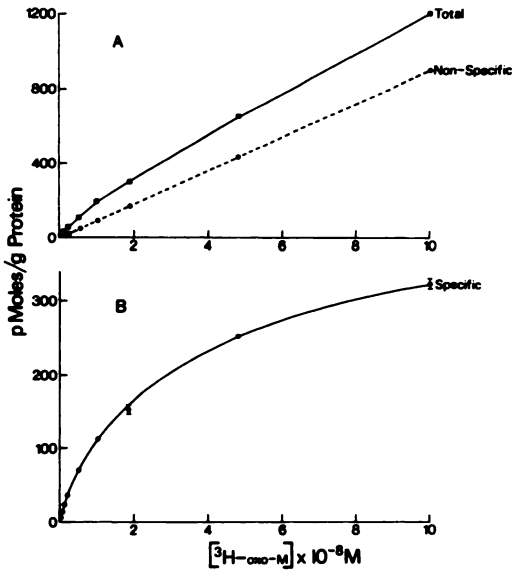

FIG. 6. *Specific and non-specific binding of [^{3}H]-oxotremorine-M*

A. ■ Binding of [^{3}H]Oxo-M in the absence of atropine (10^{-6} M). ● Binding of [^{3}H]Oxo-M in the presence of atropine (10^{-6} M). The protein concentration of the resuspended P2 pellet was 0.96 mg/ml and the total number of antagonist binding sites, measured with [^{3}H]N-methylscopolamine, was 1.49 nmoles/g protein.

B. Subtraction of the two curves in FIG. 6A gives the specific binding of [^{3}H]Oxo-M. The line through the data points is the non-linear least squares fit (discussed later in RESULTS), corresponding to 67 ± 20 pmoles/g protein with an affinity constant of 3.5 (± 1.3) × 10^8 M^{-1} and 410 ± 19 pmoles/g with a K_A of 1.8 (±0.4) × 10^7 M^{-1}. The low capacity, super high affinity site is discussed later in the Results Section.

Except where indicated, the standard errors of the mean of each data point are within the size of the symbol.

The characteristics of the binding constants for Oxo-M (Table 3) suggest that low concentrations of [^{3}H]Oxo-M will bind almost exclusively to the high affinity receptor. For instance, if the concentration of [^{3}H]Oxo-M is 10^{-8} M, less than 1% of the bound agonist is bound to the low affinity receptor. It is therefore clear that by examining the ability of unlabelled agonists to inhibit the binding of a low concentration of [^{3}H]Oxo-M (<1 × 10^{-8} M) it should be possible to study the binding properties of the high affinity receptor in isolation. Figure 9 shows agonist/[^{3}H]Oxo-M inhibition curves for a series of agonists. It is striking

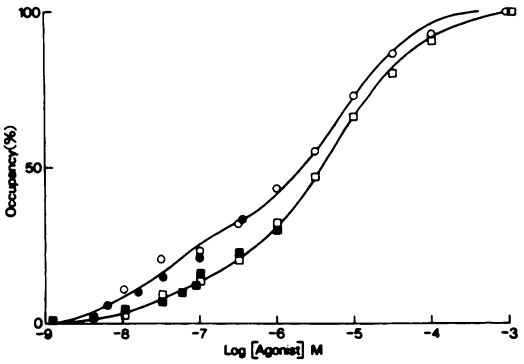

FIG. 7. *Comparison of the binding of Oxo-M and acetylcholine measured directly or by inhibition of [^{3}H]PrBCh binding*

The binding of [^{3}H]Oxo-M (●), and the Oxo-M/[^{3}H]PrBCh (10^{-10} M) (○) and the ACh/[^{3}H]PrBCh (10^{-10} M) (□) occupancy data were measured on crude synaptosome preparations; the [^{3}H]ACh binding curve (■) was measured on a purified membrane preparation. The ordinate has been normalized to the total number of the antagonist binding sites, measured with [^{3}H]N-methylscopolamine or [^{3}H]propylbenzilylcholine mustard. In the case of the [^{3}H]Ach binding curve, the capacity was 3.42 nmoles/g protein; for the other curves, the capacities were 1.4–1.7 nmoles/g protein. The curves through the data points are those predicted for (a) 30% high affinity site (K_A 3.7 × 10^7 M^{-1}), 70% low affinity site (K_A 1.7 × 10^5 M^{-1}) [Oxo-M] and (b) 25% high affinity site (K_A = 1.2 × 10^7 M^{-1}), 75% low affinity site (K_A 1.2 × 10^5 M^{-1}) [ACh].

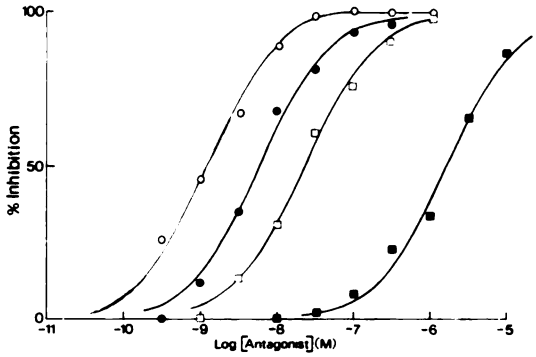

FIG. 8. *Occupancy concentration curves for unlabelled antagonists derived by inhibition of the specific binding of [^{3}H]Oxo-M*

The concentration of [^{3}H]Oxo-M was 8–9 × 10^{-9} M and the concentration of crude synaptosome suspension, 1 mg/ml (0.2 mg/ml in the case of atropine). ○ = atropine, ● = lachesine, □ = propylbenzilylcholine, ■ = decyltrimethylammonium bromide. The curves are best fit mass action curves through the experimental points.

TABLE 5

Results of antagonist/[^{3}H]Oxotremorine-M competition experiments and comparison with results of antagonist/[^{3}H]antagonist competition experiments

The concentration of [^{3}H]Oxotremorine-M was 8–9 nM and the protein concentration of resuspended P2 pellet 0.8–1.0 mg/ml. For potent antagonists the protein concentration was reduced to 0.2 mg/ml in order to reduce the depletion of the free concentration of the competing antagonist. Values are the mean of, in general, at least two separate experiments, the I_{50} values varying by less than 40%. Values for log K_{ANT} are taken from reference (6).

Antagonist	I_{50}	n_H	$\log(I_{50}^{-1})$	$\log K_{ANT}$
	M		M^{-1}	M^{-1}
PrBCh	2.2×10^{-8}	0.85	7.67	7.93
(−)-atropine	3.0×10^{-9}	0.96	9.05	9.23
lachesine	5.2×10^{-9}	1.10	8.28	8.81
benzhexol	2.5×10^{-8}	0.92	7.86	8.15
C_{10}-TMA	1.0×10^{-6}	1.00	6.00	5.68
(−)-N-methyl-atropine	7.0×10^{-10}		9.15	9.46

that these appear to be normal mass action curves of standard slope, quite unlike those curves for agonist/[^{3}H]antagonist competition. Indeed the calculated Hill slopes for the 17 agonists are all greater than 0.80 (Table 6). If one considers the five agonists at the top of Table 1, the mean Hill coefficient for inhibition of [^{3}H]PrBCh binding is 0.36 whereas when measured against [^{3}H]-oxo-M, the mean is 0.89. When the affinity constants derived from the two site analysis (K_H) are compared with the inhibition constant against [^{3}H]Oxo-M (Fig. 10) there is an excellent correlation ($r = 0.985$, $p < 0.001$) but the points are not randomly distributed about the line of equivalence. In fact the inhibition constant versus [^{3}H]Oxo-M ($K_{oxo\text{-}M}$ which is equal to I_{50}^{-1}) is on average 40% higher than that determined by the analysis of the agonist/[^{3}H]antagonist competition curves.

The explanation for these discrepancies becomes clearer on re-examination of the direct binding curve of [^{3}H]Oxo-M. A Scatchard plot of this data (Fig. 11) shows that the binding is not to a single site but may be analyzed by a non-linear least squares iterative procedure in terms of a high affinity site of capacity (410 + 19) pmoles/g protein (the number of antagonist binding sites measured using [^{3}H]N-methyl scopolamine was 1,490 pmoles/g protein) and affinity constant 1.8 (± 0.4) × 10^7 M^{-1} and a super high affinity binding site with an affinity constant 3.5 (± 1.3) × 10^8 M^{-1} and a capacity (67 ± 20) pmoles/g protein. The curves in Fig. 6 and Fig. 11 are calculated using these parameters and fit the experimental data excellently. This analysis suggests that there are three binding sites for Oxo-M consisting of 68%, 27.5% and 4.5% of the total available binding sites. A curved Scatchard plot of the same type as depicted in Fig. 11 has been found for [^{3}H]ACh binding (data not shown).

The inclusion of the minor super high affinity site rationalizes the discrepancies

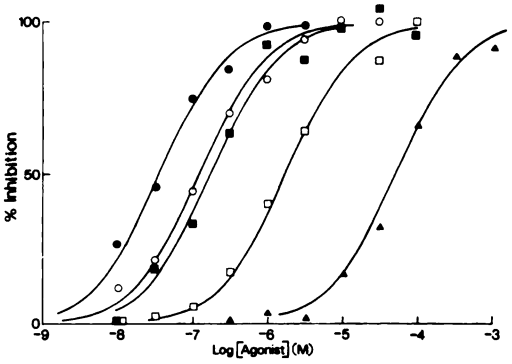

FIG. 9. *Occupancy concentration curves for unlabelled agonists derived by inhibition of the specific binding of [^{3}H]Oxo-M*

The concentration of [^{3}H]Oxo-M was 8–9 × 10^{-9} M and the concentration of crude synaptosome suspension 1 mg/ml. ● = oxotremorine, ○ = (d/l) cismethyldilvasene, ■ = acetylcholine, □ = dilvasene, ▲ = ethyltrimethylammonium bromide. The curves are best fit mass action curves through the experimental points.

TABLE 6

Results of agonist/[^{3}H]Oxotremorine-M competition experiments

The concentration of [^{3}H]Oxotremorine-M was 8–9 nM and the protein concentration of resuspended P2 pellets 0.7–1.0 mg/ml. Values are the mean of, in general, at least two separate experiments, the I_{50} values varying by less than 40%.

Agonist	I_{50}	n_H
Oxotremorine-M	2.0×10^{-8}	0.85
oxotremorine	3.8×10^{-8}	1.00
(d/l)-cis-methyldilvasene	1.1×10^{-7}	0.86
methylfurmethide	1.6×10^{-7}	0.80
acetylcholine	2.0×10^{-7}	1.06
carbachol	2.4×10^{-7}	0.89
(+)-acetyl-β-methylcholine	3.5×10^{-7}	0.84
furmethide	6.3×10^{-7}	0.81
C_5-TMA	1.3×10^{-6}	0.88
dilvasene	1.8×10^{-6}	0.84
pilocarpine	2.7×10^{-6}	0.82
C_4-TMA	2.9×10^{-6}	1.12
C_6-TMA	4.3×10^{-6}	0.87
tetramethylammonium	3.6×10^{-5}	0.88
(−)-acetyl-β-methylcholine	3.9×10^{-5}	0.96
C_3-TMA	4.7×10^{-5}	1.04
C_2-TMA	5.9×10^{-5}	0.84

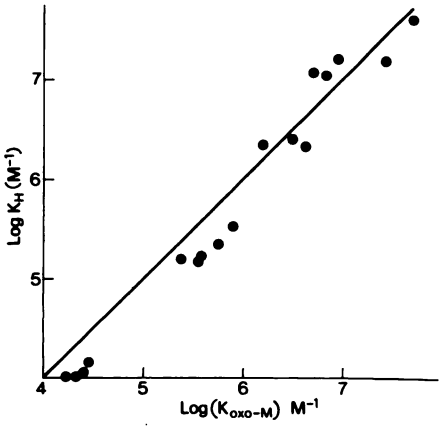

FIG. 10. *Comparison of the values of K_H and the apparent affinity constants for inhibition of [^{3}H]Oxo-M binding (K_{OXO-M}) by muscarinic agonists*

The line represents the line of equivalence. The correlation coefficient is 0.985 between the values of K_H and K_{OXO-M} ($p < 0.001$).

in affinity constants determined by competition with [^{3}H]agonists and [^{3}H]antagonists. In the [^{3}H]Oxo-M competition experiments, the concentration of agonist was 8–9 × 10^{-9} M. At this concentration over half the binding is to the super high affinity site and so inhibition experiments with other agonists examine competition at both high and super high sites. This explains why the Hill coefficients are less than 1 in these experiments. When the binding of [^{3}H]Oxo-M to the two sites is taken into account, there is excellent quantitative agreement and internal consistency between the results of both the binding of agonists and antagonists whether measured directly, or in competition with a [^{3}H]agonist or [^{3}H]antagonist.

In the light of this analysis, it is possible to understand the result of the agonist protection experiment (Fig. 5), which did not quite give the expected result in that the Hill coefficient for carbachol/[^{3}H]PrBCh competition of the alkylated membranes did not approach 1. Using affinity constants for carbachol of 1.2×10^4, 1.7×10^6 and 1.0×10^8 M^{-1}, at the concentration of carbachol used in the experiment (10^{-5} M), the occupancies of the three sites are 0.10, 0.94 and 0.999, respectively. It would therefore be expected that the PrBCM would not alkylate appreciably the super high site (SH) and would react with the high affinity site (H) at ~5% of the rate for alkylation of the

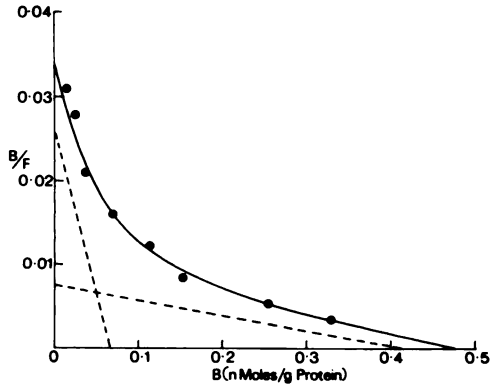

FIG. 11. *Scatchard plot of the specific [^{3}H]Oxo-M binding curve shown in Fig. 6*

The curve is the non-linear least squares fit to the experimental data with 410 ± 19 pmoles/g protein of a high affinity site [K_A (1.8 ± 0.4) × 10^7 M^{-1}] and 67 ± 20 pmoles/g protein of a super high affinity site [K_A (3.5 ± 1.3) × 10^8 M^{-1}]. The total number of antagonist binding sites, measured with [^{3}H]N-methylscopolamine was 1.49 pmoles/g protein.

low affinity site (L). Since 55% of the sites were alkylated in this experiment, the ratio of the capacities L:H:SH should have changed from 15:6:1 to 3.3:5.7:1. The main effect is a selective reduction in the L site but because of the increased proportion of SH site in the alkylated membranes, the Hill coefficient is still considerably less than 1. The agonist protection experiment using cis-methyldilvasene as the agonist also showed evidence for the presence of the SH site (data not shown).

In view of the cumulative errors in (a) the analysis of the agonist/[^{3}H]PrBCh curves, (b) the determination of $\log I_{50}^{-1}$ and n_H of agonist/[^{3}H]Oxo-M competition and (c) the analysis of the [^{3}H]Oxo-M binding curve, it is not possible from these experiments to calculate the affinity constants for nonradioactive agonists for the SH sites. In general it would be expected that an agonist with a similar pattern of binding to that of oxo-M would have $I_{50}^{-1} > K_H$ and a Hill coefficient of about 0.85 for displacement of [^{3}H]Oxo-M whereas agonists which might exhibit a behavior similar to that of antagonists for the H and SH sites would have $I_{50}^{-1} < K_H$ and a n_H of ~0.95 in competition with [^{3}H]Oxo-M.

DISCUSSION

The experiments described in this paper show complexities in the binding of agonists not apparent in the binding of antagonists to the muscarinic receptor (6). The use of irreversible antagonist PrBCM to alkylate a proportion of the binding sites has excluded the possibility that these results are

TABLE 7

Pharmacological values for agonist induced contraction and Rb^+ efflux of smooth muscle

Values of K_c are taken from references (4, 7, 13, 27–53), values of K_A and K_C/K_A from references (13, 47–55), values of K_{Rb+} from reference (47) and the mean log values calculated (where appropriate). Also included in this Table are unpublished results of Drs. C. R. Hiley and J. M. Young. Numbers in parentheses refer to the number of estimates obtained in the literature search (which is intended to be reasonably comprehensive but not exhaustive). If potencies have been expressed relative to a standard (usually acetylcholine) the K_c value has been calculated as its value relative to the mean log K_c value for the standard agonist.

Agonist	log K_c	Range	log K_A	Range	log K_C/K_A	Range	log K_{Rb}^+
Oxotremorine-M	7.11	(2) 6.99–7.20	—	—	—	—	5.89
(d/l)-cis-methyldilvasene	7.49	(6) 6.96–7.96	5.02	—	2.08	—	
carbachol	6.92	(10) 6.16–7.50	4.56	(6) 3.90–5.00	2.43	(6) 2.12–3.10	4.89
acetylcholine	7.08	(13) 6.44–7.90	5.12	(5) 4.34–5.97	1.85	(4) 1.27–2.16	3.56
methylfurmethide	7.01	(5) 5.90–7.69	5.00	—	2.00	—	5.21
furmethide	5.33	(4) 4.23–5.90	4.52	—	1.28	—	3.86
(+)-acetyl-β-methylcholine	6.93	(7) 6.32–7.48	5.61	—	2.03	—	3.74
(+)-muscarine	6.55	(3) 5.90–6.96		—		—	4.74
dilvasene	5.47	(4) 5.20–5.72	3.34	—	1.86	—	4.02
arecoline	6.50	(3) 5.79–7.40	4.63	(2) 3.95–5.31	1.51	(2) 0.99–2.03	
C_4-TMA	5.70	(2) 5.50–5.89	3.98	(2) 3.59–4.36	1.72	—	
oxotremorine	6.86	(4) 5.92–7.59	4.96	—	0.95	—	5.84
tetramethylammonium	4.03	(2) 3.70–4.36		—		—	3.20
C_5-TMA	5.43	(5) 3.85–6.22	4.18	(2) 3.92–4.45	1.30	—	3.38
(−)-acetyl-β-methylcholine	4.44	(2) 4.40–4.47		—		—	
propionylcholine	5.23	(5) 4.65–5.77	4.04	(2) 3.15–4.92	1.33	(2) 0.85–1.82	
C_2-TMA	4.30	(1) —		—		—	
C_3-TMA	3.70	(1) —		—		—	
pilocarpine	5.07	(5) 3.78–6.05	5.01	(5) 4.49–5.19	0.40	(5) −0.25–0.92	3.47
C_6-TMA	4.97	(4) 4.60–5.60	4.51	(3) 4.28–4.70	0.46	—	
butyrylcholine	4.55	(4) 3.60–5.82	4.28	—	0.54	—	

due to either fixed or mobile anticooperative assemblies. Effects due to agonist-mediated receptor transformation (so-called inactivation or desensitization) also appear to have been excluded. All our experiments point to an explanation based on the heterogeneity of the receptor binding sites, and the analysis of all the agonist binding results is internally consistent with the proposition that there is a major component with low affinity, a smaller component with higher affinity and a minor component with super high affinity. The ratio between the affinity constants is clearly dependent on the structure of the agonists, although with present methods our knowledge of the affinities for the SH binding site is very incomplete. Flattened agonist/[^{3}H]antagonist competition curves for muscarinic receptors have been reported to occur in membrane preparations from brain and smooth muscle from several species (1–3, 11) as well as in cloned neuroblastoma cells in culture (12). It is also apparent in intact, functional smooth muscle from the ileum of the rat and the guinea pig (13, 14) and in thin sections from the rat brain.[4] This suggests that this behavior is not confined to rat brain, is not an artifact due to tissue disruption nor is it a property of cell heterogeneity. Dispersity of agonist binding properties appears to be not uncommon in neurotransmitter receptors and is evident in studies of opiate (15–17), glycine (18), dopamine (19), serotonin (20), nicotinic (21, 22) and the α- and β-adrenergic receptors (23–26).

The physiological significance of these multiple binding sites is not easy to perceive. We have collected data on the K_c (EC_{50}^{-1}) values for contractile responses of smooth muscle in Table 7. It can be seen that the values are both very similar in magnitude to and correlate well with the values of K_H ($r = 0.93, p < 0.001$). However, the existence of spare receptors must cast doubt on whether K_c represents a real affinity constant. There is less data on the activity of agonists on smooth muscle after spare receptors have been eliminated (K_A) but the values correlate reasonably well with K_L ($r = 0.77, p < 0.01$). As a result of these correlations, K_c/K_A, which is equal to the efficacy, correlates with K_H/K_L ($r = 0.85, p < 0.001$) and furthermore for each agonist the values of these ratios are approximately the same for all agonists examined.

[4] Rotter, A., Birdsall, N. J. M., & Hulme, E. C., unpublished results.

We have suggested elsewhere (56) that a possible explanation for these correlations is that the L receptors have their effective affinity constant reduced by conformational coupling to an effector whereas the H receptors are not so constrained. The postulated scheme is:

$$A + R \overset{K_1}{\longleftrightarrow} AR \overset{K_2}{\longleftrightarrow} AR' \qquad \text{H}$$

$$A + RE \overset{K_1}{\longleftrightarrow} ARE \overset{\alpha K_2}{\longleftrightarrow} AR'E^* \qquad \text{L}$$

where $\alpha \ll 1$, $K_2 > 0$ for agonists and $K_2 = 0$ for antagonists. Whether or not this is a correct explanation, the quantitative correlations do seem to point to the L receptors being the ones concerned with the contractile response in smooth muscle and the scheme does provide a satisfactory explanation of how there can be receptors that can be differentiated by their affinities for agonists but not for antagonists. It is interesting that in a neuroblastoma clone, the agonist binding properties (12) are similar to those in the brain preparation described here but the activation of guanylate cyclase appears to be the exclusive property of the low affinity receptor (12). On the other hand, it has been reported that phosphatidylinositol turnover in smooth muscle (57) is activated in a way suggestive of both H and L receptors being utilized.

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