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The Importance of Thiol- and Disulfide Groups in Agonist and Antagonist Binding to the Muscarinic Receptor

BRITTA HEDLUND AND TAMAS BARTFAI

Department of Biochemistry, University of Stockholm, Arrhenius Laboratory, S-106 91 Stockholm, Sweden

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SUMMARY

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Reducing agents cysteine, glutathione and dithiothreitol in the concentration range of 10 μM-10 mM diminished binding of [^{3}H]3-quinuclidinyl benzilate ([^{3}H]3-QNB) to *in situ* muscarinic receptor sites of membranes from rat cerebral cortex. The thiol reagents *N*-ethylmaleimide, pCMB, and Cd^{2+} and the oxidizing agent 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) in low concentrations (10 μM-10 mM) have also diminished muscarinic binding causing an approximately 50% inhibition at 50 μM concentration. Treatment of membranes with reducing- or thiol-reagents diminished the number of antagonist and agonist binding sites and changed the affinity toward these ligands. The apparent K_d value for [^{3}H]3-QNB binding increased from 0.4 nM to 0.7-0.8 nM as a result of covalent modification of the membranes. The binding of carbamylcholine as determined from competition with [^{3}H]3-QNB (0.1 nM) could be described with a two-site model with apparent K_d values 95 ± 15 μM and 52 ± 8 nM, respectively. In treated membranes these values were altered to 36-230 μM and 0.5-5 μM, respectively. The results suggested that a disulfide bond, which could be reduced and reoxidized, may play an important role in ligand binding. Furthermore, experiments with *N*-ethylmaleimide, pCMB, and DTNB suggested the presence of thiol groups of importance for the binding. The finding that Cd^{2+} inhibited ligand binding in low concentrations (50 μM) suggested the presence of vicinal thiol groups. Modification of thiol-disulfide state of receptor may be involved in the control of binding activity.

INTRODUCTION

Studies on the muscarinic acetylcholine receptor (1-3) have been limited to the *in situ* receptor because solubilization and purification procedures (4-6) gave unstable preparations with low yield. Binding studies with radioactively labeled antagonists (7, 8) have shown that antagonist binding to the muscarinic receptor is a process that could be described with a single binding isotherm (2). Examination of binding of agonists and of physiological responses mediated *via* the muscarinic receptor did indicate that two classes of binding sites might be present with different affinities (2). Until recently we had no information on mechanisms that might be involved in regulating any possible interconversion of the two classes of binding sites.

Recently, Aronstam *et al.* (9) have shown that treatment of membranes from rat fore-

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brain with NEM[1] enhanced the ability of carbamylcholine to compete with [^{3}H]3-QNB for muscarinic binding sites.

Lack of preparations of solubilized and purified receptor protein has also hampered characterization of the active site(s) of the receptor. Presence of nucleophilic groups on the receptor could be inferred from the action of alkylating antagonists acetylcholine mustard (10), benzilylcholine mustard (11), propylbenzilylcholine mustard (7) and dibenamine (12). However, the nature of these groups and their importance in ligand binding have not been studied. The early theoretical models of the topology of receptor sites were prepared on the basis of ligand structure-affinity relationships (13).

This study was undertaken to examine the nature of the nucleophilic group(s) that react with NEM and to investigate the effect of reduction of disulfide bridges on ligand binding to the muscarinic receptor. Our results suggest the presence of two vicinal thiol groups and the presence of disulfide bond(s) on the muscarinic receptor. Alkylation of thiol groups or reduction of disulfide bond(s) influenced the capacity and affinity of the *in situ* receptor toward its ligands. The experiments also suggest a possible mechanism of receptor desensitization as a consequence of reduction with GSH or cysteine.

MATERIALS AND METHODS

[^{3}H]3-(±)-QNB (16 Ci/mmole) was purchased from Amersham Radiochemical Centre, U.K., or [^{3}H]3-QNB (29.4 Ci/mmole) from New England Nuclear, Boston, Mass. USA. All other reagents were of analytical grade and supplied by Sigma Chemical Company, St. Louis, Mo., USA. $CdCl_2$ was purchased from Merck, Darmstadt, FRG.

Preparation of membranes. Male Sprague-Dawley rats (200–250 g) were killed by decapitation, and the brains were rapidly removed. The cerebral cortex was dissected, and white matter was carefully cleaned away. Ten per cent (w/v) homogenate in 0.32 M ice-cold sucrose was prepared with a loose-fitting glass-teflon homogenizer at 695 rpm (15 up–down strokes). The homogenate was centrifuged at 1000 × *g* for 10 min; the supernatant was further centrifuged at 40,000 × *g* for 30 min in a Beckman Ultracentrifuge. The pellet was resuspended in Krebs-Ringer buffer (NaCl 137 mM, KCl 2.68 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.05 mM, phenylmethylsulfonylfluoride 0.1 mM, glucose 5.2 mM, HEPES 0.5 mM pH = 7.4) to yield a protein concentration of 2–3 mg/ml. All solutions were made fresh daily.

Binding studies with [^{3}H]3-QNB. In 800 µl final volume in Krebs-Ringer buffer, 0.2–0.4 mg protein was incubated with the labeled antagonist at room temperature for 60 min and filtered on Whatman GF/B filters on a Millipore filtering manifold according to Yamamura and Snyder (8). The filters were immediately rinsed with 3 × 3 ml ice-cold buffer, sucked dry and counted in a scintillation spectrometer with Aquasol scintillation liquid (source: New England Nuclear). Filtration and wash of filter was accomplished within 30 sec.

Specific [^{3}H]3-QNB binding was defined as the difference in binding of [^{3}H]3-QNB in the absence and presence of atropine sulfate (1 µM). All values were calculated from quadruplicate determinations.

The K_d values for [^{3}H]3-(±)-QNB, a racemic mixture obtained from the supplier, were determined at different protein concentrations, ranging from 0.12–0.5 mg/800 µl sample volume. The K_d^{app} is a linear function of the receptor concentration, as indicated in the inset of Fig. 5B. The data in the paper give the apparent K_d values obtained at 0.4 mg/800 µl protein content and do not take into account that the (−) isomer of [^{3}H]3-(±)-QNB is about 20 fold more active than the (+) isomer.

Agonist binding studies. In 800 µl final volume, 0.2–0.4 mg protein was incubated with [^{3}H]3-QNB (at 0.05 and 0.1 nM final concentrations) and with the agonists for

[1] The abbreviations used are: NEM, *N*-ethylmaleimide; [^{3}H]3-QNB, [^{3}H]3-quinuclidinyl benzilate; GSH, reduced form of glutathione; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); DTT, dithiothreitol; pCMB, parachloro mercury benzoic acid; GSSG, oxidized form of glutathione.

60 min at room temperature. Filtration was carried out as in [^{3}H]3-QNB binding experiments.

The on rate of [^{3}H]3-QNB in the absence and presence of various reagents was determined either by preincubating the membranes with the test reagent 30 min before addition of [^{3}H]3-QNB or adding [^{3}H]3-QNB and the reagents simultaneously. (At 1 mM concentration both NEM and cysteine almost completely inhibited [^{3}H]3-QNB binding within 30 sec; the extent of inhibition was only slightly increased by further incubation up to 1 hr (cf. Fig. 2) or by incubation with 10 mM NEM or 10 mM cysteine). Incubation with the labeled ligand was then interrupted by filtration. The off rate of [^{3}H]3-QNB was determined by adding 1 μM atropine and various reagents to a mixture of [^{3}H]3-QNB (1 nM) and membranes which were incubated 60 min at room temperature. The off rate was calculated from the loss of radioactivity at different time points after addition of atropine and different reagents. It should be mentioned that only the first part of this kinetic curve was used for evaluation of k_{-1} and that it was difficult to attain full release of [^{3}H]3-QNB. k_{+1} values varied: 0.7 ± 0.5 nM/min and k_{-1} = 0.04 ± 0.03/min, (n=3).

None of the thiol reagents or reducing agents reacted with the [^{3}H]3-QNB. Incubation of the labeled ligand and the reagents was carried out for 1 hr at room temperature in Krebs-Ringer buffer and the incubation mixture was examined by TLC chromatography. In the chromatography medium chloroform:methanol (9:1) on Suppelco cellulose plates, the [^{3}H]3-QNB the was incubated with the different reagents and the untreated [^{3}H]3-QNB had the same mobility.

Protein was determined according to Lowry *et al.* (14).

The binding data were calculated by subtracting nonspecific binding (in the presence of atropine, 1 μM) and correcting the [^{3}H]3-QNB concentration for the amount bound [^{3}H]3-QNB. The data were analyzed with an IBM 360/75 by a nonlinear-least squares program using a Gauss-Newton algorithm (BMDP-3R, University of California). The equations used for the fitting included

$$y = \frac{V[S]}{K_1 + [S]} \qquad \text{a}$$

where a single class of binding sites with affinity K_1 and with capacity V. y stands for the bound amount of ligand at any given [S]

$$y = \frac{V_1[S]}{K_1 + [S]} + \frac{V_2[S]}{K_2 + [S]} \qquad \text{b}$$

Two sites model assuming no interaction between sites.

The y values in the carbamylcholine binding experiments were calculated by determining the number of sites from which [^{3}H]3-QNB was displaced by carbamylcholine. These values of y for carbamylcholine were analyzed as a function of the corrected free concentration of this agonist.

The evaluation of the results of least squares fit was carried out as described previously (15, 16).

RESULTS

The effect of Ca^{2+} on the protein modification. Most of the physiological responses mediated by muscarinic receptors are dependent on the presence of extracellular Ca^{2+} (cf. for reviews, 1-3). Therefore this study was carried out using Krebs-Ringer buffer (Ca^{2+}: 1.8 mM) rather than phosphate buffer (8) or phosphate buffer complemented with EDTA (9). Binding of the alkylating affinity label propylbenzilylcholine mustard (7) and of the reversible antagonists benzetimide (4) and atropine (5) has been previously studied in Krebs-Ringer medium. Table 1 shows that almost twice as many [^{3}H]3-QNB binding sites could be measured at 1 nM ligand concentration in the presence as in the absence of Ca^{2+}. The omission of Ca^{2+} from the Krebs Ringer buffer changed the affinity of [^{3}H]3-QNB for the receptor. (K_d in the presence of Ca^{2+} = 0.45 ± 0.07 nM; in the absence of Ca^{2+} 0.7-0.8 nM). Thus changes in receptor concentration measured at 1 nM [^{3}H]3-QNB are reflecting the effect of Ca^{2+} on both the binding capacity and on binding affinity. Data obtained at saturating (4 nM)

TABLE 1

The effect of Ca^{2+} on the modification of muscarinic binding sites

All agents were present during the 60 min incubation with [^{3}H]3-QNB (1 nM or 4 nM) at room temperature. Values in parentheses indicate the percentage of remaining active sites

	Krebs-Ringer buffer specific [^{3}H]3-QNB binding		Krebs-Ringer's buffer[a] without Ca^{2+} (1 mM EGTA)	
	(1 nM)[b]	(4 nM)[c]	(1 nM)[b]	(4 nM)[c]
	[^{3}H]3-QNB (*picomoles/mg protein*)			
No addition	0.25 ± 0.04 (100)	0.40 ± 0.03 (100)	0.15 ± 0.04 (100)	0.30 ± 0.03 (100)
DTT (1 mM)	0.12 ± 0.02 (50)	0.27 ± 0.07 (68)	0.19 ± 0.02 (129)	0.23 ± 0.03 (77)
NEM (1 mM)	0.11 ± 0.03 (44)	0.16 ± 0.04 (39)	0.10 ± 0.02 (64)	0.17 ± 0.02 (57)
pCMB (1 mM)	0.16 ± 0.03 (62)	0.23 ± 0.02 (59)	0.05 ± 0.04 (35)	0.19 ± 0.02 (65)
DTNB (1 mM)	0.09 ± 0.02 (35)	0.23 ± 0.05 (57)	0.07 ± 0.04 (49)	0.12 ± 0.02 (39)
Cd^{2+} (1 mM)	0.13 ± 0.01 (54)	0.20 ± 0.05 (51)	0.13 ± 0.02 (88)	0.20 ± 0.02 (66)

[a] The composition of this medium was identical to that of Krebs-Ringer's buffer (cf. METHODS), but Ca^{2+} was omitted and 1 mM EGTA and 1 mM Mg^{2+} were added.

[b] Data from three independent experiments each carried out in quadruplicate on different days.

[c] Data from a single experiment carried out in triplicate.

[^{3}H]3-QNB concentration reflect only the changes in the binding capacity of the membranes. The binding capacity of membranes is 25% higher in the presence of Ca^{2+} and the inhibitory effect of NEM (1 mM) was more pronounced whereas the inhibitory effect of DTNB was somewhat lower.

The effect of reducing agents and thiol reagents on the binding of [^{3}H]3-QNB. In the presence of various concentrations of the reducing agents cysteine, glutathione and DTT, the amount of specifically bound [^{3}H]3-QNB decreased greatly while nonspecific binding changed very little. Fifty per cent inhibition was observed at 50–100 μM concentration of the reducing agents (Fig. 1A). The inhibition of the binding that was achieved in the presence of 10 mM DTT, 10 mM GSH, or 10 mM cysteine, respectively, did not differ significantly from that observed with 1 mM reagent. Thus it is concluded that the concentration of reagents was not limiting.

The alkylating reagents NEM and pCMB also diminished the specific binding of [^{3}H]3-QNB to the receptor. The thiol oxidizing agent DTNB and the mercaptyl-forming ion Cd^{2+} both inhibited specific [^{3}H]3-QNB binding in a similar manner to that of the alkylating agents. Fifty per cent inhibition occurred at approximately 50 μM concentration of NEM, pCMB, DTNB and Cd^{2+}. The highest concentration of these reagents tested was 10 nM. The extent of the inhibition at this concentration was 70% for NEM and 75% for DTNB, pCMB and Cd^{2+} (Fig. 1B).

Inhibition of binding by thiol reagents and reducing agents was a rapid process. The same percentage of [^{3}H]3-QNB binding sites was inhibited after 30 sec incubation with NEM (1 mM) or cysteine (1 mM) (Fig. 2) as after 60 min incubation with the same reagents in the presence of [^{3}H]3-QNB (Figs. 1A and 1B).

Protection experiments. To assess whether the protein modification occurred in the vicinity of the receptor or on the receptor protein itself, we examined the protection of specific binding of [^{3}H]3-QNB against reducing and alkylating agents by two ligands of the receptor, carbamylcholine and atropine. Table 2 shows that carbamylcholine protected the binding sites almost fully against the inhibitory effect of reduction by DTT. No significant protection was found by carbamylcholine against NEM and $CdCl_2$. Atropine, which binds more tightly, afforded a better protection against inhibition by both NEM and $CdCl_2$. Reduction of the membranes with DTT prior to addition of DTNB increased the efficiency of the latter agent to inhibit [^{3}H]3-QNB binding (Table 3). The inhibition of [^{3}H]3-QNB binding by NEM and $CdCl_2$ increased by 50–100% upon pretreatment of the membranes with DTT. The percentage of [^{3}H]3-QNB binding that was

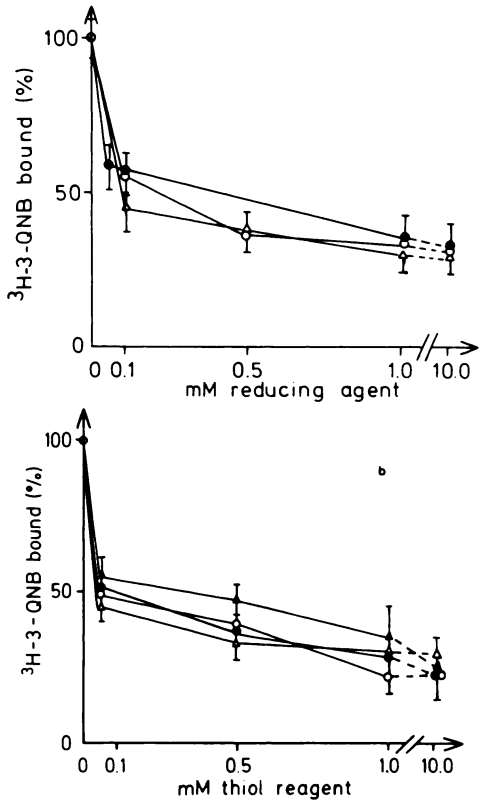

FIG. 1. *Inhibition of ^{3}H-3-QNB binding by various concentrations of reducing agents (A) and thiol reagents (B)*

A. Cysteine ●, glutathione ○ and dithiothreitol △. B. Inhibition of [^{3}H]3-QNB binding by various concentrations of Cd^{2+}, pCMB, NEM and DTNB. Cd^{2+} ▲, pCMB ●, NEM △, and DTNB ○.

The [^{3}H]3-QNB concentration was 1 nM and the reagents were present during the 60 min long incubation with the labeled ligand in all experiments.

blocked by carbamylcholine (0.1 mM) did vary with the treatment. In DTNB- and in $CdCl_2$-treated membranes the portion of [^{3}H]3-QNB that was displaced by carbamylcholine increased as compared to the nontreated membranes. Pretreatment with DTT before Cd^{2+} decreased this potentiation of the efficiency of carbamylcholine. Treatment with DTT followed by NEM also decreased the ability of carbamylcholine to compete for [^{3}H]3-QNB binding sites (Table 3).

Combinations of DTT and cysteine at 1 mM concentration yielded approximately the same level of inhibition of [^{3}H]3-QNB binding as did cysteine alone (Table 4). Combinations of alkylating, oxidizing or mercaptyl-forming agents did enhance the inhibition slightly compared to the inhibition given by these agents individually.

The reversibility of the effect of reduc-

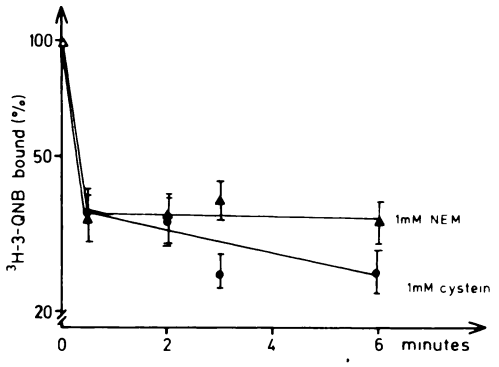

FIG. 2. *The kinetics of the loss of binding activity of the membranes upon incubation with cysteine and with NEM*

Membranes (2.0–3.0 mg/ml) were incubated with cysteine (1 mM) ● or NEM (1 mM) ▲ and aliquots removed at different time points, diluted 20 fold and incubated with [^{3}H]3-QNB (1 nM) for 60 min at room temperature.

TABLE 2

Protection of receptor sites by carbamylcholine and atropine against modification by DTT, NEM and Cd^{2+}

Preincubation period Addition to Krebs-Ringer's buffer 30 min	Total binding [^{3}H]3-QNB bound (%)		
	No drug	Carbamylcholine	Atropine
No addition	100 ± 14	100 ± 10	100 ± 15
DTT (0.1 mM)	43 ± 3	98 ± 4	105 ± 11
NEM (1 mM)	37 ± 12	43 ± 8	62 ± 9
$CdCl_2$ (1 mM)	38 ± 17	57 ± 11	50 ± 4

The membranes were preincubated for 40 min either with Krebs-Ringer medium or with Krebs-Ringer supplemented with atropine (0.1 μM) or with carbamylcholine (0.1 mM). During the last 30 min of this preincubation period DTT, NEM or $CdCl_2$ was present. Unbound atropine, carbamylcholine and reagents were removed by centrifugation for 30 min at 40,000 × *g*. The resulting pellet was resuspended and incubated with [^{3}H]3-QNB (1 nM) for 60 min. Since a portion of receptors was occupied by carbamylcholine or atropine, the total number of sites available for [^{3}H]3-QNB was 63 and 43% of that found when these ligands were absent during preincubation. This value was 0.25 picomoles [^{3}H]3-QNB/mg protein. Protection of sites was expressed as the percentage of the sites available for [^{3}H]3-QNB.

TABLE 3

The effect of reduction of membranes by DTT followed by treatment with thiol reagents on [^{3}H]3-QNB binding and on its displacement by carbachol

After the first preincubation of 30 min the samples were centrifuged and the pellet resuspended in the medium of the second preincubation period. Following the second preincubation period the samples were centrifuged and the pellets resuspended with Krebs-Ringer buffer containing [^{3}H]3-QNB (1 nM). The incubation period with this ligand was 60 min at room temperature. All centrifugations were carried out at 40,000 × *g* for 30 min. 100% [^{3}H]3-QNB corresponds to 0.40 picomoles/mg protein. The data are derived from three independent experiments, each carried out in quadruplicate (mean ± SD).

Preincubation conditions		[^{3}H]3-QNB bound (%)		Carbachol (0.1 mM) displaced part of [^{3}H]-3-QNB binding
Period 1 (30 min)	Period 2 (30 min)	No carbachol	Carbachol	
			(0.1 mM)	*(%)*
No addition	No addition	100	47.6 ± 9.2	47.6
DTT (0.1 mM)	No addition	52.5 ± 4.2	21.3 ± 4.1	40.6
No addition	DTNB (0.25 mM)	37.0 ± 14.8	19.6 ± 4.9	53.0
DTT (0.1 mM)	DTNB (0.25 mM)	26.8 ± 8.1	16.8 ± 5.3	62.7
No addition	NEM (0.25 mM)	47.5 ± 15.7	22.9 ± 5.6	48.2
DTT (0.1 mM)	NEM (0.25 mM)	30.3 ± 3.9	13.0 ± 0.3	42.9
No addition	$CdCl_2$ (0.25 mM)	42.0 ± 15.0	22.7 ± 7.7	54.0
DTT (0.1 mM)	$CdCl_2$ (0.25 mM)	15.7 ± 3.7	9.5 ± 1.6	60.5

TABLE 4

The compounded effect of the combination of two thiol reagents or of two reducing agents on [^{3}H]3-QNB binding

Values are means ± SD (n = 8–12). The reagents were present during a preincubation period of 30 min and were removed by centrifugation at 40,000 × *g* for 30 min before the incubation with [^{3}H]3-QNB (1nM) was started.

Reagents added to Krebs-Ringer buffer	[^{3}H]3-QNB binding
	(%)
—	100
DTT (1 mM)	44 ± 10
Cysteine (1 mM)	34 ± 7
NEM (1 mM)	39 ± 16
$CdCl_2$ (1 mM)	42 ± 12
pCMB (1 mM)	54 ± 17
DTNB (1 mM)	42 ± 16
DTT (1 mM) + cysteine (1 mM)	27 ± 4
NEM (1 mM) + $CdCl_2$ (1 mM)	33 ± 10
NEM (1 mM) + pCMB (1 mM)	27 ± 11
NEM (1 mM) + DTNB (1 mM)	37 ± 4
$CdCl_2$ (1 mM) + DTNB (1 mM)	28 ± 6
pCMB (1 mM) + DTNB (1 mM)	27 ± 5

tion of the membranes was examined with DTT, GSH and cysteine as reducing agents. The effect of reduction by DTT on [^{3}H]3-QNB binding was not fully reversed within three hours of the removal of the reducing agent. Removal of cysteine after the 30 min incubation resulted in a reoxidation of the membranes and a restoration of the original binding capacity 90 min after the removal of the reducing agent (Fig. 3a).

The same kinetics of the reoxidation was observed with GSH as reducing agent (Fig. 3b). The reoxidation was not accelerated by addition of GSSG; rather, an almost complete inhibition of [^{3}H]3-QNB binding was observed.

The effect of changing GSSG to GSH ratio at 10 μM GSSG concentration on [^{3}H]3-QNB binding was investigated. Figure 4 shows that GSH or GSSG alone inhibited the binding. Increasing GSSG/GSH ratio relieved the inhibition and a maximum of [^{3}H]3-QNB binding was reached at 10 μM GSSG and 0.1 μM GSH concentrations.

Effects of covalent modification of the receptor on antagonist and agonist binding. The effects of DTT, Cd^{2+}, pCMB, NEM and DTNB at 1 mM concentrations on binding of [^{3}H]3-QNB were examined in experiments in which these agents were present during the incubation (60 min) with [^{3}H]3-QNB.

Reduction of the membranes with DTT altered the capacity and the affinity of

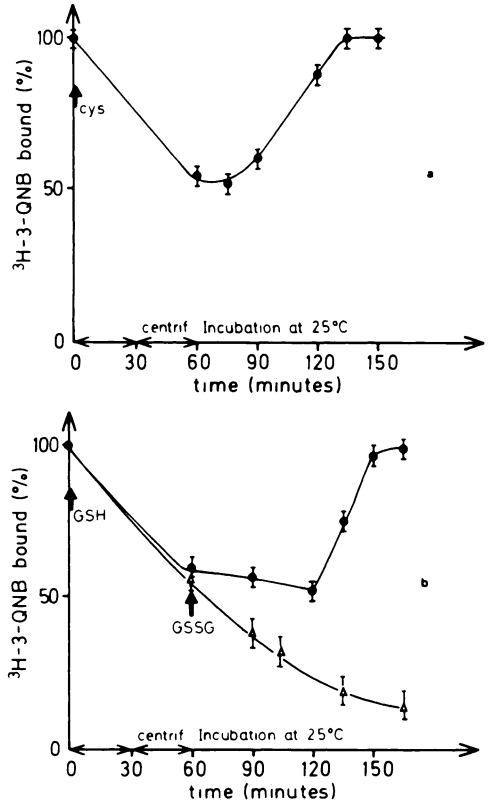

FIG. 3. *Kinetics of reduction of receptors by cysteine (A) and GSH (B).*

A. 0.1 mM cysteine was added. The membranes were resuspended after centrifugation and removal of the reducing agent. After reoxidation of receptors, incubation with [^{3}H]3-QNB for 60 min was started at different time points. B. Kinetics of reduction of receptors with GSH (0.1 mM) and reoxidation of the receptors after removal of GSH ●. Addition of GSSG (0.1 mM) after the removal of GSH △ further inhibited binding activity.

these to bind the labeled antagonist (Table 5). Cd^{2+}, pCMB, NEM and DTNB in 1 mM concentrations have also caused a loss of about 50% of the binding sites and a concomitant increase in the K_d for the receptor-[^{3}H]3-QNB complex (Table 5 and Fig. 5A).

The effects of reducing and alkylating agents on the kinetics of [^{3}H]3-QNB binding were also examined. The on rates were decreased 1.3–1.8 fold whereas the off rates were increased 3–4 fold as a result of treatment of the membranes. The K_d values derived from these kinetic experiments (data not shown) showed a reasonable agreement with the steady-state binding data and indicated that treatment of membranes decreased the affinity for [^{3}H]3-QNB. Change of the number of sites and affinity was not accompanied with any change of the Hill coefficient of binding (Fig. 5B).

The effects of covalent modifications of the membranes with DTT, Cd^{2+}, pCMB and NEM (all in 1 mM concentrations) were investigated by examining the ability of carbamylcholine to suppress binding of 0.05 nM and 0.1 nM [^{3}H]3-QNB (Fig. 6A). The Scatchard plots of the carbamylcholine binding revealed the existence of at least two binding sites. Fitting the binding data by a nonlinear regression method showed that a significantly lower residual sum of squares is obtained when the data are fitted to a two-sites model (II) than with fitting to a simple binding isotherm (model I). In each case the minimizing procedure showed convergence. The parameter values were all positive with real asymptotic standard deviation values.

The data of Table 6 indicated that the K_d value of the high affinity site increased upon treatment with DTT, Cd^{2+}, NEM and pCMB, whereas its capacity stayed below or equal to that of the low affinity site. The latter site had a K_d value of 95 ± 15 μM in untreated membranes. The affinity of this site increased upon Cd^{2+} treatment and decreased subsequent to treatment with DTT, NEM and pCMB. The capacity of the low affinity site was decreased in treated membranes. The extent of this decrease was reminiscent of that seen on antagonist binding capacity when the membranes were treated with the same reagents. pCMB and NEM treatments have also changed the relative capacities of the low and high affinity sites in such a manner that these have almost equal capacities, whereas in untreated membranes the low affinity site has twice the capacity that of the high affinity site.

DISCUSSION

The reducing agent dithiothreitol (DTT) mediates depolarization of electroplax membranes by inhibition of the nicotinic

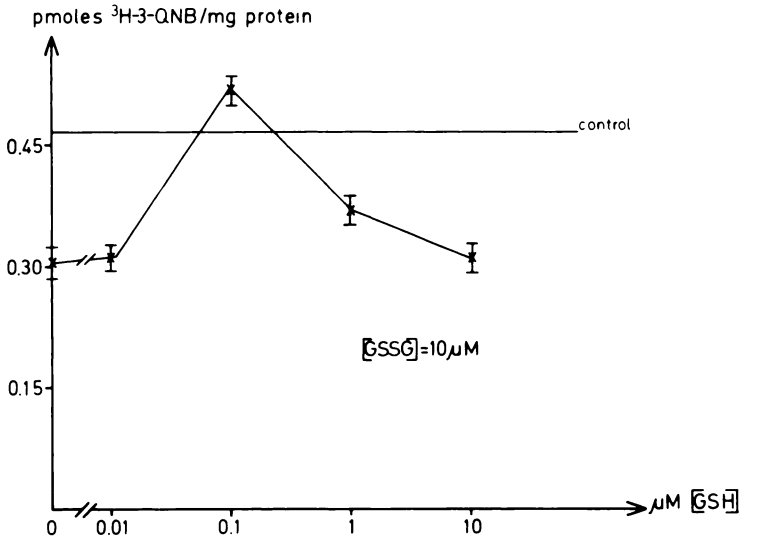

FIG. 4. *The effect of varying GSH concentration at constant GSSG (10 μM) concentration on binding of [³H]3-QNB (1 nM) to membranes from rat cerebral cortex*

TABLE 5

The effect of DTT, pCMB, NEM, Cd^{2+} and DTNB on binding of [³H]3-QNB to membranes from rat cerebral cortex

Addition to Krebs-Ringer buffer	Binding capacity	K_d (equilibrium[a] binding)
	(picomoles/mg protein)	*(nM)*
None	0.41 ± 0.02	0.45 ± 0.07
DTT (1 mM)	0.19 ± 0.01	0.70 ± 0.07
Cd^{2+} (1 mM)	0.19 ± 0.01	0.75 ± 0.05
pCMB (1 mM)	0.22 ± 0.04	0.87 ± 0.20
NEM (1 mM)	0.21 ± 0.04	0.85 ± 0.20
DTNB (1 mM)	0.18 ± 0.04	0.90 ± 0.20

[a] Binding was determined after 60 min incubation at room temperature with [³H]-3-QNB in the presence of the various reagents (see Methods).

acetylcholine receptor (Karlin and Bartels [17], Eldefrawi and Eldefrawi [18]). These observations have been extended to the demonstration of changes in ligand binding properties of the receptor as a result of reduction of a conformationally important disulfide bond (19, 20). Until recently similar information on the effect of modification of thiol and disulfide groups on the binding properties of the muscarinic acetylcholine receptor was lacking.

In this study we examined the effects of modification of thiol and disulfide groups on the activity of the *in situ* muscarinic receptor.

Two findings are important: the susceptibility of the receptor to thiol reagents was influenced by Ca^{2+} in the medium, and K_d values for [³H]3-QNB binding, even after extrapolation to zero receptor concentration, were higher in this study than the values reported by other workers who used phosphate buffer and EDTA. Use of Krebs-Ringer buffer and removal of Ca^{2+} by EGTA (1 mM) seems to have diminished the binding capacity and decreased the affinity for [³H]3-QNB. This finding is in apparent contradiction with reports that EDTA (100 mM) did not affect binding of [³H]3-QNB (21) or that EDTA enhances binding of [³H]3-QNB (22). The contradiction can be resolved by the differences in our experiment conditions: several other ions—Na^+, K^+, Mg^{2+}, Cl^-—were still present, even in the absence of Ca^{2+}. These ions may very well have specific effects on [³H]3-QNB binding. (We have repeated and confirmed the 2–5 fold lower K_d values of [³H]3-QNB binding in a phosphate buffer, pH 7.4, 37°). The values of all of our experiments refer to 25° and to a protein concentration of 0.3–0.4 mg/sample.

Effects of treatments of membranes from rat telencephalon with NEM on binding of

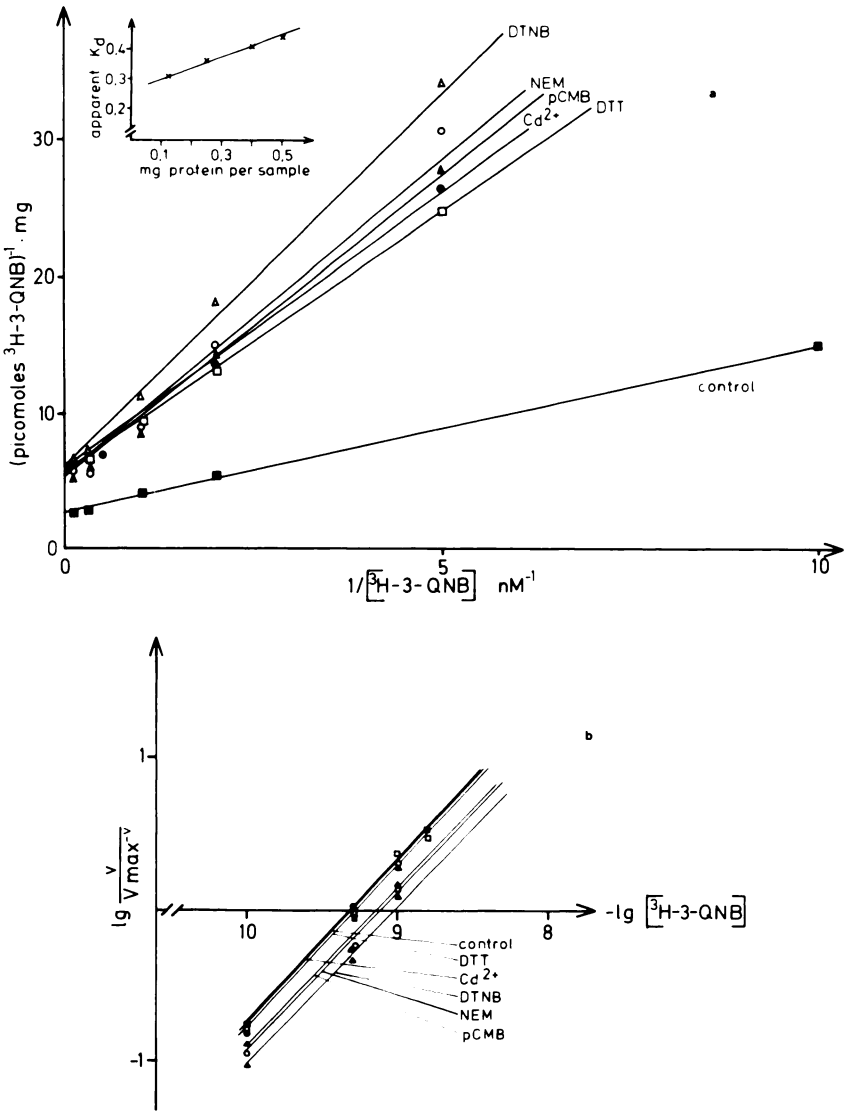

FIG. 5. *Double reciprocal (A) and Hill (B) plots of [^{3}H]3-QNB*

Double reciprocal plots of [^{3}H]3-QNB binding to membranes from rat cerebral cortex: 1/bound [^{3}H]3-QNB versus 1/free [^{3}H]3-QNB. (The concentration of the latter was corrected for that [^{3}H]3-QNB lost from the solution as a result of binding. Such corrections were negligible with 0.5 nM [^{3}H]3-QNB and higher concentrations. Binding to control membranes ■, in the presence of DTT (1 mM) □, in the presence of Cd^{2+} (1 mM) ●, in the presence of pCMB (1 mM) ▲, in the presence of NEM (1 mM) ○, and in the presence of DTNB (1 mM) △, respectively. The inset shows the dependency of the apparent K_d on the protein concentration. B. Hill plots of the data shown in Fig. 5A. Symbols are the same as in Fig. 5A.

carbamylcholine to the receptor were recently reported by Aronstam *et al.* (9). These authors, using a phosphate buffer (pH = 7.4), a shorter incubation time (20 min at 35°) and a 10 times less NEM to protein ratio, found no effect of NEM treatment on [^{3}H]3-QNB binding. The present study, using Ca^{2+} containing medium and longer incubation times (60 min at room temperature), demonstrated that NEM inhibited binding of [^{3}H]3-QNB to membranes from rat cerebral cortex. This result

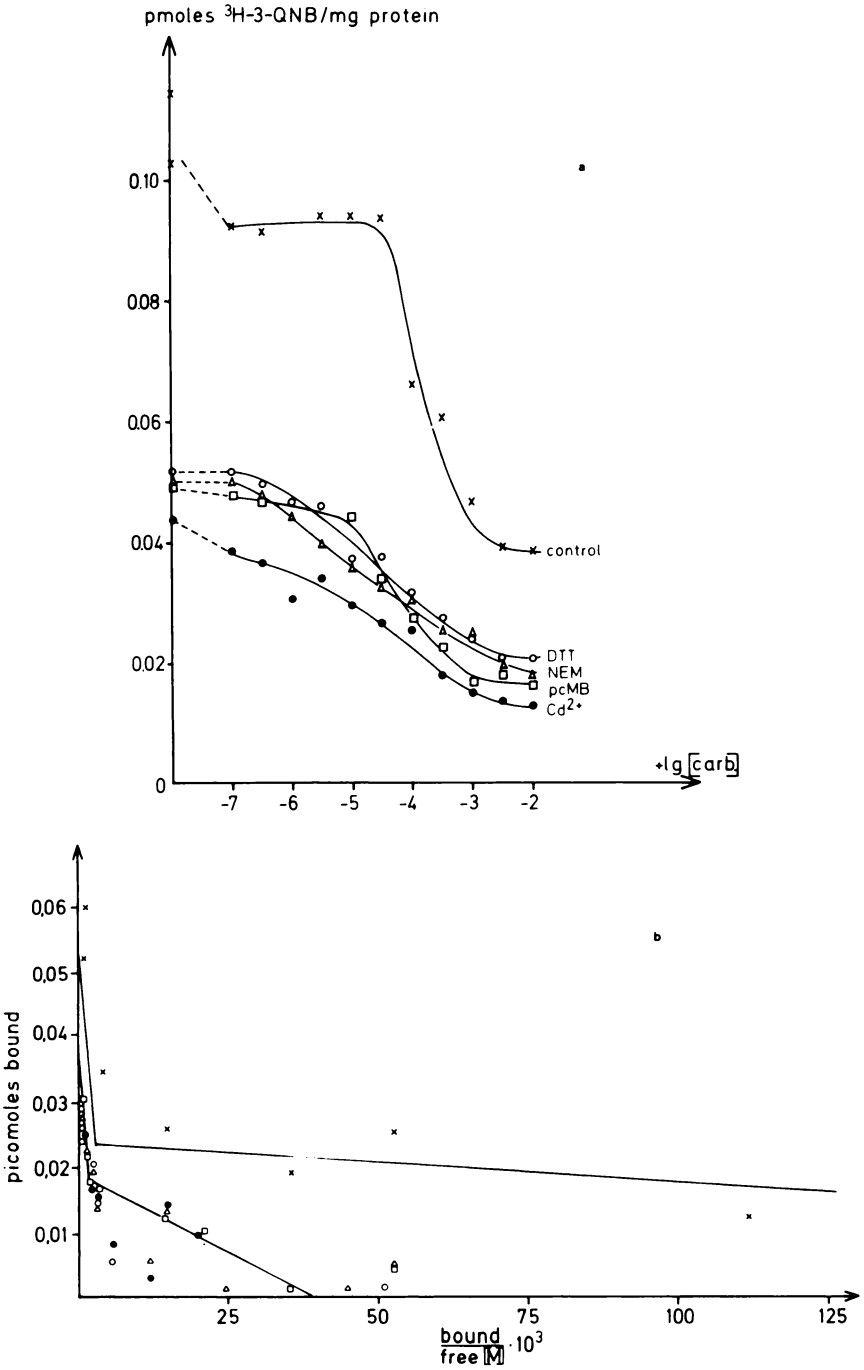

FIG. 6. *Competition of carbamylcholine and [³H]3-QNB for muscarinic binding sites (A) and Schatchard plots of data (B)*

A. Competition between carbamylcholine and [³H]-3-QNB (0.1 nM) for muscarinic binding sites of control ×; DTT treated ○; NEM (1 mM) treated △; pCMB (1 mM) treated □; and Cd^{2+} (1 mM) treated ● membranes, respectively. The various reagents were incubated with the membranes for 30 min at room temperature. The incubation was stopped by centrifugation at 40,000 × *g* 30 min, and the pellets were resuspended and incubated with 0.1 nM [³H]3-QNB in the presence of various concentrations of carbamylcholine. The incubation was carried out for 60 min. B. Schatchard plots of the data shown in A: Bound carbamylcholine (picomoles/mg) versus bound/free carbamylcholine (picomoles/mg/M).

TABLE 6

The effect of DTT, pCMB, NEM and Cd^{2+} on the dissociation constants of carbamylcholine binding to membranes from rat cerebral cortex

The data were fitted with a Newton-Gauss algorithm as described in METHODS. y denotes the amount of carbamylcholine bound (femtomoles/mg protein). V_1 and V_2 are expressed in the same units and stand for the binding capacities of sites with affinities K_1 and K_2, respectively.

Treatment	Model		Number of points	Parameter values				Residual sum of squares
				Low affinity site V_1	Low affinity site K_1	High affinity site V_2	High affinity site K_2	
				(femtomoles/mg)	*(μM)*	*(femtomoles/mg)*	*(nM)*	*(× 10^4)*
None	$y = \frac{V_1[S]}{K_1 + [S]}$	(I)	48	73 ± 4	26 ± 7.2	—	—	7.39
None	$y = \frac{V_1[S]}{K_1 + [S]} + \frac{V_2[S]}{K_2 + [S]}$	(II)	48	55 ± 4	95 ± 15	23 ± 4	52 ± 8	3.9
DTT (1 mM)	I		27	34 ± 2	5.5 ± 2.2	—	—	3.56
DTT (1 mM)	II		27	38 ± 6	230 ± 140	19 ± 2	490 ± 28	1.27
NEM (1 mM)	I		27	42 ± 3	21.5 ± 4.7	—	—	2.52
NEM (1 mM)	II		27	33 ± 3	139 ± 66	33 ± 8	2370 ± 1460	1.01
pCMB (1 mM)	I		20	26 ± 2	1.4 ± 0.7	—	—	3.19
pCMB (1 mM)	II		20	15 ± 4	202 ± 230	15 ± 4	4760 ± 400	1.72
Cd^{2+} (1 mM)	I		26	23 ± 1	17.8 ± 3.4	—	—	1.32
Cd^{2+} (1 mM)	II		26	20 ± 1	49 ± 11	5 ± 1	400 ± 30	0.49

was found both in experiments in which the alkylating agent was present together with [^{3}H]3-QNB (Fig. 1B) and in those where NEM was removed prior to incubation with [^{3}H]3-QNB (Tables 2 and 3).

The mercaptide-forming reagent, pCMB, had similar inhibitory effect on [^{3}H]3-QNB binding. Inhibition of [^{3}H]3-QNB binding by low concentrations of $CdCl_2$ suggested that vicinal thiol groups were present (23). The observation that combinations of $CdCl_2$, NEM and pCMB inhibited binding to the same extent as the individual reagents suggested that the site of action of these agents might be identical or at least mutually exclusive. The thiol-oxidizing agent DTNB or the naturally occurring disulfide GSSG could also inhibit [^{3}H]3-QNB, binding probably through formation of mixed disulfides (Figs. 1b and 3b).

Experiments with cysteine, GSH and DTT (Fig. 1a) indicated that reduction of disulfide bridges was inhibitory to [^{3}H]3-QNB binding. That physiologically important thiols such as cysteine and GSH in low concentrations 10–50 μM inhibited binding to the receptor may be of physiological significance. At repetitive stimuli GSH, which is present in millimolar concentration intracellularly (24), might escape from the presynaptic terminal and accumulate in the synaptic gap. Such an event would transiently change the affinity for carbamylcholine (Table 6). Since several biochemical and physiological responses such as phosphatidylinositol turnover (25) and generation of cGMP (2, 26, 27) correlate with the low affinity agonist binding, a shift to higher affinity might have the effect of physiological densitization. In this mechaism the "desensitized" receptor could regain full activity *via* reoxidation (Fig. 3). Desensitization of muscarinic receptors has been shown in guinea pig ileum by Young (28) and on N1E 115 neuroblastoma cells (29). In these cases oxidation rather than reduction could be the mechanism of desensitization since the experiments were carried out in the absence of any reducing agent.

The effects of Cd^{2+}, NEM, pCMB, DTNB and DTT suggested that at least one pair of vicinal thiol groups and a disulfide bridge are closely influencing binding properties. These groups could be on the receptor protein or in its vicinity in the membranes. The possibility that more than one group can be alkylated on the receptor also gains support from the work of Gupta *et al.*, who found that more than one benzilyl choline mustard could be bound to the receptor (30).

The observation that both thiol-blocking and reducing agents were effective on isoosmotically treated membranes suggested that the thiol and disulfide groups were present on a portion of the receptor facing the extracellular space and that no hypotonic treatment was required to make the receptors accessible to an attack from the inside of the cell. (In fact, hypotonic treatment (0°, 60 min) did not increase the inhibition by the reagents; data not shown). The finding that almost full protection against the effect of DTT was afforded by an agonist and an antagonist suggests that the sensitive disulfide bridge is on the receptor molecule itself and is probably localized in the binding domain. The protection against alkylation, oxidation or mercaptide formation was much poorer than that against reduction of the disulfide bond. This might indicate that the vicinal thiol groups were less closely associated with the ligand binding site.

Any interpretation of these findings based on a receptor mechanism exclusively is dangerous. It is obvious that several membrane proteins and lipids which surround the receptor could be, and most likely are modified upon treatment with reducing, oxidizing and alkylating agents. Bearing this limitation in mind, the protective effects in our experiments with specific ligands (Table 3), still allow us to assume that the inhibition data could be interpreted in terms of modifications of the receptor.

Any acceptable model must explain the following findings: a) In untreated membranes [^{3}H]3-QNB binding can be described by a single binding isotherm with a Hill coefficient of 1. (Results presented here and [2]). b) Binding of carbamylcholine to untreated membranes in the ligand concentration range of 0.1 μM–0.1 mM reveals two sites (Fig. 6B). c) Treatment of the membranes decreased the number and the affinity of binding sites for [^{3}H]3-QNB. (The binding still obeyed a single binding isotherm.) d) Treatment of the membranes decreased the number and changed the affinity of the low and high affinity binding sites for carbamylcholine.

Thus one has to assume that the receptor population can exist in at least two conformational states, which are interconvertible. In antagonist binding experiments one of these populations dominates and the binding obeys a single binding isotherm. The agonist carbamylcholine shifts the equilibrium between the two conformational states and a measurable portion (30%) of receptors assumes one conformation with K_d 52 ± 8 nM, whereas K_d for the other conformer is 95 ± 15 μM. Alkylation, oxidation or reduction will inhibit a portion of receptors dependent on the concentration of the reagent. At 1 mM DTT, pCMB, NEM and Cd^{2+} this portion was about 40–50%. The remaining portion of receptors has changed its affinity toward [^{3}H]3-QNB indicating that even this population of receptors may have been subject to covalent modification (Table 5). This modified receptor population however, retains its ability to assume agonist-induced conformation. Incubation with carbamylcholine will evoke a partition of the remaining [^{3}H]3-QNB binding sites into two populations of carbamylcholine binding sites, just as in untreated membranes.

The finding that agonist and antagonist binding was influenced differently by alkylation and oxidation resembles the finding by Rang and Ritter. These investigators found that reduction of chicken biventer muscle by DTT changed the affinity toward acetylcholine and converted hexamethonium from an antagonist to agonist (31).

Whether transitions of the type produced by DTT, pCMB, NEM, Cd^{2+} and DTNB occur *in vivo* is doubtful. The most likely action is that an equilibrium of GSSG to GSH determines the "redox state" of the thiol and disulfide groups of the receptor. Indeed at 10 μM GSSG and 0.1 μM GSH an

optimum of the binding capacity was found. These concentrations are likely to correspond to the extracellular concentrations of these compounds (in perfusates of rat liver 10 μM GSSG concentration was found [32]).

The important finding, besides the demonstration of agonist-induced split of the receptor population in the native and treated membranes (Aronstam *et al.* [9], Birdsall and Hulme [2]) was the demonstration of the strong inhibitory effect of Cd^{2+} on agonist and antagonist binding. The low concentrations required for substantial inhibition of the receptor, together with data indicating that Cd^{2+} accumulates in the brain (33, 34), make it likely that muscarinic receptor might be one of the primary targets of the neurotoxic actions of Cd^{2+} (34). Experiments to test this hypothesis are in progress.

ADDENDUM

Two months after having submitted this paper we found a detailed and elegant study on the same topic published by Aronstam *et al.* (35), whose first publication on the effects of NEM (9) drew our attention to this problem. Their study, in agreement with this report, demonstrated the presence of thiol groups of importance for antagonist and agonist binding. Some of the discrepancies between their study and this report on the inhibition of [^{3}H]3-QNB binding by NEM can be explained by bearing in mind that these investigators carried out their experiments in a medium of completely different ionic composition. We have confirmed their earlier results on the lack of NEM effect on [^{3}H]3-QNB binding in phosphate buffer (9). The differences in susceptibility to modification in the presence of Ca^{2+} and other ions emphasize the need for use of medium of more closely physiological composition in studies on muscarinic receptor.

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