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ANNUAL RESEARCH REPORT (1982/83)

THE MECHANISM OF INTERACTION OF OXIMES WITH THE MUSCARINIC-CHOLINERGIC COMPLEX IN THE CENTRAL NERVOUS SYSTEM

Contract No.: DAMD-17-81-C-1223

Contractor: Tel-Aviv University
Research Authority

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Date: November 3, 1983

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE	READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. A148398 / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) THE MECHANISM OF INTERACTION OF OXIMES WITH THE MUSCARINIC-CHOLINERGIC COMPLEX IN THE CENTRAL NERVOUS SYSTEM	5. TYPE OF REPORT & PERIOD COVERED ANNUAL--December 1982-November 1983 / 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) Mordechai Sokolovsky	8. CONTRACT OR GRANT NUMBER(s) DAMD17-81-C-1223
9. PERFORMING ORGANIZATION NAME AND ADDRESS Tel-Aviv University Research Authority 69978 Tel-Aviv, Israel	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62734A.3M162734A875.AI.096
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command Fort Detrick Frederick, Maryland 21701-5012	12. REPORT DATE November 1983 / 13. NUMBER OF PAGES 42
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified / 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE
16. DISTRIBUTION STATEMENT (of this Report) Unlimited	
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) Unlimited	
18. SUPPLEMENTARY NOTES To be published (in part) in Mol. Pharmacol.	
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) muscarinic, acetylcholine, oximes, antagonists, agonists, brain stem, cortex.	

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The studies described in the current report were aimed at exploring the mode of interaction between bisquaternary oximes and muscarinic acetylcholine (ACh) receptors in the brain.

The binding of the muscarinic antagonist [^{3}H]-N-methyl-4-piperidyl benzilate ([^{3}H]-4NMPB) to rat brain muscarinic receptors was inhibited by bisquaternary oximes, the most potent inhibitors being 1-(2-hydroxyiminoethylpyridinium) 1-(3-cyclohexyl-carboxypyridinium) dimethylether (HGG-42) and its 3-phenylcarboxy-

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pyridinum analog (HGG-12) (K_I=1.5 μM and 2.0 μM, respectively). At the μM concentration range, both oximes competed with [^{3}H]-4NMPB, and their K_I values were similar in rat brain stem and brain cortex preparations. However, at higher concentrations, the binding of the oximes and [^{3}H]-4NMPB was non-exclusive. Kinetic binding studies indicated that the oximes interact allosterically with the muscarinic antagonist site, decreasing both the association and dissociation rates of the [^{3}H]-4NMPB/receptor complex.

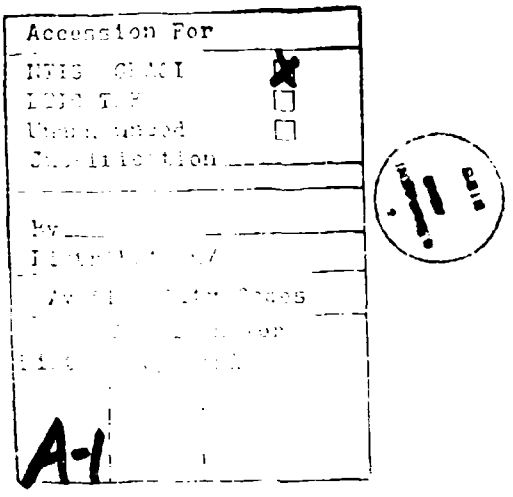
In the brain stem, HGG-12 and HGG-42 induced an irreversible loss of 30% of the muscarinic binding sites. In rat brain cortex, only 10% of the muscarinic receptors were lost under similar conditions, demonstrating regional selectivity in the effect of the oximes on [^{3}H]-4NMPB binding. The reduced binding of [^{3}H]-4NMPB in the brain stem was time- and concentration-dependent, and occured both in vitro and in vivo (following injection of oximes to rats). However, the in vivo receptor loss was not irreversible, and the muscarinic binding capacity was restored two hours after the injection.

The in vitro effects of the oximes on muscarinic agonist binding were also investigated. In both brain stem and cortex, pre-treatment with HGG-12 did not affect the binding characteristic of carbamylcholine and ACh binding to the remaining sites. However, the affinity of oxotremorine to the high affinity agonist binding sites was reduced. The relative proportions of the high affinity (M_H) and low affinity (M_L) agonist binding sites were unaltered, but the interconversion process between M_H and M_L was affected. In the brain stem, HGG-12 treatment cancelled the ability of guanosine 5'-(β,γ-imino) triphosphate (GppNHp) to induce M_H to M_L conversion. Similarly, cortex membranes treated with HGG-12 lost their response to conversion for M_L to M_H by Co^{2+}. Thus, HGG-12 inactivates a component required for both the GppNHp- and the Co^{2+}-induced interconversions between M_H and M_L.

Protection experiments with carbamylcholine indicated that this component is associated with the agonist binding sites, since, occupancy of M_H by the agonist could protect M_L from inactivation by HGG-12.

Our results suggest that bisquaternary oximes are allosteric inhibitors of the muscarinic receptors, and may induce irreversible effect on the muscarinic binding sites. They also suggest that in the presence of the oximes, the muscarinic antagonist/agonist binding ratio is increased, enhancing the blockade of muscarinic sites by the antagonist.

THE MECHANISM OF INTERACTION OF OXIMES WITH THE MUSCARINIC CHOLINERGIC COMPLEX IN THE CENTRAL NERVOUS SYSTEM



acetylcholine

SUMMARY

The purpose of the studies described in the present report was to explore the mode of interaction between oximes and ACh muscarinic receptors in the brain. A better understanding of the mechanism of these interactions could help to improve the protection of oxime-muscarinic antagonist mixtures from organophosphate poisoning.

In order to achieve these goals, we employed methods based on measurements of the binding of the radiolabeled muscarinic antagonist [^{3}H]-4NMPB. The methods employed included both direct binding studies and competition experiments between labeled and unlabeled ligands. The effects of HGG bispyridinium oximes on the binding parameters of muscarinic agonists and antagonists were investigated.

Binding of [^{3}H]-4NMPB was inhibited by bisquaternary oximes, the most potent inhibitors being HGG-42 and its analog HGG-12 (apparent K_I = 1.3-1.7 μM and 1.8-2.2 μM, respectively). At the μM concentration range, binding of these oximes and of [^{3}H]-4NMPB was mutually exclusive, and their K_I values were similar in brain stem and cortical preparations. Analysis of the binding properties at higher concentrations suggested that binding of the muscarinic antagonist and the bisquaternary pyridinium oximes was non-exclusive. Kinetic binding data provide evidence that the drugs inhibit binding of muscarinic antagonists in an allosteric manner, decreasing the rates of both association of [^{3}H]-4NMPB to the receptor and its dissociation from it (1).

The selectivity of HGG-12 and HGG-42 was apprent from their irreversible effects on the number of muscarinic binding sites. In brain stem the presence of these drugs resulted in a loss of about 30% of binding sites, which accounts in part for the apparent decrease in maximal binding capacity observed in the equilibrium binding of [^{3}H]-4NMPB. In the cortex, however, only ∿ 10% of the muscarinic receptors were lost upon exposure to these drugs. The decrease in the muscarinic receptor population of the brain stem was dependent on both concentration and time and occurred both in vitro and in vivo following injection of HGG-12 into rats. Unlike the in vitro loss of receptor sites which was irreversible, the in vivo effect was restored two hours after the injection.

In vitro studies were conducted on the binding of muscarinic agonists in brain stem and cortex homogenates pre-treated with HGG-12. The binding characteristics of carbamylcholine and ACh, when examined in the remaining sites in the washed membranes, were similar to those in control membranes. The affinity of the agonist oxotremorine towards the high affinity binding sites was reduced. No change was observed in the relative proportions of high affinity agonist binding sites (M_H) and low affinity binding sites (M_L). However, the inhibitor affected the processes of interconversion between these sites. In HGG-12-treated brain stem membranes, GppNHp could not induce conversion of M_H to M_L as it did in control membranes, where the proportion of M_H was reduced from 60 to 30%. Similarly, in HGG-12-treated cortical membranes Co^{2+} could not induce conversion of M_L to M_H as it did in control membranes, where the proportion of M_H was increased from 30 to 60%. These results suggest that HGG-12 inactivates a component which is involved in both the GppNHp induced conversion of M_L to M_H. Protection experiments with the agonist carbamylcholine

indicated that this component is associated with the agonist binding sites. Induced interconversion between M_H and M_L was not affected in membranes treated with HGG-12 in the presence of carbamylcholine in concentrations at which the agonist occupies mainly M_H. This suggests that the occupation of M_H induced a conformational change which is in turn reflected in the sites of oximes and effectors. Evidently, occupation of M_H results in protection of M_L from the oxime. The latter sites can become converted to M_H despite the oxime treatment, provided that any preexisting M_H have been occupied by agonist. It thus appears that there is an association between M_H and M_L.

Taken together, the results suggest that the bisquaternary oximes are allosteric inhibitors of the muscarinic ACh receptor, and are capable of distinguishing between receptor states and inducing specific irreversible effects. They also point out important aspects of the therapeutic effect of oxime-muscarinic antagonist mixtures in organophosphate poisoning. The overall effect of the oxime is to increase the ratio of antagonist/agonist binding to the muscarinic receptors, thus enabling the antagonist to block the muscarinic cholinergic sites more efficiently. Moreover, the in vivo effects of the oximes examined in this study on rat brain muscarinic receptors suggest that the proposed mechanism can also operate in the intact animal.

FORWARD

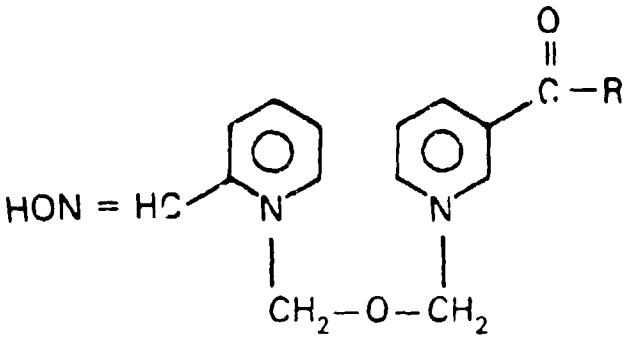
Organophosphates, when interacting with the enzyme acetylcholinesterase, inhibit its activity by the formation of phosphoryl-enzyme adduct (2,3), thereby increasing levels of endogenous acetylcholine. In the absence of protecting drugs, organophosphate poisoning can be lethal. Recovery from such poisoning can be achieved by administration of a mixture of muscarinic antagonists and oximes, provided that the phosphoryl-enzyme can be reactivated by the latter. Several organophosphates (such as soman) form a phosphoryl-enzyme adduct which is not reactivated by oximes (4, 5), yet the mixture of some oxime/antagonists is still useful in partial protection against soman intoxication (6-10). This phenomenon led to the idea that oximes may act via the muscarinic cholinergic receptor, in conjunction with the muscarinic antagonist.

Previous reports (11-13) indicated that oximes possess mild antimuscarinic activity and inhibit the binding of [^{3}H]-4NMPB, a potent muscarinic antagonist. Their interaction with the muscarinic system could not be described as if they simply bind to the acetylcholine binding sites on the muscarinic receptor (11, 13). Together with the cooperative protecting activity of muscarinic antagonists and oximes against organophosphate poisoning (14), this phenomenon prompted us to a detailed study on the direct effects of oximes on the muscarinic cholinergic receptor in rat brain.

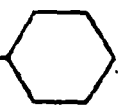
Our recent studies on the interaction of oximes with the muscarinic receptors (1) indicate that this group of drugs inhibits binding of the muscarinic antagonist, [^{3}H]-4NMPB, in a complex manner. The two simplest models, which were based on mutually exclusive binding or on the formation of ternary oxime-receptor-NMPB complexes, do not adequately explain the complex interactions between oximes and the muscarinic receptors. Two other possibilities were therefore considered: (1) Interaction of oximes with heterogeneous populations of muscarinic receptors, and (2) irreversible inactivation of the receptor by oximes. The first of these was examined in comparative binding studies using preparations of pure presynaptic muscarinic receptors from synaptosomes of Torpedo electric organ and heterogeneous receptors from rat brain stem. We have reported that oximes inhibit a higher affinity towards presynaptic as compared with postsynaptic receptors. Such behavior is expected to lead to heterogeneous binding of oximes to rat brain preparations. However, the complex "non-competitive" binding of [^{3}H]-4NMPB and oximes was apparent even in the purely presynaptic receptor preparation.

In the present study we summarize our results on the interactions of two potent oximes, HGG-12 and HGG-42 (see structure in Scheme I), with muscarinic receptors in rat brain stem and cortex, and in Torpedo electric organ synaptosomes. Our results indicate strong interference of these oximes with the interactions of muscarinic antagonists and agonists with muscarinic acetylcholine receptors, as well as effects on the action of modulators of the muscarinic system.

The HGG Bispyridinium Oximes



HGG--12, R = 

HGG—42, R = 

HGG—52, R = $-CH_2-CH(CH_3)_2$

Scheme I

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MATERIALS AND METHODS

[^{3}H]-4NMPB (70 Ci/mmol) was prepared by catalytic tritium exchange as described previosuly (15). Its purity was > 97%. HGG-12 (m.p. 157-159), HGG-42 (m.p. 130-134) and HGG-52 (m.p. 142-145) were prepared accordin to published procedures (16), and were > 96% pure as determined by t.l.c. in n-butanol:acetic acid:H_2O (4:1:1). GppNHp was from Sigma.

Methods

Adult male and female rats of the CD strain were obtained from Levinstein's Farm (Yokneam, Israel) and maintained in an air-conditioned room at 24± 2° for 14 hr (5 a.m. - 7 p.m.) under fluorescent illumination and in darkness for 10 hr. Rats of either sex were employed in the experiment, since no sex differences were observed throughout the experiments with oximes. Food from Assia Maabarot Ltd. (Tel Aviv, Israel) and water were supplied ad libitum. The rats were then 3-4 months old and weighted 190-250 g. They were decapitated (between 10 a.m. and noon) and their brains were rapidly removed. Brain regions were dissected out in a cold room after identification with the aid of a stereotaxic atlas (17). Brain regions were homogenized in 0.32 M sucrose as described in detail previously (18, 19) to yield a 3% homogenate (w/v).

These homogenates were used for binding assays with [^{3}H]-4NMPB. When tissues were used for [^{3}H]-acetylcholine binding assays, the homogenates were prepared in 50 mM Tris/HCl buffer pH 7.4. These homogenates were incubated for 30 min at room temperature and the membranes then precipitated (30,000 x g 20 min). After the addition of 200 µM diisopropylfluorophosphate in order to inhibit endogenous acetylcholine esterase, membranes were used for the binding assays with [^{3}H]-acetylcholine. This treatment with the inhibitor does not affect the muscarinic receptors, as judged by its lack of effect on the binding of [^{3}H]-acetylcholine to these receptors (20). For pretreatment of membranes with oximes, homogenates were incubated at room temperature for 60 min in the presence of 200 µM HGG-12, unless otherwise indicated. The homogenates were then centrifuged (30,000 x g, 20 min) and the membranes resuspended and washed three times with the incubation Krebs buffer by repeated centrifugations. The final membrane pellet was resuspended in the incubation buffer and used for binding assays.

For in vivo experiments with HGG-12, rats (males, 180-230 g) were given s.c. injections of various doses of HGG-12 in saline. Control rats were given s.c. saline injections. All the injection doses (3-20 mg/kg) were much lower than the LD_{50} dose, so that no death of rats occurred. At various times after the injection (0.5-2 h) the rats were killed by decapitation and their cerebellar cortex and brain stem were dissected out and homogenized as described for the in vitro studies. The resulting homogenates were washed three times with buffer as in our in vitro experiments (30,000 g xm 20 min each). The final membrane suspension was assayed for muscarinic receptor binding as described above.

Preparation of Torpedo synaptosomes

Torpedo ocellata were caught live off the coast of Tel Aviv and maintained in sea water aquaria up to 4 months prior to use. Synaptosomes from the electric organ of the elasmobranch Torpedo were prepared (21) by the following procedure. The electric organs, which weigh 38±11 g, were excised from fish which had been prechilled at 4°C for 30 min. The excised tissue, 10-15% w/v, in 0.8 M glycine - 1 mM EGTA, pH 6.6 was belnded in an omnimixer (Sorvall) for 0.5 min and then homogenized in a Lucite glass homogenizer with a clearance of 0.8% of the pestle's diameter (6 passages at 880 rev./min). The homogenized material (H) was centrifuged for 10 min at 1000 g. The resulting pellet (P_1) was discarded, and the supernatant (S_1) was subjected to further centrifugation at 17,500 g for 1 h. The fast centrifugation pellet (P_2) was resuspended by homogenization with the Lucite glass homogenizer (one passage at 150 rev./min) and loaded onto the density gradient. The supernatant (S_2) was discarded. Density gradient centrifugation was performed with a discontinuous gradient in SW40 rotor spun at 110,000 g for 1 h. The loaded gradient tubes contained six layers (a_1 to a_6), all of which contained 1 mM EGTA and one of the following concentrations of sucrose: 0, 0.15, 0.3, 0.55, 0.8, 1.6 M. Layers a_1 to a_4 also contained glycine so that their osmolarity was equal to that of the homogenization buffer. After centrifugation, the material, which separated into bands at the interfaces of the gradient, was carefully collected with a Pasteur pipette starting at the lighter fractions. Fractions a_2 and a_3, which are the most enriched in synaptosomes and muscarinic receptors, were pooled, diluted with 4 vol. of the homogenization buffer, and then further centrifuged at 17,500 x g for 20 min. The supernatant was discarded and the pellet resuspended so that each ml contained 1.2 - 2.4 mg protein. This purified presynaptic preparation, which we shall refer to as the synaptosomal fraction, was used for the binding assays.

Binding assay for [^{3}H]-4NMPB

Homogenates were used for binding assays as follows: 50 µl of tissue preparation were incubated at 25° in 2 ml of modified Krebs-Hanseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.69 mM KCl, 1.9 mM $CaCl_2$, 0.54 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11.1 mM glucose), pH 7.3, containing varying amounts of the labeled ligand and the oximes. After the required period of incubation, ice-cold Krebs solution (3 ml) was added and the contents were passed rapidly by suction through a glass filter (GF/C, Whatman, 25 mm diameter). The filters were washed three times in 3 ml ice-cold Krebs solution. The filtration and washing procedures were completed within less than 10 sec. Binding assays were performed in triplicate, together with triplicate control samples containing 5×10^{-5} M unlabeled atropine. In the absence of homogenate, the adsorption of [^{3}H]-4NMPB to the filters was negligible (19) The filters were placed in vials containing 4 ml of scintillation liquid (Hydro-Luma, Lumac Systems, Inc., Titusville, Fla.) and were maintained at 25° for 30 min; the radioactivity was then measured by liquid scintillation spectrometry (Packard Tri-Carb 300.) with a counting efficiency of 40-45%.

Protein was determined by the method of Lowry (22) using bovine serum albumin as a standard.

Specific binding was defined as the total binding minus the nonspecific binding, i.e., binding in the presence of 5×10^{-5} M unlabeled atropine. Direct binding studies, competition experiments, and kinetic experiments were carried out as described in detail in previous report (1).

Binding assay for [^{3}H]-acetylcholine

Aliquots of 20 µl of homogenates (equivalent to 3-5 mg of original tissue weight) were added to tubes containing 20 µl of modified Krebs buffer, 200 µM DFP, and the indicated concentrations of [^{3}H]-ACh (20). Following 1 h incubation with gentie shaking at 25°C, 4 ml of ice cold modified Krebs buffer was added and the contents of the tubes were filtered under high pressure through GF/C filters (Whatman, 25 mm diameter). The filters were immeidately washed with an additional 2 ml of buffer. Filtration took 2-2.5 sec. Filters were then counted for tritium. Specific binding was calculated as the difference between the total binding to control membranes and measured non-specific binding, i.e. binding to membranes after adding 20 µM atropine during the last 10 min of the preincubation step. The same values for non-specific binding were obtained when 20 µM oxotremorine was substituted for atropine. All determinations were carried out in quadruplicate, each one varying by < 15%. Centrifugation assays were carried out using a similar protocol (20), but bound and free ligand were separated by centrifugation in an Eppendorf microcentrifuge.

Data analysis

We have described in detail in our Annual Report 1981/82 (1) the analysis of binding data. The two site non-competitive model (23) for [^{3}H]-4NMPB/oxime interactions was described as well (1, 23, 24). In the present work we have also used muscarinic agonists. Their equilibrium binding with the muscarinic receptor is best described by binding to two sites (high and low affinity). Antagonists bind with equal affinity to these two sites (25, 26, 27). The two-site model for agonist/[^{3}H]-antagonist binding is shown below:

$$M_H + A \overset{K_H}{\rightleftharpoons} M_HA$$

$$M_L + A \overset{K_A}{\rightleftharpoons} M_LA$$

$$M + [^3H]\text{-4NMPB} \overset{K}{\rightleftharpoons} M[^3H]\text{-4NMPB}$$

Where M_H and M_L are the high and low affinity agonist binding sites and K_H and K_L the dissociation constants of the agonist (A) from these sites, respectively. With respect to antagonists (as [^{3}H]-4NMPB) $M_L \equiv M_H \equiv M$. In a

typical [^{3}H]-4NMPB/agonist competition experiment, the fraction of bound [^{3}H]-ligand is described by:

Eq. 1

$$\frac{(M - [^3H]\text{-}4NMPB)_i}{(M - [^3H]\text{-}4NMPB)_o} = \frac{\alpha}{1 + K_{Happ}(A)} + \frac{1 - \alpha}{1 + K_{Lapp}(A)}$$

Where $(M - [^3H]\text{-}4NMPB)_i$ and $(M - [^3H]\text{-}4NMPB)_o$ are the specific bound [^{3}H]-4NMPB measured in the presence and in the absence of the agonist, respectively. α is the fraction of high affinity agonist binding sites. K_{Happ} and K_{Lapp} are the apparent affinity constant of A to the high and low affinity binding sites, where:

$$K_H = \frac{K_{Happ}}{1 + K([^3H]\text{-}4NMPB)} \; ; \quad K_L = \frac{K_{Lapp}}{1 + K([^3H]\text{-}4NMPB)}$$

K is the affinity constant of [^{3}H]-4NMPB. The competition binding data were analyzed according to Eq. 1, using a non-linear least squares regression computer program (26).

RESULTS

1. Interactions of HGG-42 with presynaptic receptors

As discussed in previous works, synaptosomes prepared from Torpedo electric organ are ideal for research on presynaptic muscarinic receptors. Unlike brain synaptosomes, the synaptosomal preparation from electric organs contains purely presynaptic structures. The muscarinic receptors in these synaptosomes can regulate the release of ACh in vitro (21).

We have studied the inhibition of binding of [^{3}H]-4NMPB to receptors by HGG-42 (Tables I and II). As shown in Fig. 1a, 5-25 μM of the drug strongly inhibited the binding of the muscarinic antagonist. The inhibition of [^{3}H]-4NMPB binding to the presynaptic receptors by HGG-42 was similar to that observed in rat brain preparations (see previous report). That is, Scatchard plots are curvilinear (Fig. 2), and Hill slopes are lower than 1.0 (Fig. 3). In spite of these similarities, we have observed that HGG-42 is a more potent inhibitor of [^{3}H]-4NMPB binding in the Torpedo preparation than in rat brain preparations (Fig. 1). However, the apparent dissociation constant of HGG-42, calculated from the double reciprocal plots (Fig. 4, 5) according to the two-site model (see ref. 1), are very similar to those observed in rat brain stem or cortex (Table III).

We have discussed in our previous report the two-site non-competitive model in detail. With respect to the data obtained in brain preparations, it was mentioned that the model is used merely to approximate the inhibitory potency of the oximes. For example, the model cannot account for the deviations of the Scatchard plots from linearity, neither can it account for

Table I: HGG-42 inhibition of [^{3}H]-4NMPB binding to muscarinic receptors in Torpedo synaptosomes.

A. Binding in the absence of inhibitor

([^{3}H]-4NMPB) free (nM)	([^{3}H]-4NMPB) bound (cpm)
5.04	675 ± 50
3.75	745 ± 49
2.50	696 ± 60
1.25	563 ± 25
0.514	276 ± 20
0.25	188 ± 22
0.133	111 ± 15
0.052	62 ± 17

B. Binding in the presence of 5 μM HGG-42

([^{3}H]-4NMPB) free (nM)	([^{3}H]-4NMPB) bound (cpm)
5.04	457 ± 30
3.75	423 ± 23
2.50	380 ± 12
1.25	347 ± 39
0.514	103 ± 20
0.25	60 ± 15
0.133	41 ± 10
0.052	31 ± 12

C. Binding in the presence of 10 μM HGG-42

5.04	273 ± 12
3.75	276 ± 25
2.50	278 ± 13
1.25	262 ± 20
0.514	70 ± 9
0.25	36 ± 14
0.133	30 ± 12
0.052	20 ± 10

D. Binding in the presence of 25 μM HGG-42

5.04	190 ± 15
3.75	197 ± 9
2.50	170 ± 12
1.25	159 ± 9
0.514	34 ± 12
0.25	29 ± 11
0.133	19 ± 9
0.052	16 ± 5

Binding was determined as described in detail earlier (1). Each tube contained 0.05 mg protein.

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the apparent reduction in [^{3}H]-4NMPB binding at high concentrations (see Figs. 1 and 4). The latter could be due to reduction in the number of available binding sites (B_{max}), but this has yet to be verified. Whether this explanation is correct or not, it is clear that a factor related to B_{max} is affected by HGG-42. A plot of B_I/B_o versus HGG-42 concentrations, where B_o and B_I represent the maximal binding in the absence and presence of oximes, is shown in Fig. 6. This plot reveals a marked difference between the inhibitory potency of HGG-42 in Torpedo and in the brain preparation. In Torpedo 50% inhibition was observed at ~ 6 μM HGG-42, while 60 μM HGG-42 were required to produce this effect in the latter preparation. These findings were confirmed by 3 sets of experiments in the Torpedo, and 5 sets of experiments in the brain stem.

These findings led to the following conclusions: (a) HGG-42, and possibly other oximes, exhibit higher affinity towards presynaptic muscarinic receptors as compared with postsynaptic receptors. (b) The complex

Table II: Replicate experiment of inhibition of [^{3}H]-4NMPB binding by HGG-42

A. Binding in the absence of inhibitor

([^{3}H]-4NMPB) free (nM)	([^{3}H]-4NMPB) bound (cpm)
6.63	1874 ± 95
4.2	1701 ± 115
2.14	1344 ± 35
0.89	1071 ± 120
0.45	708 ± 72
0.23	397 ± 40

B. Binding in the presence of 2.5 µM HGG-42

([^{3}H]-4NMPB) free (nM)	([^{3}H]-4NMPB) bound (cpm)
6.63	1651 ± 89
4.2	1356 ± 60
2.14	958 ± 100
0.89	479 ± 37
0.46	349 ± 25
0.23	158 ± 33

C. Binding in the presence of 5 µM HGG-42

6.63	1584 ± 150
4.2	963 ± 60
2.14	671 ± 41
0.89	415 ± 36
0.46	252 ± 31
0.23	126 ± 16

D. Binding in the presence of 7.5 µM HGG-42

6.63	1683 ± 125
4.2	865 ± 72
2.14	527 ± 29
0.89	230 ± 22
0.46	167 ± 19
0.23	96 ± 17

E. Binding in the presence of 10 µM HGG-42

6.63	1369 ± 74
4.2	748 ± 39
2.14	406 ± 27
0.89	237 ± 30
0.46	141 ± 17
0.23	107 ± 22

F. Binding in the presence of 15 µM HGG-42

6.63	1064 ± 56
4.2	688 ± 58
2.14	369 ± 31
0.89	155 ± 20
0.46	76 ± 15
0.23	49 ± 11

G. Binding in the presence of 25 µM HGG-42

6.63	812 ± 53
4.2	504 ± 62
2.14	366 ± 27
0.89	139 ± 16
0.46	96 ± 14
0.23	54 ± 19

Binding was determined as described in detail in (1). Each tube contained 0.086 mg protein.

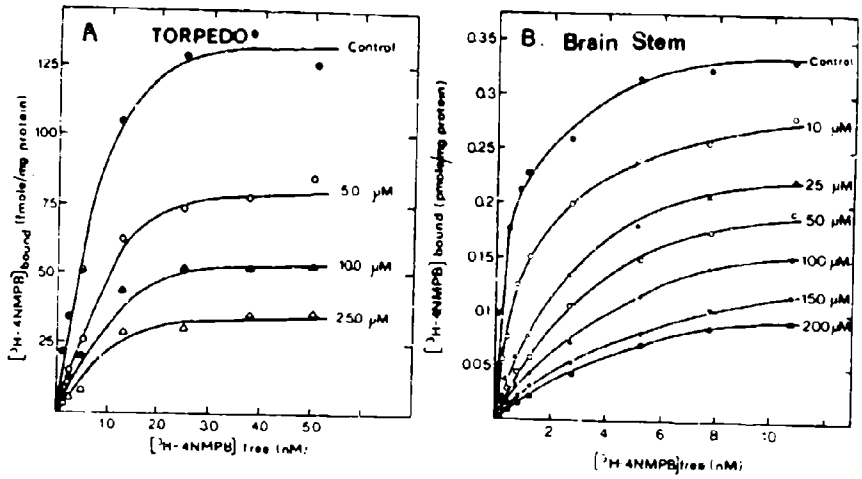

Figure 1: Binding of [^{3}H]-4NMPB to muscarinic receptors in the presence of various concentrations of HGG-42.
A. Torpedo synaptosomes. B. Rat brain stem preparation.

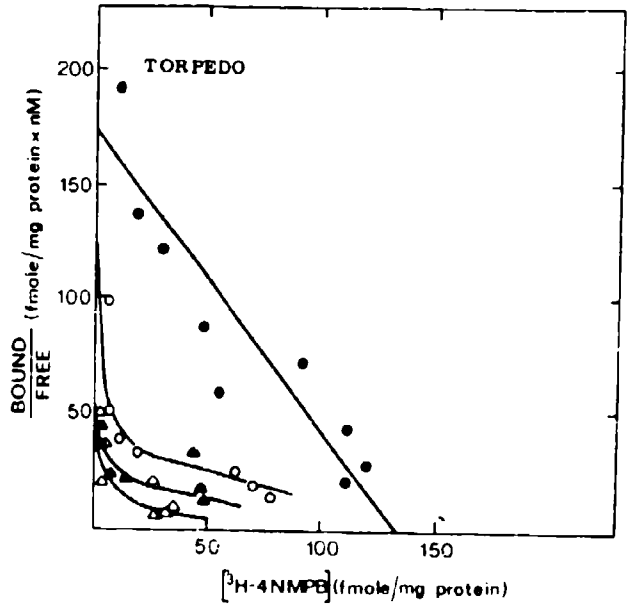

Figure 2: Scatchard plot of data shown in Figure 1.

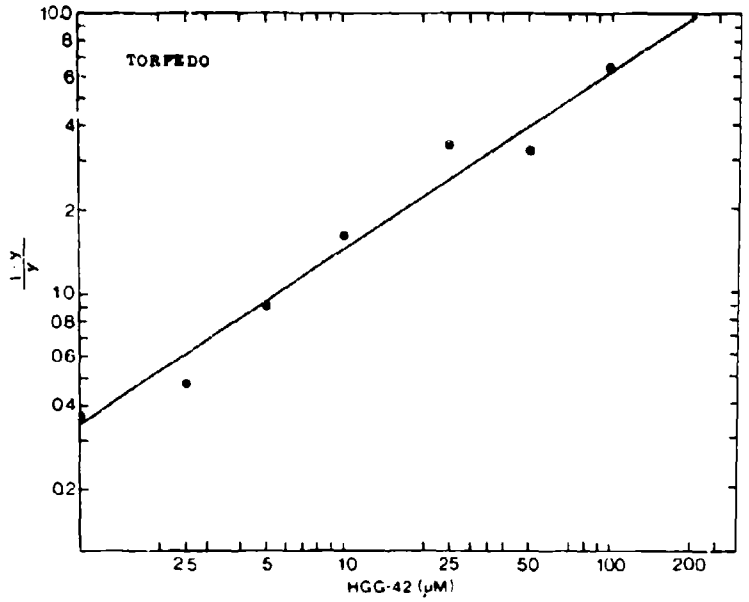

Figure 3: Hill plot of inhibition of [^{3}H]-4NMPB binding (2.5 nM) to Torpedo synaptosomes by various concentrations of HGG-42.

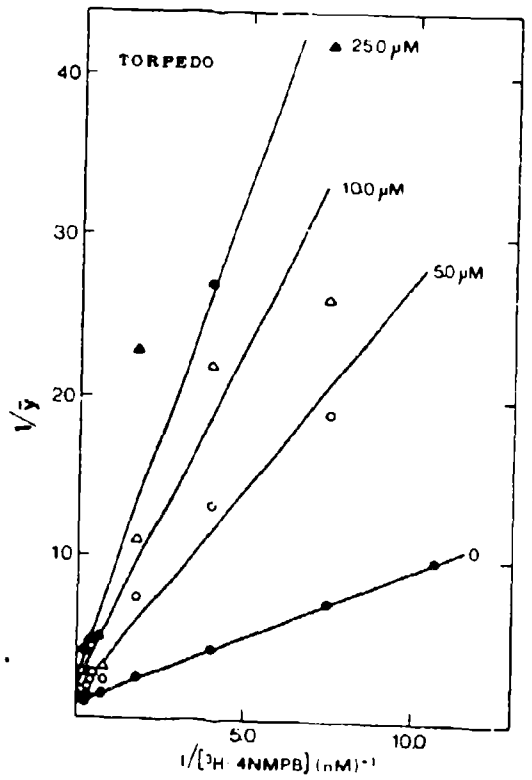

Figure 4: Double reciprocal plot of [^{3}H]-4NMPB binding to Torpedo synaptosomes in the absence and presence of HGG-42.

$\bar{Y}$ = fractional occupancy.

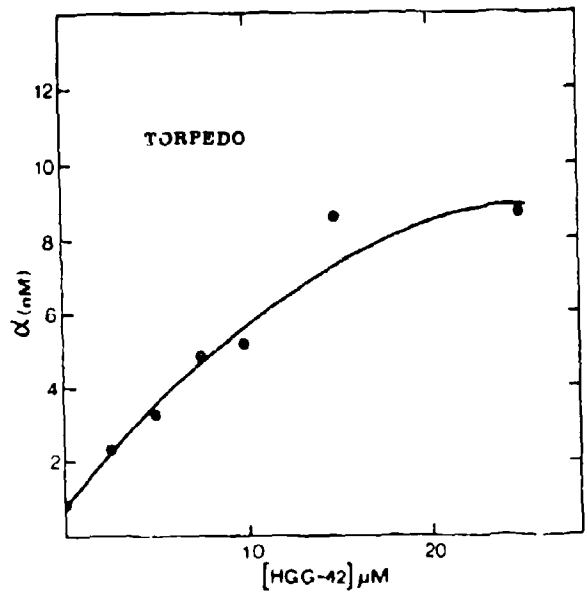

Figure 5: Dependence of the apparent dissociation constant (α) of [^{3}H]-4NMPB on the concentration of HGG-12.
α was calculated from double reciprocal plots shown in Fig. 4.

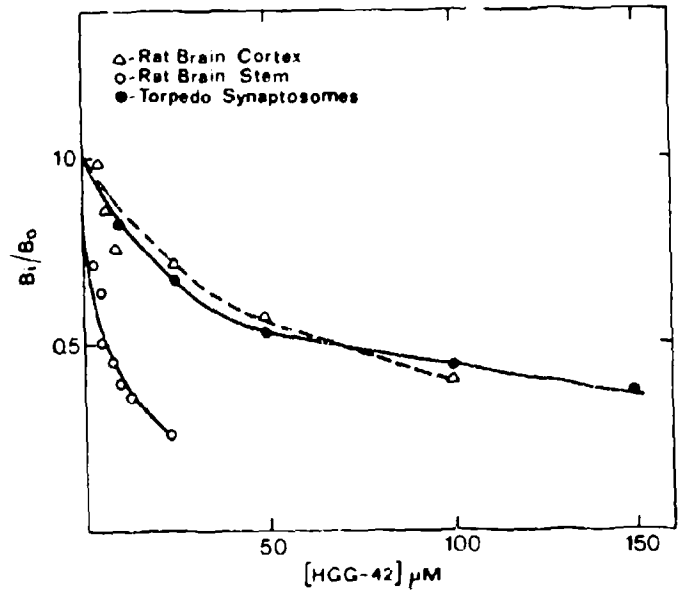

Figure 6: Dependence of the apparent maximal binding capacity of [^{3}H]-4NMPB on the concentration of HGG-42.
Maximal binding in the absence (B_o) and in the presence (B_I) of HGG-42 were derived from double reciprocal plots.

Table III: Dissociation constants of HGG-42 determined by inhibition of [^{3}H]-4NMPB binding according to a two-site non-competitive model

		Brain stem	Cortex	Torpedo synaptosomes
K_2	(μM)	40	20	18
K_3	(μM)	1.0	1.5	1.6

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interaction of oximes with the muscarinic receptors from rat brain is not only due to the presence of pre- and postsynaptic receptors. This suggestion is supported by the finding that the complex interactions are observed also in the pure presynaptic preparation of Torpedo synaptosomes. (c) Interactions of oximes with presynaptic muscarinic receptors should now be considered as one plausible mechanism by which these drugs may protect against organophosphorous poisoning.

In the following chapters we describe our attempts to determine the nature of the apparently "non-competitive" binding of oximes and of [^{3}H]-4NMPB.

2. Inactivation of muscarinic antagonist binding by oximes

In the presence of high oxime concentrations, full saturation of the muscarinic receptors by [^{3}H]-4NMPB is not reached (see ref. 1). This could indicate either that much higher [^{3}H]-4NMPB concentrations are needed to overcome the inhibition of the oxime, or that receptors have been inactivated. It should be noted that in direct binding measurements the apparent "non-competitive" inhibition (manifested by a reduced Bmax) has a different meaning from that of non-competitive inhibition in enzymatic measurements. In the latter case one measures only the active complexes formed; a reduction in the maximal velocity could thus reflect true non-competitive inhibition. In binding studies all receptor-ligand complexes are measured; thus reduced Bmax could be only due to the two reasons mentioned above. It is difficult to measure [^{3}H]-4NMPB binding at very high concentrations (say, 100 times Kd) because of the high non-specific binding. We therefore used a difference procedure in which membranes were preincubated in the presence of oxime (HGG-12 or HGG-42) for 60 min, then precipitated and washed three times in buffer. Binding of [^{3}H]-4NMPB was then measured in the usual way. The results of an experiment in which rat brain stem membranes were exposed to 100 μM HGG-42 are presented in Fig. 7. As shown, pretreatment with the oxime caused a 30% reduction in Bmax. Note that this inhibition of [^{3}H]-4NMPB binding was observed in spite of the repeated washings of the membranes. Furthermore, the Kd of [^{3}H]-4NMPB from the residual binding sites remained unaltered; the presence of unwashed oxime would have caused a change in the Kd. We therefore conclude that HGG-42 caused

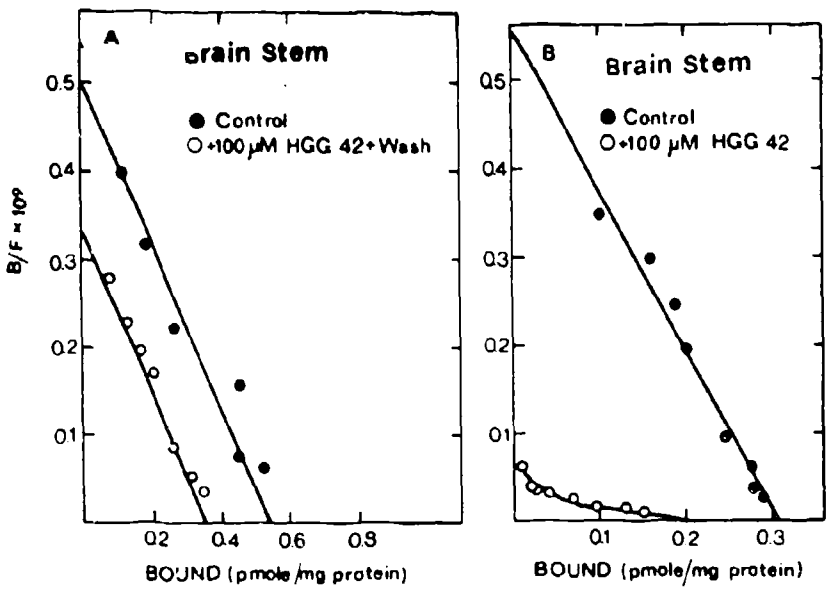

Figure 7: (A) Scatchard plot of [^{3}H]-4NMPB binding to rat brain stem homogenate pretreated with 100 μM HGG-42 for 60 min, then precipitated and washed three times with buffer. (B) Scatchard plot of [^{3}H]-4NMPB binding in the presence and absence (control) of 100 μM HGG-42.

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an irreversible inactivation of about 30% of the muscarinic receptors in the brain stem. When the oxime (100 μM) was not washed, inhibition of [^{3}H]-4NMPB binding was much stronger but the reduction in Bmax was not altered (30%, Fig. 1B). Thus, the oxime possesses at least two modes of interaction with the muscarinic receptor in the brain stem.

Results similar to those obtained with HGG-42 were observed with its analog HGG-12 (Fig. 8). In preparations from rat cortex, however, inactivation by HGG-12 and HGG-42 was almost undetectable (~ 10%) (Fig. 9). It should be noted that in our previous studies (see ref. 1) we could not detect marked differences between the interactions of oximes with muscarinic receptors from the brain stem and from the cortex. This is because the models used to analyze the data did not fully explain the interactions between the oximes and the receptor. However, the simple technique presented here for inactivation of the receptor is evidently sensitive enough to detect variations between the interactions of oximes with receptors in the brain regions under study.

The process of inactivation of brain stem muscarinic receptors by the oximes is dependent upon both time (Fig. 10) and concentration (Fig. 11).

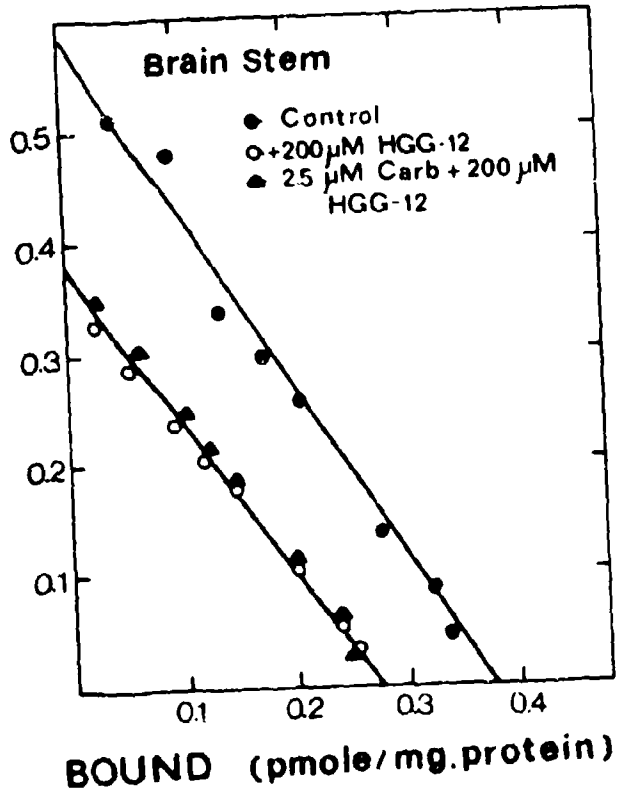

Figure 8: Scatchard plot of [^{3}H]-4NMPB binding to rat brain stem preparation pretreated with 200 μM HGG-12 in the presence and absence of 2.5 μM carbamylcholine as described in Fig. 1A.

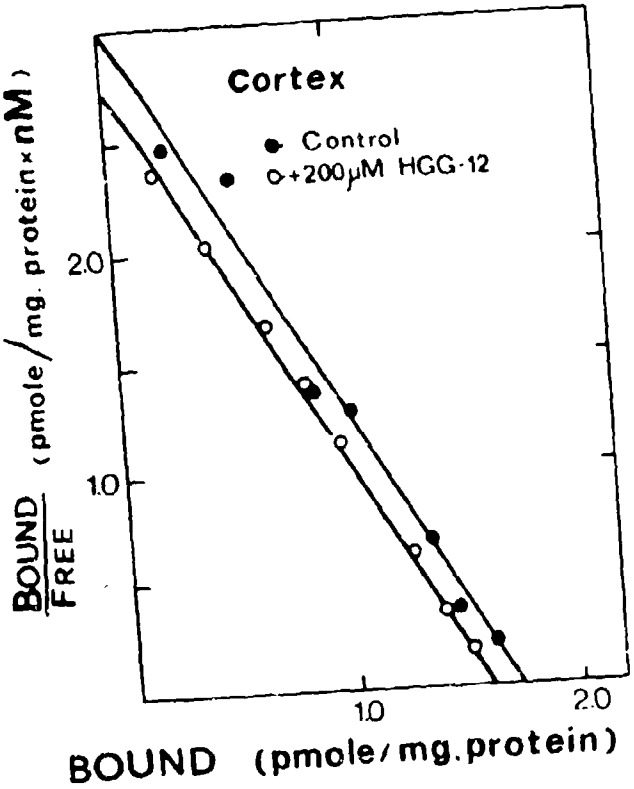

Figure 9: Scatchard plot of [^{3}H]-4NMPB binding to rat cortex homogenate, pretreated with 200 μM HGG-12 for 60 min, then precipitated and washed three times with buffer.

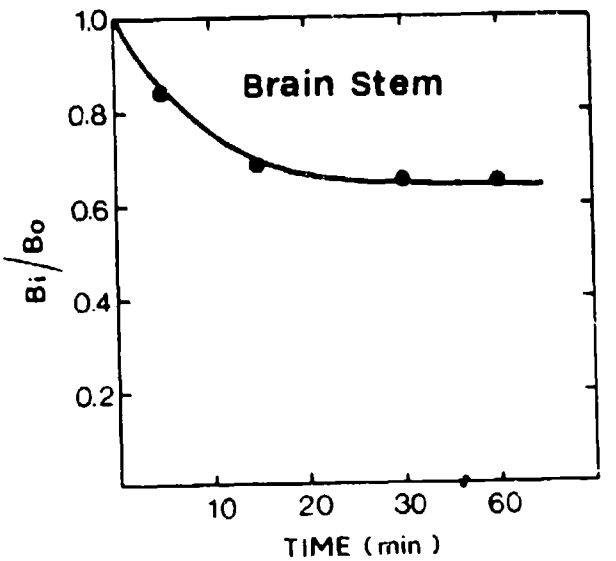

Figure 10: Time-dependence of the inactivation of brain stem muscarinic receptors by 200 μM HGG-12.
Bo = binding capacity of the control (Bmax); Bi = binding capacity after pretreatment with the oxime.

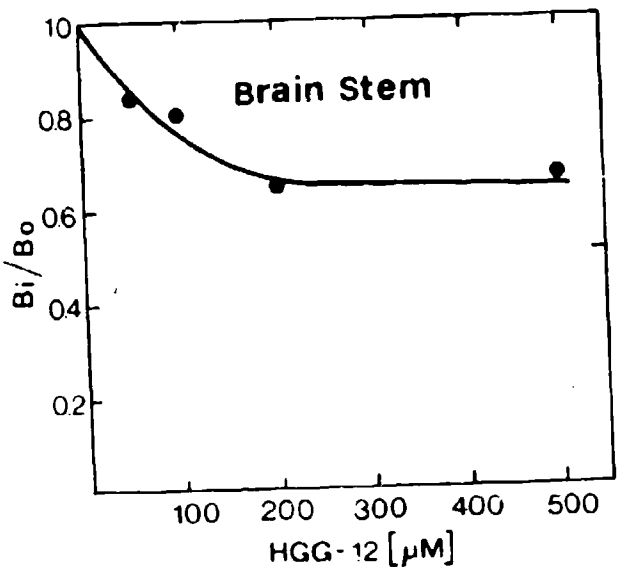

Figure 11: Concentration-dependence of the intactivation of brain stem muscarinic receptors pretreated with oxime for 60 min.

The effect appeared after about 5 min of preincubation with e.g. HGG-12, and reached a maximal level after 15-30 min. At a concentration of 50 μM HGG-12 the degree of inactivation was low; maximal level was achieved at 200 μM. Note that the highest degree of inactivation by the oxime was ∿30%. At concentrations lower than 50 μM, inactivation was almost undetectable. This is in accord with our previous observations that the strongest anomalies in [^{3}H]-4NMPB-oxime-receptor interactions occur at high oxime concentrations (> 50 μM).

3. Inactivation of high affinity muscarinic agonist binding sites by HGG-12

The marked differences between oxime-dependent activation of muscarinic receptors in the brain stem and cortex, as well as the fixed degree of inactivation, suggested that the oximes may exert their effect on a distinct population of receptors. Since muscarinic agonists can distinguish between at least two apparent classes of muscarinic receptors, we examined the effects of oxime on muscarinic agonist binding. Competition binding experiments using [^{3}H]-4NMPB and the agonist oxotremorine were performed following preincubation with 200 μM HGG-12 and washings as described above. The ability of oxotremorine to inhibit [^{3}H]-4NMPB binding was affected only slightly when cortical membranes were used, mostly at low oxotremorine concentrations (Fig. 12A). The effect of oxime on oxotremorine binding in brain stem

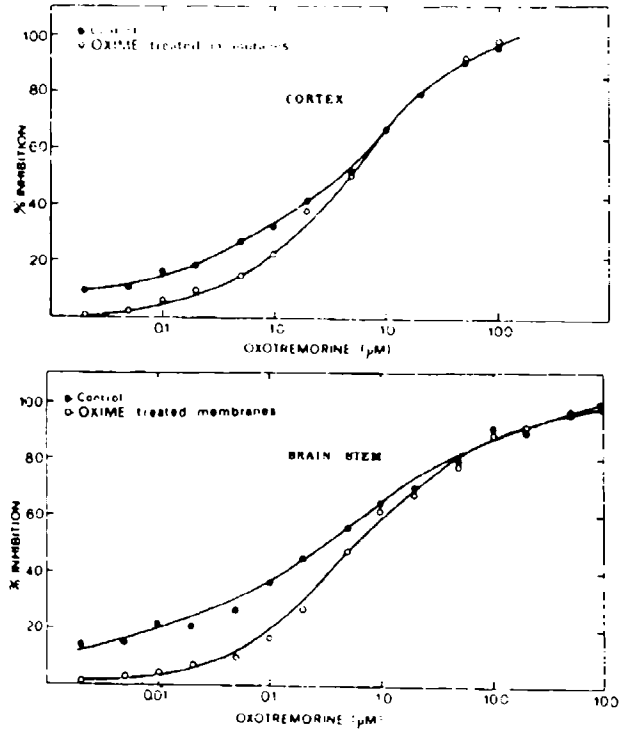

Figure 12: (A) Inhibition curve of [^{3}H]-4NMPB binding to cortical membranes by the muscarinic agonist oxotremorine. (●) - control; (○) - membranes pretreated with 200 μM HGG-12 for 60 min, then precipitated and washed three times with buffer.

(B) Inhition of [^{3}H]-4NMPB binding to brain stem preparation by oxotremorine.

preparations was much stronger (Fig. 12B); here too the effect was almost exclusively restricted to low concentrations of oxotremorine. These results suggest that the oxime specifically inactivates high affinity muscarinic agonist binding sites, whose proportion is low in the rat brain cortex and high in the rat brain stem. Computer analysis of the competition bidning data confirmed that the affected sites were those of the high affinity class (Table IV).

Table IV: Effect of GppNHp on agonist binding parameters in control and in oxime treated rat brain stem membranes

Control membranes	Proportion of high affinity agonist binding sites (%)	K_H (nM)	K_L (μM)
No effector	57.2 ± 6.8	10.0 ± 3.3	0.1 ± 0.1
+ 100 μM GppNHp	27.9 ± 2.5	12.2 ± 1.6	0.3 ± 0.1
Oxime treated membranes (200 μM HGG-42)			
No effector	60.0 ± 4.2	16.9 ± 3.0	0.5 ± 0.1
+ 100 μM GppNHp	56.0 ± 7.4	18.8 ± 5.1	0.6 ± 0.2
Oxime treated membranes (200 μM HGG-42) in the presence of 2.5 μM carbamylcholine			
No effector	58.0 ± 2.6	18.3 ± 4.6	0.5 ± 0.0
+ 100 μM GppNHp	26.3 ± 6.1	17.7 ± 5.2	0.4 ± 0.0

Binding parameters were calculated according to a two site model (see Methods, Eq. 1), describing binding of oxotremorine to high and low affinity sites. K_H and K_L are the dissociation constants of oxotremorine from the high and low affinity binding sites, respectively.

4. Oxime-dependent and GppNHp-dependent inactivation of high affinity muscarinic agonist binding sites.

High affinity binding sites for agonists in rat brain stem preparations are strongly affected by GppNHp (28), in a manner similar to that described here for HGG-12. However, unlike the oxime, GppNHp does not affect antagonist binding in this preparation. This suggests that two different mechanisms could be responsible for the inactivation. In order to examine this possibility we first studied the effect of GppNHp on oxime-treated membranes. As shown in Fig. 13, treatment with HGG-12 abolished to ability of GppNHp to reduce high

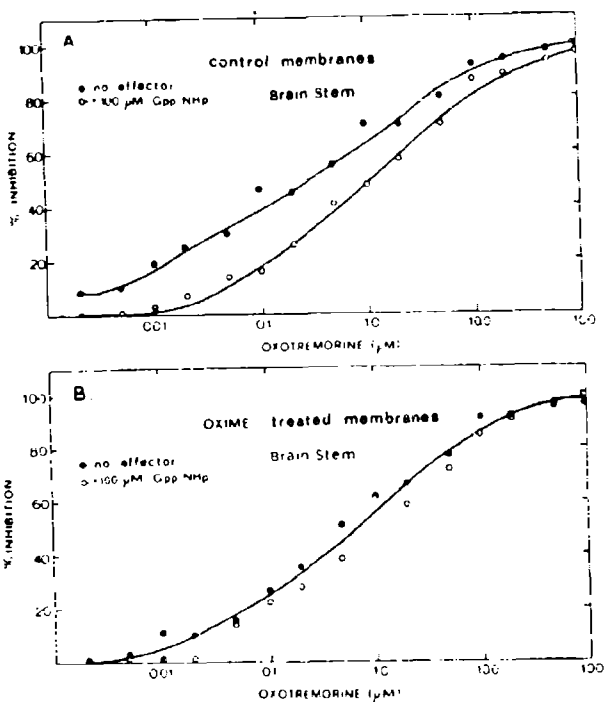

Figure 13: Effect of 100 μM GppNHp on the inhibition curve of [^{3}H] 4NMPB binding to brain stem preparation by oxotremorine. (A) Effect of GppNHp on control membranes; (B) Effect of GppNHp on membranes pretreated with HGG-12.

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affinity oxotremorine binding (in contrast with the GppNHp effect in control membranes). These results indicate that the two drugs indeed inactivate a common population of sites.

Evidence for different bu dependent mechanisms of action for oxime and GppNHp comes from protection experiments using the agonist carbamylcholine. In the brain stem, K_H and K_L for carbamylcholine are 0.14 μM and 30 μM respectively (29). Thus, 2.5 μM of the agonist will block the high affinity sites, leaving the low affinity sites almost unoccupied. Membranes were treated with 200 μM HGG-12 in the presence and in the absence of 2.5 μM carbamylcholine. One hr later, the membranes were washed and used in competition binding studies. The results indicate that the carbamylcholine did not protect the high affinity binding sites from the oxime effect (Fig. 14). It was clear, however, that when carbamylcholine was present during the period of inactivation by HGG-12, it protected those sites normally affected by GppNHp, thus releasing that drug for the alternative activity of decreasing high affinity oxotremorine

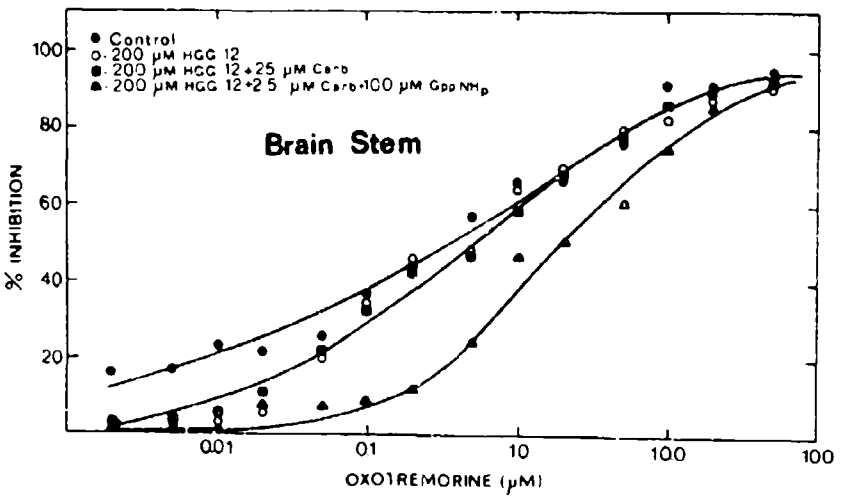

Figure 14: Protection by carbamylcholine of GppNHp effect on brain stem muscarinic receptors against inactivation by HGG-12. Membranes were first treated with 2.5 μM carbamylcholine for 15 min, room temperature, then treated fro 60 min with 200 μM HGG-12 and washed as described above. Binding was performed on membranes treated and untreated with 100 μM GppNHp.

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binding (Fig. 14). Such activity could not be manifested in the absence of carbamylcholine (Fig. 13B). It should be noted that carbamylcholine did not protect oxime-dependent inactivation of antagonist binding sites (See Fig. 7). We therefore suggest that by protecting the sites by carbamylcholine, GppNHp must affect either the residual high affinity sites which were not inactivated by the oxime, or additional high affinity sites derived from low affinity sites that became converted in the presence of carbamylcholine. These possibilities are discussed in the following chapters. It is clear, however, that protection by 2.5 μM carbamylcholine induces an additive inactivation of the high affinity muscarinic binding sites by oxime and GppNHp.

5. High affinity agonist binding sites in rat brain cortex membranes are affected by oximes

In our previous report we have demonstrated that brain cortex membranes lose only about 10% of their muscarinic receptors after pretreatment with 100 μM HGG-42. A small effect was also observed on the binding of the agonist

oxotremorine. As in the brain stem preparation, the oxime affected mainly the high affinity agonist binding sites. We have performed analogous experiments where the agonist-binding properties were measured employing a second drug, carbamylcholine. Membranes were incubated with 200 μM HGG-42 for 1 hr at 25°C, washed three time with buffer and taken for the binding studies. As shown in Fig. 15, no effect was observed on the binding of carbamylcholine or acetylcholine. These results, as compared to those observed with oxotremorine (Fig. 12), gave us the first indication that in the cortex (as in the brain stem) oximes affect a component which is directly related to a conformation of the agonist-occupied receptor. We have noticed in the past that various agonists exert different effects on the state of the cortical muscarinic receptors, as

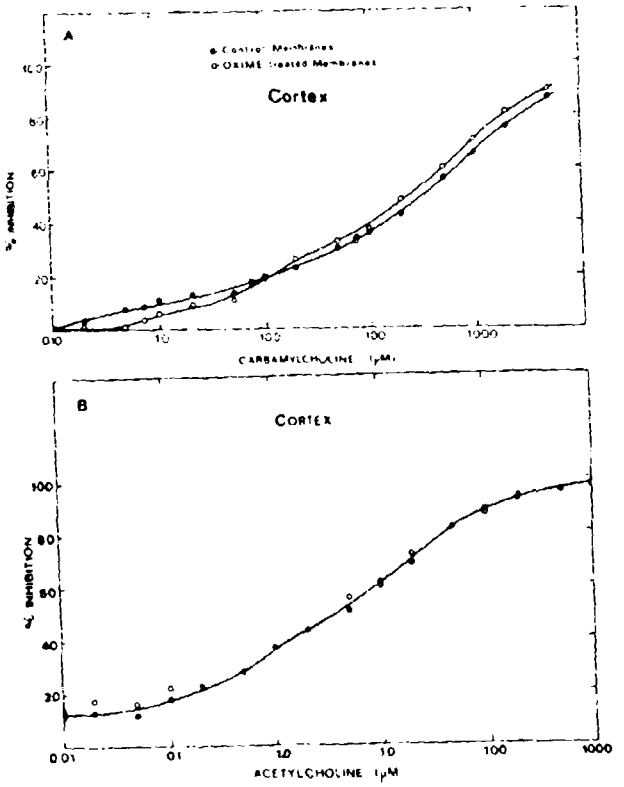


Figure 15: Effect of oxime treatment on agonist binding to rat brain cortex muscarinic receptors.
Membranes were preincubated with 200 μM HGG-12 for 1 hr, precipitated and washed three times with buffer and used in competition binding experiments with 2.0 nM [^{3}H]-4NMPB and carbamylcholine (A) or acetylcholine (B). Data presented are % inhibition of [^{3}H]-4NMPB binding in control (●) and oxime treated (o) membranes as a function of agonist concentration.

shown by the different proportions of high to low affinity sites revealed in their binding patterns. For example, oxotremorine binds to about 15-20% high affinity binding sites, whereas the percentage of high affinity sites increases to 25-30% for carbamylcholine and to 30-40% for ACh. The effect of oximes on cortical muscarinic receptors is thus inversely correlated with the agonist state of the receptor; the less agonist high affinity binding sites are manifested, the stronger is the apparently observed effect of oxime (oxotremorine vs carbamylcholine) (Fig. 15 and 12).

The nature of the oxime effect on agonist binding was further studied by direct binding studies using [^{3}H]-ACh. We have recently characterised the binding of [^{3}H]-ACh to high affinity muscarinic receptors in rat brain cortex membranes (20). Using the same procedure (see Methods), we examined the binding of [^{3}H]-ACh to brain cortex muscarinic receptors in membranes pretreated with 200 μM HGG-42. As shown in Fig. 16, binding of [^{3}H]-ACh was strongly affected. Apparently, more than 70% of the high affinity binding sites were affected as judged by

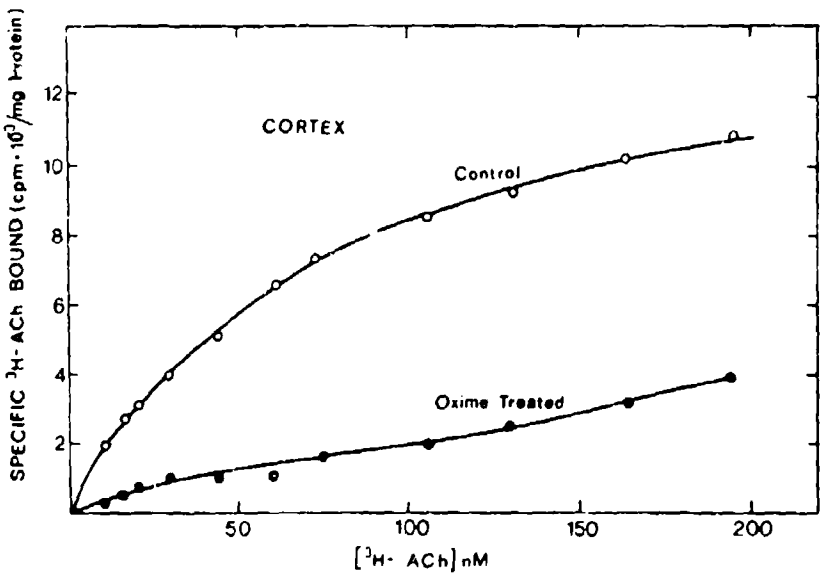


Figure 16: Specific [^{3}H]-ACh binding (CPM/mg protein) to rat cerebral cortex membranes with and without pretreatment of 200 μM HGG-42. Membranes were treated with 200 μM HGG-42 and washed as described in Methods, and subsequently washed twice in 50 mM Tris-HCl (pH = 7.4). The pellets were suspended in Krebs buffer containing 200 μM DFP (see Methods) and assayed for specific [^{3}H]-ACh binding to control (o) and oxime treated (•) membranes, using rapid filtration technique (see Methods). Non-specific binding (not shown) was measured using 10 μM atropine and was the same for control and oxime treated membranes.
Note: Scatchard analysis for control membranes yielded a straight line with $Kd_{[^3H]\text{-ACh}}$ = 83 nM; for oxime treated membranmes. no straight line could be drawn.

the reduction in [^{3}H]-ACh binding (Fig. 16). It should be noted that in the competition binding experiments, using the antagonist [^{3}H]-4NMPB various concentrations of carbamylcholine or ACh, we did not observe this effect. The simplest explanation for this apparent discrepancy would be that the oxime induced a change in the dissociation rate of [^{3}H]-ACh - receptor complexes, which is not apparent in the [^{3}H]-antagonist/agonist competition experiments. If both association and dissociation rates of [^{3}H]-ACh to and from the receptor were enhanced by the oxime, it is plausible that [^{3}H]-ACh - receptor complexes would dissociate during the rapid filtration (used to separate bound from free lidand). We examined therefore binding of [^{3}H]-ACh to oxime treated membranes using a centrifigation method, in which dissociation of bound ligand is eliminated. As shown in Table V, under these conditions the oxime effect on [^{3}H]-ACh binding was less pronounced, supporting our hypothesis. These results are preliminary and more detailed studies should be undertaken before a final conclusion can be drawn. At any rate, the results indicate that most of the high affinity agonist binding sites are affected allosterically by the oxime; under the conditions used about 90% of the muscarinic recognition sites were free to bind ligand, yet more than 70% of the high affinity agonist binding sites were affected.

As pointed out earlier, the agonist state of the cortical muscarinic receptor can be manipulated in vitro by transition metal ions (20, 30). We have therefore studied the effect of oximes on the Co^{2+}-induced conversion of low to high affinity agonist binding sites.

Table V: Specific binding of 50 nM [^{3}H]-ACh to control and oxime treated cerebral cortex membranes measured by filtration and centrifugation techniques

	Specific [^{3}H]-ACH bound (cpm)	
	Filtration	Centrifugation
Control membranes	459 ± 172	490 ± 282
Oxime treated membranes (200 μM HGG-42)	270 ± 160	492 ± 170

Membranes were treated with the oxime as described in Methods. Control membranes received the same incubations, precipitations and washing treatments in the absence of oxime. Washed membranes were suspended in Krebs buffer containing 200 μM DFP and assayed for specific [^{3}H]-ACh binding using 10 μM atropine for non-specific binding (see Methods). Filtration and centrifugation assays were carried out simultaneously with the same preparations, using the same assay volume (40 μl) and incubation (1 hr, 25°C).
Note: S.D. is higher in centrifugation assays.

6. Co^{2+}-induced conversion of low affinity cortical muscarinic receptors to their high affinity state is inactivated by oximes

In the following experiments we have pretreated brain cortex membranes with 200 μM HGG-12 as described before. These membranes were employed in competition binding studies performed in the presence and absence of 2 mM Co^{2+}. As shown in Fig. 17A, the incubation of control membranes with Co^{2+} yielded receptors which exert higher affinity towards carbamylcholine, due to conversion of low to high affinity agonist binding sites (20, 30 and Table VI). In oxime-treated membranes, however, Co^{2+} could no longer induce its effect (Fig. 17B, Table VI), although the apparent affinity of carbamylcholine was not changed (Fig. 17A). Thus the component through which Co^{2+} acts was affected by the oxime.

It is therefore possible that the muscarinic recognition site is associated with another site (the Co^{2+} site), and the two affect each other. As a positive effector, Co^{2+} converts low to high affinity agonist binding sites, while the oxime inactivates the Co^{2+} site or the interaction between this site and the muscarinic recognition site. As in a typical allosteric system, the effect of the oxime on its sites would be manifested on the second site and vice versa. Namely, muscarinic agonists should affect the oxime site as well.

Table VI: Effect of Co^{2+} and GppNHp on agonist binding parameters in control and oxime treated rat brain cortex membranes

Control membranes	Proportion of high affinity agonist binding sites (%)	K_H (μM)	K_L (μM)
No effector	26	0.59	145
+ 2 mM Co^{2+}	59	1.1	310
+ 2 mM Co^{2+} + 100 μM Gpp NHp	28	1.05	132
Oxime treated membranes (200 μM HGG-12)			
No effector	29	0.86	105
+ 2 mM Co^{2+}	29	1.1	147
+ 2 mM Co^{2+} + 100 μM GppNHp	27	0.64	85
Oxime treated membranes (200 μM HGG-12) in the presence of 25 μM Carb			
No effector	27	0.68	131
+ 2 mM Co^{2+}	47	0.59	137
+ 2 mM Co^{2+} + 100 μM GppNHp	31	0.77	90

Binding parameters were calculated according to a two site model describing binding of carbamylcholine to high and low affinity binding sites. K_H and K_L are the dissociation constants of carbamylcholine (Carb) from the high and low affinity binding sites respectively.

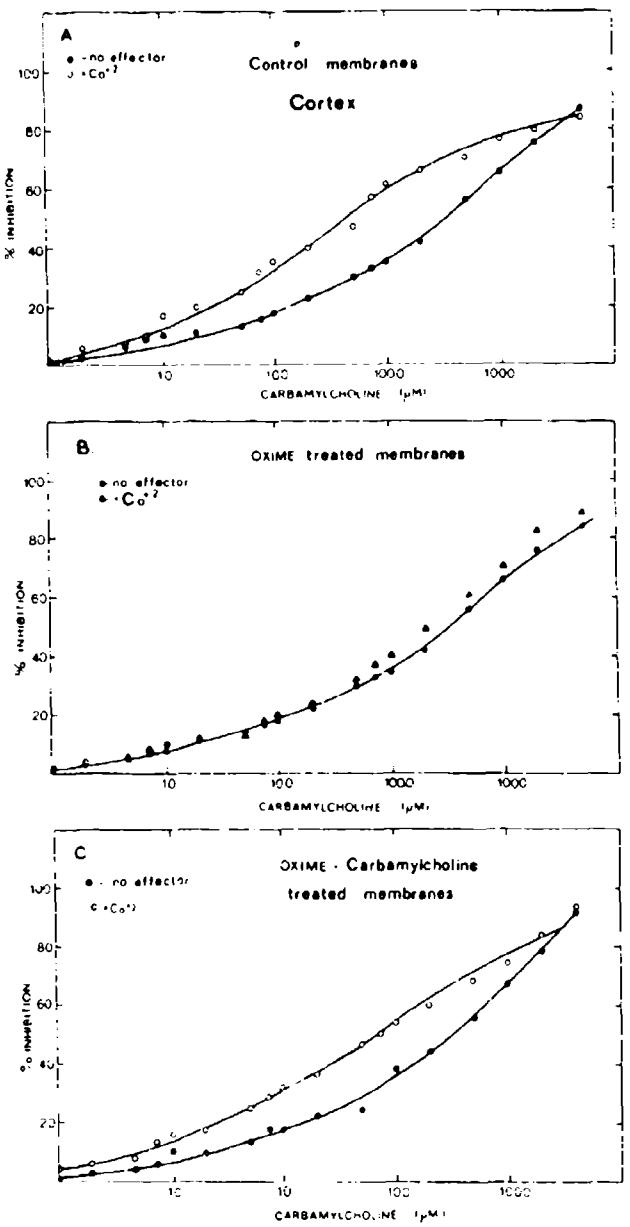


Figure 17: Effect of oxime treatment on Co^{2+} induced conversion of low to high affinity agonist binding sites.

Membranes were treated with 200 μM HGG-12 as described in Fig. 15.

A. Effect of 2 mM Co^{2+} in control membranes.

B. Effect of 2 mM Co^{2+} in oxime treated membranes (▲ + Co, ● - Co). For comparison Co^{2+} effect on control membranes is also given (o).

C. Protection of the Co^{2+} effect by 25 μM carbamylcholine against HGG-12. Membranes were incubated in the presence of 200 μM HGG-12 and 25 μM carbamylcholine, then washed as described in Fig. 1 and used for competition binding studies with 2.0 mM [^{3}H]-4NMPB and carbamylcholine (● - Co^{2+}, o + Co^{2+}).

The inverse relationship between the effects of oxime and the agonist state of the muscarinic receptor can be explained by these allosteric terms: Carbamylcholine induced more high affinity sites than oxotremorine upon binding to the receptor, thus producing a stronger allosteric effect on the sites occupied by oxime. We have therefore examined whether indeed carbamylcholine can affect the oxime activity.

7. Protection of Co^{2+}-induced conversion of low to high affinity agonist sites from oxime inactivation by carbamylcholine

In the experiment described here we have examined whether the presence of carbamylcholine during exposure of membranes to oxime can protect the muscarinic sites from the oxime. Results of these experiments are summarized in Fig. 17A-C and Table VI. Fig. 17C demonstrates that when membranes were treated with 200 μM HGG-12 in the presence of 25 μM carb, the Co^{2+} effect was very similar to that observed in control membranes (Fig. 17A), indicating that occupation of the muscarinic sites by the agonist protected the Co^{2+} sites from incactivation by the oxime. The effective concentration of carbamylcholine was very low (< 2.5 μM). Therefore, it is clear that occupation of the high affinity agonist binding sites was sufficient to protect the rest of the sites (low affinity sites) from the oxime. These sites can now convert to the high affinity state through the binding of Co^{2+}. These results show that indeed carbamylcholine affects allosterically the oxime sites. It is important to note that the effects of oximes on GppNHp-induced conversion of high to low affinity sites could not be tested in the cortical membranes under the conditions employed. This is because in these membranes GppNHp acts only on Co^{2+}-induced high affinity agonist sites (Fig. 18A). Moreover, we have shown that this conversion to high affinity state is inactivated by the oxime. Indeed, oxime treated membranes lost their ability to respond to Co^{2+} or to Co^{2+} + GppNHp in terms of carbamylcholine binding (Fig. 18B).

8. GppNHp effect on Co^{2+}-induced high affinity agonist muscarinic binding sites in cortical membranes is blocked by oximes

Rat brain cortex low affinity agonist binding sites are converted to their high affinity state in the presence of 2 mM Co^{2+} (20, 30 and Fig. 17B). This effect of Co^{2+} is reversed if 100 μM GppNHp is present in the incubation media (20, 30, Fig. 18A). In an additional set of experiments, we have pretreated cortical membranes (30 min at room temperature) with 2 mM Co^{2+} in order to induce conversion of low to high affinity binding sites without the interference of oximes. The membranes were than treated with 200 μM HGG-12 for 1 hr at room temperature, precipitated and washed with buffer (containing 2 mM Co^{2+}) as described before. These membranes and Co^{2+}-treated membranes which were not treated with oxime (control) were used in competition binding studies ([^{3}H]-4NMPB/carbamylcholine), in the absence and presence of 100 μM GppNHp, Co^{2+} (2.0 mM) was included in all incubation media. The results of these experiments are summarized in Table VII. As expected,

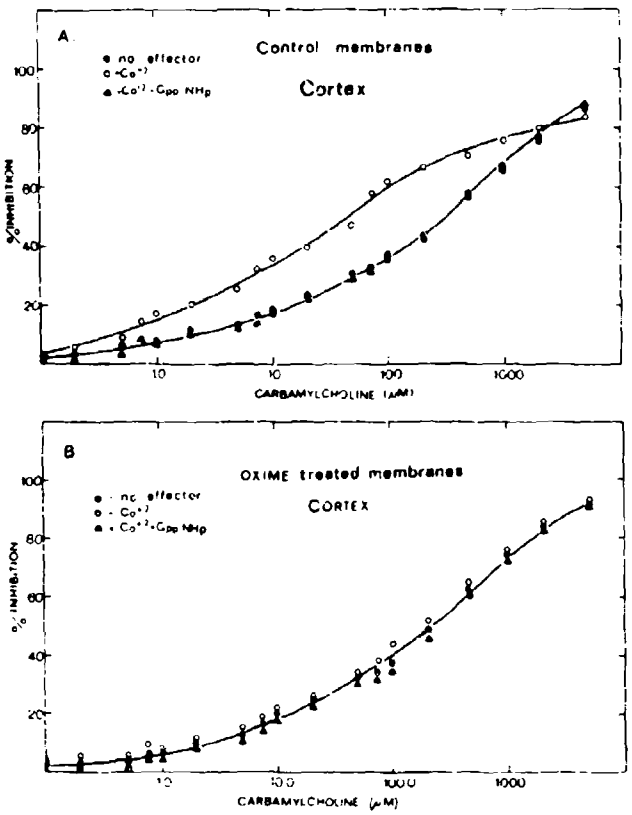


Figure 18: Effect of Co^{2+} and GppNHp on control and oxime treated membranes.

Membranes were treated with 200 μM HGG-12 as described in Fig. 15, and then incubated with 2.0 nM [^{3}H]-4NMPB and various concentrations of carbamylcholine in the presence (o) and absence (●) of 2.0 mM Co^{2+} or 2 mM Co^{2+} + 100 μM GppNHp (▲). A. Control membranes. B. Oxime treated membranes.

Table VII: GppNHp effect on Ca^{2+}-induced high affinity agonist sites in cortical membranes

	Proportion of high affinity agonist binding sites (%)	K_H (μM)	K_L (μM)
No effector (control)	38.6	3.4	228
+ 100 μM GppNHp (control)	14.1	4.2	141
No effector, HGG-12 treated	38.3	1.4	157
+ 100 μM GppNHP, HGG-12 treated	31.9	5.0	210

Membranes were preincubated with 2 mM Co^{2+}.
Binding parameters were evaluated as in Table VI.

pretreatment with Co^{2+} induced an increase in the proportion of high affinity agonist binding sites (from 28% to 38%). Treatment with oxime did not reconvert the 38% high affinity binding sites induced by Co^{2+}. GppNHp, as expected, reconverted the Co^{2+}-induced high affinity sites (the percentage of the high affinity sites decreased from 38% to 14%). In the oxime treated membranes the GppNHp was almost completely blocked (reconversion was only from 38% to 31%). These results indicate that in control membranes (as in brain stem membranes) oximes block the GppNHp induced conversion of high to low affinity binding sites.

9. In vivo inactivation of muscarinic binding sites by HGG-12 is reversed

From our in vitro binding studies we could conclude that oximes affect the muscarinic receptors by an allosteric and probably an irreversible mechanism. We therefore decided to test whether similar effects are exerted by the drugs when administered to the animals. For these experiments, we injected s.c. various doses of HGG-12 to rats. All of the injection doses were much lower than LD_{50} doses (the conditions used did not cause any death of the rats). At various times after the injection the rats were killed, and their brain cortex and brain stem were homogenized. The resulting membranes were washed three times with buffer as in our in vitro experiments, and than used in the binding studies. Results of these experiments are shown in Fig. 19 and 20.

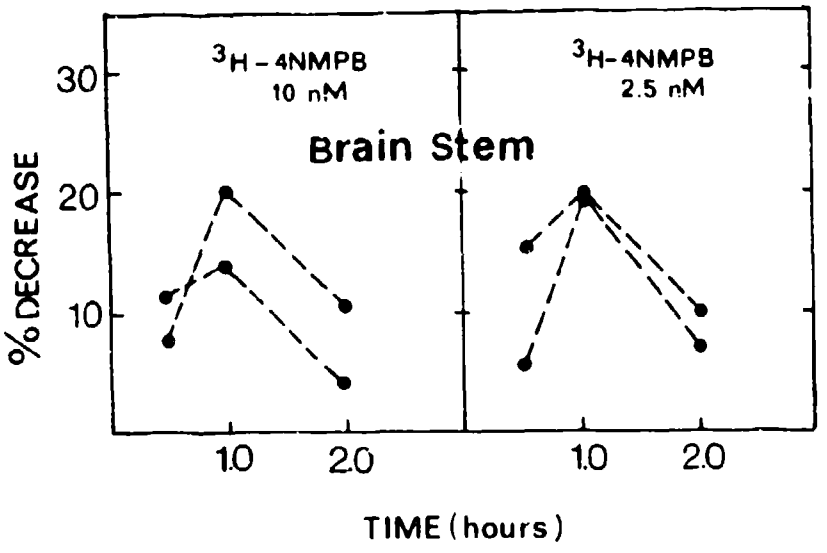


Figure 19: Time course of the effect of HGG-12 on brain stem muscarinic receptors after s.c. injection of the drug.
Rats received s.c. injection of 10 mg/kg HGG-12 and were sacrificed at the indicated times. The brain stem was removed, homogenized and the membrane washed three times with buffer. Membranes were than tested for their binding capacity with 2.5 mM and 10.0 mM [^{3}H]-4NMPB.

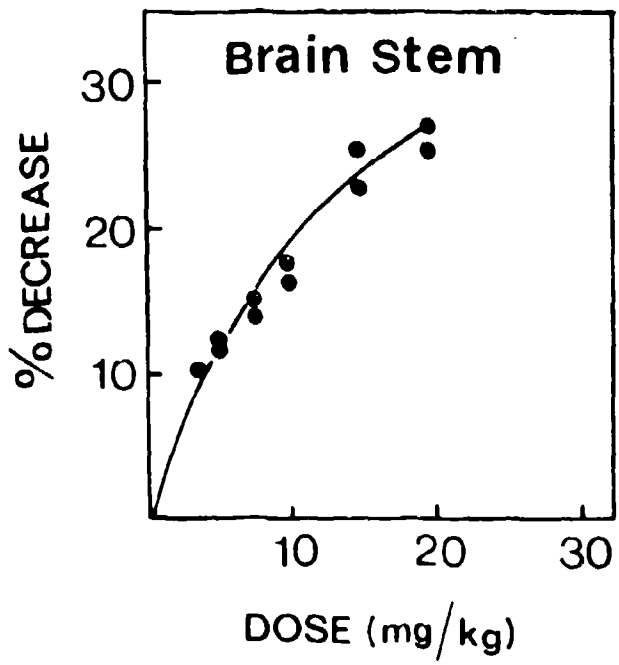


Figure 20: Dose dependence of oxime-induced decrease in brain stem muscarinic receptors following s.c. injection of the drug.
Rats received s.c. injection of the indicated dose of HGG-12 and were sacrificed after 1 hr. Muscarinic receptors in the brain stem were determined with 10 nM [^{3}H]-4NMPB in membranes prepared as described in Methods.

As shown in Fig. 19, injection of 10 mg/kg HGG-12 induced a time-dependent loss of muscarinic binding sites in the brain stem. This effect was observed already after 30 min, reached a peak value after 1 hr, and then declined during the next hour. These results indicate that in the intact animal (as under in vitro condition) oximes inactivate muscarinic receptors. Unlike the in vitro system, in the intact animal there are mechanisms by which the damage is repaired. Under the conditions used, we did not observe reduction in cortical muscarinic receptors - a finding which is in accord with the phenomenon observed in vitro. In the brain stem, however, a dose-dependent loss of receptors was observed (Fig. 20).

DISCUSSION

Oximes possess a mild antimuscarinic activity: They inhibit the acetylcholine-induced contraction of guinea pig ileum (11, 13) as well as the binding of the potent and specific muscarinic antagonist [^{3}H]-4NMPB (13). In both cases, their activities can not be described simply by competitive blockade of the muscarinic receptor recognition site. Several characteristics of the interactions of oximes with the muscarinic receptor (both in vitro and in vivo) justify the notion that these drugs have an allosteric effect on the receptor:

1) At low oxime concentration (< 50 μM), the binding of oximes and [^{3}H]-4NMPB to the muscarinic receptors is mutually exclusive. In spite of this, at this concentration range, oximes reduce the dissociation rate of [^{3}H]-4NMPB-receptor complexes. The latter observation is typical for heterotrophic allosteric effect.

2) At higher oxime concentrations (> 50 μM), the oximes simply inactivate [^{3}H]-4NMPB recognition sites on the receptor. This effect appears as a reduction in binding capacity either when [^{3}H]-4NMPB binding is measured in the presence of oxime or following removal of the drug from the incubation medium. The strongest inactivation occurs in rat brain stem preparations (up to 30%), but a slight reduction in the number of sites (∿ 10%) was observed also in rat brain cortex preparation.

3) The oxime specifically inactivates those receptors which exhibit a high affinity state towards muscarinic agonist. This is shown by: (a) weak inactivation of cortical receptors, which are present mostly in the low affinity state, and strong inactivation of brain stem receptors, most of which are in the high affinity state and (b) the marked reduction in high affinity oxotremorine binding sites observed in the brain stem after oxime inactivation.

4) Concomitantly with the loss of brain stem muscarinic receptors, agonist binding is also affected, and the allosteric effector GppNHp can no longer affect agonist binding. This effector is known to convert high affinity brain stem muscarinic receptors to the low affinity state, but has no effect on the binding of antagonists. The muscarinic agonist carbamylcholine, at a concentration range which occupies mostly high affinity agonist binding sites, could protect the GppNHp effect from inactivation by the oximes, but could not prevent the reduction in the number of binding sites.

5) Rat brain cortex muscarinic agonist binding sites are affected by oximes: (a) conversion of low to high affinity agonist binding sites induced by transition metal ions in control membranes is inactivated by pretreatment with oximes; (b) high affinity binding sites for the agonist oxotremorine are reduced after oxime treatment, a phenomenon which was not apprent with the agonists carbamylcholine and acetylcholine; (c) high

affinity binding of [^{3}H]-ACh, measured in direct binding studies, is strongly affected by oxime treatment, even though ~ 90% of the total muscarinic recognition sites remain free for [^{3}H]-antagonist binding.

6) Occupation of high affinity cortical muscarinic receptors by agonist during preincubation with oximes, protected the receptors from inactivation of the Co^{2+} effect by oxime. Namely, the high affinity agonist binding sites occupied by agonist protected the low affinity agonist binding sites which can now interconvert in spite of the oxime treatment.

7) Cortical muscarinic receptors that have been converted by Co^{2+} to their high affinity agonist state were not affected by oxime treatment. Namely, the proportion of high affinity agonist binding sites was higher in Co^{2+} pre-treated membranes compared with non-treated membranes, and remained so even after treatment with oxime.

8) The Co^{2+} -induced high affinity agonist binding sites in cortical membranes were interconverted to their low affinity state in the presence of GppNHp. Treatment of the Co^{2+}-treated membranes with oxime blocked the GppNHp effect. This oximes can block GppNHp-induced conversion of high to low affinity agonist bidning sites in either case: (a) Pre-existing high affinity agonist binding sites (brain stem); (b) Co^{2+}-induced high affinity agonist binding sites (brain cortex).

9) Inactivation of brain stem muscarinic antagonist binding sites by HGC-12 occurs in untreated rats as it does in vitro. However, the effect in vivo is reversed by endogenous mechanisms which do not operate in vitro under the conditions used.

The results summarized support the notion that oximes interact with a component associated with the polypeptide which possesses the muscarinic recognition site. We can not exclude as yet a possible direct interaction of oximes with the recognition site itself. Even if they do, the interaction of oximes with a neighboring site seems to be the more important one when considering the therapeutic effects of oximes after organophosphorous intoxication. Also, it seems that this group of drugs is a unique tool for studies on structure and function of the muscarinic receptor and possibly of other receptors as well. These two issues (therapy and receptor structure) will be discussed separately.

Predicted structure of the muscarinic receptor

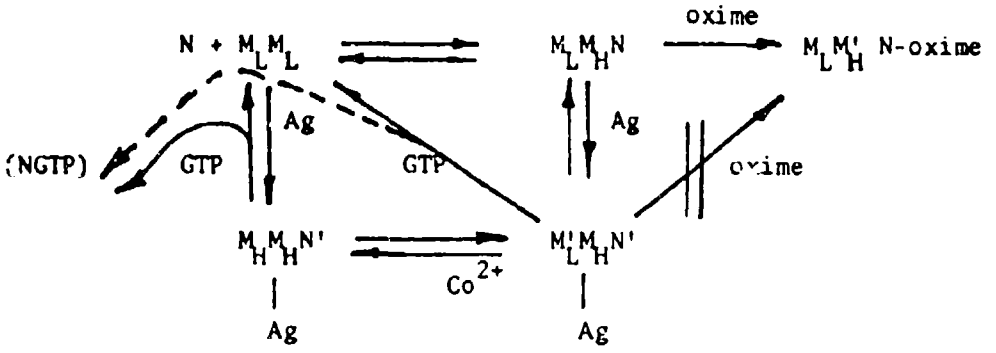
Muscarinic receptors in most tissues exist in apparent multiple forms as judged by the different affinity states towards agonists (25-27). It was suggested by several groups that these phenomena are related to different states of the same muscarinic receptor recognition site (25-27). Evidence in support of this hypothesis came mainly from studies on the effects of GTP (or GppNHp) (27, 28), and transition metal ions (20, 30), which modulate agonist binding properties in vitro. Given that GTP converts high to low affinity binding sites, and transition metal ions convert low to high affinity binding sites, the following model is proposed: GTP modulates a nucleotide

binding protein which is coupled to the muscarinic recognition sites. The coupled complex is manifested as high affinity agonist binding sites, whereas the uncoupled receptor is manifested as low affinity binding sites (31). Transition metal ions bind (or activate enzymatically) either the nucleotide binding protein or the low affinity binding sites, thereby enabling formation of the coupled complex (reassociation of low affinity binding sites with nucleotide binding protein). Molecular data on the postulated muscarinic recognition site - nucleotide binding protein interactions are not yet available. Nevertheless, if such interactions indeed exist, then allosteric interactions between those sites should be apprent. Such itneractions, well known in many enzyme and receptor systems (32), are a strong indication for the existence of multi-subunit structures. Heterotrophic allosteric interactions can be demonstrated with the appropriate allosteric effectors, i.e., aminated local anesthetics and phencyclidine for the nicotinic receptor. Thus, the effects of heterotrophic allosteric effectors may help to elucidate the receptor structure. Our data indicate that oximes inactivate both the process of conversion from low to high affinity binding sites and the process of conversion of high to low affinity sites. This strongly suggests that there is a common component involved in the two processes. Moreover, in either case, occupation of high affinity agonist binding sites by agonist protected this component against inactivation by oximes. This indicates that binding of agonist to muscarinic recognition site induces a conformational change, which propagates to the sites of oxime action. As expected in such site-site interactions, occupation of the oxime sites induces changes in the muscarinic recognition sites, as evidenced by changes in the mode of antagonist and agonist binding. Since GTP (or GppNHp) binds to a nucleotide binding protein and affects high affinity muscarinic binding sites, it is assumed that the high affinity sites are coupled to this protein. The effect of GTP could be described by the following simple model (22):

$$M_H N + Ag \rightleftharpoons Ag\text{-}M_H N \xrightleftharpoons{GTP} M_L + N\text{-}GTP + Ag$$

Scheme II

Where M_H, M_L are the high and low affinity binding sites and N is the nucleotide binding protein. Ag is the agonist. The formation of $M_H N$ would follow the scheme $M_L + N \rightarrow M_H N$. In vitro this postulated process is forced by transition metal ions. The data presented on the interaction of oximes with this system indicate that the scheme described above is too simple: The scheme should include the agonist-induced conformational change which enables either GTP or Co^{2+} to interact with their site, as we now recognize via the interactions of oximes. Moreover, the fact that occupation of high affinity binding sites by agonists protects the low affinity binding sites, strongly suggests that the two sites are associated. This should also be considered in a model describing muscarinic receptor - ligand interactions. We propose the following plausible model:



Scheme III

As in the former model (Scheme II), the nucleotide binding protein (N) is coupled to the high affinity binding sites (M_H). Upon binding the GTP, they dissociate to form the low affinity binding sites. Unlike the case in the former model (Scheme II), we assume here: (1) association of low and high affinity binding sites; (2) agonist-induced conformational changes, which propagate to the N and M_L subunits and induce formation of $M'_L M_H AgN'$. Combining these two assumptions, we can now accommodate all the ligand - muscarinic receptor interactions described here. In the absence of cholinergic ligands the receptor is found in two pre-existing forms: $M_L M_L$ and $M_L M_H N$. Binding of agonist to the high affinity sites induces a conformational change, yielding the $M'_L M_H AgN'$ conformer. Binding of GTP to this conformer releases the N-GTP concomitantly with the formation of $M_L M_L$. Transition metal ions lead to the formation of $M_H M_H AgN'$ conformer, which is also suceptible to GTP action. The oxime can act only on the $M_L M_H N$ conformer. Thus, pretreatment with oxime in the absence of agonist would lead to an $M_L M'_H$-oxime complex, thereby yielding: (1) A change in high affinity agonists binding sites; (2) inactivation of N. Pretreatment with oxime in the presence of low agonist concentrations (occupying mainly high affinity sites) will protect N and M_L, since the agonist induces a conformer ($M'_L M_H AgN'$) to which the oxime can not bind.

Although tentative, the model described in Scheme III should be helpful for further characterization of the muscarinic receptors.

Therapeutic aspects related to interactions of oximes with the muscarinic receptor

The results described on the interactions of oximes with the muscarinic receptors may have some applications for the effects of the drugs on live rats or humans. We have shown that administration of HGG-12 to rats yields a reversible decrease in muscarinic binding sites in the brain stem, as was

observed in vitro, though the latter was irreversible. We have not yet studied in vivo the effects of the oxime on agonist binding properties or on their modulation by GppNHp and Co^{2+}. Nevertheless, the in vitro results suggest strong effects of oximes on muscarinic receptors, that may occur in vivo as well. Two interesting points should be mentioned: (1) the effect on the rates of agonist and antagonist binding; (2) the plausible effect on the N protein.

In the presence of oximes the dissociation rate of antagonist - muscarinic receptor complexes is decreased (1). An inverse effect is observed on the dissociation rate of acetylcholine-muscarinic receptor complexes, which is increased. Therefore, it is expected that administration of the combination oxime/antagonist (HS-6/benactyzine) would lead to a favorable situation, in which receptors are primarily occupied by antagonists. This would occur even if high acetylcholine concentrations are present due to organophosphate poisoning. If the model for the interaction of muscarinic receptors with oximes applies also in vivo, a completely different situation would be expected when oximes are injected prior to organophosphate poisoning. In such a case the oxime is capable of interacting with the M_LM_HN species, thereby inactivating the N. Preoccupation of the M_LM_HN conformer by acetylcholine (after poisoning would yield an $M_LN_H(Ag)N'$ which is not affected by the oxime. Large doses of antagonist should free the binding sites from acetylcholine and possibly enable the oxime to attack N. Since it is expected that the antagonist-oxime mixture would be administered normally after organophosphate poisoning, the fate of the activated N (due to formation of $M_LM_H(Ag)N'$ should be considered. We do not know whether oximes can interact with free N and what the relative quantities are of N and the muscarinic receptor. We do know, however, that in heart cells the negative ionotropic effects of muscarinic agonists are at least in part due to a GTP and Na^+-dependent inhibition of adenylate cyclase activity (both basal and β-adrenergic-stimulated (33). The effects of oximes on the muscarinic receptor-nucleotide binding proteins in conjugation with effects on the β-adrenergic receptor - adenylate cyclase system in brain stem should give important information on the activity of these drugs.

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