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The effects of *p*-chloromercuribenzoate on muscarinic receptors in the cerebral cortex

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1 The action of *p*-chloromercuribenzoate (PCMB) on the ligand binding properties of the muscarinic receptors in the rat cerebral cortex has been examined.

2 At low concentrations, PCMB produces a selective change in the binding of agonists without any effect on the binding of antagonists.

3 At higher concentrations, the structure-binding profile for binding antagonists is changed. The affinity of agonists is greatly reduced and the heterogeneity of binding eliminated.

4 The effects of both high and low concentrations of PCMB can be reversed by dithiothreitol.

5 Inactivation of receptors proceeds in parallel and is kinetically complex. It can only be partially reversed by dithiothreitol.

6 Evidence is presented connecting the low affinity agonist binding site with the high affinity pirenzepine binding site.

7 The changes produced by PCMB have been interpreted in terms of the modification of receptor conformation.

Introduction

It has been shown by Aronstam, Abood & Hoss (1978) that a number of heavy metals and other chemicals that react with sulphydryl groups can produce substantial changes in the binding properties of muscarinic receptors. The effects were surveyed at single concentrations of muscarinic ligands and have not been studied in further detail with the exception of several investigations which have shown that N-ethylmaleimide produces a selective change in agonist binding (Aronstam, Hoss & Abood, 1977; Hedlund & Bartfai, 1979; Salvaterra, 1980; Carson, 1980; Wei & Sulakhe, 1980; Ehlert, Roeske & Yamamura, 1980; Ikeda, Aronstam & Eldefrawi, 1980; Aronstam & Carrier, 1982; Vauquelin, Andre, De Backer, Laduron & Strosberg, 1982). In this paper and its companion (Birdsall, Burgen, Hulme & Wong, 1983) we have examined in detail the effects of another sulphydryl reagent, *p*-chloromer curibenzoate (PCMB) on the muscarinic receptors in the rat cerebral cortex.

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Methods

A crude synaptosomal fraction (P_2) was prepared from rat cerebral cortex as described by Hulme, Birdsall, Burgen & Mehta (1978). One rat cortex provides 30–35 mg membrane protein after this procedure. The membranes were resuspended to a concentration of 1 mg ml^{-1} in 100 mM NaCl, 20 mM HEPES-Na (pH 7.0). (Some ^{3}H-antagonist binding curves were determined at 0.2–0.3 mg ml^{-1} to minimize depletion of the free concentration of the ^{3}H-ligand.) Binding studies were always performed on fresh material, usually within 2 h of preparation of the P_2 fraction.

For the binding assays, the resuspended P_2 fraction was preincubated at 30°C for 10 min. PCMB was then added and at the end of the treatment period (generally 15 min although the exact conditions varied according to the nature of the experiment, see Figure legends), unless otherwise specified, 1 ml aliquots were pipetted into plastic microcentrifuge tubes containing the ^{3}H-ligand and competing drug if appropriate. The tubes were incubated at 30°C for 15 min, unless otherwise specified, and were centrifuged at 14,000 *g* for 2 min. After decanting the supernatants, the pellets were rapidly and superficially washed with 3 × 1.5 ml 150 mM NaCl to remove radioactivity adhering to the side of the tube.

The radioactivity in the pellets was solubilized and counted as described previously (Hulme *et al.*, 1978). Non-specific binding in all experiments was defined as the radioactivity bound to, or entrapped within, the membrane pellet when the incubation medium contained 10^{-6}M 3-quinuclidinylbenzilate (QNB). It should be noted that PCMB was more potent in affecting muscarinic receptor binding if the incubations were carried out at < 1 mg protein ml^{-1}. This was probably due to depletion of the free concentration of PCMB by its non-specific mercuration of membrane proteins.

Binding curves were analysed by non-linear least-squares regression analysis (see e.g., Hulme *et al.*, 1978; Birdsall, Burgen & Hulme, 1978). Protein samples were analysed by the Lowry method using bovine serum albumin as standard. The sulphydryl content of the P_2 membrane fraction under non-denaturing conditions was estimated using 5,5'-dithio-*bis* (2-nitro-benzoic acid) and the method of Sedlak & Lindsay (1968).

PCMB was obtained from Sigma Chemical Co. Ltd. All reagents were of the highest grade available. (+)-Atropine and 4-diphenylacetoxy-N-methylpiperidine methiodide were kind gifts of Dr R.B. Barlow. Pirenzepine was a generous gift from Dr R. Hammer. Lachesine, 3-quinuclidinylbenzilate and oxotremorine-M were synthesized in our laboratory. (−)-[^{3}H]-3-quinuclidinylbenzilate ([^{3}H]-QNB) was purchased from Amersham International. (−)-[^{3}H]-N-methylscopolamine of high specific activity was obtained from New England Nuclear. Other tritiated ligands: [^{3}H]-propylbenzilylcholine ([^{3}H]-PrBCh 40 Ci mmol^{-1}), [^{3}H]-(±)-N-methylatropine ([^{3}H]-NMA 2.2 Ci mmol^{-1}) and [^{3}H]-oxotremorine-M (10 Ci mM^{-1}) were synthesized in our laboratory.

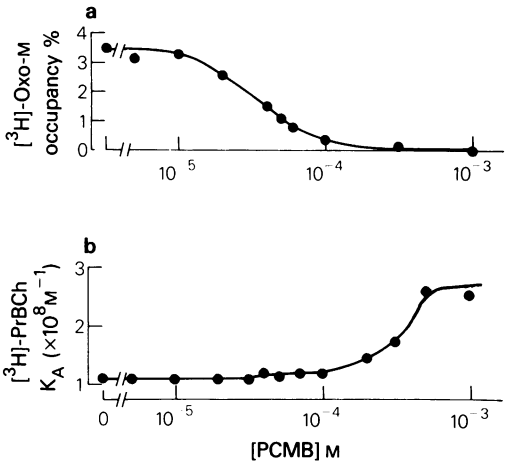

Figure 1 Effects of *p*-chloromercuribenzoate (PCMB) on the binding of (a) [^{3}H]-oxotremorine-M (3 nM) and (b) [^{3}H]-propylbenzilylcholine (1 nM) to muscarinic receptors from the rat cerebral cortex. Membrane preparations (1 mg protein ml^{-1}) were incubated with different concentrations of PCMB for 15 min at 30°C followed by addition of the radioligand and further incubation for 15 min. The total concentration of binding sites was estimated in a parallel experiment by incubating the membranes for 15 min with a receptor saturating concentration (20 nM) of (±)-[^{3}H]-N-methylatropine after treatment with an appropriate concentration of PCMB.

The binding of [^{3}H]-oxo-M is expressed as % receptor occupancy and that of [^{3}H]-PrBCh as the affinity constant, calculated from the single point estimate of receptor occupancy.

The data points represent the mean of 2–15 independent experiments.

Results

Effects of various concentrations of p-*chloromercuribenzoate (PCMB)*

The effects of PCMB on muscarinic receptors are not singular, but are rather complex. A preliminary separation of the effects can be achieved by examining the changes in binding as a function of exposure to increasing concentrations of PCMB for a standard time (15 min).

(a) *Effect of the binding of [^{3}H]-oxotremorine-M* At the concentration used (3 nM) the binding is almost exclusively to superhigh (SH) class of agonist binding sites (Birdsall, Hulme & Burgen, 1980). At concentrations of PCMB less than 10^{-5}M, no effect was seen, but in the range 10^{-5}–10^{-4}M PCMB the binding of oxo-M was reduced to such an extent that it was not significantly greater than the non-specific binding (Figure 1a).

(b) *Effect on the binding of [^{3}H]-propylbenzilylcholine ([^{3}H]-PrBCh)* The affinity of [^{3}H]-PrBCh was determined at a number of PCMB concentrations (Figure 1b). In the control the affinity was 1.1×10^8M^{-1} and this was unaffected by any concentration up to 10^{-4}M PCMB, but above this concentration the affinity rose to 2.7×10^8M^{-1} (Figure 1b).

(c) *Effects on the competition between carbachol and [^{3}H]-PrBCh* In the control experiment carbachol (10^{-4}M) inhibited the binding of [^{3}H]-PrBCh by 66%. In the range of 10^{-5}–10^{-4}M PCMB this inhibition rose to 76% and then in the range 10^{-4}–10^{-3}M PCMB the carbachol inhibition of binding approached zero (Figure 2). Since we have shown that there is no change in PrBCh affinity in the PCMB concentration range 10^{-5}–10^{-4}M, the increased in-

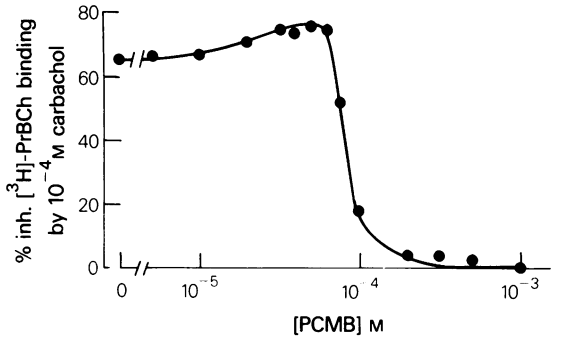

Figure 2 Effects of *p*-chloromercuribenzoate (PCMB) on the binding of 10^{-4}M carbachol to muscarinic receptors from the rat cerebral cortex. Membrane preparations (1 mg protein ml^{-1}) were incubated with different concentrations of PCMB for 15 min at 30°C followed by addition of [^{3}H]-N-propyl-NN-dimethyl-2 aminoethyl-benzilate ([^{3}H]-PrBCh) (1 nM) and 10^{-4}M carbachol (as appropriate) and further incubation for 15 min. The percentage inhibition by 10^{-4}M carbachol of the receptor-specific binding of [^{3}H]-PrBCh is a reasonable approximation to the receptor occupancy. The data points represent the mean of up to 18 independent experiments.

hibition is an indication that the affinity of carbachol has increased. In the concentration range 10^{-4}–10^{-3}M, the effect of PCMB on carbachol inhibition has decreased far more than can be accounted for by the rather modest increase in the affinity of PrBCh and this means that there has been a large decrease in the affinity of carbachol.

(d) *Effect on total binding capacity* The total binding capacity can be measured by using a relatively high concentration of a strongly binding antagonist whose binding constant is not much affected by PCMB. We have used [^{3}H]-N-methylatropine (NMA) whose binding constant of the pharmacologically active (−)-isomer for the receptor is $4.1 \times 10^9 M^{-1}$. At a concentration of 20 nM (±)-NMA the receptor is 99% saturated. At concentrations of PCMB greater than 10^{-4}M this binding constant is reduced to $1.8 \times 10^9 M^{-1}$ (see later), but this will have the effect of reducing the occupancy only to 97% so that any changes in binding of NMA will be due almost entirely to a change in the binding capacity.

In Figure 3 it can be seen that with increasing concentrations of PCMB there is a gradual loss of capacity which at 10^{-4}M is about 20% and at 10^{-3}M about 40%.

These results show that there is a change in the range 10^{-5}–10^{-4}M PCMB affecting agonist binding (both carbachol and oxotremorine-M, although in different ways), but without apparent effect on the binding of the antagonist PrBCh, whereas in the range 10^{-4}–10^{-3}M PCMB, while further changes in agonist binding occur, there is also a change in binding of the antagonist PrBCh. We have called the

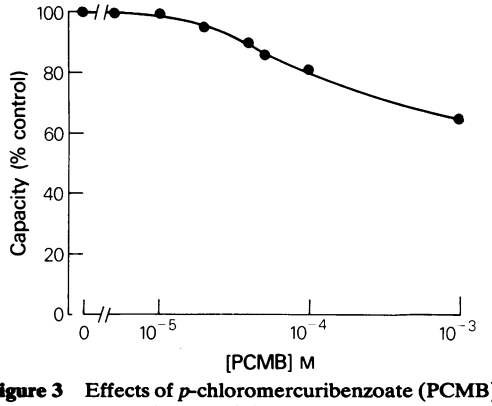

Figure 3 Effects of *p*-chloromercuribenzoate (PCMB) on the number of muscarinic binding sites in the rat cerebral cortex. Membranes (1 mg protein ml^{-1}) were incubated with different concentrations of PCMB for 15 min at 30°C followed by addition of 20 nM (±)-[^{3}H]-N-methylatropine ([^{3}H]-NMA) and further incubation for 15 min. This concentration of NMA occupies > 97% of the receptors under these conditions and therefore the specifically bound [^{3}H]-NMA is a good estimate of numbers of binding sites. The data points represent the mean of up to 13 independent experiments.

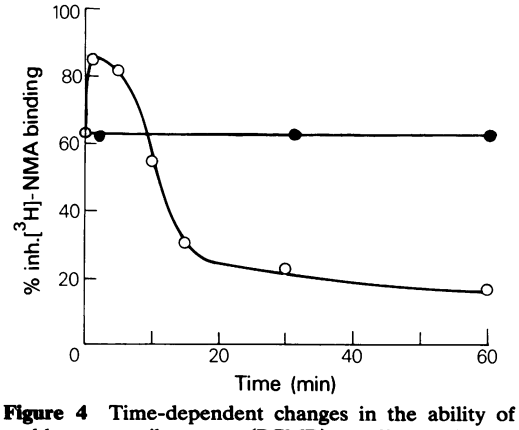

Figure 4 Time-dependent changes in the ability of *p*-chloromercuribenzoate (PCMB) to affect carbachol binding to muscarinic receptors from the rat cerebral cortex. Membranes (1 mg protein ml^{-1}) were treated with 10^{-4}M PCMB at 30°C and the ability of carbachol (3×10^{-3}M) to inhibit (±)-[^{3}H]-N-methylatropine ((±)-[^{3}H]-NMA) (20 nM) binding (○) was measured in a 5 min incubation with the muscarinic drugs after the designated time of treatment with PCMB. The closed circles (●) represent control experiments in which no PCMB was added. The high concentrations of muscarinic ligands were required to inhibit phase II reactions of PCMB after incubation with PCMB.

effects seen in these two concentration ranges Phase I and Phase II respectively.

Time-course of PCMB action

During exposure to 10^{-4}M PCMB the increase in carbachol binding appears very rapidly and reaches a peak within a minute or two, then declines as the effect of decreased carbachol binding supervenes and reaches a steady state (Figure 4). Phase I effects can be produced in a stable and reproducible fashion by exposure to 10^{-4}M PCMB for 1 min followed by a four fold dilution with buffer or addition of [^{3}H]-NMA (20 nM); these procedures prevent the development of Phase II. We can now proceed to an analysis of Phase I effects.

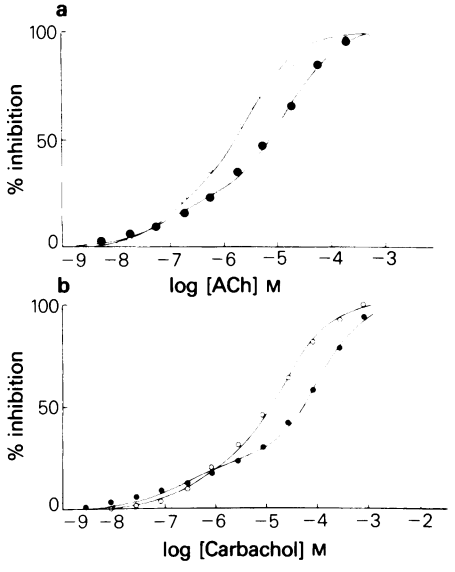

Figure 5 The phase I effect of *p*-chloromercuribenzoate (PCMB) on the inhibition of (±)-[^{3}H]-N-methylatropine ([^{3}H]-NMA) binding by (a) acetylcholine and (b) carbachol. Membranes (1 mg protein ml^{-1}) were incubated at 30°C for 1 min in the presence (○) or absence (●) of 10^{-4}M PCMB, before addition of (±)-[^{3}H]-NMA (20 nM) and non-radioactive muscarinic drugs, as appropriate. Incubation was continued for 5 min. All data points were corrected for the radioligand occupancy by (−)-[^{3}H]-NMA ($K_A = 5 \times 10^9$M^{-1}, determined independently). In the experiment (a) neostigmine (10^{-6}M) was added 5 min before the binding assay to inactivate acetylcholinesterase. The curves through the data points are non-linear least squares fits to the data of a 2-site model with (a) 75±2% low affinity sites, log affinity constant 4.82±0.05 (●), 5.48±0.05 (○); 25% high affinity sites, log affinity constant 6.97±0.13 (●), 6.99±0.16 (○); (b) 78±2% low affinity sites, log affinity constant 4.18±0.04 (●), 4.83±0.03 (○); 22% high affinity sites, log affinity constant 6.94±0.15 (●), 6.67±0.12 (○). The estimated Hill Coefficients are (a) (●) 0.45; (○) 0.64; (b) (●) 0.37; (○) 0.51.

Phase I Figure 5a shows the effect of Phase I treatment with PCMB on the binding of acetylcholine to the muscarinic receptors. It can be seen that there is an effect largely confined to the high concentration end of the curve in which the curve is shifted to a concentration about five times lower than in the control. The Hill coefficient is increased by 0.2. Analysis of the curves shows that the change in the curve can be attributed primarily to a change in the affinity of the low affinity form of the receptor from 6×10^4M^{-1} to 2.5×10^5M^{-1} without any change in the proportion of high and low affinity forms. In the case of carbachol (Figure 5b), there is a similar shift at the high concentration end of the curve but there is also a significant decrease in affinity at the low concentration end of the curve. The result seen in Figure 2 was obtained at 10^{-4}M carbachol and corresponds to the change seen at this concentration in Figure 5b. These results, together with the effects on oxo-M (Figure 1a) show that the effects on agonists are selective as far as the (three) SH, H and L subtypes are concerned. By contrast with these effects of PCMB on agonist binding, no significant effect was found in the binding of any of seven antagonists (Table 1).

Phase II Effects on antagonist binding In confirmation of the preliminary experiments (Figure 1b), exposure to PCMB (10^{-3}M; 15 min) leads to an approximately 4 fold increase in the affinity of PrBCh (Figure 6a). As can be seen, the Scatchard plot remains linear, i.e. there is a total conversion of the receptors to the new binding state. On the other hand, the affinity of NMA is considerably decreased from 4.1 to 10^9M^{-1} to 1.8×10^9M^{-1} (Figure 6b). Larger changes in affinity were found with some other antagonists. The detailed changes in structure-binding relationships are reported in the following paper (Birdsall *et al.*, 1983).

More extreme treatment with PCMB reduced capacity further but did not change the affinity of antagonists for the remaining sites.

Phase II Effects on agonist binding The effects on agonist binding under Phase II conditions were very dramatic (Figure 7). The binding curve for acetylcholine (Figure 7a) was shifted far to the right. The IC_{50} increased from 8×10^{-6} to 2×10^{-3}M and at the same time the heterogeneity of binding completely disappeared so that the Hill slope has become 1.0. Since the control affinities for the SH, H and L sites were 5×10^7, 3.2×10^6, and 5×10^4M^{-1} (Birdsall *et al.*, 1980) and after PCMB have all become 5×10^2M^{-1}, the affinities have been reduced by 2–5 orders to magnitude. Once again more extensive

Table 1 Lack of change of antagonist affinity constants under Phase I conditions

Antagonist	Affinity constant ($\times 10^9 M^{-1}$) Control	PCMB treated	% inhibition of 10^{-9}M [^{3}H]-PrBCh binding Control	PCMB treated
[^{3}H]-PrBCh	0.13	0.14[d]	—	—
[^{3}H]-NMA	2.8	3.2[d]	—	—
[^{3}H]-NMS	3.3	3.1[d]	—	—
Pirenzepine[a]	—	—	68	69
4-Diphenylacetoxy-N-[b] methyl piperidine methiodide	—	—	54	53
Lachesine[b]	—	—	63	65
(+)-Atropine[c]	—	—	53	49

[a] 10^{-7}M; [b] 2×10^{-9}M; [c] 2 $>10^{-7}$M; [d] Under these conditions there was a $16 \pm 2\%$ (mean $\pm$ s.e.mean, $n = 10$) decrease in capacity

PCMB treatment did not cause further changes in the affinity. Very similar changes occurred in the binding of oxotremorine-M (Figure 7b) and oxotremorine, arecoline and methacholine (Birdsall *et al.*, 1983).

Reversibility of PCMB effects

The Phase I effects were not reversed by extensive washing but they were fully reversed by dithioerythritol (DTE 10 mM; 15 min). Phase II effects on both antagonist and agonist binding was also readily and almost completely reversed (Figures 8a and 9a). This included the restitution of the agonist binding heterogeneity. However, only part of the loss of capacity found under Phase II conditions could be recovered. For instance, in one experiment after treatment with 10^{-3}M PCMB for 15 min, 45% of the capacity had been lost. Treatment with DTE (10 mM)

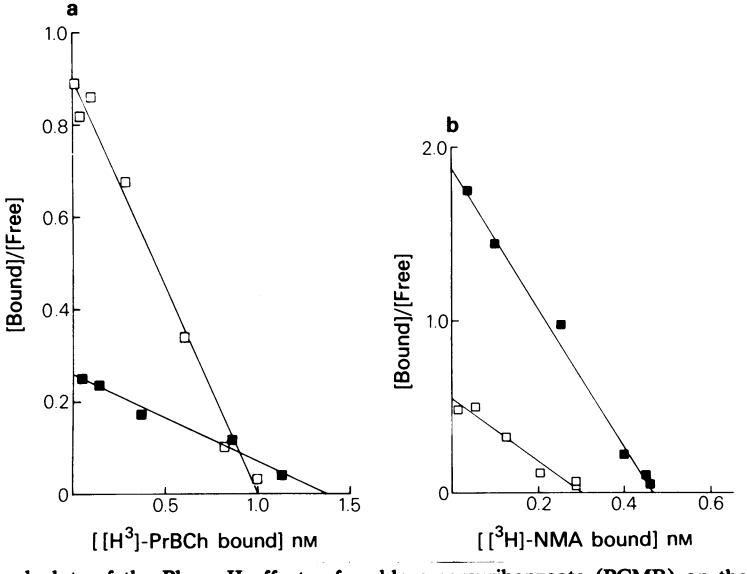

Figure 6 Scatchard plots of the Phase II effects of *p*-chloromercuribenzoate (PCMB) on the binding of (a) [^{3}H]-propylbenzilylcholine ([^{3}H]-PrBCh) and (b) [^{3}H]-N-methylatropine ([^{3}H]-NMA) to muscarinic receptors from the rat cerebral cortex. Membranes were incubated at 30°C for 15 min in the presence (□) or absence (■) of 10^{-3}M PCMB, before the start of the 15 min binding assay. The membrane protein concentration was (a) 0.97 mg ml^{-1} (b) 0.32 mg ml^{-1}. The straight lines are the non-linear least squares fit to the untransformed data. (a) Control = 1.4 pmol mg^{-1} protein, $K_A = 1.8 \times 10^8 M^{-1}$; PCMB-treated 1.0 pmol mg^{-1} protein, $K_A = 9.0 \times 10^8 M^{-1}$; (b) control, 1.5 pmol mg^{-1} protein, $K_A = 4.1 \times 10^9 M^{-1}$; PCMB-treated 1.0 pmol mg^{-1} protein, $K_A = 1.8 \times 10^9 M^{-1}$. In the latter experiment, it is assumed that only the pharmacologically more active (−)-NMA is binding and the calculated affinity constant is that of this enantiomer.

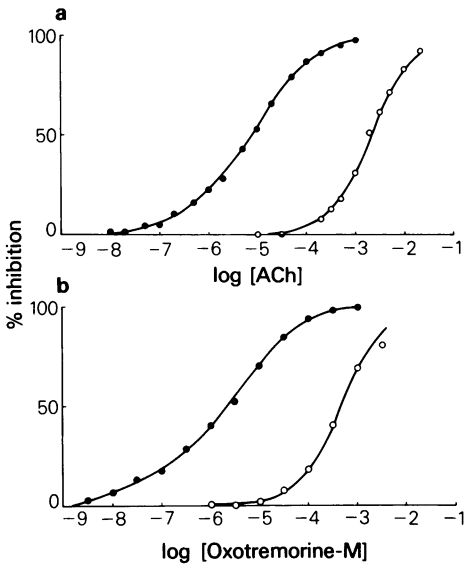

Figure 7 The Phase II effect of *p*-chloromercuribenzoate (PCMB) on the inhibition of [^{3}H]-propylbenzilylcholine ([^{3}H]-PrBCh) binding by (a) acetylcholine and (b) oxotremorine-M to muscarinic receptors from the rat cerebral cortex. Membranes (1 mg protein ml^{-1}) were incubated at 30°C for 15 min in the presence (○) or absence (●) of 10^{-3}M PCMB, before the start of the 15 min binding assay. Neostigmine (10^{-6}M final concentration) was added 5 min before the start of the binding experiment. The curves through the open circles are simple mass action curves. The concentration of [^{3}H]-PrBCh (1 nM) was such that the agonist inhibition curves approximate closely to the agonist occupancy isotherms. The curves through the closed circle data points are non-linear least squares fits of a 2-site model with (a) 73 ± 2% low affinity sites, log affinity constant, 4.76 ± 0.05; 27% high affinity sites, log affinity constant 6.77 ± 0.09; (b) 65 ± 7% low affinity sites, log affinity constant 5.07 ± 0.05; 35% high affinity sites, log affinity constant 7.01 ± 0.11.

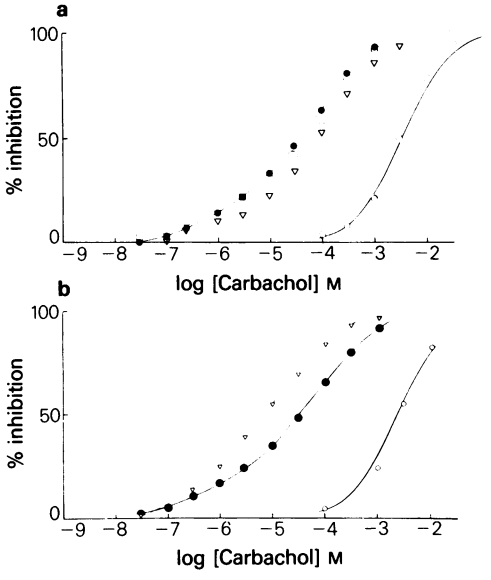

Figure 8 The reversal by dithioerythritol of the effects of (a) short and (b) long incubations with PCMB (10^{-3}M) on the inhibition by carbachol of [^{3}H]-PrBCh (1 nM) binding. Membranes (1 mg protein ml^{-1}) were incubated in the presence (○) or absence (●) of 10^{-3}M PCMB for (a) 15 min and (b) 2 h. Reversibility of the effects of PCMB (▽) and the effects of dithioerythritol on the control membranes was assessed by the addition of DTE (30 min) prior to the binding assay. The curves through the closed circle data points are non-linear least squares fits of a 2-site model with (a) 68 ± 2% low affinity sites, $\log K_A = 3.91 \pm 0.04$, 32% high affinity sites, $\log K_A = 5.83 \pm 0.09$; (b) 69 ± 4% low affinity sites, $\log K_A = 3.95 \pm 0.08$, 31% high affinity sites, $\log K_A = 6.04 \pm 0.17$. The curve through the triangles in (b) is the non-linear least squares fit with 41 ± 4% low affinity sites, $\log K_A = 4.10 \pm 0.06$; 59% high affinity sites, $\log K_A = 5.95 \pm 0.07$. In this experiment there was a 75% loss in concentration of binding sites after treatment with PCMB for 2 h, followed by DTE. These data are compatible with a 7 fold decrease in concentration of low affinity sites for carbachol and only a 2 fold decrease in concentration of high affinity sites. The curves through the open circles are the best fit simple mass action binding curves with (a) $\log K_A = 2.49 \pm 0.05$, (b) 2.58 ± 0.04.

caused a slow recovery to a 25% loss after 30 min, but thereafter the binding capacity started to decline again. However, it was found in membranes not treated with PCMB that DTE also produced a delayed slow decrease in capacity. This is probably due to an endogenous sulphydryl activated protease (Hulme *et al.*, unpublished results). These results suggested that the reversal effects on the receptor population were not homogeneous and that more than one population of sulphydryl group might be involved.

As pointed out earlier, more extreme treatment with PCMB, for instance 10^{-3}M for 2 h, reduced the capacity of 20% of the control but did not produce any further changes in binding affinity. However, after prolonged PCMB treatment, reversal with DTE (10 mM, 30 min) returned the affinity for PrBCh to normal but that for carbachol was reversed to higher affinity (Figure 8b). Analysis of the curve suggests that the proportion of L receptor sites has been reduced.

Anomalous results were also obtained with the selective antagonist pirenzepine. Pirenzepine normally binds to two populations of receptor in the cerebral cortex (Hammer, Berrie, Burgen & Hulme, 1980). The binding is not mass action and has a Hill slope of 0.75 (Figure 9a).

Treatment with PCMB (1 mM, 15 min) shifts the curve to the right and converts it into a mass action

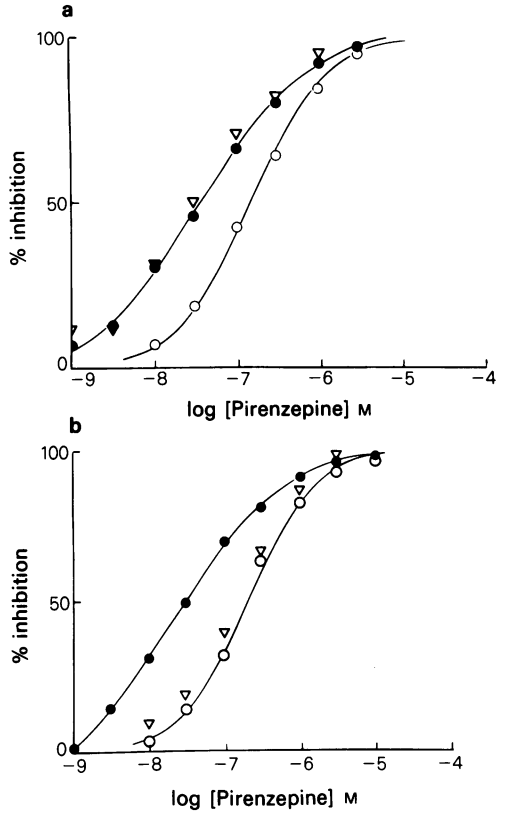

Figure 9 Effects of *p*-chloromercuribenzoate (PCMB) on pirenzepine inhibition of [^{3}H]-propylbenzilylcholine ([^{3}H]-PrBCh) binding to muscarinic receptors from rat cerebral cortex and the reversal by dithioerythritol (DTE) after (a) 15 min and (b) 2 h PCMB treatment. Membranes were incubated at 30°C in the presence (○) or absence (●) of 10^{-3}M PCMB for 15 min (a) or 120 min (b) before initiation of the 15 min binding assay. Reversibility (▽) was assessed by the addition of dithioerythritol (10 mM, final concentration) to the PCMB-treated membranes and incubation for 30 min before the binding assay. The concentration of [^{3}H]-PrBCh (10^{-9}M) was such the pirenzepine inhibition curve approximates closely to the receptor occupancy curve for pirenzepine. The curves through the closed circle data points are non-linear least squares fits of a 2-site model with (a) 36±7% low affinity sites, $\log K_A = 6.60 \pm 0.17$, 64% high affinity sites, $\log K_A = 7.72 \pm 0.07$ (b) 30±8%, $\log K_A = 6.45 \pm 0.15$, 70% high affinity sites, $\log K_A = 7.75 \pm 0.06$. The analysis of the data on DTE reversal (▽) after 2 h treatment with PCMB (b) gave 73±5% low affinity sites for pirenzepine. Under these conditions there was a 65% loss of binding sites. These data are compatible with a 7 fold decrease in concentration of high affinity sites for pirenzepine and only a 1.4 fold decrease in concentration of low affinity sites. This analysis and that described in the legend to Figure 8 indicate the greater sensitivity of the low affinity carbachol sites and high affinity pirenzepine sites to PCMB. The curves through the open circles are least squares fits to a one-site model with (a) $\log K_A = 6.84 \pm 0.06$; (b) $\log K_A = 6.73 \pm 0.04$.

curve; in this sense it is like an agonist curve but the shift is relatively small. These changes are fully reversible by DTE. However, after the more extensive PCMB treatment (1 mM, 2 h) and attempted reversal with DTE, there was little increase in pirenzepine affinity or recovery of binding heterogeneity (Figure 9b). The simplest explanation of this effect is that the high affinity pirenzepine sites are selectively inactivated and are not reactivated by DTE after prolonged PCMB treatment. These two long-term effects of PCMB link together the properties of the low affinity carbachol binding sites and the high affinity pirenzepine binding sites in the cortex which are also present in approximately equal amounts.

Protection against PCMB by antagonists and agonists

The effects of incubation of membranes with PCMB under Phase I conditions were totally unaffected by the presence of atropine 10^{-6}M or carbachol 10^{-3}M. These are concentrations that occupy 99.9% and >95% of receptors respectively. However, the usual small loss (~15%) of receptor capacity was prevented.

Under Phase II conditions (10^{-3}M PCMB, 15 min) either pretreatment or simultaneous addition of an antagonist such as NMA (2×10^{-8}M) or N-methylscopolamine (NMS, 10^{-8}M) almost totally prevented the development of the changed receptor affinity or loss of receptor capacity. Carbachol (10^{-3}M) was less effective but did slow down the development of Phase II characteristics. However, protection against higher concentrations or longer exposures to PCMB was limited. If we were dealing with an absolute steric barrier to PCMB action by occupation of the receptor we would expect the rate of reaction to be proportional to the residual fraction of unoccupied receptors. Using (±)-NMA at a concentration of 10^{-6}M this fraction would be 0.0005, yet the rate of inactivation of receptors was not reduced by anything like this amount; at the very most it was reduced by a factor of 10.

Changes in membrane bound thiols

The membrane thiols were measured under non-denaturing conditions (Table 2). The control value was 6.5±1.0 mol g^{-1} protein, a value similar to that reported by Aronstam *et al.* (1978). Under Phase I conditions 40–50% of the sulphydryl groups in the membrane had been rendered unavailable. Under Phase II conditions there was no significant amount of residual thiol detectable. It should be noted that the concentration of thiols in the membrane is very high and far exceeds the likely number of thiol groups associated with the receptors, and indeed it is known that other membrane proteins such as ATPases are rich in SH groups. The measurements thus tell us

Table 2 Estimation of membrane bound protein thiol groups after reaction with *p*-chloromercuribenzoate (PCMB)

[PCMB]M	*% control*
6×10^{-5}	62
10^{-4}	49
10^{-3}	<3

* Control values were 6.5 ± 1.0 (s.e.mean, $n=5$) nmol mg^{-1} protein

what is happening to the bulk of SH groups in the membrane and may give little indication of the behaviour of the groups in the receptor. It is, however, clear that some of the SH groups responsible for the loss of receptor capacity must be exceptionally highly hindered or unreactive.

Discussion

From the known chemistry of PCMB and its reaction with proteins, it is reasonable to assume that the effects on ligand binding that we have observed are due to reaction with sulphydryl groups, either on the receptor protein itself (i.e. the ligand binding subunit), or on associated proteins. The differences in reaction rate and other parameters have enabled us to discriminate between three groups of action, each of which may be due to the inactivation of one or more sulphydryl groups. The criteria distinguishing these three actions are summarised in Table 3. For convenience of description, the sulphydryl groups are termed A, B and C. Phase I effects are caused predominantly by reaction of the A sulphydryl group(s), Phase II by reaction of the B sulphydryl group(s), although reaction of C sulphydryls occurs in Phases I and II. It should be borne in mind that because of the temporal sequence, the effects described in Phase II (B group modification) are really Phase I (A group modification) plus a new effect. We cannot isolate this new effect because at present we have no means for blocking the reaction of the A sulphydryl group(s).

In the next section we will attempt to provide an interpretation of the three groups of effects in terms of molecular changes at the receptor level.

Phase I (A group modification)

In this Phase there are no effects on antagonist binding but selective changes affecting agonist binding which affect the SH, H and L sites unequally. Since we cannot protect by either agonists or antagonists, we must conclude that the A sulphydryl group(s) involved do not lie within the ligand binding site. The effect must be a communicated conformational (allosteric) effect derived either from a part of the receptor protein not directly involved in ligand binding, or from an associated conformationally coupled protein.

If one considers the simplest model for receptor activation (Burgen, Birdsall & Hulme, 1979).

$$\begin{array}{ccc} & \overset{K_1}{} & \\ L + R & \rightleftharpoons & LR \\ K_\alpha \downdownarrows & & \downdownarrows K_\beta \\ & \underset{K_2}{} & \\ L + R^* & \rightleftharpoons & LR^* \end{array}$$

where R represents the ground state of the receptor and R* its active conformation (the latter is poorly populated except in the presence of an agonist), then the lack of effect on antagonist binding requires that K_1 remains unchanged. Selective effects on agonist binding could be produced by a change in either K_2 or in K_α (which may be regarded as a set of constants corresponding to the SH, H and L receptors). A change in the K_α's could account for the selective effects on the SH, H and L receptors, but this would have to be similar on all potent agonists (i.e. those in

Table 3 The effects of *p*-chloromercuribenzoate (PCMB) on the postulated classes of sulphydryl groups on muscarinic receptors in the cerebral cortex

Effect	*Sulphydryl group*		
	A	*B*	*C*
Kinetics of PCMB reaction	Rapid	Intermediate	Complex
Agonist binding	Affected	Affected	Abolished
Antagonist binding	Unchanged	Affected	Abolished
Existence of agonist binding heterogeneity	Altered but still present	Absent	—
Protection against PCMB reaction by muscarinic drugs	None	Yes	Yes
Reversibility of PCMB effect by DTE	Rapid	Rapid	Complex

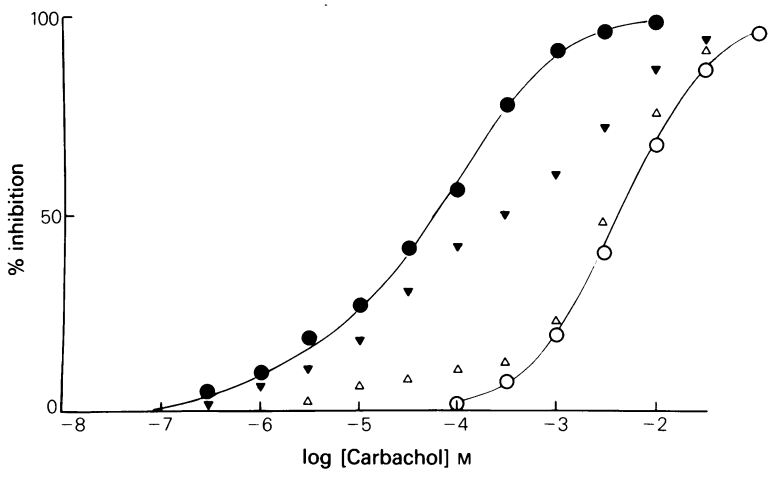

Figure 10 The effects of increasing concentrations of *p*-chloromorcuribenzoate (PCMB) on the inhibition of [^{3}H]-propylbenzilylcholine [^{3}H]-PrBCh (1 nM) binding to muscarinic receptors in rat cortical membranes by carbachol. The concentrations of PCMB used were (●), 0; (▼), 7×10^{-5}M; (△) 10^{-4}M; (○) 10^{-3}M, and the duration of incubation 15 min prior to the binding assay.

which $K_2 >> K_1$) but there are clear indications, even in Figure 5, that this is not so. An alternative way of looking at the problem is to assume that it is not K_α that varies with the subtype but K_2, which is a family (K_{2SH}, K_{2L}, K_{2L}). The action of PCMB would thus be to change the R* conformation and hence its structure-binding profile, which would entail a change in the quantitative values of K_{2SH}, K_{2H} and K_{2L}.

It should be noted that Phase I effects are superficially similar to the results reported by Aronstam *et al.* (1977, 1982) for NEM, but in fact there are differences which will be examined in a later paper.

Phase II (A and B group modification)

In this Phase, major changes are produced in the binding of antagonists, and of agonists and the heterogeneity of binding of agonists is eliminated. There is evidence that this is due either to a single or a very limited number of sulphydryl groups because if one examines the development of Phase II in the intermediate stages before it is fully developed, irregular binding curves are seen that appear to be a superimposition of the normal (actually Phase I) and Phase II curves (Figure 10).

One possibility to explain these results is that in Phase II a change in the conformation of the R state occurs which changes its structure-binding profile for antagonists and at the same time prohibits the R→R* conformational transition. Under these circumstances the residual binding of agonists will be weak, since it will be to the modified ground state R′ and since heterogeneity is a consequence of the R→R* transition, this will no longer be a feature of agonist binding. This explanation, however, does not readily account for the non-stoichiometric features of the protection experiments with agonists and antagonists.

An alternative possibility is that PCMB reacts selectively with and traps a minor conformational state of the receptor which thus becomes dominant and effectively becomes the R′ state mentioned above. The effect of agonists or antagonists would therefore be to populate R* or R at the expense of R′ so that the reaction with PCMB would be reduced but not completely blocked.

It will be noted that once again the evidence points away from the SH group responsible for creation of the Phase II state being within the receptor ligand binding domain.

Loss of binding capacity (C group modification)

The loss of binding capacity follows multiphase kinetics; there is a rapid inactivation of some 15% of the binding sites under very mild PCMB treatment (~1 min, 6×10^{-5}M), a further loss of 55–60% at an intermediate rate (~30 min, 10^{-3}M) and a final fraction of 20–25% that is very resistant to inactivation. These must represent receptor subpopulations in different states or environments, yet they show little connection with the generation of the Phase I or Phase II states. The protection experiments showed that the loss of capacity could be prevented by antagonists or agonists, whereas the development of Phase I could not. These changes must therefore be due to the inactivation of distinct sets of sulphydryls

(C group). There is likewise no connection between the generation of Phase II and moderate inactivation of binding capacity. On the other hand, the reactivation experiments after extensive loss of capacity do point to a selective action on C sulphydryl groups of low affinity carbachol and high affinity pirenzipine binding sites and suggests as other evidence does that these criteria may be delineating the same receptor subtype.

The same limitatiion to protection against loss of binding capacity apply as to Phase II; the protection is not stoichiometric to the occupation of receptors and this suggests that inactivation is due to generation of receptor conformations in which the binding site is no longer complementary to either antagonists or agonists.

This chemical exploration of the muscarinic receptor in the central cortex by PCMB has revealed a number of interesting effects which seem to be explained by conformational modifications – a conformation change in response to ligands is, of course, at the heart of receptor action. In the following paper the details of the changes in structure-binding profiles produced by PCMB are examined.

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