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Influence of Sulfhydryl Reagents and Heavy Metals on the Functional State of the Muscarinic Acetylcholine Receptor in Rat Brain

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SUMMARY

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Membranes from rat brain contain several groups that react with sulfhydryl reagents to influence muscarinic acetylcholine receptor binding. *p*-Chloromercuribenzoate (PCMB) reacts with a group(s) within or under the allosteric control of the receptor binding site to inhibit both agonist and antagonist binding. Receptors can be protected from PCMB inactivation by the presence of receptor ligands (agonists or antagonists), and the inactivation can be reversed by subsequent treatment with organic sulfhydryls. Reductive alkylation of neural membranes with *N*-ethylmaleimide (NEM) can prevent much of the inhibition of antagonist, but not agonist, binding by subsequent PCMB treatment, suggesting that it is the presence of a mercuribenzoate, but not an ethylmaleimide residue, within the binding site that is inimical to receptor binding. NEM treatment increases agonist binding by converting receptors from a state of low agonist affinity to high agonist affinity, whereas both states have the same high affinity for receptor antagonists. The presence of agonists but not antagonists during the NEM treatment enhances the ability of NEM to increase agonist affinity. Prior treatment with low concentrations of PCMB abolishes the ability of NEM subsequently to increase agonist binding, even when binding site-saturating concentrations of receptor ligands are included during the PCMB treatment to protect the binding site—a finding which suggests that NEM exerts its influence on agonist binding through interaction with a group contiguous to the receptor binding site. Transition metal ions, which appear to interact with all of the NEM- and PCMB-reactive moieties, increase agonist binding, decrease agonist binding, or decrease both agonist and antagonist binding, depending on the concentration of metal. Membranes from various areas of the brain contain receptors that differ in their distribution between the high- and low-agonist-affinity forms; in terms of the affinity of muscarinic receptors for agonists, brain stem≫telencephalon>hippocampus. Receptors from different brain areas also display different sensitivities to sulfhydryl reagents and heavy metals.

INTRODUCTION

Binding studies on the muscarinic acetylcholine receptor of mammalian brain indicate the presence of discrete populations of receptors that differ in their affinities for

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receptor agonists but have a uniformly high affinity for receptor antagonists (1, 2). We have recently shown that the lower-agonist-affinity form is converted to the high-affinity form by alkylation of a membrane sulfhydryl group(s) (3). The total number of receptors and their affinity for antagonists are unaffected by the alkylation. We report here the presence of several groups in neural membranes that react with sulfhydryl reagents and heavy metals, thereby influencing muscarinic binding. Sulfhydryl groups that affect binding are located both within and contiguous to the receptor binding site. Under different conditions sulfhydryl reagents may inhibit all binding, inhibit agonist binding preferentially, or enhance agonist binding.

The existence of disulfide and sulfhydryl groups in both the binding and ion translocation mechanisms of nicotinic acetylcholine receptors has been demonstrated. Karlin has used affinity labels directed at sulfhydryl groups, which are produced by the reduction of a nearby disulfide bond, to map the binding site of fish electric organ receptors (4). The ability of electroplax receptors to bind cholinergic ligands is reduced by both disulfide and sulfhydryl reagents (5). Súarez-Isla and Hucho have demonstrated that prior treatment of *Torpedo* electroplax membranes with carbamylcholine results in a decrease in the reactivity of receptor sulfhydryl groups toward DTNB[3] (6). Time-dependent modification of the binding properties of electric fish receptors, which involves an increase in agonist binding and is induced by prior exposure to agonists, but not antagonists, has been reported (7, 8). It would appear that in the conversion of these nicotinic receptors to the desensitized, higher-agonist-affinity state, a conformational change occurs that sequesters sulfhydryl groups in the region of the receptor.

The effects of disulfide- and sulfhydryl group-modifying reagents on the pharmacological responses of smooth muscle to muscarinic stimulation have also been investigated (9, 10). Stubbins and Hudgins (9) found that while both NEM and PCMB reduce the response of intestinal smooth muscle to acetylcholine, both reagents are also irreversible inhibitors of muscle contraction in response to nonspecific potassium stimulation. In addition, the receptor could not be protected from NEM action by including muscarinic drugs during the treatment with NEM. These findings suggest that while both sulfhydryl reagents can react with sulfhydryl groups involved in the production of contractions, the groups are not necessarily involved with the receptor binding site. Stubbins and Hudgins (9) also reported that DTT inhibits acetylcholine-induced smooth muscle contraction. DTT converts bromoacetylcholine from a reversible to an irreversible activator of nicotinic electroplax receptors (3), but not of muscarinic receptors, in smooth muscle (9). This indicates a lack of a disulfide bond in the immediate vicinity of the muscarinic receptor. Fleisch *et al.* (10) came to a similar conclusion regarding the lack of a disulfide bond near the acetylcholine binding site of rat trachea.

MATERIALS AND METHODS

2-Mercaptoethanol was obtained from Eastman Organic Chemicals; NEM, from Mann Research Laboratories; L-cysteine, PCMB, DTNB, and DTT, from Sigma Chemical Company; cadmium chloride, ferric ammonium sulfate, mercuric chloride, and zinc sulfate, from J. T. Baker Chemical Company; gold chloride, lead chloride, manganese chloride, and silver nitrate, from Fisher Scientific Company; and cobalt chloride, cupric nitrate, and nickel chloride, from Mallinckrodt Chemical Works.

Membranes were prepared from Sprague-Dawley rat forebrains (telencephalon and diencephalon) by homogenization in 10 volumes of 50 mM sodium–potassium phosphate buffer, pH 7.4. The supernatant from a 10-min centrifugation at 1000 × *g* was centrifuged at 50,000 × *g* for 1 hr, and the pellet was resuspended in phosphate buffer and used without further treatment. In certain experiments, as noted, the starting material was rat brain

[3] The abbreviations used are: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); NEM, *N*-ethylmaleimide; PCMB, *p*-chloromercuribenzoate; DTT, dithiothreitol; QNB, [^{3}H]quinuclidinyl benzilate.

stem (medulla oblongata, pons, and midbrain), telencephalon, or hippocampus.

The binding of QNB (16 Ci/mmole, Amersham/Searle, batch 2), a specific and potent muscarinic antagonist, was measured by a modification of the filtration assay introduced by Yamamura and Snyder (11). A suspension containing 0.3 mg of membrane protein, 50 mM sodium-potassium phosphate buffer, pH 7.4, and QNB, with or without unlabeled atropine, was incubated for 40 min at 20° and then filtered by suction through a Whatman GF/B glass fiber filter, which was then washed with 7 ml of phosphate buffer. The filters were placed in plastic vials, and 10 ml of scintillation fluid (10 g of 2,5-diphenyloxazole, 0.5 g of 1,4-bis[2-(5-phenyloxazolyl)] benzene, 1 liter of Triton X-100, and 2 liters of toluene) were added. The vials were held for at least 12 hr before being counted in a Beckman LS-233 counter at an efficiency of 24%. Specific binding was operationally defined as the total binding minus the binding that could not be displaced by 1 μM unlabeled atropine. The ability of various drugs to compete with 1 nM QNB for muscarinic binding sites was determined by including appropriate amounts of the drugs in the incubation media, as noted in the tables and figure legends. The effects of heavy metals on muscarinic binding were determined by including the metals in the incubation media, which contained 50 mM Tris buffer, pH 7.4, instead of phosphate buffer. All assays were performed in triplicate, except for binding curves, which were determined in duplicate.

The association of carbamylcholine with the receptor was determined from its ability to displace the binding of 0.05 nM QNB (2, 3). In determining binding curves, the concentration of muscarinic binding sites was kept at less than 1/10 of the true QNB dissociation constant (0.1–0.2 nM) and the concentration of free ligand was corrected for by the amount of bound ligand. To meet the former requirement, the amount of membrane assayed was reduced while the total incubation medium volume was increased to 25 ml.

The effects of protein-modifying reagents on muscarinic receptors were determined by incubating membranes (2 mg of protein per milliliter, except as noted) with the reagents for 20 min at 35°. The reagents were removed by centrifugation at 35,00 × *g* for 30 min. The pellets were rinsed with buffer and resuspended and recentrifuged prior to use. When treatment with a second modifying reagent followed, the incubation and washing procedures were repeated. In the study of the kinetics of the development of increased receptor affinity for agonists induced by NEM treatment, the NEM reaction was rapidly quenched at the appropriate time by the addition of 5 mM DTT. The membranes were then extensively washed before the binding was assayed.

The protein content of the membranes was determined by ultraviolet absorbance in 2% sodium dodecyl sulfate (12). Membrane sulfhydryl content was assayed spectrophotometrically with DTNB by the procedure suggested by Habeeb (13).

RESULTS

The effects of sulfhydryl-alkylating and -oxidizing reagents and disulfide-reducing reagents on the specific binding of 1 nM QNB in rat forebrain membranes are presented in Table 1. After treatment of neural membranes with 1.0 mM PCMB for 20 min at 35°, QNB binding was reduced 96%. All other modifying reagents tested were without effect. The presence of 0.5 M guanidine during the membrane treatments did not alter the ability of any reagent to affect muscarinic binding.

The PCMB-mediated inhibition of QNB binding (Fig. 1) was partially reversed by subsequent incubation of the membranes with various organic sulfhydryls for 30 min at 30°. For example, after incubation of 5 mg/ml of membrane protein with 1 mM PCMB for 20 min at 35°, QNB binding was reduced to 20.1% of control levels. Subsequent treatment with 5 mM cysteine, 2-mercaptoethanol, or DTT increased binding to 49.3%, 50.9%, or 48.2% of control levels, respectively. Similar recoveries were observed in four separate experiments using varied conditions of membrane treatment, although complete recovery was never observed. The presence of the muscarinic agonist carbamylcholine or arecoline at 2 mM

TABLE 1

Effects of disulfide- and sulfhydryl-modifying reagents on muscarinic binding in membranes from rat forebrains (telencephalon and diencephalon)

Values are means ± standard errors.

Reagent	Blockade of specific QNB binding	Blockade of 1 nM QNB binding by:			
		40 μM carbamylcholine	20 μM arecoline	100 μM nicotine	0.04 μM atropine
	%	%	%	%	%
Control	0	41.3 ± 0.3	60.0 ± 0.8	7.9 ± 1.8	44.3 ± 0.9
1 mM dithiothreitol	0	40.0 ± 0.7	61.8 ± 1.4	10.2 ± 2.1	47.9 ± 1.3
1 mM *N*-ethylmaleimide	0	50.6 ± 0.8	70.2 ± 1.2	12.9 ± 1.6	47.4 ± 1.6
1 mM dithiobis (2-nitrobenzoate)	0	48.7 ± 1.8	64.2 ± 0.8	7.8 ± 1.0	43.9 ± 1.4
0.1 mM *p*-chloromercuribenzoate	2.2				
1 mM *p*-chloromercuribenzoate	96.1			60.1 ± 3.1	10.9 ± 1.4
5 mM 2-mercaptoethanol	0	42.5 ± 1.1	61.4 ± 0.5	9.8 ± 1.2	44.6 ± 1.6

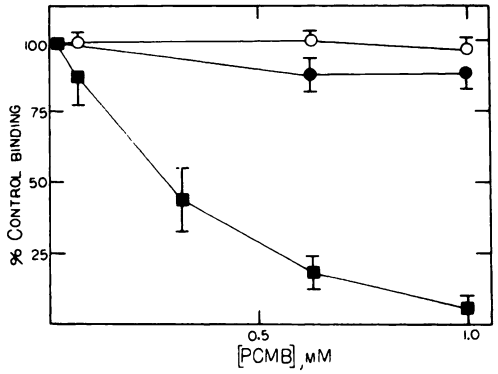

FIG. 1. *Inhibition of [^{3}H]QNB binding by PCMB*

Membranes (3 mg of membrane protein per milliliter) were treated with various amounts of PCMB for 20 min at 35° in the presence of 2 mM carbamylcholine (○), 0.1 μM atropine (●), or no ligand (■). The membranes were washed by centrifugation prior to being assayed for [^{3}H]QNB binding. Control binding values represent membranes incubated in the presence (or absence) of the appropriate ligand with no PCMB present. The control binding levels were reduced 10% by the atropine incubation, indicating incomplete removal of the drug by the wash.

or the antagonist atropine or scopolamine at 0.1 μM during the incubation with PCMB prevented much of the QNB binding inactivation. There is a discrepancy between the effectiveness of atropine in protecting QNB binding from inactivation by PCMB in the experiments reported in Fig. 1 and Table 2; the reason for this discrepancy is not clear. It is possible that the higher tissue concentrations used in the experiments reported in Fig. 1 compared with those in Table 2 (3 mg of membrane protein per milliliter vs. 2 mg/ml) and the use of tissues from different sources (telencephalon plus diencephalon vs. telencephalon alone) may account for some of the differences. Both studies were repeated at least three times on different material on separate days; however, the two series of experiments were performed several months apart. It is possible that a minor adjustment in some unidentified aspect of our protocols is responsible for the difference.

Treatment of the membranes with NEM increased the abilities of carbamylcholine and arecoline, but not of atropine or unlabeled QNB, to compete with QNB for the muscarinic receptor (Table 1). DTNB was somewhat less effective than NEM, increasing the displacement of QNB by agonists by about 10%. Prior incubation of the membranes (2 mg of protein per milliliter) with 1 mM DTT (20 min at 35°) to reduce disulfide bonds did not potentiate the ability of NEM to enhance agonist affinity; for example, 40 μM carbamylcholine inhibited 1 nM QNB binding 59.4% ± 0.8% without, and 58.7% ± 1.2% with, prior DTT treatment. The effects of various combinations of membrane treatments on QNB binding and on the ability of carbamylcholine to block QNB binding under various assay condi-

TABLE 2

Effects of protein-modifying reagents and heavy metals on muscarinic binding in membranes from different brain areas

Values are means and standard errors from two to six experiments, performed in triplicate. When specific QNB binding was reduced to below 20% of control levels, the measure of agonist binding became variable and difficult to reproduce. In both treatments 1 and 2, membranes (2 mg of protein per milliliter) were treated with 1 mM NEM or 0.5 mM PCMB for 20 min at 35°. Carbamylcholine (100 μM) or atropine (0.1 μM) was included in some treatments, as shown. All reagents and ligands were washed away prior to the binding assay or the second treatment. Metal (100 μM zinc or 10 μM silver) was added just before binding was to be assayed.

Membrane source	Treatment 1	Treatment 2	Metal	QNB binding[a]	Agonist binding[b]
				%	%
Telencephalon				100 ± 0.8	38.2 ± 0.5
			Ag	7.3 ± 4.2	61.2 ± 30.2
			Zn	99.7 ± 0.9	45.9 ± 1.6
	NEM			96.9 ± 1.2	59.0 ± 0.8
	NEM		Ag	5.2 ± 2.2	51.6 ± 20.2
	NEM		Zn	95.8 ± 2.4	57.6 ± 1.6
	PCMB			27.1 ± 2.0	6.5 ± 2.6
	PCMB		Ag	13.4 ± 4.2	11.5 ± 5.3
	PCMB		Zn	28.2 ± 2.3	10.2 ± 4.1
	PCMB + carbamylcholine			42.7 ± 2.8	4.8 ± 3.0
	PCMB + carbamylcholine		Ag	21.6 ± 3.2	0 ± 2.6
	PCMB + carbamylcholine		Zn	47.3 ± 2.7	2.4 ± 3.6
	PCMB + atropine			48.0 ± 1.8	5.1 ± 2.4
	PCMB + atropine		Ag	19.8 ± 3.2	−0.3 ± 1.7
	PCMB + atropine		Zn	49.9 ± 3.5	4.5 ± 6.0
	NEM	PCMB		43.6 ± 2.1	6.6 ± 5.9
	PCMB	NEM		25.0 ± 1.7	0 ± 2.7
	PCMB + carbamylcholine	NEM		38.1 ± 2.6	−0.4 ± 2.8
	PCMB + atropine	NEM		45.6 ± 3.0	0.1 ± 4.1
Brain stem				100 ± 0.7	77.6 ± 0.9
			Zn	104.5 ± 1.2	69.9 ± 1.3
	NEM			90.1 ± 0.9	90.6 ± 1.4
	NEM		Zn	91.7 ± 1.3	80.5 ± 1.7
	PCMB + carbamylcholine			55.7 ± 2.6	11.5 ± 1.6
	PCMB + carbamylcholine		Zn	57.3 ± 1.9	5.7 ± 1.3
Hippocampus				100 ± 0.6	30.2 ± 0.8
			Zn	95.1 ± 1.4	38.6 ± 0.6
	NEM			93.3 ± 0.5	50.9 ± 1.0
	NEM		Zn	90.0 ± 1.1	56.2 ± 2.1
	PCMB + carbamylcholine			58.7 ± 2.3	10.1 ± 4.3
	PCMB + carbamylcholine		Zn	50.6 ± 1.7	3.7 ± 3.6

[a] Specific binding of 1 nM [^{3}H]QNB, expressed as a percentage of "no treatment" control binding.

[b] Percentage of specific binding by 1 nM [^{3}H]QNB that is blocked by 40 μM carbamylcholine.

tions are presented in Table 2. Prior treatment with PCMB abolished the ability of NEM to alter any remaining agonist binding, even when saturating concentrations of receptor ligands were included during the treatment with PCMB to protect the binding site. A similar result was obtained with PCMB at concentrations (0.05–0.2 mM PCMB, 3 mg of membrane protein per milliliter) that decreased receptor-agonist interactions by less than 50%. In this range QNB binding was inhibited 0–25%. Treatment with NEM prior to PCMB greatly mitigated the inhibitory action of PCMB on the binding of QNB, but not carbamylcholine. QNB binding after PCMB treat-

ment was significantly ($p < 0.005$) greater in membranes that had been treated with NEM (43.6% vs. 27.1% of control QNB binding, average of four experiments) (Table 2). This finding indicates that while both NEM and PCMB have access to sulfhydryl groups associated with the binding site, only the presence of a mercuribenzoate, and not an ethylmaleimide, residue within the binding region decreases ligand binding.

As previously described (3), the enhancement of carbamylcholine binding by NEM is consistent with a model that predicts a conversion of receptors from a state of low agonist affinity to one of high agonist affinity, whereas the affinity of receptors in both states for receptor antagonists is identical. (That there are multiple receptor populations with respect to agonist binding is clear; that there are only two such populations is still speculative.) The distribution of receptors between these states is different in membranes from different regions of the brain (Fig. 2). Based on a two-state model with previously determined carbamylcholine dissociation constant values of 0.13 and 50 μM for the high- and low-agonist-affinity receptor forms (3), respectively, Scatchard analyses of carbamylcholine binding indicate that the proportion of muscarinic receptors in the high-affinity form is about 60% in brain stem membranes, but only about 35% in telencephalic membranes. NEM treatment causes an apparent increase in the proportion of high-affinity receptors to essentially 100% in the brain stem and to about 50% in the telencephalon. The ratio of carbamylcholine to QNB affinity was found to be much lower in the hippocampus than in the whole telencephalon or brain stem (Table 2). In six brains carbamylcholine was at least 20% less effective in blocking QNB binding in the hippocampus than in the whole telencephalon.

The kinetics of the development of enhanced carbamylcholine binding by NEM treatment is depicted in Fig. 3. The effect of NEM on the muscarinic receptor was temperature-dependent, being considerably reduced at 18° compared with 35°. The reaction was unaffected by the presence of 5 mM $CaCl_2$ or $MgCl_2$. Monovalent cations inhibited the development of the NEM effect without affecting the NEM-induced reduction of available membrane thiol groups, as measured by reaction with DTNB under nondenaturing conditions. Inhibition of NEM enhancement of carbamylcholine binding ranged from 10% to 47% in the presence of 50 mM and from 36% to 75% in the presence of 400 mM monovalent cations. At all concentrations tested, the

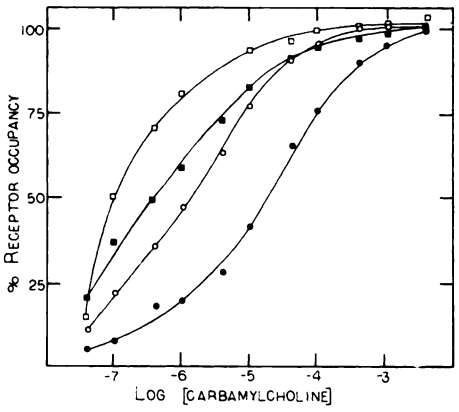

FIG. 2. *Binding of carbamylcholine to neural membranes from rat telencephalon (circles) and brain stem (squares) that were untreated (solid symbols) or treated with 1 mM NEM for 20 min at 35° (open symbols)*

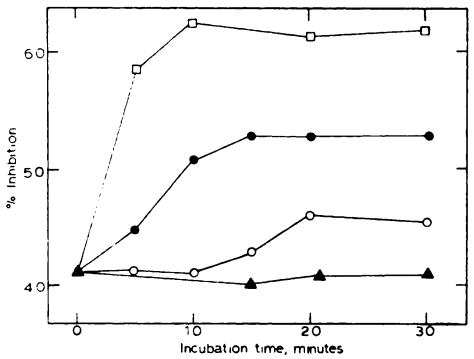

FIG. 3. *Kinetics of development of increased receptor affinity for carbamylcholine induced by NEM treatment*

The percentage inhibition of specific binding of 1 nM [^{3}H]QNB by 40 μM carbamylcholine is shown for prior membrane treatment with 1 mM NEM at 35° (●), 1 mM NEM at 18° (○), 1 mM NEM + 10 μM carbamylcholine at 35° (□), or no added reagents (▲), is shown. The values depicted are averages from three experiments performed in triplicate on separate membrane preparations, which agreed within 10%.

order of ability of the cations to inhibit the NEM effect was $Li^+ > Cs^+$, $Na^+ > Rb^+ > K^+$.

The total sulfhydryl content of forebrain membranes as assayed spectrophotometrically with DTNB in 2% sodium dodecyl sulfate was 27 nmoles/mg of protein. Only 29.6% of these groups reacted with DTNB under nondenaturing conditions. NEM, however, reacts with 47.3% of the total membrane sulfhydryl groups (2 mg/ml of membrane protein treated with 5 mM NEM for 20 min at 35°) under nondenaturing conditions, as indicated by the subsequent decrease in DTNB interaction with sulfhydryl groups in 2% sodium dodecyl sulfate. All of the "available" sulfhydryl groups (those groups which react with DTNB under mild conditions) were alkylated during the preliminary treatment with NEM. The greater ability of NEM to enhance agonist binding compared with DTNB may be related to its greater degree of sulfhydryl group alkylation. Perhaps the relevant sulfhydryl group is partially sequestered in interior membrane regions and is only susceptible to the influence of the more lipophilic NEM molecule.

The presence of muscarinic agonists during the NEM treatment caused a further increase in the ability of carbamylcholine to inhibit QNB binding subsequently (Fig. 3). The muscarinic agonists arecoline, pilocarpine, and carbamylcholine doubled the NEM effect, while the agonists methacholine and oxotremorine were less effective, and muscarinic antagonists and the nicotinic cholinergic ligands nicotine and *d*-tubocurarine had no effect (Table 3).

The effects of various concentrations of a number of metals on QNB binding and on carbamylcholine inhibition of QNB binding are presented in Fig. 4. All of the transition metals tested were effective both in inhibiting QNB binding and in increasing the proportion of 1 nM QNB binding susceptible to blockade by 40 μM carbamylcholine. The only exception was mercury, which decreased QNB binding without increasing the relative amount of agonist binding. Several metals, including cadmium, copper, lead, and zinc, increased carbamylcholine binding at concentrations that caused no decrease in QNB binding. No metal increased agonist binding in membranes from the telencephalon that had been treated with NEM (the data for zinc are presented in Table 2), a finding that suggests that both metals and NEM affect agonist binding by a common mechanism, possibly involving sulfhydryl groups. Prior treatment of membranes with 0.5 mM PCMB in the presence of receptor-saturating concentrations of muscarinic ligands to protect the binding site abolished the ability of zinc to stimulate the surviving agonist binding, a finding that again suggests a sulfhydryl-mediated response (Table 2).

TABLE 3

Effect of cholinergic ligands on NEM enhancement of receptor agonist affinity

Values are means ± standard errors.

Preliminary membrane treatment	Blockade of 1 nM [^{3}H]QNB binding by 40 μM carbamylcholine
	%
Control	41.3 ± 0.3
1 mM NEM	50.6 ± 0.8
+10 μM carbamylcholine chloride	60.8 ± 1.1
+10 μM arecoline HCl	61.0 ± 1.4
+10 μM pilocarpine HCl	59.6 ± 1.7
+10 μM oxotremorine	54.9 ± 1.0
+10 μM methacholine chloride	55.9 ± 0.9
+10 μM neostigmine bromide	50.0 ± 1.2
+10 μM *d*-tubocurarine chloride	52.6 ± 1.4
+10 μM nicotine	50.3 ± 1.4
+0.1 μM atropine	50.7 ± 1.4
+0.1 μM scopolamine HBr	50.4 ± 1.3

Muscarinic receptors in membranes from different areas of the brain responded differently to the presence of heavy metals. Zinc (100 μM) stimulated agonist binding in telencephalic and hippocampal receptors and inhibited agonist binding in brain stem receptors. Since the inhibition was not prevented by prior treatment with NEM, it is conceivable that brain stem receptors possess an additional, non-sulfhydryl moiety that interacts with zinc but is absent from muscarinic receptors of other brain areas.

Carbamylcholine binding curves in the absence and presence of two metals are presented in Fig. 5. The presence of 10 μM cadmium and 20 μM copper shifted the curves to lower concentrations. The ID_{50}

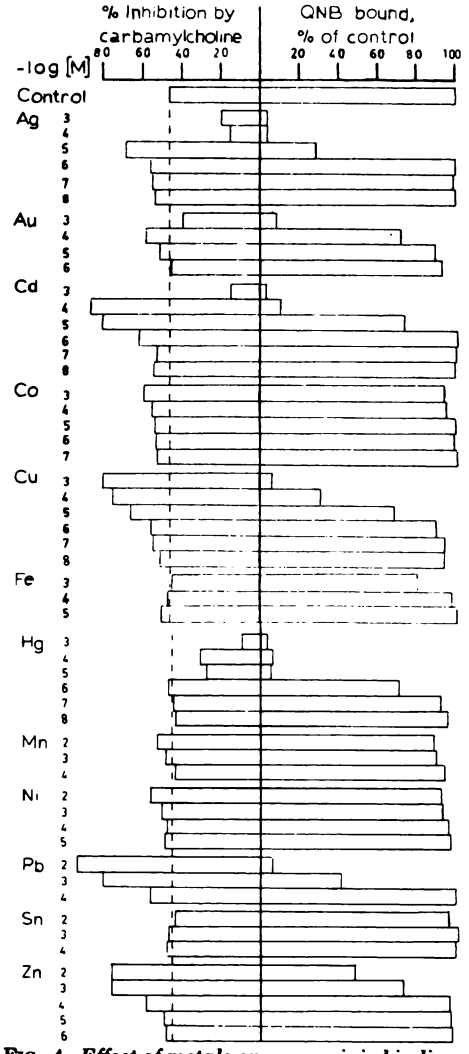

FIG. 4. *Effect of metals on muscarinic binding*
The influence of metals on specific [^{3}H]QNB binding (1 nM) and on the inhibition of 1 nM QNB binding by 40 μM carbamylcholine binding is shown.

values for carbamylcholine inhibition of QNB binding were reduced from 17.8 to 8.9 μM by cadmium and to 3.2 μM by copper. QNB binding in the absence of carbamylcholine was decreased 60% by 20 μM copper and 8% by 10 μM cadmium. Scatchard plots of carbamylcholine binding in the presence and absence of 10 μM cadmium are shown in the inset of Fig. 5. There was an increase in the number of binding sites in the high-affinity state(s) when cadmium was present, even though the total number of sites decreased by 8%.

DISCUSSION

Role of sulfhydryl groups in brain muscarinic receptors. The present results indicate the existence of several groups in the muscarinic receptor that react with sulfhydryl reagents. The alkylation or binding of heavy metals to these groups influences the binding properties of the receptor, including the distribution of receptors between high- and low-agonist-affinity forms. A diagram of the muscarinic receptor, indicating possible locations of sulfhydryl groups with relation to agonist and antagonist binding sites, is presented in Fig. 6. The present results do not allow a unique interpretation, however; the diagram is offered only as an aid to understanding some complicated data and is not intended to represent a demonstrated "molecular map" of the receptor.

PCMB treatment of neural membranes inhibits the binding of all muscarinic ligands. That this inhibition involves sulfhydryl groups is suggested, but not proven, by (a) the specificity of PCMB for sulfhydryls, (b) the partial reversal of PCMB inhibition by treatment with cysteine, DTT, or 2-mercaptoethanol, and (c) the partial protection of QNB binding from PCMB by prior treatment with NEM, a sulfhydryl-alkylating reagent. Inhibition of QNB binding by PCMB treatment can be prevented by including receptor-saturating concentrations of antagonists (atropine, scopolamine) or agonists (arecoline, carbamylcholine) during the treatment of membranes with PCMB, an observation indicating that the sulfhydryl group [—$SH_{(1)}$ in Fig. 6] is in or under the allosteric control of the receptor binding site. The observation that alkylation of the receptor by NEM decreases the ability of PCMB subsequently to inhibit receptor binding suggests that NEM is capable of reacting with the same sulfhydryl in the binding site with which PCMB reacts when it inhibits QNB binding. At the concentrations used, however, NEM does not decrease binding; consequently the inhibition must be attributable to the group itself. One possible mechanism may involve the formation of coordination complexes between mercury and functional groups in the vicinity of the binding site (represented as

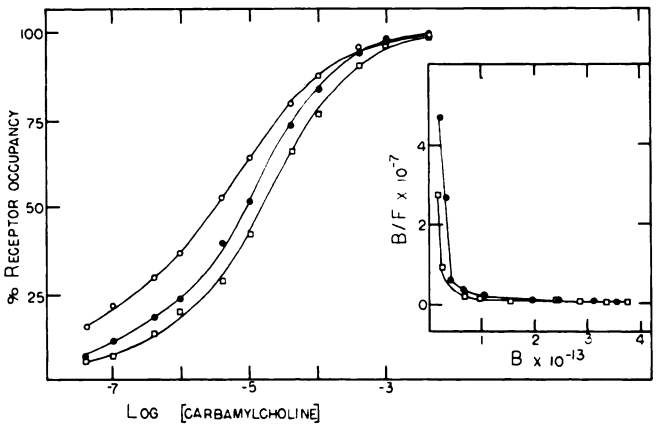

FIG. 5. *Binding of carbamylcholine to neural membranes in the presence of copper and cadmium*

Receptor occupancy by carbamylcholine was measured in telencephalic membranes in the presence of 20 μM copper (○), 10 μM cadmium (●), or the absence of any heavy metal (□). A Scatchard plot of the binding in the presence (●) and absence (□) of 10 μM cadmium is shown in the inset. *B* is the amount of carbamylcholine bound in moles per milligram of membrane protein, and *F* is the molar concentration of unbound carbamylcholine.

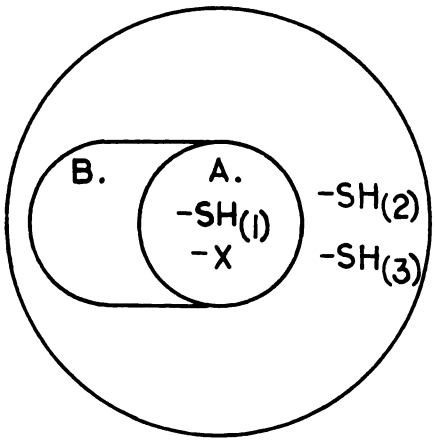

FIG. 6. *Schematic diagram of muscarinic acetylcholine receptor, depicting possible locations of protein moieties that interact with sulfhydryl reagents and heavy metals to alter muscarinic binding*

The diagram is not intended as a literal molecular description of the receptor, but rather as an aid in organizing the present experimental results. The muscarinic agonists and antagonists used in this study are all small molecules with numerous structural similarities and are depicted as binding to overlapping sites. Antagonists differ from agonists in that they contain large lipophilic moieties that participate in strong hydrophobic interactions with membranes, and it is these forces which dominate antagonist binding (14). A, agonist binding site; B, antagonist binding site; —SH, sulfhydryl group; —X, membrane group that participates in a coordination bond with mercury.

—X in Fig. 6). The findings also indicate that the sulfhydryl group is not directly involved in receptor-ligand interactions, since its alkylation by NEM has no adverse effect on binding.

Agonist binding is much more susceptible to inhibition by PCMB than is antagonist binding. Treatment with concentrations of PCMB that produce a 50% decrease in QNB binding will almost completely abolish carbamylcholine binding, regardless of the presence of agonists or antagonists during the PCMB treatment. Furthermore, prior treatment with 1 mM NEM, which mitigates PCMB inhibition of QNB binding, does not protect carbamylcholine binding. Consequently there appears to be an additional sulfhydryl reagent-reactive group(s) [—$SH_{(2)}$ in Fig. 6] contiguous to the agonist and antagonist binding sites. The binding of PCMB to this group results in allosteric inhibition of agonist binding, while its alkylation by NEM either does not occur or is also inimical to agonist binding. In the latter case, NEM must have a lower affinity for the contiguous sulfhydryl, since no decrease in agonist binding results from treatment with NEM under conditions that afford protection of the sulfhydryl at the binding site from PCMB.

NEM treatment leads to a change in the muscarinic receptor to a form characterized

by an increased affinity for muscarinic agonists (3). Receptor affinity for antagonists is unchanged by this treatment. Evidently this NEM effect involves alkylation of a sulfhydryl group [—$SH_{(3)}$ in Fig. 6] that is contiguous to the binding site, since treatment of the receptor with low concentrations of PCMB completely destroys the ability of NEM subsequently to alter the surviving agonist binding, even in the presence of an excess of muscarinic ligands. A further indication that the NEM effect is due to reaction with a contiguous sulfhydryl is that the effect is not blocked by the presence of large concentrations of receptor ligands during the treatment with NEM (indeed, receptor agonists actually increase the effect).

Consequently there appear to be at least two reactive sulfhydryl moieties that can exert allosteric effects on muscarinic binding. Reaction of PCMB with one of these groups [—$SH_{(2)}$] inhibits agonist binding but has no effect on antagonist binding. PCMB reacts more readily with this site than with the sulfhydryl at the binding site [—$SH_{(1)}$], since agonist inhibition is always seen at lower PCMB concentrations than is antagonist inhibition. NEM provides no protection of agonist binding from PCMB inhibition at this site. PCMB reaction at the second contiguous site [—$SH_{(3)}$] has no influence on receptor binding but prevents NEM from subsequently reacting at the site. NEM binding at the second contiguous site, however, stabilizes the receptor in a high-agonist-affinity state.

Heavy metals affect muscarinic binding in a manner which suggests that they are capable of interacting with all three of the distinct sulfhydryl groups. As the metal concentration is increased, the first effect on muscarinic binding observed is an increase in agonist binding, while inhibition of agonist and then antagonist binding emerges only at higher metal concentrations. At the high concentrations used, metals will bind to other protein residues, such as carboxyl and imidazole groups (15). $^{65}Zn^{++}$ binds with high affinity (K_D = 2.5 μM) to purified nicotinic acetylcholine receptors from *Torpedo californica* (16). Cadmium, cobalt, and manganese compete with zinc at low concentrations for these binding sites, and PCMB inhibits 30% of the total zinc binding, indicating a partial role for sulfhydryl groups in the metal binding (16).

Muscarinic receptors from different brain areas exhibit different pharmacological and biochemical properties. Although receptors from all brain regions bind QNB with the same high affinity, their affinities for carbamylcholine differ markedly. The order of binding affinity for carbamylcholine is brain stem $\gg$ telencephalon > hippocampus. These differences can be accounted for by different distributions between high- and low-agonist-affinity receptors according to the model of Birdsall and Hulme (2, 3). There are also differences in the reactivity of receptors from different brain regions toward protein-modifying reagents, the most outstanding difference being the sensitivity of agonist binding in brain stem receptors to the presence of zinc. Other apparent differences include a greater sensitivity of QNB binding to NEM treatment in hippocampus and brain stem receptors compared with telencephalon receptors and a greater sensitivity of QNB to PCMB treatment in telencephalon receptors than in receptors from the other two areas.

Multiple configurational states of the muscarinic receptor. Biochemical measurements on several neural receptors indicate the existence of agonist and antagonist conformations (17), and the suggestion that an agonist-antagonist state model also applies to the muscarinic receptor has been made (18). The present results, however, indicate a different model of the receptor. The receptors appear to exist in "high-affinity agonist" and "low-affinity agonist" forms, as first suggested by Birdsall and Hulme (2). Antagonists have the same high affinity for the receptor in all configurations, and receptors can be converted from low- to high-agonist-affinity forms (3). Although the present results are discussed in relation to a two-agonist-state model, the binding data are not inconsistent with models incorporating more than two binding states.

A scheme that is based on the Katz-Thesleff (19) cyclic model of end plate receptor function and incorporates the present ex-

perimental results is the following:

$$\begin{array}{ccc} L + & R'' \overset{b}{\rightleftharpoons} & LR'' \\ \text{NEM} \uparrow & & \uparrow \ \text{NEM?} \\ L + & R \overset{a}{\rightleftharpoons} & LR \\ & \Downarrow\Uparrow & \Downarrow\Uparrow \\ L + & R' \overset{b}{\rightleftharpoons} & LR' \end{array}$$

where R and R' represent the low- and high-agonist-affinity states, respectively. R'' is receptors that have been converted to the high-affinity form by NEM alkylation and have the same binding properties as R'. L is a ligand, and a and b are the association constants of the ligand with the receptor in the different states.

It is assumed that under the conditions of the present experiments there is insignificant interconversion between the two states; hence the flattened agonist binding curves. Reductive alkylation of certain sulfhydryl groups stabilizes the receptors in the high-affinity form. The marked temperature dependence of the NEM effect may indicate that the relevant sulfhydryl moiety is buried within the receptor molecule, being exposed to the action of exogenous NEM only at elevated temperatures, which increase the conformational possibilities of the receptor. NEM-dependent conversion to the high-affinity form is more nearly complete in the presence of an agonist than an antagonist or no receptor ligand, although exposure of the receptor to agonists alone does not alter subsequent agonist binding properties. It is possible that agonist binding induces a conformational change in the receptor protein that exposes buried sulfhydryl groups to NEM. Antagonist binding does not induce this sulfhydryl-exposing conformational change, which may be related to the physiological response of the receptor to an agonist.

The model of the muscarinic receptor presented here is an extension of the cyclic model proposed by Katz and Thesleff (19) for the motor end plate. Based on kinetic studies of muscle desensitization, they proposed a model in which R' (in the present model) would refer to the refractory state of the receptor. Pharmacological responses could be elicited only by the association of an appropriate ligand with a receptor in the active R state. In the Katz-Thesleff model, the inactive state had the higher affinity for receptor agonists. The observation that agonist preconditioning of electric fish nicotinic receptors results in a higher receptor affinity for agonists led Weber *et al.* (7) to correlate the higher-agonist-affinity state with a desensitized state, in agreement with the Katz-Thesleff model.

The situation is somewhat different in the case of smooth muscle. Two populations of muscarinic receptors, which differ in their affinities for agonists, can be detected (20), and it is possible to equate the production of the contractile response with occupancy of the lower-agonist-affinity sites (21). In addition, physiological desensitization can be elicited by treating guinea pig ilea with sulfhydryl group-blocking reagents such as PCMB and the toxin from *Naja nigricollis* (22). Agonist preconditioning, however, resulted in a decrease in agonist binding relative to antagonist binding (23).

REFERENCES

1. Birdsall, N. J. M., Burgen, A. S. V., Hiley, C. R. & Hulme, E. C. (1976) *J. Supramol. Struct.*, **4**, 367–371.
2. Birdsall, N. J. M. & Hulme, E. C. (1976) *J. Neurochem.*, **27**, 7–16.
3. Aronstam, R. S., Hoss, W. & Abood, L. G. (1977) *Eur. J. Pharmacol.*, **46**, 279–282.
4. Karlin, A. (1969) *J. Gen. Physiol.*, **54**, 245s–264s.
5. Eldefrawi, M. E. & Eldefrawi, A. T. (1972) *Proc. Natl. Acad. Sci. U. S. A.*, **69**, 1776–1780.
6. Súarez-Isla, B. A. & Hucho, F. (1977) *FEBS Lett.*, **75**, 65–69.
7. Weber, M., David-Pfeuty, T. & Changeux, J.-P. (1975) *Proc. Natl. Acad. Sci. U. S. A.*, **72**, 3443–3447.
8. Weiland, G., Georgia, B., Wee, V. T., Chignall, C. F. & Taylor, P. (1976) *Mol. Pharmacol.*, **12**, 1091–1105.
9. Stubbins, J. F. & Hudgins, P. M. (1971) *Experientia*, **27**, 669–670.
10. Fleisch, J. H., Krzan, M. C. & Tisus, E. (1974) *Am. J. Physiol.*, **227**, 1243–1248.
11. Yamamura, H. I. & Snyder, S. H. (1974) *Proc. Natl. Acad. Sci. U. S. A.*, **71**, 1725–1729.
12. Warburg, O. & Christian, W. (1941) *Biochem. Z.*, **310**, 384–421.
13. Habeeb, A. F. S. A. (1972) *Methods Enzymol.*, **25**, 457–464.
14. Aronstam, R. S., Abood, L. G. & Baumgold, B.

(1977) *Biochem. Pharmacol.*, **26**, 1689–1695.
15. Valee, B. L. & Ulmer, D. D. (1972) *Annu. Rev. Biochem.*, **41**, 91–128.
16. Eldefrawi, M. E., Eldefrawi, A. T., O'Brien, J. A. & Van Campen, D. (1975) *Life Sci.*, **16**, 925–936.
17. Snyder, S. H. & Bennett, J. P. (1976) *Annu. Rev. Physiol.*, **38**, 153–175.
18. Snyder, S. H., Chang, K. J., Kuhar, M. J. & Yamamura, H. I. (1975) *Fed. Proc.*, **34**, 1915–1921.
19. Katz, B. & Thesleff, S. (1957) *J. Physiol. (Lond.)*, **138**, 63–80.
20. Burgen, A. S. V. & Hiley, C. R. (1974) *Br. J. Pharmacol.*, **51**, 127P.
21. Birdsall, N. J. M. (1977) *Biochem. Soc. Trans.*, **5**, 74–76.
22. Kuhnen-Clausen, D. (1976) *Naunyn-Schmiedebergs Arch. Pharmacol.*, **294**, R9.
23. Young, J. M. (1974) *FEBS Lett.*, **46**, 354–356.