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The effect of *p*-chloromercuribenzoate on structure-binding relationships of muscarinic receptors in the rat cerebral cortex

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1 Muscarinic receptors in the rat cerebral cortex, reacted with *p*-chloromercuribenzoate (PCMB) under different conditions (Phase I and II), have modified binding sites.

2 These exhibit remarkable changes in the structural dependence of the binding of drugs.

3 In Phase I, the structure-binding profile of agonists for both the high and low affinity agonist sites are altered.

4 In Phase II, the structure-binding profile of antagonists is also observed.

5 In Phase II, the ability of potent agonists to discriminate between sub-classes of agonist binding sites is eliminated. There is also a loss of heterogeneity in the binding of the selective antagonist pirenzepine.

6 Of the 16 agonists examined, only pilocarpine has a heterogeneous binding profile in Phase II, the dispersity of binding being increased.

7 The changes in binding properties of the receptors are discussed in terms of general theories of drug-receptor interactions.

Introduction

In the preceding paper (Birdsall, Burgen, Hulme & Wong, 1983) we outlined the qualitative changes in the binding of agonists and antagonists to muscarinic receptors in the rat cerebral cortex produced by *p*-chloromercuribenzoate (PCMB). In this paper we describe in detail the striking changes in the structure profile of binding produced by these modifications.

Methods

The preparation of a crude synaptosomal fraction (P_2) from rat cerebral cortex is described elsewhere (Hulme, Birdsall, Burgen & Mehta, 1978), as is the general protocol for the centrifugation binding assay (Hulme *et al.*, 1978; Birdsall *et al.*, 1983) and the non-linear least squares analysis of the binding curves (Hulme *et al.*, 1978; Birdsall, Burgen & Hulme, 1978). The buffer used was 100 mM NaCl, 20 mM HEPES-Na (pH 7.0) and incubations were all carried out at 30°C on freshly prepared membranes. When necessary, acetylcholinesterase was inhibited by neostigmine (10^{-6}M) added 5 min before the binding assay. The conditions used for producing the Phase I and Phase II effects (Birdsall *et al.*, 1983) were as follows:

Phase I effect

Method A Membranes (1 mg protein ml^{-1}) were pre-incubated at 30°C for 10 min before addition of PCMB (10^{-4}M, final concentration). Incubation was continued for 1 min before addition of (±)-[^{3}H]-N-methylatropine ([^{3}H]-NMA) (20 nM) and non-radioactive muscarinic drugs, as appropriate. Incubation was continued for 5 min before termination by centrifugation. The short incubation time and the high concentration of [^{3}H]-NMA were used to minimize Phase II effects which are inhibited by antagonists. All data points were corrected for the radioligand occupancy by (−)-[^{3}H]-N-methylatropine (the pharmacologically more active enantiomer, $K_A = 5 \times 10^9 M^{-1}$).

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Method B This method differs from that described above in that the membranes were incubated with 6×10^{-5}M PCMB for 1 min and then were immediately diluted 3 fold with buffer before addition of [^{3}H]-PrBCh (10^{-9}M, final concentration) and competing non-radioactive drugs, as appropriate. Incubation was continued for 15 min before termination by centrifugation. In some experiments a 1 min incubation with 10^{-4}M PCMB was used followed by a 4-fold dilution of the membranes. This dilution method effectively quenches any further reaction of PCMB and the inhibition curves using non-radioactive drugs approximate closely to the occupancy curves because of the low receptor occupancy by [^{3}H]-PrBCh (~10%).

Methods A and B gave essentially identical occupancy-concentration curves for the binding of agonists (and antagonists) to muscarinic receptors in PCMB-treated membranes or control (no PCMB added) membranes. There was a 16±2% (mean±s.e.mean, $n=10$) decrease in receptor binding sites using this protocol.

Phase II effect

Membranes (1 mg protein ml^{-1}) were preincubated at 30°C for 10 min. PCMB (10^{-3}M, final concentration) was added and the incubation continued for 15 min. The binding assay was initiated by addition of the membrane suspension (1 ml) to [^{3}H]-PrBCh (10^{-9}M, final concentration) and competing drugs as appropriate. Incubation was then continued for 15 min and terminated by centrifugation. The membrane protein concentration was diluted to 0.2–0.3 mg ml^{-1} after incubation with PCMB, in the instances when full binding curves of potent ^{3}H-antagonists were measured or when the competing antagonist had a high affinity (> 10^9M^{-1}). This latter procedure minimized problems of data analysis caused by depletion of the free concentration of the antagonist by its binding to the receptors.

Accompanying the production of a Phase II effect was a decrease in concentration of binding sites of 23±2% (mean±s.e.mean, $n=35$, range 9–44%).

Oxotremorine-M, aceclidine (3-acetoxyquinuclidine), hexyltrimethyl ammonium bromide, dodecyltrimethylammonium bromide, furmethide, lachesine, 3-quinuclidinylbenzilate and 2-dimethylamino-ethylbenzilate were synthesized in our laboratory. The N-ethyl analogues of acetylcholine, the optical isomers of atropine and scopolamine and 4-diphenyl acetoxy-N-methylpiperidine methiodide were generous gifts from Dr R.B. Barlow. Pirenzepine (Dr R. Hammer) and (+)- and (−)-acetyl-β-methyl choline (Dr J.M. Young) were also kind gifts. (−)-[^{3}H]-3-quinuclidinylbenzilate was purchased from Amersham International and (−)-[^{3}H]-N-methylscopolamine (10 Ci mmol^{-1}), (±)-[^{3}H]-N-methylatropine (2 Ci mmol^{-1}) and [^{3}H]-propylbenzilylcholine ([^{3}H]-PrBCh, 40 Ci mmol^{-1}) were synthesized in our laboratory. *p*-Chloromercuribenzoic acid was obtained from Sigma Chemical Co. Ltd. All other drugs or reagents were of the highest grade available.

Results

In the accompanying paper (Birdsall *et al.*, 1983) we described qualitatively the selective action of short incubations of low concentrations of PCMB (10^{-5}–10^{-4}M) on the agonist binding properties of muscarinic receptors. These incubation conditions are called Phase I conditions and the effect is ascribed

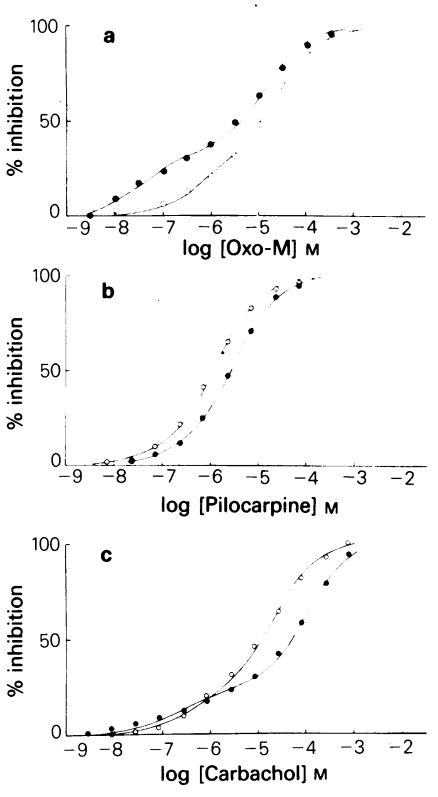

Figure 1 Effect of *p*-chloromercuribenzoate (PCMB) (Phase I conditions) on the binding of (a) oxotremorine-M, (b) carbachol and (c) pilocarpine to muscarinic receptors from the rat cerebral cortex. The conditions are given in the experimental section (Phase I, method B). (○): PCMB treated; (●): control (no PCMB added). The curves are non-linear least squares fits to a two-site model in which it is assumed that the proportions of high and low affinity sites are unaltered after PCMB treatment. (See text and Table 1).

Table 1 Effect of Phase I *p*-chloromercuribenzoate (PCMB) on agonist binding

	$\log K_H$			$\log K_L$		
	Control (C)	*Treated (T)*	*T − C*	*Control (C)*	*Treated (T)*	*T − C*
Oxotremorine	7.58	6.53	−1.05	6.24	6.18	−0.06
Oxotremorine-M	7.44	6.15	−1.39	4.88	4.53	−0.35
Acetylcholine	6.97	6.99	0.02	4.82	5.48	0.66
Carbachol	6.94	6.67	−0.27	4.18	4.83	0.65
Pilocarpine	6.83	7.19	0.36	5.55	5.92	0.37
Furmethide	6.09	5.47	−0.62	4.32	4.25	−0.07

C = control, T = PCMB-treated. Standard errors in the estimates of $\log K_H$ and $\log K_L$ were in the range, (0.08–0.29) and (0.02–0.08) log units respectively. Estimates of T − C varied by less than ± 0.2 log unit between individual experiments.

to the action of PCMB on a sulphydryl group (or groups), termed A. Whereas the binding of antagonists to muscarinic receptors is unaffected under these conditions, higher concentrations of PCMB (10^{-4}–10^{-3}M, 15 min, Phase II conditions) result in a change in antagonist binding and further changes in agonist binding, caused by mercuration by PCMB of a second population of sulphydryl groups, termed B.

In the following sections, the quantitative changes in the structure-binding profile of drugs after Phase I and Phase II treatments with PCMB are described.

Changes in structure-binding profile of agonists under Phase I conditions

In Figure 1 the different effects of PCMB on the binding of oxotremorine-M (oxo-M), carbachol and pilocarpine are shown. The binding of oxo-M is decreased at all concentrations but the effects are proportionally greater in the low concentration (high affinity) region (Figure 1a). By contrast, carbachol shows increased affinity in the high concentration (low affinity) region, but a modest fall in affinity in the low concentration range (Figure 1b). Pilocarpine shows a more or less uniform increase over the whole concentration range. It is clear, therefore, that the three agonist binding curves are affected in different ways, although there is a general tendency for the Hill coefficients for the binding curves to increase. The effects on the affinities of high and low affinity agonist binding sites (H and L sites; Birdsall *et al.*, 1978) are presented in Table 1. The data could not be satisfactorily fit if it was postulated that PCMB induced an interconversion between H and L sites but the model in which only changes in affinity of the H and L sites had occurred did provide excellent fits (Figure 1). On the high affinity receptor site, the largest change was the reduction in oxo-M binding by a factor of 25, but on the other hand, the binding of pilocarpine increased 2.3 fold. On the low affinity receptor, the binding of oxotremorine was hardly affected, whereas that of acetylcholine and carbachol was increased 4.5 fold. Looked at from the point of view of rank order of binding, the changes are visualized in Figures 2a and b. It is clear that the structure-binding profile has been changed for both high and

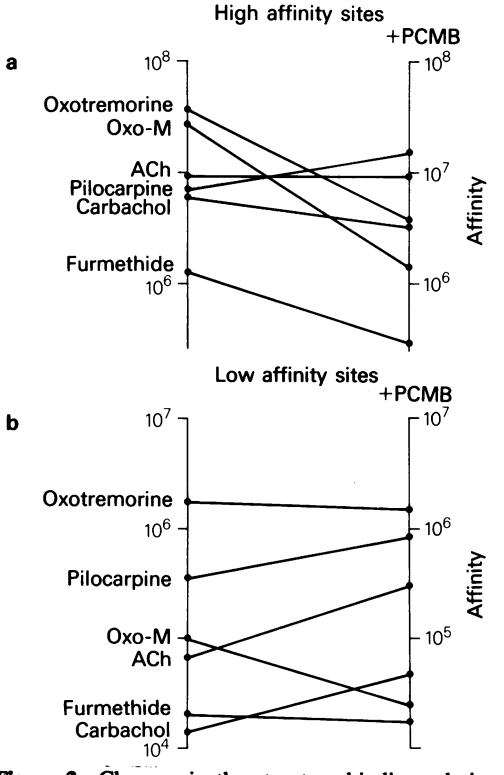

Figure 2 Changes in the structure-binding relationships for the binding of agonists to the (a) high and (b) low affinity agonist sites on muscarinic receptors in the rat cerebral cortex after *p*-chloromercuribenzoate (PCMB) Phase I treatment. In this type of plot, both the quantitative changes in affinity and the qualitative changes in rank order of potency are visualized, by the gradient of the tie-lines and the cross-over of these lines, respectively.

Table 2 Effects of Phase II *p*-chloromercuribenzoate (PCMB) treatment on the antagonist binding properties of muscarinic receptors in cortical membranes

Antagonist	*log (Affinity constant)* Control (C)	Treated (T)	T − C
(−)-[^{3}H]-QNB	9.97[a]	9.63[a]	−0.34
(−)-[^{3}H]-NMA	9.71[a]	9.25[a]	−0.46
(−)-[^{3}H]-NMS	9.61	7.63[a]	−1.98
(−) Scopolamine	9.14	7.42	−1.72
Di-4	9.00	7.12	−1.88
(−) Atropine	8.98	8.13	−0.85
Lachesine	8.84	9.13	+0.29
(+) Scopolamine	8.38	6.92	−1.46
[^{3}H]-PrBCh	8.17[a]	8.71[a]	+0.54
2-Dimethylaminoethyl-benzilate	7.92	8.06	+0.14
Pirenzepine (high affinity sites)	7.72	6.87	−0.85
(+)-NMA	7.40	7.10	−0.30
(+)-Atropine	6.82	5.80	−1.02
Pirenzepine (low affinity sites)	6.40	6.87	+0.47
Dodecyl-NMe$_3$	5.26	4.89	−0.37

[a] Derived from saturation binding curves

QNB, 3-quinuclidinylbenzilate; NMA, N-methylatropine; NMS, N-methylscopolamine; Di-4, 4-diphenylacetoxy-N-methyl-piperidine methiodide; PrBCh, propylbenzilylcholine

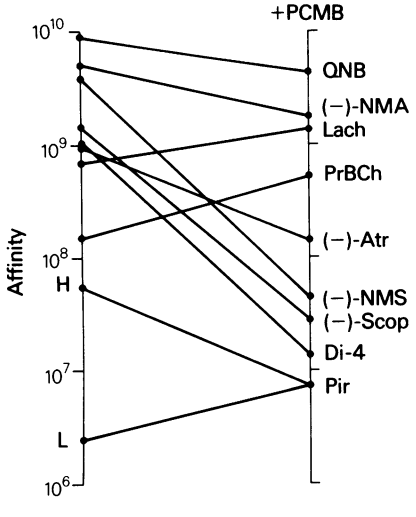

Figure 3 Changes in the structure-binding relationships of antagonists for muscarinic receptors in the rat cerebral cortex after *p*-chloromercuribenzoate (PCMB) II treatment. H and L denote the high and low affinity pirenzepine binding sites found in the control membranes. Lach, lachesine; Atr, atropine; Scop, scopolamine; Pir, pirenzepine; QNB, 3-quinuclidinylbenzilate; NMA, N-methylatropine; NMS, N-methylscopolamine; Di-4, 4-diphenylacetoxy-N-methyl-piperidine methiodide.

low affinity sites but in a differential fashion. In all the agonists studied the ratio of the binding constants for the H and L sites is reduced compared with the ratio present in the controls. It should be noted that neither in the absence nor presence of PCMB do the H and L sites have the same structure-activity relationships for agonists.

It is not possible to extract superhigh (SH) binding affinities from the antagonist inhibition curves in the cerebral cortex because of the low abundance of this receptor sub-type (Birdsall, Hulme & Burgen, 1980). The alternative reagent, [^{3}H]-oxo-M, has its affinity so much reduced under Phase I conditions (Birdsall *et al.*, 1983) that it cannot be used to estimate the binding of other agonists by competition.

Changes in structure-binding profile of antagonists under Phase II conditions

The effects on the binding of antagonists are shown in Table 2. The changes in affinity range from a 4 fold increase to a 100 fold decrease; the differences in order of potency that are produced are illustrated in Figure 3.

The scopolamines showed especially large decreases in affinity, in marked contrast to the small effects on the binding of the atropines which are closely related in structure. The effects on the three

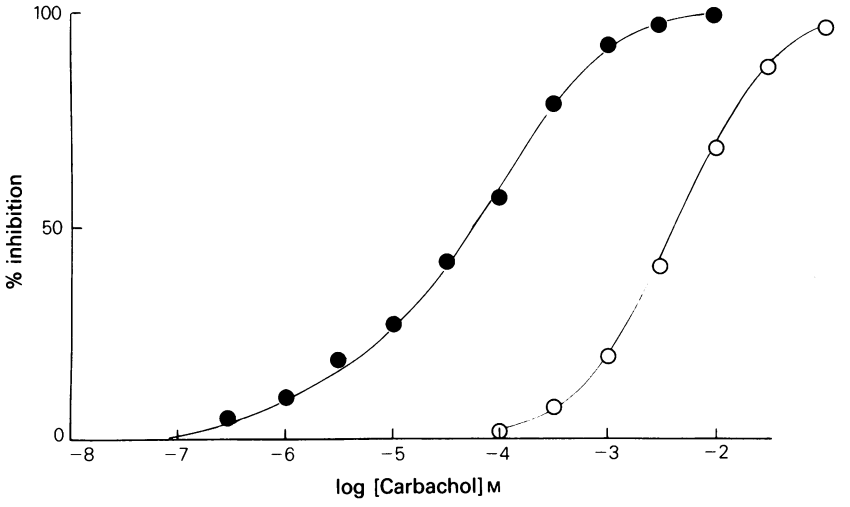

Figure 4 Effect of *p*-chloromercuribenzoate (PCMB) (Phase II conditions) on the binding of carbachol to muscarinic receptors from the rat cerebral cortex. (○): Data from PCMB II-treated receptors; (●): data from control membranes. The curve through the closed circles is a non-linear least squares fit with 74 ± 3% low affinity sites, log $K_A = 3.87 \pm 0.04$ and 26% high affinity sites, log $K_A = 5.76 \pm 0.12$. The curve through the open circles is a simple mass action curve, log $K_A = 2.33 \pm 0.03$.

stereoisomeric pairs investigated did not show important differences.

Pirenzepine is an antagonist which discriminates between two sub-populations of receptors in the cerebral cortex (Hammer, Berrie, Birdsall, Burgen & Hulme, 1980). After PCMB treatment, the population of binding sites has apparently become homogeneous, the affinity of pirenzepine for the lower affinity site has been increased and that for the higher affinity decreased.

Changes in structure-binding profile of agonists under Phase II conditions

Under these conditions massive changes occurred in the affinity of agonists. An example of the effect on carbachol binding is shown in Figure 4. Not only are the affinities strikingly reduced but the curves become mass action with Hill coefficients close to 1. The changes are recorded in Table 3 and Figure 5. It is clear that the order of potency of the agonists has changed considerably for both high and low affinity sites. For instance, oxotremorine-M is ranked second at the high affinity site and is only ranked seventh after PCMB and pilocarpine, ranked 6th, is in the first place after PCMB treatment.

Pilocarpine has a special interest since it is the only agonist whose Hill slope is not unity after PCMB treatment and has in fact decreased from 0.91 to 0.67 (Figure 6). This is the only anomaly in the simple picture of conversion of heterogenous agonist (and antagonist) binding to homogeneous binding under Phase II conditions. Analysis of the binding data (see legend to Figure 5) suggests that the pilocarpine affinity for the high affinity (H) sites is decreased to a lower extent that that for the low affinity (L) sites.

Discussion

The results presented in this paper reveal that remarkable changes in the structural dependence of the binding of drugs to the muscarinic receptor are produced by PCMB. In the previous paper (Birdsall *et al.*, 1983) we have presented evidence that the changes in binding in both Phase I and Phase II are not due to modification within the binding site and hence to direct steric effects. The effects are produced indirectly through mercuration of distal sites on the receptor molecule or on its associated subunits, which causes a conformational change in the receptor binding site, modifying the binding of agonists and/or antagonists.

There has been general acceptance of the idea that a second binding site in a receptor complex can modify the binding of antagonists or agonists to the appropriate binding site. This is, for instance, the basis of the modulation by GTP of binding of ligands to a number of receptors (including the muscarinic receptor). This modulation is known to be due to the binding of GTP to a separate nucleotide binding subunit, which is conformationally coupled to the

Table 3 Effects of Phase II *p*-chloromercuribenzoate (PCMB) treatment on agonist binding

	n_H[a]		$\log K_H$[a]			$\log K_L$[a]		
	C	*T*	*C*	*T*	*T*−*C*	*C*	*T*	*T*−*C*
Oxotremorine	0.66	1.00	7.68	3.50	−4.18	6.24	3.50	−2.74
Oxotremorine-M	0.44	1.03	7.01	3.25	−3.76	5.07	3.25	−1.82
Carbachol	0.48	1.00	6.22	2.42	−3.80	3.93	2.42	−1.51
Bethanechol	0.66	0.9	5.42	2.68	−2.74	3.74	2.68	−1.06
Aceclidine	0.86	1.00	5.75	3.46	−2.29	4.80	3.46	−1.34
Arecoline	0.73	1.01	6.92	3.38	−3.54	4.98	3.38	−1.60
Pilocarpine	0.91	0.67	6.32	5.49	−0.83	5.50	3.89	−1.61
Acetylcholine	0.58	1.04	6.86	2.72	−4.14	4.72	2.72	−2.00
ACh NMe_2Et	1.0	1.0	4.51	4.10	−0.41	4.51	4.10	−0.41
ACh $NMeEt_2$	1.0	1.0	4.16	3.73	−0.43	4.16	3.73	−0.43
ACh NEt_3	0.96	1.0	3.60	3.04	−0.56	3.60	2.04	−0.56
(+)-Methacholine	0.48	0.97	6.45	3.38	−3.07	4.54	3.38	−3.07
(−)-Methacholine	0.94	0.9	3.32	2.72	−0.60	3.32	2.72	−0.60
Choline	0.99	1.0	3.04	2.05	−0.99	3.04	2.05	−0.99
TMA	0.76	0.9	4.28	1.96	−2.32	2.82	1.96	−0.86
Hexyl-NMe_3	1.00	1.1	5.18	3.81	−1.37	5.18	3.81	−1.37

[a] n_H, Hill coefficient; K_H and K_L are the affinity constants for the high and low affinity agonist sites.

receptor subunit. The assumption is that the equilibrium between two conformations of the receptor subunit can be displaced by the occupation of the nucleotide subunit, and that these conformations have different affinities for the ligand.

As the specificity of the receptor binding site is due to the complex disposition in space of various amino acid residues, and the overall binding energy of the ligand for the receptor is due to the summation of the many interactions of the ligand with these residues, it is evident that when the binding energy for the ligand is changed by spatial displacement of one or more of the residues in the receptor, the structure-binding characteristics of a range of ligands must be modified. Thus a conformational change must result in a change in the structure-binding profile, except in the case where one of the conformers is non-binding.

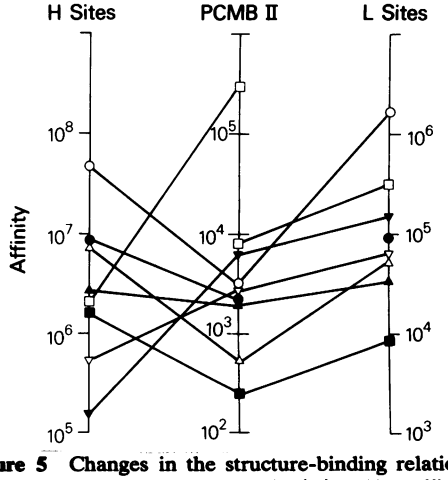

Figure 5 Changes in the structure-binding relationships of agonists for the high affinity (H) and low affinity (L) binding sites on muscarinic receptors in the rat cerebral cortex after PCMB II treatment. Note the differences in the scales on the 3 axes. (○) Oxotremorine; (●) arecoline; (△) acetylcholine; (▲) (+)-acetyl-β-methylcholine; (□) pilocarpine; (■) carbachol; (▽) aceclidine; (▼) hexyltrimethylammonium.

Indeed this principle is implicit in the widely-held theory that the distinction between antagonists and agonists is that whereas the former binds to a ground state of the receptor, the latter binds to an active state of the receptor which is coupled to the agonist response. The logic of this system is that the ground state of the receptor is complementary to the three-dimensional structure of antagonists, that of the active state is complementary to the three-dimensional structure of agonists. The actions of PCMB can be understood within this logical framework.

In Phase I no changes are produced in the binding of antagonists, so the ground conformation of the receptor is unaffected, but considerable changes are produced in the binding of agonists and therefore the active conformation has been changed. The effects on the high and low affinity forms of the receptor differ. For example, with acetylcholine there is no change in K_H but an increase in K_L, whereas for oxotremorine there is a ten fold reduction in K_H and virtually no change in K_L, and with carbachol there are yet further differences in that K_H is decreased and K_L increased. Figure 2 makes it clear that the structural requirements for binding agonists to the high and low affinity receptor sites are not only different

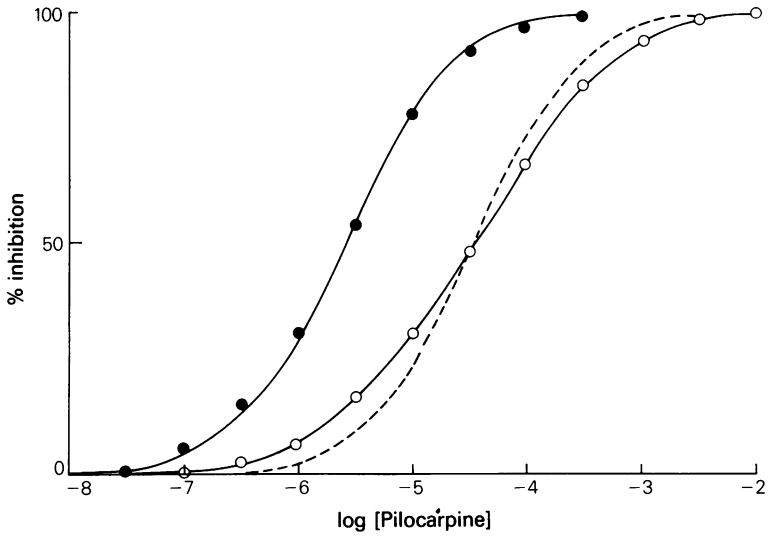

Figure 6 Effect of *p*-chloromercuribenzoate (PCMB) (Phase II conditions) on the binding of pilocarpine to muscarinic receptors from the rat cerebral cortex. The data from control (●) and PCMB-treated (○) membranes represent the mean of five experiments, the maximum variation being ±6%. The dashed curve and the curve through the control curve are simple mass action curves; the curve through the open circles is a non-linear least squares fit with 58±3% low affinity sites, $\log K_A = 3.90 \pm 0.03$ and 42% high affinity sites, $\log K_A = 5.20 \pm 0.04$. The Hill coefficient of the PCMB-treated curve was 0.67 compared with an estimate of 0.91 for the control curve.

before PCMB treatment but they are also changed in different ways by PCMB. This emphasises that the changed conformation of the high and low affinity forms of the receptor remain distinctive. Unfortunately, we do not have available at present a method to evaluate the changes in the superhigh affinity binding sites.

In Phase II, since the antagonist-binding profile is changed, we must presume that the conformation of the ground state of the receptor has been changed, but the drastic changes in agonist binding may result either from changes in the active conformation or the failure to generate any active states which are now forbidden. The low affinity residual binding of the agonists could then be to the ground state of the receptor which presents a far from ideal complementary structure for agonists. This explanation would provide an explanation of the loss of the multiple binding states for agonists which we have suggested (Birdsall, Burgen & Hulme, 1977) may be due to differences in the active state of the receptor. This receives some support from the binding profile of the effect of N-ethyl substitutions of acetylcholine. In the untreated high and low affinity agonist binding sites, the affinity falls progressively with each ethyl group, whereas after treatment with PCMB the affinity is increased by substitution of the first ethyl group as is commonly found with antagonists (Holton & Ing, 1949; Barlow, Scott & Stephenson, 1963). If this is the mechanism of the effect on agonist binding in Phase II it implies that there must be at the same time a loss of agonist activity at the receptor.

This would then represent a novel form of antagonism. It should be added that since sulphydryl reagents have been reported to uncouple receptors from such effector systems as adenylate cyclase (Ross, Howlett, Ferguson & Gilman, 1979; Harden, Scheer & Smith, 1982; Andre, Vauquelin, Severne, De Backer & Strosberg, 1982), it would not be unexpected that agonist activity was lost after PCMB whether or not the active state is forbidden.

An interesting anomaly in our results is that of pilocarpine, in that a dispersity of binding is not only retained in Phase II but actually increased. So far this is the only ligand that we have examined that shows dispersity of binding in Phase II. Pilocarpine is structurally rather different from other muscarinic agonists and is presumably sensing a feature of the receptor not pertinent to the binding of other agonists, and it may be noted that its affinity for the high affinity agonist site is much higher than that of any other agonist. The behaviour of pilocarpine argues against the proposition that the receptor is in an homogenous state after treatment with PCMB when sensed by agonists or antagonists. This is especially interesting in view of the loss of heterogeneity to pirenzepine.

It is interesting to compare the effects of Phase II PCMB with gallamine which we have recently shown

(Stockton, Birdsall, Burgen & Hulme, 1983) to modify the binding of ligands for the muscarinic receptor as a result of its binding to a secondary site on the receptor. In this case the general effect is to reduce the affinity of both agonists and antagonists with changes in the structure-binding profile that is quite unlike that of Phase II PCMB. Also in this case the agonist heterogeneity is retained. Gallamine evidently selects or induces yet another conformation of the ground state of the receptor, which also effects the binding of agonists to the active states.

When a receptor conformation is considerably altered one must bear in mind the possibility that the change may be great enough to make it complement an entirely different ligand structure. It is then no longer appropriate to confine one's attention to a group of drugs that have been clustered into the type 'muscarinic agonists' or 'muscarinic antagonists' as a result of studies on the native receptor. To discover whether this is so would require a broad screen, which is being undertaken.

One of the conclusions of this study must be that receptors, as molecular structures that are designed to generate signals in response to ligand binding by conformational change, may not be restricted in their repertoire of responses to an either/or production of one or other conformations. They may be inherently conformationally labile structures that can respond in multiple ways to a variety of perturbants in addition to the conventional agonists and antagonists. This property opens the way to new approaches to drug action as we have pointed out in connection with the action of gallamine (Stockton *et al.*, 1983).

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