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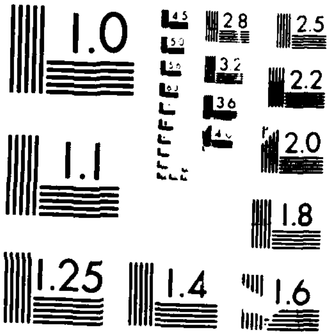

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THE MECHANISM OF INTERACTION OF OXIMES WITH THE MUSCARINIC CHOLINERGIC COMPLEX IN THE CENTRAL NERVOUS SYSTEM

Annual/Final Report

Mordechai Sokolovsky

31 March 1985

Supported by

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The present work was aimed at examining the significance of the oxime moiety and other substituents on the pyridinium rings of bisquaternary pyridinium analogs for the interactions of these drugs with rat brain muscarinic receptors.

The bisquaternary pyridinium oximes allosterically inhibited binding of the muscarinic antagonist [^{3}H]-N-methyl-4-piperidyl benzilate ([^{3}H]-4NMPB) to rat brainstem homogenates. The inhibition was reversible. The apparent inhibition constants obtained were close to those determined for binding of the oximes to postsynaptic muscarinic receptors in the guinea pig ileum, where these drugs block the activity of acetylcholine. The most potent inhibitors were 1-(2-hydroxyiminoethylpyridinium) 1-(3-cyclohexylcarboxypyridinium) dimethylether (HGG-42) and its 3-phenylcarboxypyridinium analog (HGG-12) ($K_I \approx 2$ μM). The analogs HGG-52 and the tert-butyl analog of Toxogonin, SAD-128, were less potent inhibitors (K_I 7-15 μM), and the least potent drugs were toxogonin, TMB-4 and HI-6 ($K_I > 40$ μM).

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19. Abstract (continued)

In addition to their allosteric inhibitory effect, which was reversible, the HGG pyridinium oximes were found to induce irreversible loss of 30% of brainstem muscarinic receptors and loss of sensitivity of these receptors to guanine nucleotides. Such an effect was also observed with HI-6 but not with SAD-128, Toxogonin or TMB-4. It follows that the above irreversible effects appear to be due to the presence of an oxime moiety at position 2 of the pyridinium ring.

Bisquaternary pyridinium oximes were found to inhibit the K^+-evoked release of $[^3H]$-acetylcholine from brain slices in a dose-dependent manner. This effect was blocked by atropine. Both the extent of inhibition of $[^3H]$-acetylcholine release (40-60%) and the time course of the inhibitory response were similar to those observed with the muscarinic agonist oxotremorine. It thus appears that at presynaptic muscarinic receptors the bisquaternary oximes are cholinomimetic, in contrast to their antagonistic behavior at postsynaptic receptors. The analog TMB-4 was the most potent inhibitor of $[^3H]$-acetylcholine release (IC_{50} = 8 μM), whereas the HGG oximes were the least potent ($IC_{50} \approx$ 100 μM). Toxogonin and HI-6 were equipotent ($IC_{50} \approx$ 50 μM).

Taken together, our results indicate that TMB-4 could serve as a selective presynaptic cholinomimetic that would act synergistically with acetylcholine to block acetylcholine release. At the same time, the HGG oximes are postsynaptic allosteric antagonists, capable of increasing the ratio of antagonist to agonist binding and consequently of enhancing the blockade of muscarinic postsynaptic receptors by antagonist. It thus seems likely that a mixture of TMB-4, HGG-12 and a muscarinic antagonist would be therapeutically effective in cases of organophosphate poisoning.

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SUMMARY

An understanding of the mechanisms by which the bisquaternary pyridinium oximes affect brain muscarinic receptors could lead to the improved design of mixtures of oximes and muscarinic antagonists for pharmaceutical protection against organophosphate poisoning. Accordingly, in the present study we explored the role of functional substituents on the pyridinium rings of these oximes in their interactions with brainstem muscarinic receptors. More specifically, we aimed to assess the significance of the oxime moiety and hydrophobic substituents for the interactions of bisquaternary pyridinium analogs with post- and presynaptic muscarinic receptors.

Seven bisquaternary pyridinium analogs were studied (see Scheme I). In all of them, one pyridinium ring contained an oxime moiety at position 2 or 4 and the second ring contained a substituent at position 3 or 4. The modes of inter action of these drugs with muscarinic receptors were studied by methods based on measurement of binding between the receptor and the radiolabeled muscarinic antagonist [^{3}H]-N-methyl-4-piperidyl benzilate ([^{3}H]-4NMPB) (1, 2) and of potassium-evoked release of [^{3}H]-acetylcholine from brain slices.

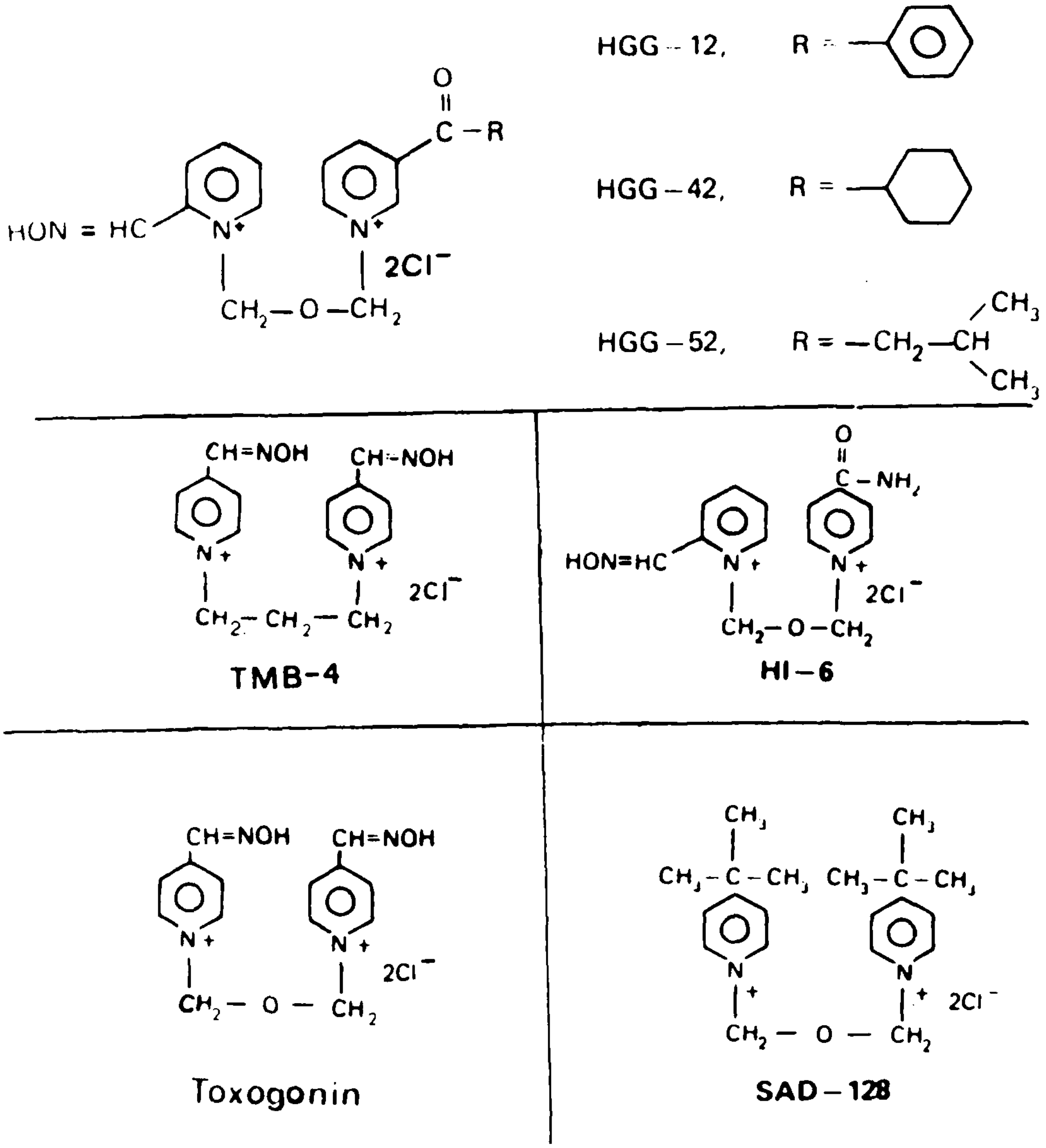
All of the bisquaternary drugs studied allostericlly inhibited the binding of [^{3}H]-4NMPB to muscarinic receptors in the brainstem. The effect was reversible. The inhibitors HGG-12, HGG-52, HGG-42 (Ki = 2-15 μM) and SAD-128 (Ki= 6 μM) were more potent than Toxogonin, TMB-4 and HI-6 (K_I > 40 μM), indicating that a hydrophobic moiety at position 3 or 4 of the pyridinium ring increases the apparent affinity of the drug toward the muscarinic receptor. The apparent affinity constants of the bisquaternary pyridinium analogs determined in rat brainstem homogenates are of the same order as those determined previously (3-6) by measuring the antagonism to acetylcholine-induced contraction in the guinea pig ileum. We concluded that the most potent inhibitors (i.e., HGG-12, HGG-42, HGG-52 and SAD-128) are likely to reversibly inhibit postsynaptic muscarinic receptors in the brainstem.

Two other properties of some bisquaternary pyridinium oximes are evident from their interactions with brainstem muscarinic receptors: a) they induce an irreversible loss of ~30% of muscarinic binding sites: b) they induce loss of sensitivity of the muscarinic receptors to guanine nucleotides. Our results indicate that these effects are induced only by drugs which contain an oxime moiety at position 2 of the pyridinium ring (e.g., HGG-12, HGG-42 HI-6). The analogs which contain an oxime moiety at position 4 (Toxogonin and TMB-4), or possess no oxime moiety (SAD-128) could not induce loss of receptors or loss of sensitivity to guanyl nucleotides.

The bisquaternary pyridinium oximes were found to inhibit K^+-evoked release of [^{3}H]-acetylcholine from rat brain slices in a dose-dependent and atropine-sensitive manner. Inhibition by oximes of [^{3}H]-acetylcholine release was similar to that induced by the muscarinic agonist oxotremorine. These results indicate that the oximes studied here act as cholinomimetics at presynaptic autoreceptors,

since at these receptors muscarinic agonists are known to inhibit release of acetylcholine (7, 8). The most potent inhibitor of [^{3}H]-acetylcholine release was TMB-4 (IC_{50} = 8 μM). Toxogonin and HI-6 were less potent (IC_{50} = 50 μM), and the HGG pyridinium oximes were the least potent inhibitors (IC_{50} = 100 μM).

A comparison between the potencies of the bisquaternary oximes at what appear to be postsynaptic and presynaptic muscarinic receptors suggests that some analogs act as antagonists at postsynaptic sites while others selectively affect presynaptic sites. The more strongly hydrophobic analogs, such as HGG-12 or HGG-42, preferentially antagonize acetylcholine at the postsynaptic receptor (K_I = 1-4 μM), while at presynaptic receptors the presence of these drugs in micromolar concentrations does not inhibit acetylcholine release (IC_{50} = 100 μM). TMB-4, on the other hand, can inhibit acetylcholine release (IC_{50} = 8 μM) but has only a small effect on the postsynaptic receptors (K_I = 50 μM). Taken together, our results indicate that bisquaternary pyridinium oximes behave as allosteric antagonists of postsynaptic muscarinic receptors and act as cholinomimetics at presynaptic autoreceptors. It thus seems likely that treatment with a mixture of certain analogs (i.e., TMB-4 and HGG-12) in conjunction with a muscarinic antagonist might be therapeutically effective in cases of organophosphate poisoning.


HGG-12, R =
HGG-42, R =
HGG-52, R = —CH₂—CH(CH₃)₂
HON = HC
C − R
O
2Cl⁻
CH₂—O—CH₂
TMB-4
CH=NOH
CH₂— CH₂— CH₂
HI-6
HON=HC
C − NH₂
Toxogonin
SAD-128
CH₃−C−CH₃

Scheme I

FOREWORD

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INTRODUCTION

Bisquaternary pyridinium oximes represent a group of drugs which were originally designed and synthesized as reactivators of the enzyme acetylcholinesterase following organophosphate poisoning (9-17). Several organophosphates (such as soman) form a phosphoryl-enzyme adduct which cannot be reactivated by oximes (11, 12); nevertheless, the mixture of some bisquaternary oximes and muscarinic antagonists still affords some partial protection against soman intoxication (14-15). This phenomenon led to the hypothesis that bisquaternary pyridinium oximes may also act, in conjunction with muscarinic antagonists, on the muscarinic cholinergic receptor. Indeed, several reports have indicated that these oximes possess mild antimuscarinic activity (3-6) and inhibit the binding of [^{3}H]-4NMPB, a potent muscarinic antagonist. However, the interaction between bisquaternary pyridinium oximes and the muscarinic receptors could not be described in terms of simple binding to the acetylcholine-binding sites on the receptor (6). Kuhnen-Clausen (3), using a smooth muscle preparation, showed that the bisquaternary pyridinium oximes may block the acetylcholine-induced response in a noncompetitive manner and suggested the possible operation of an allosteric mechanism. We have previously compared the potency of several bisquaternary pyridinium analogs (e.g., Toxogonin) as blockers of the acetylcholine-induced response of smooth muscle and as inhibitors of [^{3}H]-4NMPB binding to muscarinic receptors in whole mouse brain (6). Although their apparent potencies both in the binding and in the functional assays were similar, the experimental data could not be explained in terms of a simple competitive interaction between the drugs and a homogeneous population of muscarinic receptors. A new class of bispyridinium oximes (HGG oximes, see Scheme I), first described by Hagedorn, were also shown to possess antimuscarinic activity (5 and references therein); like Toxogonin they inhibit binding of [^{3}H]-4NMPB to rat brain muscarinic receptors (1, 2, 18, 19).

The experiments described in the present work were designed to examine the mode of interaction of bisquaternary pyridinium oximes with muscarinic receptors. For this purpose, we investigated: 1. the effects of bisquaternary oximes on muscarinic antagonist binding and on the antagonist-induced isomerization of the muscarinic sites; 2. the effects of bisquaternary pyridinium oximes on muscarinic agonist binding and on the modulation of agonist binding by guanine nucleotides and by transition metal ions; 3. the effects of oximes on presynaptic muscarinic autoreceptors. Our efforts during the previous terms of this work were aimed at examining the effects of HGG oximes on the binding of antagonists and agonists to the muscarinic receptors. Results of these studies have been detailed earlier (see refs. 1, 2, 18, 19), and are also summarized in the present final report. The overall conclusions are discussed as well (see Discussion).

Our recent equilibrium and kinetic studies with [^{3}H]-4NMPB indicate that in rat brain and in Torpedo electric organ synaptosomes, the bisquaternary pyridinium oximes bind to a site distinct from the antagonist binding site (2, 18). In addition, treatment of rat brainstem homogenates for 30-60 min with an oxime such as HGG-12 resulted in irreversible loss of ∿ 30% of the muscarinic receptors; in the cerebral cortex only ∿ 10% of the receptors were lost under the same conditions. Thus, HGG-12 apparently exerts two distinct effects on antagonist binding to brainstem muscarinic receptors, namely, reversible allosteric inhibition and irreversible loss of binding sites. The binding of muscarinic agonist to rat brain muscarinic receptors is also irreversibly affected by oximes; treatment of brain muscarinic receptors with oximes results in a loss of sensitivity to modulation of agonist binding by guanine nucleotides and transition metal ions (19). The degree of reversible inhibition determined in competition binding experiments appears to be similar (K_I values are identical) in membranes treated with oximes and in untreated membranes (18). The irreversible effects are detected in oxime-treated washed membranes and can therefore be measured separately. The purpose of the last part of our studies was to explore the role of the functional substituents on the pyridinium ring in exerting these effects on antagonist binding to rat brainstem muscarinic receptors, as well as their effects on presynaptic mechanisms. We used seven bisquaternary pyridinium analogs (see Scheme I) and examined their potency in: 1) reversible inhibition of [^{3}H]-4NMPB binding; 2) irreversible inhibition of [^{3}H]-4NMPB binding; and 3) inhibition of [^{3}H]-acetylcholine ([^{3}H]-ACh) release from rat brainstem slices.

We present our results on the activity of these oximes on brainstem muscarinic receptors. We also report on the attempts to synthesize radiolabeled HGG-12 and soman; it was hoped that these derivatives will enable the direct evaluation of possible interactions of organophosphates with brain muscarinic receptors.

MATERIALS AND METHODS

[^{3}H]-Choline (80 Ci/mmole) was purchased from Amersham, [^{3}H]-4NMPB (70 Ci/mmole) was prepared by catalytic tritium exchange as described previously (20). 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 according to published procedures (21), and were > 96% pure as determined by thin-layer chromatogrphy in n-butanol:acetic acid:H_2O (4:1:1). TMB-4 was a gift from Dr. C. Broomfield, Gpp(NH)p and diisopropylfluorophosphate (DFP) were from Sigma. All other drugs have been described previously (1, 2).

Methods

Adult male and female rats of the CD strain were obtained from Levinstein's Farm (Yokneam, Israel) and maintained in an air-conditions room at 24±2°C for 14 hr (0500-1900) under fluorescent illumination and in darkness for 10 hr. Rats of both sexes 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 weighed 190-250 g. They were decapitated (between 1000 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 (22). Brain regions were homogenized in 0.32 M sucrose as described in detail previously (23-26) to yield a 3% homogenate (w/v). These homogenates were used for binding assays with [^{3}H]-4NMPB. For pretreatment of membranes with oximes, homogenates were incubated in 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.

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

Homogenates were used for binding assays as follows: 50 µl of tissue preparation were incubated at 25°C 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 of 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 homogenates, the adsorption of [^{3}H]-4NMPB to the filters was negligible (24). The filters were placed in vials containing 4 ml of scintillation liquid (Hydro-Luma, Lumac Systems, Inc., Titusville, Fla.) and were maintained at 25°C 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 (27) 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 and competition experiments were carried out as described in detail in the previous report (1).

[^{3}H]-acetylcholine release from brain slices

The amount of [^{3}H]ACh release was measured by employing previously published methods (28, 29). Rats were killed and brain slices (200 µM) immediately prepared using a tissue chopper. The slices were placed in preoxygenated Krebs solution containing 25 mM HEPES, pH 7.4 (buffer A), and then transferred to small plastic baskets (2-4 slices/basket). The baskets were then placed in 50 ml of buffer A containing 1 µM unlabeled choline and 1 µCi/ml [^{3}H]-choline (specific activity 80 Ci/mmole) and incubated for 45 min at 37°C with constant oxygen bubbling. The slices were then washed three times at 10 min intervals by successively transferring the baskets into 50 ml of fresh buffer A. Following these washings each basket was placed in a vial containing 2 ml of buffer A (zero time) and transferred into other vials at the times indicated. The vials were kept at 37°C and contained either 4 ml of buffer A (basal release) or 4 ml of buffer B, which included 25 mM KCl (K^+-evoked release). The tissue slices were exposed simultaneously to 25 mM KCl and to the drugs. At the end of the experiment, a 400-µl sample was collected, subjected to digestion by 1% sodium dodecyl sulfate (SDS) and 50-µl samples were counted. The total radioactivity (i.e., that present in the tissue and released into the buffer) was taken to reflect total [^{3}H]-choline plus [^{3}H]-ACh present in the tissue at zero time. From previous studies (28) we know that most of the radioactive material released into the medium upon K^+-depolarization originates from $[^3H]$-ACh. The percentage of release is thus given by: 100 × cpm present in the medium at time t/total cpm.

Synthesis of tritium-labeled derivatives

The specific aims of the synthesis of tritium-labeled HGG-12 and soman were to determine, both in vitro and in vivo: (i) regional distribution in rat brain of possible binding sites for these reagents; (ii) evaluation of the possibility of irreversible labeling of muscarinic receptors by soman as one of the reasons for its toxicity.

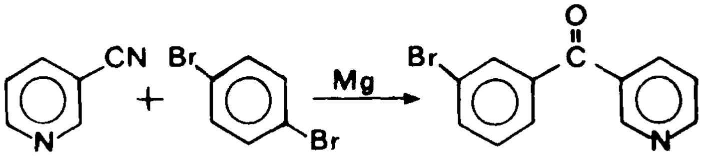
a. Attempts to prepare labeled HGG-12

In the initial set of experiments we attempted to prepare tritium (T)-labeled HGG-12 via general labeling using exchange of T_2O in the presence of Pd. Unfortunately this method, although easy to perform, was ineffective for our purpose, namely, for obtaining $[^3H]$-HGG with a specific activity of at least 10 Ci/mmol. General labeling using $T_2/Pd/C$ could not be employed either, since the oxime group would have been reduced. We therefore had to resort to the tedious method of synthesizing fragments and then coupling them to yield the desired HGG-12, according to the synthetic scheme(1). We decided to prepare T-labeled 3-benzoylpyridine and then couple it to the chloroethyl ether derivative. In order to obtain $[^3H]$-3-benzoylpyridine we prepared bromobenzoylpyridine via several routes:

(i) Bromination with Br_2/CCl_4;

(ii) Bromination in acetic acid using $AlCl_3$ as catalyst;

(iii)



In each case the resulting bromoderivative was tritiated using $T_2/Pd/C$, yielding the following specific activities of $[^3H]$-benzoylpyridine from the respective reactions: (i) 53 mCi/mmol, (ii) 15 Ci/mmol, (iii) 23 Ci/mmol. We therefore decided to adopt method (iii) for the routine preparation of $[^3H]$-benzoylpyridine.

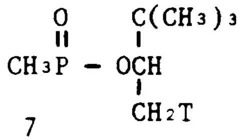
Our attempts to couple the chloroethyl ether derivative of pyridine aldoxime with $[^3H]$-benzoylpyridine have so far been unsuccessful. The $[^3H]$-derivative obtained from the coupling mixture did not show the expected chromatographic behavior. We suspect that something went wrong in the preparation of the chloroethyl ether derivative. It should be mentioned that small-scale synthesis such as the one described here is often beset by difficulties not experienced with large-scale preparative techniques.

b. Attempts to prepare labeled and unlabeled soman

Labeled soman has been prepared previously from labeled CH_3POF_2 (30), but the yield of radioactivity of methyldifluorophosphonate was low (only one-third of the label in methyl idodide was incorporated). We therefore attempted to prepare labeled soman via another route, i.e., by labeling at either the acidic or the alcoholic moiety, as follows:

$$\underset{1}{(CH_3)_3CCOCH_3} \xrightarrow{Br_2} \underset{2}{(CH_3)_3CCOCH_2Br} \text{ or } \underset{3}{(CH_3)_3CCOCHB_2} \xrightarrow{T_2/Pd/C}$$

$$\underset{4}{(CH_3)_3CCHOHCH_2T} \text{ or } \underset{5}{(CH_3)_3CCHOHCHT_2}$$



$$\underset{6}{CH_3POF_2} + \underset{4}{(CH_3)_3CCHOHCH_2T} \xrightarrow{Et_3N} \underset{7}{CH_3\overset{\overset{O}{\|}}{P} - O\underset{CH_2T}{\overset{C(CH_3)_3}{CH}}}$$

Unlabeld soman [7] was prepared in the laboratory according to Ashani and Catravas (31). Bromomethyl tert-butyl ketones [2, 3] according to Bayer and Straw (32), and methylphosphonyl difluoride [6] according to Monard and Quinchon (33).

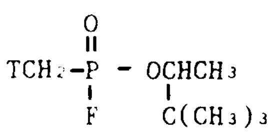
However, when the product of reduction of the bromoketone [3] was reacted with the fluoride [6], no toxic substance was obtained.

Introduction of tritium into the phosphonyl moiety:

$$\underset{8}{ClCH_2P(O)Cl_2} \longrightarrow \underset{9}{ClCH_2P(O)(OH)_2} \xrightarrow{T_2/Pd/C}$$

$$\underset{10}{TCH_2P(O)(OH_2)} \xrightarrow{SOCl_2} \underset{11}{TCH_2P(O)Cl_2}$$

$$\underset{11}{TCH_2P(O)Cl_2} + \underset{6}{CH_3P(O)F_2} + 2\ \underset{12}{(CH_3)_3CCHOHCH_3} \longrightarrow$$



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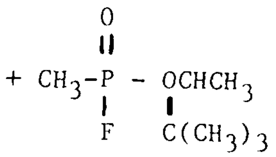

$$\underset{13}{TCH_2-\overset{\overset{O}{\|}}{\underset{F}{P}} - \underset{C(CH_3)_3}{OCHCH_3}} + CH_3-\overset{\overset{O}{\|}}{\underset{F}{P}} - \underset{C(CH_3)_3}{OCHCH_3}$$

NMR studies showed that reduction of the acid [9] with hydrogen results in about 80% yield of the desired compound [10]. However, the toxicity studies were not encouraging since the product was found to be much less toxic than expected, thus indicating the possibility that under our experimental conditions only the difluoride was reacting.

Preparation of methyl difluorophosphonate, CH_3POF_2

Molten methylphosphoric dichloride (82 g, 0.62 mol) (Aldrich) was added in portions to 84 g of sodium fluoride in a 500 ml round-bottomed flask equipped with a distillation adapter. The flask was heated (mantle). At 100-105°C a liquid started to pass and was collected until the temperature reached 135°C. This fraction was preheated overnight to 125°C (internal temperature) and a second fraction was obtained at 105-120°C. This second distillate was redistilled after addition of 10 g of NaF and boiled at 97-99°C. The yield was 13.2 g (21%).

Preparation of O-pinacolyl methylphosphonofluoridate (soman)

(i) From methyldifluorophosphonate
A solution of 0.54 g of methyldifluorophosphonate in 5 ml of abs. ether was added dropwise to 0.58 g (5% excess) of pinacolyl alcohol (Aldrich) and 0.55 g of purified triethylamine in 10 ml of ether. After 45 min the precipitate (triethylamine hydrofluoride) was filtered off and the filtrate was concentrated under reduced pressure (25 mm Hg) at 30°C to give 0.87 g of an oily substance. The LD_{50} of this product was about 0.05-0.1 mg/kg (mice), as compared with a reported value for soman (Merck index) of 0.62 mg/kg.It is possible that the strain we used was a highly sensitive one, and that this could account for the discrepancy.

(ii) From methyldichloro- and methyldifluorophosphonate
A mixture of 91 mg (0.684 mM) of the dichloride and 71 mg of the difluoride in 0.5 ml of ether was treated overnight with 155 mg pinacolyl alcohol in 5 ml of ether. The ether was then removed and the residue was heated to 90°C, cooled and evacuated for 3 min at room temperature (water pump). The residue (218 mg) was not toxic.

(iii) From methyldichlorophosphonate and sodium fluoride
A mixture of 71 mg of the dichloride, 45 mg of sodium fluoride (100% excess) and 67 mg pinacolyl alcohol (20% excess) was stirred for 1.5 hr at 100°C and left overnight. The mixture was extracted with ether and the extract concentrated as above. The residue (74 mg), however, was not toxic.

(iv) By reductive dehalogenation of chloromethylphosphoric acid
Chloromethylphosphoric acid, $ClCH_2P(O)(OH)_2$, was prepared by hydrolysis of chloromethyldichlorophosphonate, m.p. 87-90°. A solution of 138 mg of the acid in 4 ml of 1 N ethanolic potassium hydroxide was hydrogenated for 1.5 hr in the presence of 20 mg of 10% Pd/C. The solution was then diluted with 10 ml of ethanol, filtered, acidified with dilute HCl, filtered again and concentrated. After trituration of the residue with 2 ml of ethanol, a small amount of undissolved material was removed by centrifugation and concentrated to give 71 ml of a material having an NMR spectrum identical with that of methylphosphoric acid.

RESULTS

1. Summary of previous results of this contract.

a. Bisquaternary pyridinium oximes as allosteric inhibitors of rat brain muscarinic receptors

The mode of interaction of bisquaternary pyridinium oximes with rat brain muscarinic receptors in cerebral cortex and brainstem preparations was studied by the use of the tritium-labeled muscarinic antagonist [^{3}H]-4NMPB. Binding of the labeled muscarinic antagonist was inhibited by these drugs, the most potent inhibitors being HGG-42 and its 3-phenylcarboxypyridinium analog (HGG-12) (apparent K_I = 1.3-1.7 and 1.8-2.2 µM, respectively). Analysis of the binding properties suggested that binding of the muscarinic antagonist and the bisquaternary pyridinium oximes was nonexclusive. Kinetic binding data provide evidence that the drugs inhibit binding of muscarinic antagonists in an allosteric manner, with a resulting decrease in the rates of both association of [^{3}H]-4NMPB to the receptor and its dissociation from it. These effects were observed both in brainstem and in cortical preparations even after pretreatment and washing out of the inhibitors. The selective natures of HGG-12 and HGG-42 were apparent from their irreversible effects on the number of muscarinic binding sites. In brainstem, 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 $\sim$ 10% of the muscarinic receptors were lost upon exposure to these drugs. The decrease in the muscarinic receptor population of the brainstem was dependent on both concentration and time and occurred both <u>in vitro</u> and <u>in vivo</u> following injection of HGG-12 into rats. Unlike the <u>in vitro</u> loss of receptor sites, which was irreversible, the <u>in vivo</u> effect was restored 2 hr after the injection. Taken together, the results suggest that the bisquaternary oximes are allosteric inhibitors of the muscarinic acetylcholine receptor and may be capable of distinguishing between receptor states and inducing specific irreversible effects. Because of these properties, the drugs may prove extremely useful as sensitive probes in studies on the nature of the agonist-receptor-effector relationship.

b. Allosteric interactions between muscarinic agonist binding sites and effector sites demonstrated by the use of bisquaternary pyridinium oximes

Agonist binding to muscarinic receptors from rat brainstem and cerebral cortex was studied using bisquaternary pyridinium oximes for detecting possible interaction between agonist binding sites and sites of the effector guanosine 5'(β,γ-imino) triphosphate (Gpp(NH)p) and Co^{2+}. Pretreatment of either brainstem or cortical homogenates with 200 µM HGG-12 reduced the affinity of muscarinic agonists. No change was observed in the relative proportion of high (R_H) and low (R_L) affinity agonist binding sites. However, the oxime affected the processes of interconversion between these sites. Thus, unlike control membranes, in HGG-12- treated brainstem membranes, Gpp(NH)p could not induce conversion of R_H to R_L, and in cortical membranes Co^{2} could not induce conversion of R_L to R_H. These results suggest that HGG-12 inactivates a component which is involved in both processes of induced interconversion. Induced interconversion between R_H and R_L was not affected in membranes treated with HGG-12 in the presence of carbamylcholine in concentrations at which mainly R_H is occupied by the agonist. The occupation of R_H by carbamylcholine protected both R_H and R_L from the effects of the oxime. The possible role of the molecular events involved is discussed.

2. Effects of HI-6, TMB-4 and Toxogonin on antagonist and agonist binding to rat brain muscarinic receptors

Interactions of the oximes HI-6, TMB-4 and Toxogonin with muscarinic receptors were studied in detail, using the labeled muscarinic antagonist [^{3}H]-4NMPB. Results shown in Figure 1 demonstrate concentration-dependent inhibition of [^{3}H]-4NMPB binding (2.0 nM) in rat brain cortex, brainstem and atrium by TMB-4.

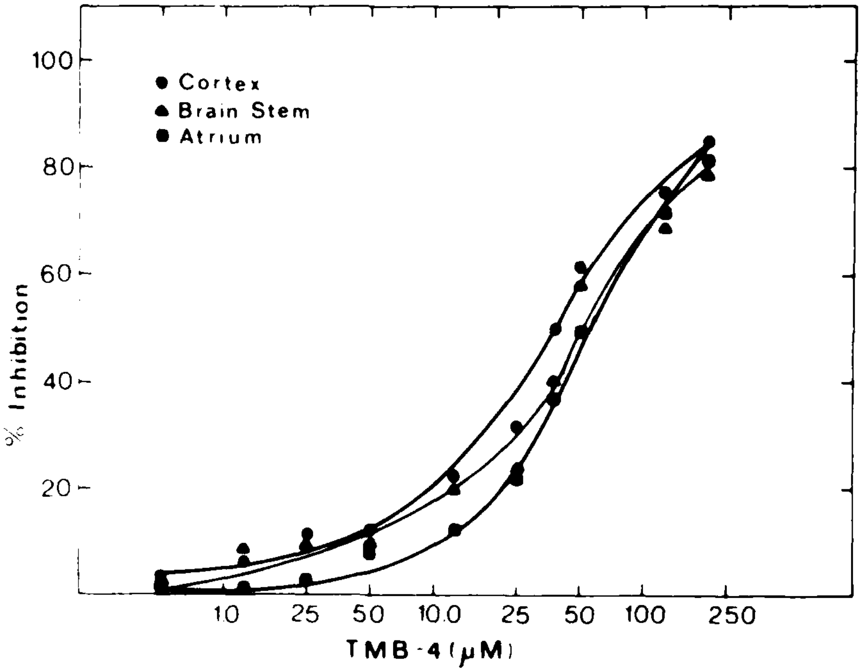

Figure 1: Concentration-dependent inhibition of [^{3}H]-4NMPB binding (2 nM) by TMB-4.
Binding of [^{3}H]-4NMPB to rat brain cortex (●), brainstem (▲) and atrium (■) was measured in the absence and in the presence of various concentrations of TMB-4 at 25°C. Results are presented as percent inhibition as a function of the concentration of TMB-4.

As shown, in all cases, TMB-4 inhibited binding of 2.0 nM [^{3}H]-4NMPB with IC_{50} values of 40-60 μM. Thus the apparent affinity of muscarinic receptors for TMB-4 is lower than those for HGG-12 or HGG-42, as recorded in our previous reports (1, 2) (See Table I).

Table I: Inhibition constants and irreversible effects of bisquaternary pyridinium analogs on brainstem muscarinic receptors

Drug	Position of oxime moiety	Reversible effect K_I apparent (μM)	Irreversible effect % loss of muscarinic binding sites
HGG-42	2	1.7 ± 0.2	30 ± 3
HGG-12	2	3.3 ± 0.1	28 ± 3
HGG-52	2	15.5 ± 0.2	29 ± 5
HI-6	2	110.0 ± 10.0	26 ± 4
Toxogonin	4	42.0 ± 6.0	0
SAD-128	no oxime moiety	6.0 ± 2.0	0
TMB-4	4	50.0 ± 12.0	0

Apparent K_I values were calculated from concentration-dependent inhibition of [^{3}H]-4NMPB binding (1, 2). Each value represents the mean ± S.D. of 3 or 4 determinations. Loss of binding sites (percent reduction in Bmax) was calculated from [^{3}H]-4NMPB binding curves measured in control membranes and in membranes treated with the bisquaternary analogs, as shown in Figure 3. Values represent the means ± S.D. of three separate experiments.

In order to further study the interactions of TMB-4 with the muscarinic receptor, we examined the effects of the drug on the binding isotherms of [^{3}H]-4NMPB. Results of typical experiments performed with homogenates of rat brainstem and cortex are depicted in Figure 2. As shown, the presence of TMB-4 (5-125 μM) results in a reduction in the apparent affinity of [^{3}H]-4NMPB as well as an apparent reduction in the maximal binding capacity (Bmax). The latter phenomenon is more pronounced in the brainstem than in the cortex. These results are similar to our previous findings with HGG-12 and HGG-42 (2,18). In the case of these two drugs, the reduced Bmax was in part due to irreversible loss of receptor binding sites; we therefore proceeded to determine whether a similar irreversible loss is induced by TMB-4.

Figure 3 summarizes experiments designed to examine the reversibility of the TMB-4 effects on [^{3}H]-4NMPB binding. Homogenates of rat brain cortex or brainstem were preincubated with 200 μM TMB-4 for 60 min at 25°C and their membranes were then precipitated and washed three times with ligand-free buffer. The final membrane preparation was used for binding studies. Pretreatment with TMB-4 did not affect binding of [^{3}H]-4NMPB to the receptors of either the cortex (Figure 3A) or the brainstem (Figure 3B). However, pretreatment of brainstem membranes with either HGG-12 or HI-6 did result in an irreversible loss of muscarinic antagonist binding sites. Toxogonin, like TMB-4, could not induce this effect (Figure 3B, Table I).

We have previously reported (2) that the bisquaternary analog SAD-128, which does not possess an oxime group, cannot induce an irreversible loss of receptors, suggesting that the presence of the oxime group is essential in order to induce this effect. Results presented here suggest that it is not only the

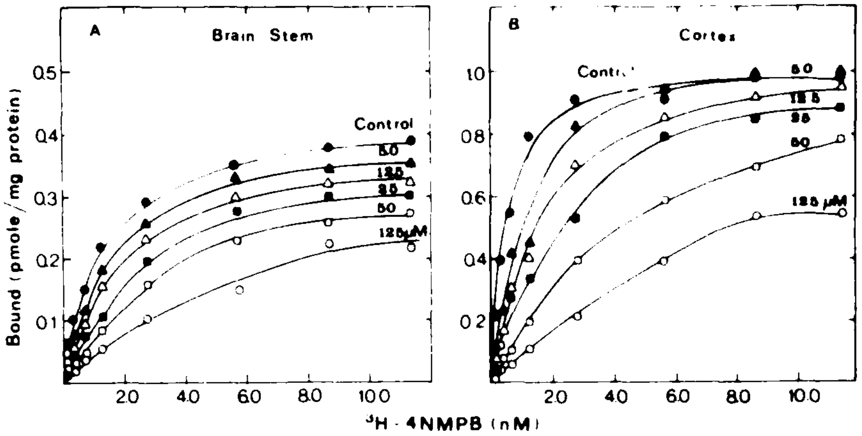

Figure 2: Binding isotherms of $[^3H]$-4NMPB in the absence and in the presence of TMB-4

Binding was measured at 25°C in Krebs/Tris buffer, pH 7.4, in the absence (control) and in the presence of the indicated concentrations of TMB-4. Results are expressed in the form of $[^3H]$-4NMPB bound as a function of its concentration. A. Brainstem preparation. B. Brain cortex preparation.

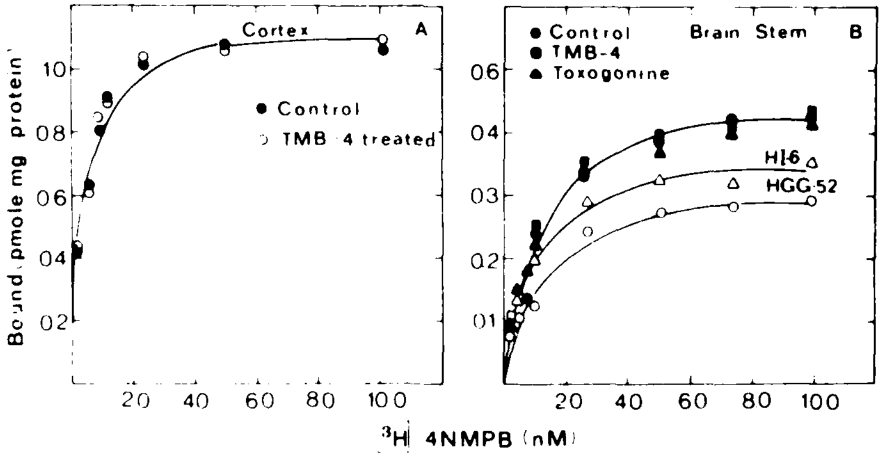

Figure 3: Reversible and irreversible effects of oximes on $[^3H]$-4NMPB binding. Cortical (A) or brainstem membranes (B) were incubated for 60 min with 200 μM of the indicated oxime. The membranes were washed three times in oxime-free buffer and then used for binding assays. Results are expressed in the form of bound $[^3H]$-4NMPB as a function of its concentration.

presence of an oxime moiety that is important but also its position on the pyridinium ring: i.e., only derivatives with an oxime moiety at position 2 (HGG-12, HGG-42, HGG-52, HI-6) can induce irreversible loss of brainstem receptors. Toxogonin, in which the oxime moiety is at position 4, cannot induce this effect.

Irreversible effects of HGG-12 and HGG-42 on brainstem muscarinic receptors were also apparent in studies on the properties of agonist binding (2, 18). We therefore proceeded to determine whether such effects are also induced by TMB-4. Brainstem membranes were pretreated with 200 μM TMB-4, washed and then used in competition experiments with the agonist carbamylcholine. Results (Figure 4) indicate that TMB-4 did not induce an irreversible effect on agonist binding. Furthermore, it did not affect the GPP(NH)p-induced conversion of binding sites from the high affinity to the low affinity state, a process which is abolished in membranes pretreated with bispyridinium compounds with oxime moieties in position 2, e.g., HGG-12.

5. Inhibition by oximes of [^{3}H]-acetylcholine release from brain slices

Our previous data suggested the possibility that bisquaternary pyridinium oximes may affect presynaptic mechanisms, since they inhibit the binding of [^{3}H]-4NMPB to presynaptic muscarinic autoreceptors (2). We therefore examined the effects of these drugs on the release of [^{3}H]-ACh from rat brain stem slices.

Following incubation for 45 min with 1 uM [^{3}H]-choline, the tissue slices were washed with Krebs buffer and subjected to the release experiments (see Methods). In a typical experiment the total radioactivity in the tissue ([^{3}H]-choline plus [^{3}H]-ACh) was 100,000-121,000 cpm. During the first 5 min of incubation in the Krebs buffer, the amount of radioactive material released into the medium was 2319 cpm (2.1% of the total radioactivity present in tissue). During each of the two subsequent 5 min intervals this basal release was 1.6% to 1.7% (Figure 5A). Exposure of the tissue slices to 25 mM KCl after 10 min in Krebs buffer triggered the release of radioactive material into the medium (3.3% and 3.4% during the next two 5 min intervals). During the subsequent 10-15 min the release returned to basal levels (Figure 5A). The K^+-evoked release was Ca^{2+}-dependent as it could be blocked by omission of Ca^{2+} from the medium (Figure 5A). From previous studies (28) we know that most of the radioactive material released into the medium upon K^+ depolarization originates from [^{3}H]-ACh. Indeed, in experiments where K^+-evoked release was measured in the presence of 100 μM DFP (Figure 5B), the release pattern was similar to that shown in Figure 5A. Under these conditions no acetylcholinesterase activity, as determined by the methods of Ellman et al. (34) was detected in brainstem homogenates prepared from tissue taken at the end of the release experiment. Although the presence of DFP could have resulted in an inhibition of [^{3}H]-ACh release (due to accumulation of ACh) such an effect was not observed (compare Figure 1A and 1B). The most probable explanation for this phenomenon is that very low concentrations of ACh were accumulated in the medium.

In additional control experiments it was found that when 100 μM DFP was present during K^+ depolarization more than 80% of the released radioactivity was in the form of [^{3}H]-ACh as determined by teteraphenylboron/hepatone extraction after

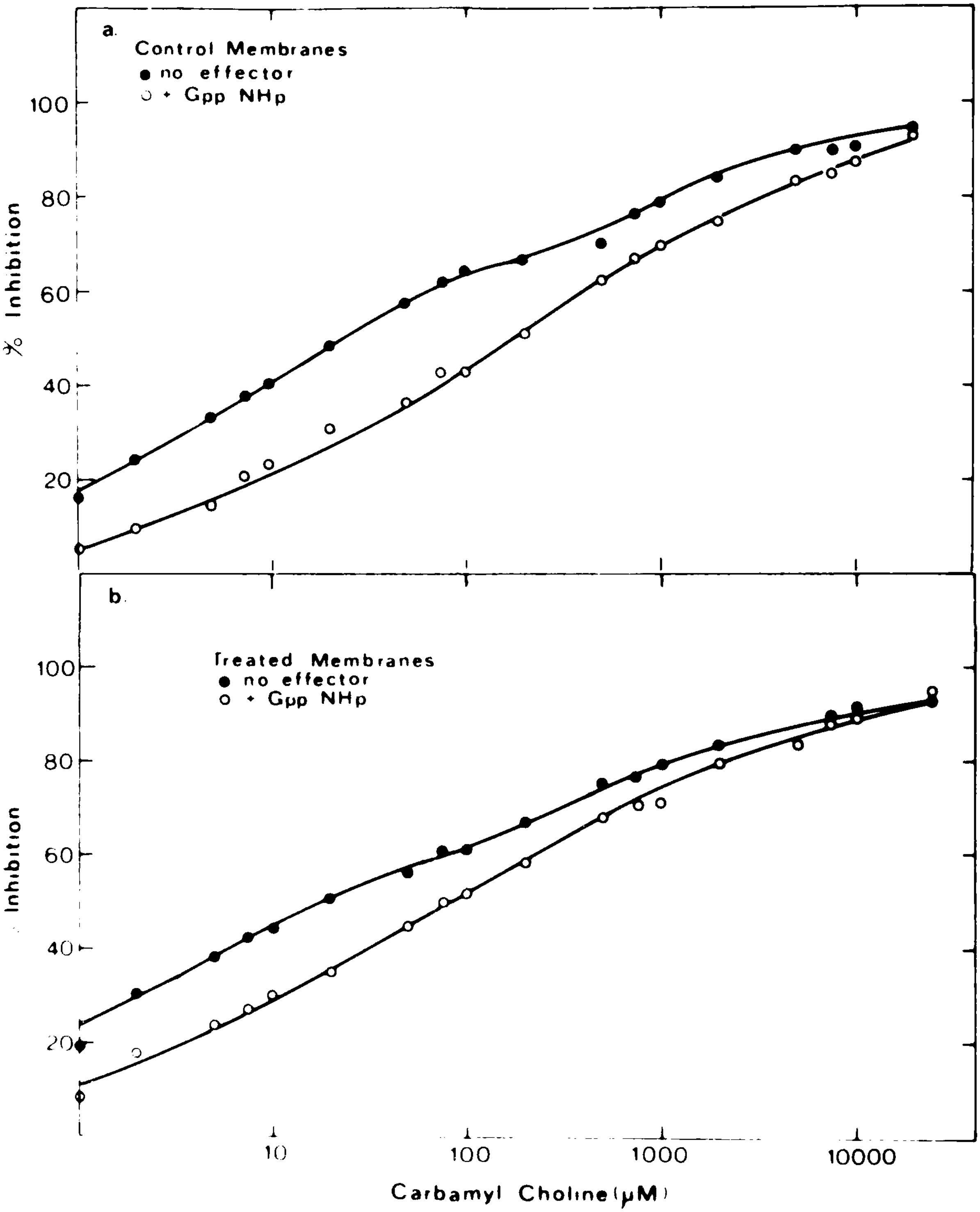

Figure 5: Effect of Gpp(NH)p on carbamylcholine binding to control (a) and TMB-4 treated (b) brainstem membranes. The inhibition of [^{3}H]-4NMPB binding is presented as a function of carbamylcholine concentration. Binding was measured in the presence (○) and in the absence (●) of 200 μM Gpp(NH)p.

phosphorylation of [^{3}H]-choline (35). Basal release was mostly (95%) [^{3}H]-choline. In the absence of DFP the total radioactivity released into the medium was identical with that observed in its presence, although under the former conditions [^{3}H]-ACh was in part degraded to [^{3}H]-choline. We preferred to avoid the use of anticholinesterases, since the drugs under study are known to be reactivators of acetylcholinesterase (17), and since the presence of anticholinesterases would complicate experiments aimed at studying the presynaptic effects of muscarinic agonists (36). The above experiments thus demonstrate that the radioactivity in the medium represents mostly [^{3}H]-ACh released in the medium upon K$^+$ depolarization.

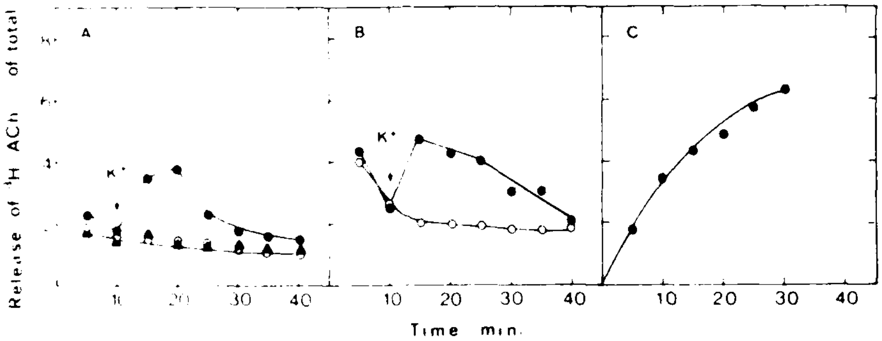

Figure 5: Release of [^{3}H]-acetylcholine from rat brainstem slices. [^{3}H]-acetylcholine release was measured as a function of time (see text). Data are presented as percentage of total radioactivity released during each 5 min time interval (A and B) and as cumulative percent of K$^+$-evoked release of radioactivity (C). The tissue slices were first incubated in the original Krebs solution. The arrows indicate the time at which they were exposed to the release solution (25 mM KCl) or to Krebs solution (Basal release). A. Data shown are for K$^+$-evoked release in the presence (●) and in the absence (o)of 2 mM Ca^{2+}, and for the basal release in the absence of K$^+$ (▲). B. K$^+$-evoked (●) and basal (o) release measured in the presence of 100 µM DFP. C. Data from A (values of basal release have been subtracted). Zero time corresponds to the time of exposure to 25 mM KCl.

The cumulative percent of K$^+$-evoked [^{3}H]-ACh release (calculated for each 5 min interval by subtracting the percent basal release from the release in the presence of 25 mM KCl) was plotted by adding values for 5 min periods (Figure 5C). Both the time course and the extent of [^{3}H]-ACh release from rat brainstem slices were similar to those described in previous studies (28, 29). The presence of presynaptic muscarinic receptors which modulate ACh release (7, 8, 29) could also be demonstrated, since the muscarinic agonist oxotremorine (10 µM) inhibited the K$^+$-evoked release of [^{3}H]-ACh (Figure 6). This effect was blocked by 1 µM atropine, although atropine by itself potentiated the K$^+$-evoked release (Figure 6). It has been suggested that the latter phenomenon reflects antagonism by atropine of the inhibition of [^{3}H]-ACh release by endogenous ACh (7, 8).

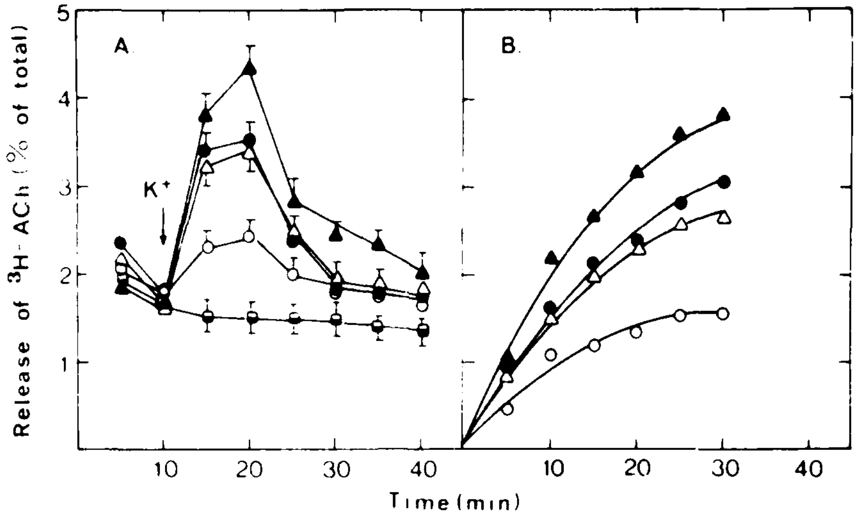

Figure 6: Inhibition of K$^+$-evoked [^{3}H]-acetylcholine release by oxotremorine and its potentiation by atropine.

A. Acetylcholine release was measured as percent of total radioactivity released per 5 min, as described in the text, in the absence of atropine or oxotremorine (●) and in the presence of 1 μM atropine (▲), 10 μM oxotremorine (o), or 10 μM oxotremorine + 1 μM atropine (Δ). (■) = basal release in the absence of K$^+$, (□) = release in the absence of Ca^{2+}. The arrow indicates the time at which slices were exposed to 25 mM KCl. B. Data from A replotted as accumulative percent of K$^+$- evoked release of radioactivity (values of basal release have been subtracted). Zero time corresponds to the time at which slices were exposed to 25 mM KCl.

The effects of bisquaternary pyridinium oximes on K$^+$-evoked [^{3}H]-ACh release were studied under the conditions described above for oxotremorine and atropine. Preliminary experiments had indicated that the oximes (100-200 μM) inhibited K$^+$-evoked [^{3}H]-ACh release but had no effect on the basal release. In some experiments the oxime was added 5 min prior to K$^+$ depolarization, while in others tissue slices were exposed simultaneously to 25 mM KCl and to the oxime. The extent of the inhibition of K$^+$-evoked [^{3}H]-ACh release by the oximes was similar under both sets of experimental conditions. In subsequent experiments the tissue was exposed simultaneously to the oxime and the depolarizing medium.

As shown in Figure 7A, HGG-12 (200 μM) resembled oxotremorine with respect to the manner in which it inhibited K$^+$-evoked [^{3}H]-ACh release (Figure 6), but was much less potent than the muscarinic agonist (compare Figure 6A with Figure 7A). The inhibitory effect of HGG-12 was completely blocked by 1 μM atropine, suggesting that the oxime affects the release via the muscarinic receptor.

It should be pointed out that only partial inhibition of K$^+$-evoked [^{3}H]-ACh release could be induced both by oxotremorine and by the oxime derivative. This was also noted in previous reports on the inhibition by muscarinic agonists of

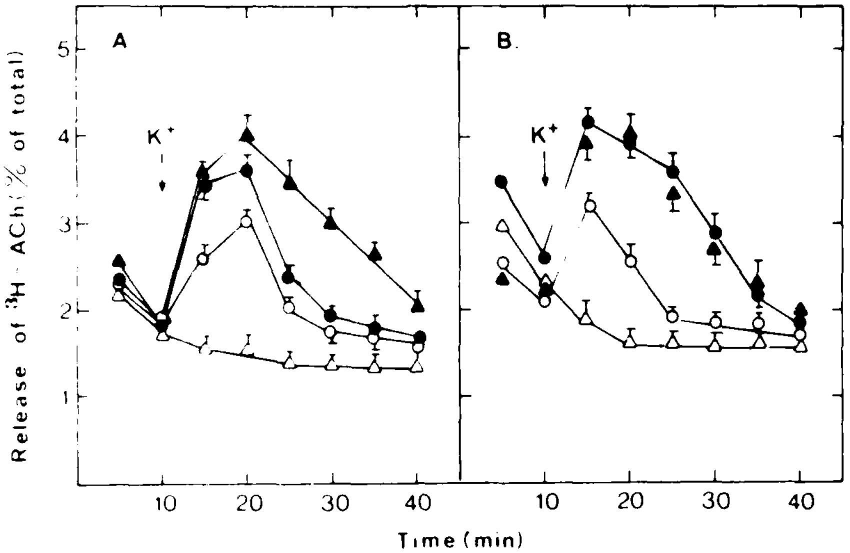

Figure 7: Inhibition of K$^+$-evoked [^{3}H]-ACh release by HGG-12 (A) and TMB-4 (B). [^{3}H]-ACh release was measured as described in the text. Data represent percent of total radioactivity released during each 5 min interval. K$^+$-evoked release was examined in the absence of added compounds (●) and in the presence of: A. 200 μM HGG-12 (o), and 200 μM HGG-12 + 1 μM atropine (▲); B. 50 μM TMB-4 (o) and 50 μM TMB-4 + 1 μM atropine (▲). (Δ) = basal release in the absence of K$^+$. The arrows indicate the time at which slices were exposed to 25 mM KCl.

[^{3}H]-ACh release (7, 8, 28, 29). As shown in Figure 7A, the inhibitory effect of HGG-12 was already apparent during the first 5 min of K$^+$ depolarization and remained unchanged during the subsequent 5 min. The inhibitory effect of HGG-12 was not as pronounced at 15 and 20 min after KCl addition as it was 5 and 10 min after KCl addition. In the presence of the oxime (or oxotremorine), the total amount of [^{3}H]-ACh released into the medium was lower than that in controls (maximal inhibition of K$^+$-evoked [^{3}H]-ACh release was 40-60% of that of controls).

In additional experiments we have studied the effects of HI-6 (Figure 8A), TMB-4 (Figure 7B and Figure 8B) and Toxogonin (Figure 9). All of these bisquaternary pyridinium oximes inhibited K$^+$-evoked [^{3}H]-ACh release in a dose-dependent manner, and the effects could be blocked by 1 μM atropine (Figures 7A and 7B).

The most potent inhibitor of [^{3}H]-ACh release was TMB-4; this drug inhibited release at the micromolar concentration range, whereas the other drugs were effective only at concentrations higher than 10μM. Dose-dependent inhibition of K$^+$-evoked [^{3}H]-ACh release by TMB-4, HI-6 and Toxogonin is shown in Figures 8 and 9. The IC$_{50}$ values (IC$_{50}$ = drug concentration at which 50% of the maximal effect is observed) for the oximes under study are summarized in Table II.

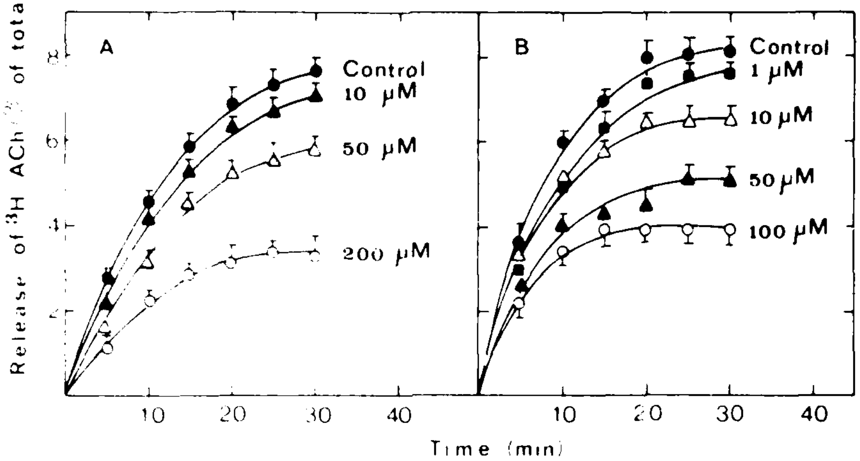

Figure 8: Inhibition of K$^+$-evoked [^{3}H]-ACh release by HI-6 (A) and TMB-4 (B). Data are presented as the cumulative percent of K$^+$-evoked release of radioactivity in the absence of added drug (control) and in the presence of the indicated concentration of the drugs. Values of basal release (in the absence of K$^+$) have been subtracted.

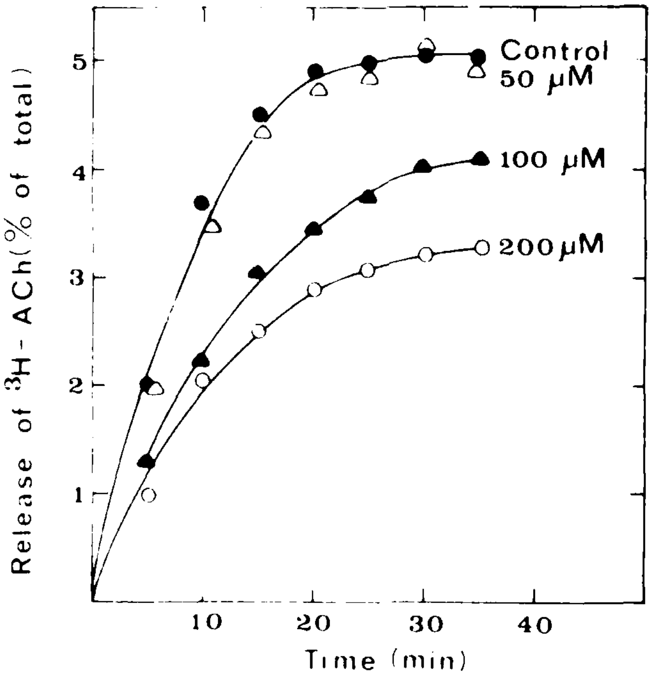

Figure 9: Inhibition of K$^+$-evoked [^{3}H]-ACh release by Toxogonin. Data are presented as the cumulative percent of K$^+$-evoked release in the absence of Toxogonin (control) and in the presence of the indicated concentrations of the drug. Values of basal release (in the absence of K$^+$) have been subtracted.

Table II: Inhibition constants of bisquaternary pyridinium analogs for presynaptic muscarinic receptors

Drug	IC_{50} (μM) presynaptic receptors
HGG-42	150 ± 40
HGG-12	150 ± 25
SAD-128	80 ± 20
Toxogonin	80 ± 20
TMB-4	8 ± 3
HI-6	50 ± 10

Drug concentration producing 50% of the maximal inhibition of $[^3H]$-ACh release (IC_{50}) was evaluated from dose-response curves as shown in Figures 8 and 9. Data are the mean value of two separate determinations.

The drugs show the following rank order of potency for inhibition of ACh release: TMB-4 > HI-6 ∿ Toxogonin > HGG-12 ∿ HGG-42.

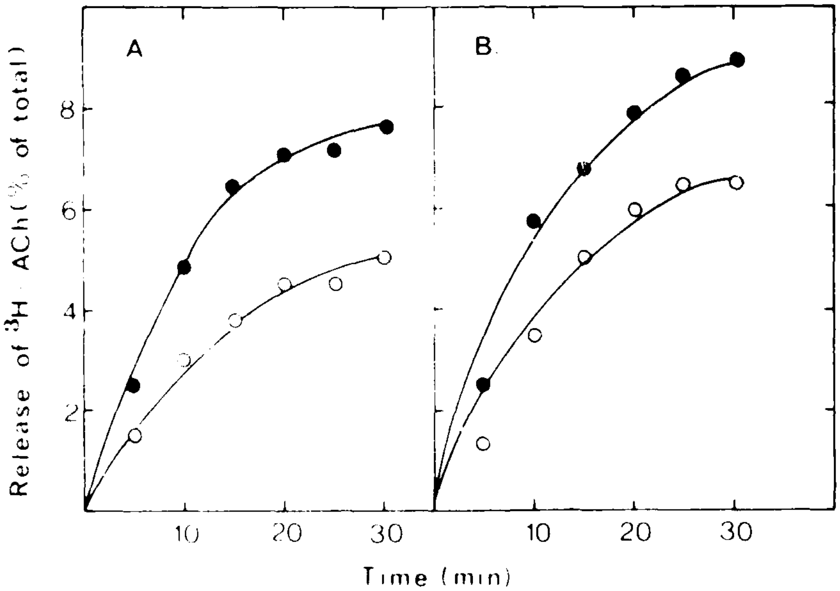

Figure 10: Inhibition of K^+-evoked $[^3H]$-ACh release by TMB-4 in the absence (A) and in the presence (B) of 100 μM DFP.

Data are presented as the cumulative percent of K^+-evoked release. Values of basal release (in the absence of K^+) have been subtracted. (●) - control; (o) - + 50 μM TMB-4.

It seems unlikely that the oximes affect [^{3}H]-ACh release by interacting with endogenous acetylcholinesterase, but in order to rule out this possibility, we measured the release in the presence of organophosphate. 100 µM DFP was added 30 min prior to K^+ depolarization and was present during the release experiment. As shown in Figure 10, inhibition of release by TMB-4 was not altered by the presence of the organophosphate. This finding, as well as the ability of atropine to block the inhibitory effects of the oximes, suggests that the bisquaternary pyridinium oximes inhibit the release of [^{3}H]-ACh via presynaptic muscarinic autoreceptors and that in this sense they are presynaptic muscarinic agonists.

DISCUSSION

The purpose of the present work was to explore the mode of interaction between the bisquaternary pyridinium oximes and muscarinic receptors in the brainstem. In our previous reports (1, 2, 18, 19), we have demonstrated that these drugs inhibit the binding of the potent muscarinic antagonist [^{3}H]-4NMPB to brainstem muscarinic receptors by an allosteric mechanism. The potent inhibitors HGG-12 and HGG-42 were shown to reduce both the association and dissociation rates for [^{3}H]-4NMPB binding to the receptors, and to reduce the capacity to bind the labeled antagonist (1, 2). The latter phenomenon is due both to reduction in the affinity of the muscarinic receptor towards the antagonist and to partial inactivation of the receptor. Inactivation of muscarinic receptors is selective, occurring in the brainstem and to a smaller extent in the cerebral cortex. The reversible and irreversible effects of various bisquaternary pyridinium oximes, which can be measured separately, were further investigated. In the final report we discuss in detail our overall results on the interaction between the bisquaternary pyridinium oximes and rat brain muscarinic receptors.

1. Bisquaternary pyridinium oximes as allosteric inhibitors of rat brain muscarinic receptors

In the present study, we have demonstrated that inhibition of muscarinic antagonist binding by bisquaternary pyridinium oximes does not conform to a scheme of simple competitive antagonism. This is shown by the partial reversibility of the inhibitory effects of the oximes, by the nonlinear dependence of the effect on the oxime concentration, and by the change in the Scatchard plots of [^{3}H]-4NMPB binding from simple linear to curvilinear curves. Thus, the bisquaternary pyridinium oximes induce a perturbation that results in apparent heterogeneity of [^{3}H]-4NMPB binding. This effect was observed in both brainstem and cerebral cortex preparations. It can be a result of preferential binding of the oximes to a subtype of muscarinic receptors or a result of allosteric interactions. Kinetic experiments indicated that the bisquaternary pyridinium oximes affect [^{3}H]-4NMPB binding in an allosteric manner. This is evidenced by the kinetic experiments on the dissociation and association rates of [^{3}H]-4NMPB from and to the muscarinic sites in the presence and absence of the oximes. The time courses of both association and dissociation of [^{3}H]-4NMPB were strongly affected by the presence of HGG-12 or HGG-42 in both brainstem and cortical preparations. Clearly, the decreased dissociation rate could not be explained in terms of direct interaction between the oxime and the [^{3}H]-4NMPB sites. Such a situation can arise only in the case of allosteric inhibition. A very similar situation has been encountered with gallamine (37, 38), pancuronium (37) and quinidine (31). The Scatchard plots of antagonist binding to the muscarinic receptor became nonlinear in the presence of, for instance, gallamine and pancuronium (37) and both binding inhibition and decreased dissociation rates from the muscarinic sites were observed in the presence of gallamine (37, 38) and quinidine (39). Another type of allosteric interaction with the muscarinic sites was observed with clomiphene (40), for which the data were interpreted in terms of positive cooperativity. Dunlap and Brown (37) have shown that gallamine displayed weak protection of heart muscarinic receptors from alkylation by propylbenzilylcholine mustard, suggesting that the drug acts via a secondary site. In our experiments, we demonstrate that atropine failed to protect against the irreversible loss of brainstem muscarinic receptors by oximes, suggesting that this effect is also via a secondary site. Consideration must be given to the possible existence of a common site for noncompetitive inhibitors on the muscarinic acetylcholine receptor, analogous to that known to be present on the nicotinic acetylcholine receptor (41). Stockton et al. (38) showed that gallamine affects muscarinic binding in a soluble receptor preparation and suggested that this activity could be exploited to distinguish between the effects of gallamine and guanine nucleotides. The bisquaternary pyridinium oximes do, however, interfere with the effects of guanine nucleotides.

The time course of the binding of muscarinic antagonists to their receptors proceeds according to a two-step isomerization pattern (23, 24). We have therefore investigated the effects of the oximes on the isomerization step. Our results demonstrate that HGG-12 alters the rates of isomerization of [^{3}H]-4NMPB-receptor complexes both in cortical and in brainstem preparations. The kinetic parameters are increased 3- to 5-fold (k_2) and 2-fold (k_{-2}) according to the scheme (where RL is the receptor ligand complex and R*L is the isomerized complex):

$$RL \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} R^{*}L$$

The ratio of k_{-2}/k_2 is therefore decreased by roughly 4- to 6-fold because of the oximes resulting in a higher proportion of slowly dissociating [^{3}H]-4NMPB-receptor complexes. The effect of HGG-12 on the isomerization step was observed even after extensive washing of the oxime, indicating that an apparently irreversible process is involved in this activity of the drug.

The exact mechanism of the irreversible effects of oximes is not yet known. In the absence of labeled oximes, we cannot distinguish between the following possibilities: (i) the oxime remains tightly (noncovalently) bound to its sites; (ii) the oxime is attached covalently to its sites; (iii) the oxime affects the [^{3}H]-4NMPB-binding sites by chemical modification. In view of the well known phenomenon of reactivation of the phosphorylacetylcholinesterase adduct (10), the last possibility represents an attractive hypothesis. The function of the oxime group in quaternary oximes is to bring about the nucleophilic displacement of the phosphoryl moiety from the covalent conjugate phosphorylcholinesterase (10). This reactivation process involves interaction between the oximate anion and the positively charged phosphorus atom, yielding an unstable phosphoryloxime intermediate. Thus, oximes generally interact with esters and show a preference for phosphate esters. A variety of esters are present in membranes, and one might thus expect to find such interactions of oximes with membranal components. Thus, the partial inactivation of the muscarinic receptor might be attributable to a nucleopholic attack on an ester bond by the oxime. The ineffectiveness of SAD-128 (which does not contain the oxime moiety) in reducing the number of brainstem receptors is consistent with this suggestion.

The regional heterogeneity of the oxime effect is apparent when comparing the extent of irreversible loss of receptor sites ($\sim$ 30%) in the brainstem to the significantly smaller loss ($\sim$ 10%) in the cortex. This difference as well as the fixed and final number of receptors lost in the brainstem suggest that a specific receptor subpopulation is sensitive to the oxime effect. The bisquaternary pyridinium oximes cause a similar loss of brain stem receptors when injected into rats, indicating that this sensitive subpopulation of receptors exists _in situ_ in the intact cells. From previous studies, we know that higher affinity toward agonists is exhibited by muscarinic receptors from the brainstem than from the cerebral cortex. This property of agonist binding reflects the apparent existence of a higher proportion of high affinity agonist-binding sites in the brainstem than in the cortex. Thus, the oxime-induced loss of receptors can be correlated with the existence of high affinity agonist-binding sites. In this context, it should be noted that differences in muscarinic antagonist activities were reported in several cases. Thus, gallamine was shown to selectively antagonize heart (38) and brainstem (39, 40) muscarinic receptors, as opposed to pirenzepine, which selectively blocks muscarinic receptors in peripheral ganglia and cerebral cortex.

In conclusion, the results presented here point to the possible existence on the musarinic receptor of a second site/component through which bisquaternary oximes act.

2. Allosteric interactions between muscarinic agonist binding sites and effector sites demonstrated by the use of bisquaternary pyridinium oximes

As discussed above, bisquaternary oximes such as HGG-12 are allosteric inhibitors of muscarinic receptors which may be capable of distinguishing between receptor states. Moreover, these oximes were found to induce specific irreversible effects - namely, a loss of about 30% of antagonist binding sites in the brainstem and $\sim$10% in the cortex. The loss of antagonist binding sites does not correspond specifically to a loss of one class of agonist sites. This is evidenced by the lack of effect of the oxime treatment on the proportion of high affinity agonist binding sites. This phenomenon is especially indicative in the brainstem, where more sites are lost upon oxime treatment than in the cortex.

Agonist binding sites were affected in the two brain regions under investigation by pretreatment with HGG-12 (2, 19). Since in these experiments no free oxime was present, it is safe to conclude that the observed decrease in oxotremorine affinity was due to an irreversible effect during the preincubation with oxime rather than due to an apparent reduction in the agonist equilibrium binding constant. The fact that pretreatment with oxime alters the affinity of agonists to the muscarinic sites, but not the proportions of high and low affinity sites, suggests that the bisquaternary pyridinium oximes do not act as, e.g., guanine nucleotides. The latter induce conversion of high affinity binding sites to the low affinity state (42-44).

Bisquaternary pyridinium oximes inactivate the process of conversion of high affinity binding sites (R_H) to the low affinity state (R_L)and vice versa (2, 19). This suggests that a common step is involved in these two interconversion processes. In both cases, occupation of high affinity agonist binding sites by the agonist protects a component involved in these processes against inactivation by oximes (2, 19). This indicates that the binding of agonist to the muscarinic recognition site induces a conformational change which is in turn reflected in the site of oxime action. As expected in such site-site interactions, occupation of the oxime site also induces changes in the muscarinic recognition site, as evidenced by changes in the modes of antagonist and agonist binding.

It should be noted that in the interconversion of R_L to R_H induced by Cu^{2+} ions, the newly formed high affinity sites are clearly different from the preexisting ones; i.e.,they lack the sensitivity to guanine nucleotides ((45) and references therein). It is not known whether high affinity agonist binding sites induced by transition metal ions (43) are identical with the native R_H; however, they appear to behave similarly, at least in their responsiveness to Gpp(NH)p-induced interconversion. It seems therefore reasonable to assume that a nucleotide binding protein is coupled to these sites (R_H sites), especially in light of the recent demonstration of pertussis toxin-induced conversion of high affinity binding sites to the low affinity state (46). The site of action of the transition metal ions is not known. Since Co^{2+}-induced R^H can be reconverted to R_L in the presence of guanine nucleotides, it is felt that divalent transition metal ions bring about an association between the low affinity state and a nucleotide binding protein, resulting in the formation of high affinity state. The inactivation by oxime of both the Co^{2+} and the Gpp(NH)p effects (2, 19) provides evidence for the suggested association between R_H sites and the nucleotide binding protein. Moreover, by the use of the oxime an agonist-dependent mechanism can be demonstrated; that is, occupation of high affinity sites by agonist protects both conversion of R_H to R_L (brainstem) and conversion of R_L to R_H (cortex) from inactivation by oxime. The findings are inconsistent with a hypothesis in which R_H and R_L are independent of each other, and suggest close association between the two sites. One would otherwise have to speculate that agonist-occupied R_H sites protect the conversion of R_L to R_H from inactivation by oximes by means of an intermediate formed as a consequence of R_H occupation. There is no evidence for such an intermediate; however, there are data which support both heterotrophic and homotrophic site-site interactions in the muscarinic receptor (47).

Several important characteristics that one would expect to find in a system in which site-site interactions occur, have yet to be demonstrated for the muscarinic receptor. The postulated steps of association and dissociation between the muscarinic recognition sites and the nucleotide binding protein have not been directly demonstrated. Nor do we have any data on the stoichiometry of ligand binding to the muscarinic receptor molecule. In spite of these gaps in our knowledge, data on the inactivation by oximes of the interconversion process and the protection against such inactivation by the agonist carbamylcholine (2, 19) provide supportive evidence for the existence of site-site interactions in the muscarinic acetylcholine receptor.

Finally, it is important to note that the bisquaternary pyridinium oximes should not be regarded as drugs which interact only with the muscarinic receptor. It is well know, for example, that they interact with acetylcholinesterase. It is reasonable to assume that they may also interfere with the activity of other receptors, as they do with the muscarinic receptors, via sites distinct from the agonist binding site.

[illegible]. Rat brainstem muscarinic receptor loss and inhibition of binding are related to different substitutions of the pyridinium rings

Each of the bisquaternary drugs under study was tested for its ability to induce loss of muscarinic receptors in the brainstem. All of the drugs which proved effective in this respect resulted in a similar irreversible loss (25%-30%) of these receptors (Table I). Preincubation of membranes in the presence of an antagonist (1 μM atropine) or agonist (25 μM carbamylcholine) did not protect the receptors from inactivation by the oximes. None of the oximes could induce a further decrease in [^{3}H]-4NMPB binding sites, even when the concentration of the oxime was increased, its preincubation period extended, or the membranes subjected to repeated tratment with the oxime. This suggests that a distinct subpopulation of brainstem muscarinic receptors is sensitive to the bisquaternary pyridinium oximes. Experiments using 200 μM SAD-128, a bisquaternary pyridinium analog which lacks the oxime moiety, showed that this drug did not induce loss of brainstem muscarinic receptors (Table I). Moreover, the bisquaternary oximes Toxogonin and TMB-4, drugs with an oxime residue at position 4, also failed to induce this effect (Table I). The results obtained with HGG-12, HGG-42, HGG-52 and HI-6 are consistent with the conclusion that the oxime moiety at position 2 of the pyridinium ring is responsible for the selective loss of brainstem muscarinic receptors. We have previously shown (2, 19) that concomitantly with the loss of brainstem muscarinic receptors, agonist binding can no longer be modulated by the allosteric guanine nucleotide effector Gpp(NH)p (35-37). Thus oxime induced loss of receptors and loss of receptor sensitivity to guanine nucleotides seem to be related phenomena. Additional support for this suggestion comes from experiments with TMB-4. This oxime does not induce loss of binding sites, nor does it induce loss of sensitivity to guanine nucleotides. It is worth noting that oximes generally interact with esters, and that a variety of ester bonds are present in membranes; thus the partial inactivation of the muscarinic receptor might be attributable to nucleophilic attack on an ester bond by the oxime.

The potency of the bisquaternary pyridinium analogs in reversibly inhibiting $[^3H]$-4NMPB binding is reflected in their apparent inhibition constants (Table 1). The most potent inhibitors were HGG-42, HGG-12 and SAD-128 ($K_I = 1.5 - 7.0$ μM). Toxogonin, TMB-4 and HI-6 were far less potent ($K_I = 40-100$ μM). The higher potency of the analogs in the first group points to the significance of the hydrophobic substituent at position 3 (HGG-12, HGG-42) or position 4 (SAD-128) of the pyridinium ring. Interestingly, the K_I values obtained in several previous studies (3-6) using the guinea pig ileum are very close to those described here. Kuhnen-Clausen et al. (5) also referred to the importance of the hydrophobic substituent. Comparison between the reversible and the irreversible activities of the bisquaternary pyridinium analogs reveals different structural requirements for the two effects: the former requires a hydrophobic residue at position 3 or 4 of the pyridinium ring and the latter an oxime residue at position 2. Thus, an analog such as SAD-128 binds to the muscarinic receptor with higher affinity than HGG-52, but unlike the latter, it does not induce loss of receptors. The analog HI-6, unlike Toxogonin, induces loss of binding sites, but its muscarinic binding affinity is lower than that of Toxogonin.

The results described here for brainstem muscarinic receptors suggest that bifunctional oximes such as HGG-12 act at two different sites of the receptor. These sites clearly do not include the antagonist binding site, since atropine did not protect against the loss of receptor binding sites. Moreover, the reversible inhibition of bispyridinium derivatives is allosteric in character (2, 18). Although we do not know whether this twofold property of the drug would be beneficial in cases of organophosphorus poisoning, its irreversible effects on rat brainstem muscarinic receptors have also been observed *in vivo* (2). This, as well as the relatively high blood levels of bisquaternary pyridinium derivatives (50-100 μM) achieved at therapeutic doses (16), suggests the possibility that the synergistic effects of oximes and muscarinic antagonists such as atropine (15, 17) may reflect their simultaneous activity on the postsynaptic muscarinic receptors. Clearly, the oxime-induced loss of receptor sites, which is not blocked by atropine or carbamylcholine, occurs in the presence of both drugs. Also, it is likely that the bisquaternary oximes will not inhibit antagonist binding to the receptors (as they do *in vitro* when the receptors are exposed simultaneously to the oxime and antagonist). This possibility is supported by kinetic data which indicate that oximes reduce the dissociation rate of receptor-antagonist complexes (2, 18); namely, it is expected that in the presence of oximes, the off-rate of the atropine-receptor complex will be reduced, resulting in a long-lasting antagonist receptor complex.

In summary, all of the bisquaternary derivatives tested could reversibly inhibit binding of the muscarinic antagonist $[^3H]$-4NMPB, with higher inhibitory potency being exerted by drugs possessing hydrophobic substituents at position 3 or 4 of the pyridinium ring. In addition to their reversible effects, the bisquaternary drugs possessing an oxime moiety at position 2 of the pyridinium ring could also induce irreversible loss of about 30% of brainstem muscarinic receptors. Thus the structural correlates of the reversible and irreversible effects of these drugs are different. Bifunctional oximes, possessing one pyridinium ring with hydrophobic moiety at position 3 and a second pyridinium ring with an oxime moiety at position 2, are thus likely to affect the muscarinic receptor at two different sites.

4. Bisquaternary pyridinium oximes as postsynaptic muscarinic antagonists and presynaptic muscarinic agonists

Bisquaternary pyridinium oximes have been shown to possess mild antimuscarinic activity (3-6), i.e., these drugs inhibit the ACh-induced contraction of the guinea pig ileum. Accordingly, at postsynaptic receptors they mimic the activity of muscarinic antagonists such as atropine. The apparent inhibition constants of the oximes determined in the guinea pig ileum preparation are close to those determined in binding experiments performed with brain homogenates prepared from rats (2,8, 19) or mice (6) (see also Table I). These results were to be expected, since most of the muscarinic receptors in the brain are postsynaptic, and since in both the guinea pig ileum (3-6) and the brainstem (2, 18), the oximes behave as allosteric inhibitors. Previous results have indicated that oxime inhibit the binding of [^{3}H]-4NMPB to presynaptic receptors (2), although the nature of their activity at these receptors was unknown. We examined this activity by investigating the effects of the drugs on the release of [^{3}H]-ACh from rat brainstem tissue slices. All of the bisquaternary pyridinium drugs under study, like the muscarinic agonists, inhibited the K^+-evoked release of [^{3}H]-ACh in a dose-dependent manner, and this activity could be blocked by atropine. We therefore conclude that at presynaptic muscarinic autoreceptors, the bisquaternary pyridinium drugs are cholinomimetic. Thus a unique property of these nonclassical muscarinic drugs makes them both postsynaptic antagonists and presynaptic agonists. Although we know from binding studies (2) that they inhibit binding of [^{3}H]-4NMPB to both pre- and postsynaptic muscarinic receptors in an allosteric manner, we do not know the exact mechanism of their presynaptic cholinomimetic activity. They could be interacting either dirctly with the ACh binding site or at a site distinct from it; the blocking effect of atropine on the inhibition by oximes of K^+-evoked [^{3}H]-ACh release supports the first possibility.

The exact structural requirements for the cholinomimetic activity of oximes at presynaptic receptors are not yet known. However, a comparison between the potencies of the various analogs at post- and presynaptic muscarinic receptors (Tables I and II) reveals some interesting differences between the activities at the two locations. For postsynaptic antimuscarinic activity, a hydrophobic moiety at position 3 or 4 of the pyridinium ring is important (K_I values of HGG-12, HGG-42, SAD-128 are lower than thos of TMB-4 and Toxogonin).

On the other hand, the presence of these hydrophobic moieties makes the drug (SAD-128, HGG-12, HGG-42) a weak cholinomimetic at the presynaptic receptors. The [illegible] oximes (TMB-4, Toxogonin and HI-6) are better inhibitors of K^+-evoked [^{3}H]-ACh release than HGG-12 or HGG-42. It is important to note that the most potent inhibitor of [^{3}H]-ACh release, TMB-4 (K_I = 8.0 μM), is [illegible] more potent than HGG-42 or HGG-12 (Table II), while the latter are [illegible] than TMB-4 at postsynaptic receptors (Table I). It follows that TMB-4 is a rather selective cholinomimetic at presynaptic autoreceptors, whereas HGG-42 and HGG-12 are selective postsynaptic antagonists.

[illegible] and HI-6 were found to be equipotent as inhibitors of [^{3}H]-ACh release, [illegible] is [illegible] a higher significance for such inhibition to the [illegible] at position [illegible] than at another position of the pyridinium ring.

As discussed in the previous section, the presence of an oxime moiety at position 2 of the pyridinium ring is necessary for the induced loss of sensitivity of muscarinic receptors to guanine nucleotides. This, together with the pronounced activity of TMB-4 at presynaptic muscarinic receptors, indicates that inhibition of [^{3}H]-ACh release by oximes does not involve inactivation of the interaction between guanine nucleotides binding protein and the muscarinic receptor.

This conclusion is in line with previous data on the mechanism of muscarinic inhibition of [^{3}H]-ACh release: in presynaptic preparations from Torpedo electric organ, inhibition of ACh released by muscarinic agonists does not proceed via inhibition of adenylate cyclase activity (38).

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