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Bisquaternary Pyridinium Oximes as Allosteric Inhibitors of Rat Brain Muscarinic Receptors

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SUMMARY

The mode of interaction of bisquaternary pyridinium oximes with rat brain muscarinic receptors in cerebral cortex and brain stem preparations was studied by the use of the tritium-labeled antagonist *N*-methyl-4-piperidyl benzilate ([^{3}H]4NMPB). Binding of the labeled muscarinic antagonist was inhibited by these drugs, the most potent inhibitors being 1-(2-hydroxyiminoethylpyridinium)-1-(3-cyclohexylcarboxypyridinium)dimethyl-ether (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 brain stem 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 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 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.

INTRODUCTION

Bisquaternary pyridinium oximes represent a group of drugs which were originally designed and synthesized as reactivators of the enzyme acetylcholinesterase following organophosphate poisoning (1, 2). Several organophosphates (such as soman) form a phosphoryl-enzyme adduct which cannot be reactivated by oximes (3, 4); nevertheless, the mixture of some bisquaternary oximes and muscarinic antagonists still affords some partial protection against soman intoxication (5, 6). 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 (7–9) and inhibit the binding of [^{3}H]4NMPB,[1] a potent muscarinic antagonist (10, 11). 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 (9). Kuhnen-Clausen (8), 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

[1] The abbreviations used are: 4NMPB, *N*-methyl-4-piperidyl benzilate; QNB, quinuclidinyl benzilate; HGG-42, 1-(2-hydroxyiminoethylpyridinium)-1-(3-cyclohexylcarboxypyridinium)dimethyl ether; HGG-12, 1-(2-hydroxyiminoethylpyridimium)-1-(3-phenylcarboxypyridinium)dimethyl ether; HGG-52, 1-(2-hydroxyiminoethylpyridinium)-1-(3-isobutylcarboxypyridinium)dimethyl ether; HI-6, 1-(2-hydroxyiminoethylpyridinium)-1-(4-carboxyaminopyridinium)dimethyl ether; SAD-128, bis-1,1′-(4-tertbutylpyridinium)dimethyl ether.

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potency of several bisquaternary pyridinium analogs 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 (9). 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.

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 the effects of bispyridinium oximes on muscarinic antagonist binding and on the antagonist-induced isomerization of the muscarinic sites (12–16). The drugs under study represent a new class of oximes which were recently shown to possess antimuscarinic activity (17).

EXPERIMENTAL PROCEDURES

Materials. [^{3}H]4NMPB (70 Ci/mmol) was prepared by catalytic tritium exchange as described previously (14). Its purity was >97%. SAD-128 and HI-6 were prepared as described previously (9) L-[^{3}H]QNB (33.2 Ci/mmol) and *N*-[*methyl*^{3}H]scopolamine (50 Ci/mmol) were from New England Nuclear. 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 (18); the purity in each case was >96% as determined by TLC in 1-butanol:acetic acid:H_2O (4:1:1). Fresh solutions of the bisquaternary pyridinium analogs were prepared by dissolving the drugs in water prior to each experiment. We were unable to detect degradation products of the drugs by means of TLC during incubation with tissue homogenates.

Methods. Cerebral cortex and brain stem of 3–4-month-old CD rats were dissected out in a cold room as described (9). The two brain regions were each pooled and homogenized in 0.32 M sucrose to yield a 3–10% homogenate (w/v). Whole homogenates were used for binding assays as follows: 50 μl of tissue preparation (containing 0.2–0.5 mg of protein) were incubated at 25° in 2 ml of modified Krebs-Henseleit 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 7.4, containing varying amounts of labeled ligand and oximes. After incubation for 60 min (unless otherwise indicated), ice-cold Krebs solution (3 ml) was added and the contents were collected rapidly by suction through glass filters (Whatman GF/C, 25-mm-diameter). The filters were washed three times in 3 ml of ice-cold Krebs solution. The filtration and washing procedures were completed in less than 10 sec. Binding assays were performed in triplicate, together with triplicate samples containing 50 μM unlabeled atropine. The filters were placed in vials containing 4 ml of scintillation liquid (Hydro-Luma, Lumac Systems, Titusville, FL) 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%.

Specific binding was defined as total binding minus nonspecific binding, i.e., binding in the presence of 50 μM unlabeled atropine. Variations between triplicate determinations were less than 10%, unless otherwise indicated.

Both in binding studies at equilibrium and in kinetic binding studies, inhibitors and labeled ligands were added simultaneously except where membranes were pretreated with the inhibitor or when dissociation kinetics were measured. In the latter case, the inhibitor was added at zero dissociation time to membranes which had been equilibrated for 30 min with 5 nM [^{3}H]4NMPB, then precipitated at 30,000 × *g* for 20 min, and resuspended in the Krebs buffer.

The procedure for pretreatment of membranes with bisquaternary pyridinium analogs was as follows. Homogenates were incubated in Krebs buffer in the presence of the indicated concentration of inhibitor for 60 min (unless otherwise indicated) at 25°. Protein concentration was 2.0–2.5 mg of protein/ml. Following incubation, the membranes were precipitated at 30,000 × *g* for 20 min and the supernatant was discarded. The membranes were resuspended in Krebs buffer 3 times the volume of the original incubation medium and precipitated again. This washing procedure was repeated three times. The final pellet was resuspended in a small volume of Krebs buffer to yield a membrane preparation of 5–6 mg of protein/ml, which was used for binding assays as described above. Control homogenates received similar treatment in inhibitor-free Krebs buffer.

For *in vivo* experiments with HGG-12, male rats (180–230 g) were injected subcutaneously with various doses of HGG-12 in saline. Control rats were injected subcutaneously with saline. All of the injected doses (3–20 mg/kg) were much lower than the LD_{50} dose, so that none of the rats died. At various times following the injection (0.5–2 hr), the rats were killed by decapitation, and in each case the cerebral 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 the *in vitro* experiments (30,000 × *g*, 20 min each). The final membrane suspension was assayed for muscarinic receptor binding as described above.

Analysis of kinetic binding data. Data relating to the time course of [^{3}H]4NMPB binding to the receptor were initially plotted in the form of first order plots for a simple bimolecular reaction (Eq. 1), as described in previous reports (12, 16, 19):

$$(B_{eq} - B_t)/B_{eq} = e^{-(k_2 + k_1 L)t} \quad (1)$$

B_t is bound labeled ligand at time t, B_{eq} is bound labeled ligand at equilibrium, k_1 is the second order rate constant for association of [^{3}H]4NMPB (L), and k_2 is the first order dissociation rate constant. In agreement with previously described data on the binding of [^{3}H]4NMPB (13, 14, 19) or [^{3}H]QNB (12, 15, 16), the pseudo-first order plots could not be fitted to a simple bimolecular reaction curve since they deviated from linearity. Instead, the data were fitted to the isomerization model (see Eq. 4 in Results), using Eq. 2 according to Frost and Pearson (20) as described in a simplified form by Galper *et al.* (16):

$$B_{eq} - B_t/B_{eq} = Ae^{-\lambda_2 t} + (1 - A)e^{-\lambda_3 t} \quad (2)$$

where B_{eq} and B_t are defined as in Eq. 1; $\lambda_2 = (p + q)/2$; $\lambda_3 = (p - q)/2$; $p = k_1' + k_{-1} + k_2 + k_{-2}$; $q = [p^2 - 4(k_1' k_2 + k_{-1}k_{-2} + k_1' k_{-2})]^{1/2}$; $A = \lambda_3(\lambda_2 - k_2 - k_{-2})/q(k_2 + k_{-2})$; k_1 and k_{-1} are respectively the second order and first order rate constants for the rapid step of the formation of receptor-ligand (RL) complex, while k_2 and k_{-2} are the first order rate constants of the forward and backward isomerization steps, respectively. k_1' is a pseudo-first order rate constant which is given by the product of k_1 and the initial ligand concentration.

The time course of dissociation of the receptor-ligand complexes was analyzed as described previously (14, 16), according to Eq. 3:

$$B_t/B_0 = \alpha e^{k_{-1} t} + (1 - \alpha)e^{-k_{-2} t} \quad (3)$$

where B_0 is bound ligand at zero dissociation time, B_t is bound ligand at time t, k_{-1} and k_{-2} are the first order rate constants as in Eq. 2, and $\alpha = k_{-1}/(k_{-1} - k_{-2}) \times [RL]/([RL] + [R'L])$. RL and $R'L$ are the concentrations of the rapidly and slowly dissociating complexes, respectively, at zero dissociation time. Kinetic parameters were calculated by the use of a nonlinear regression computer program, as described in detail previously (14).

RESULTS

Concentration dependent inhibition of [^{3}H]4NMPB binding by bisquaternary pyridinium oximes. In the first part of the study, we examined the effects of several bisquaternary pyridinium oximes (Fig. 1) on binding of the antagonist [^{3}H]4NMPB to muscarinic receptors in two distinct brain regions of the rat, brain stem and

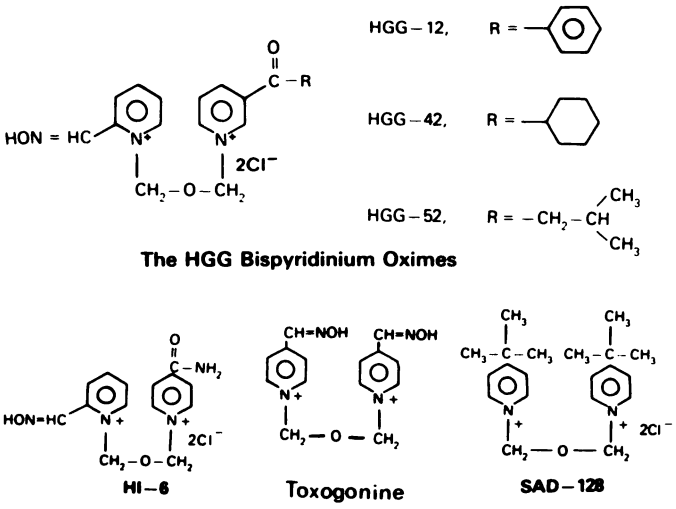

FIG. 1. *Structure of bisquaternary pyridinium analogs*

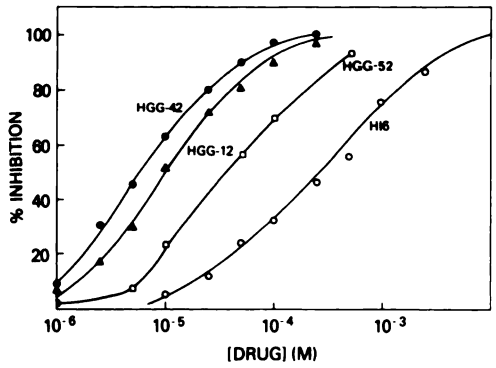

FIG. 2. *Concentration-dependent inhibition of [³H]4NMPB binding to muscarinic receptors in the rat brain stem*

Binding of 2.0 nM [³H]4NMPB to rat brain stem homogenates (0.2 mg of protein) in the presence of various concentrations of bispyridinium oximes, HGG-42, HGG-12, HGG-52, and HI-6, was determined as described in Methods. Percentage of inhibition is shown in relation to the inhibitor concentration.

cerebral cortex. We compared muscarinic receptors in these two regions because they show different characteristics with respect to both agonist and antagonist binding (19, 21–26). Thus, antagonist dissociation kinetics are faster in the brain stem, and this region displays a higher proportion of high affinity agonist-binding sites.

Several bisquaternary pyridinium analogs (structure shown in Fig. 1) were tested as inhibitors of [³H]4NMPB binding. In these experiments, the concentration of [³H]4NMPB was held constant (2.0 nM) and the concentration-dependent inhibition of binding of labeled antagonist was measured. Preliminary experiments indicated that the binding of [³H]4NMPB approached equilibrium more slowly in the presence than in the absence of the inhibitors. Because of this effect on the kinetics of antagonist binding (discussed later), it was necessary to incubate the reaction mixtures for 60 min in order for equilibrium to be reached. Typical inhibition curves obtained with brain stem preparation are presented in Fig. 2; similar curves were obtained with cerebral cortex preparation. For both preparations, the rank order of potency was HGG-42 > HGG-12 > HGG-52 > HI-6. Pseudo-Hill coefficients of the inhibition curves were only slightly less than 1.0, and the binding of [³H]4NMPB could be completely blocked at high oxime concentrations (Fig. 2).

Effect of bisquaternary pyridinium oximes on [³H]-4NMPB-binding isotherms. Typical curves of [³H]-4NMPB binding in the absence and in the presence of various concentrations of HGG-42 and HGG-12 are shown in Fig. 3, A and B (brain stem preparation), and in C and D (cerebral cortex preparation). These binding curves have important characteristics, as shown by their rightward shift as compared to controls as well as by an apparent tendency to reach saturation at values lower than the maximal binding capacity (B_{max}) at high inhibitor concentration. Similar phenomena were observed with HGG-12 (5–100 μM), when another muscarinic antagonist [³H]QNB was used in the binding assays (not shown). Fig. 4, A and B, depicts Scatchard plots of [³H]4NMPB-binding data. These plots of [³H]4NMPB binding to muscarinic receptors in either the brain stem (Fig. 4A) or the cortex (Fig. 4B) were linear both in the absence and in the presence of low inhibitor concentrations (<10 μM). The experimental data readily allow extrapolation of the Scatchard plots to B_{max} values. We could therefore estimate the K_I values from the apparent dissociation constants of [³H]4NMPB (K_{app}), assuming mutually exclusive binding, according to Eq. 4 ($K_{app} = K_d + K_d \cdot I/K_I$). These values were 1.0 ± 0.5 and 2.6 ± 0.3 μM for HGG-42 and HGG-12, respectively (cortex), and 1.5 ± 0.5 and 4.0 ± 1.9 μM for HGG-42 and HGG-12 (brain stem).

In the presence of oxime concentrations higher than 10 μM, [³H]4NMPB binding tended to become saturated at values lower than B_{max} (Fig. 4). This phenomenon could not be simply due to competitive inhibition by the high oxime concentration present, since the hypothetical [³H]4NMPB-binding isotherms calculated from K_I values estimated on the basis of a model of mutually exclusive binding did not fit the observed binding isotherms (Fig. 3A). Moreover, the Scatchard plots were curvilinear and demonstrated an apparent decrease in B_{max}. It should be noted that the reduction in B_{max} is only apparent, since an accurate extrapolation to B_{max} under conditions such as those obtained in the presence of high inhibitor concentrations is not possible (27).

Reversible and irreversible effects of bisquaternary pyridinium oximes on muscarinic antagonist binding. In order to determine whether the apparent reduction in B_{max} following oxime treatment is reversible, cortical membranes were incubated for 60 min in the presence of 200 μM HGG-12 and then washed with inhibitor-free buffer. After three washings, the membranes were used for binding experiments. Scatchard plots of [³H]4NMPB binding to these membranes were linear, parallel to those observed with untreated membranes, and extrapolated to B_{max} values ~10% lower than those of controls (Fig. 5A). In contrast to these findings, muscarinic receptors present in the brain stem were irreversibly affected by both HGG-42 and HGG-12. After preincubation with either 200 μM HGG-12 (Fig. 5B) or 200 μM HGG-42 (Fig. 5C), about 30% of the muscarinic binding sites could no longer

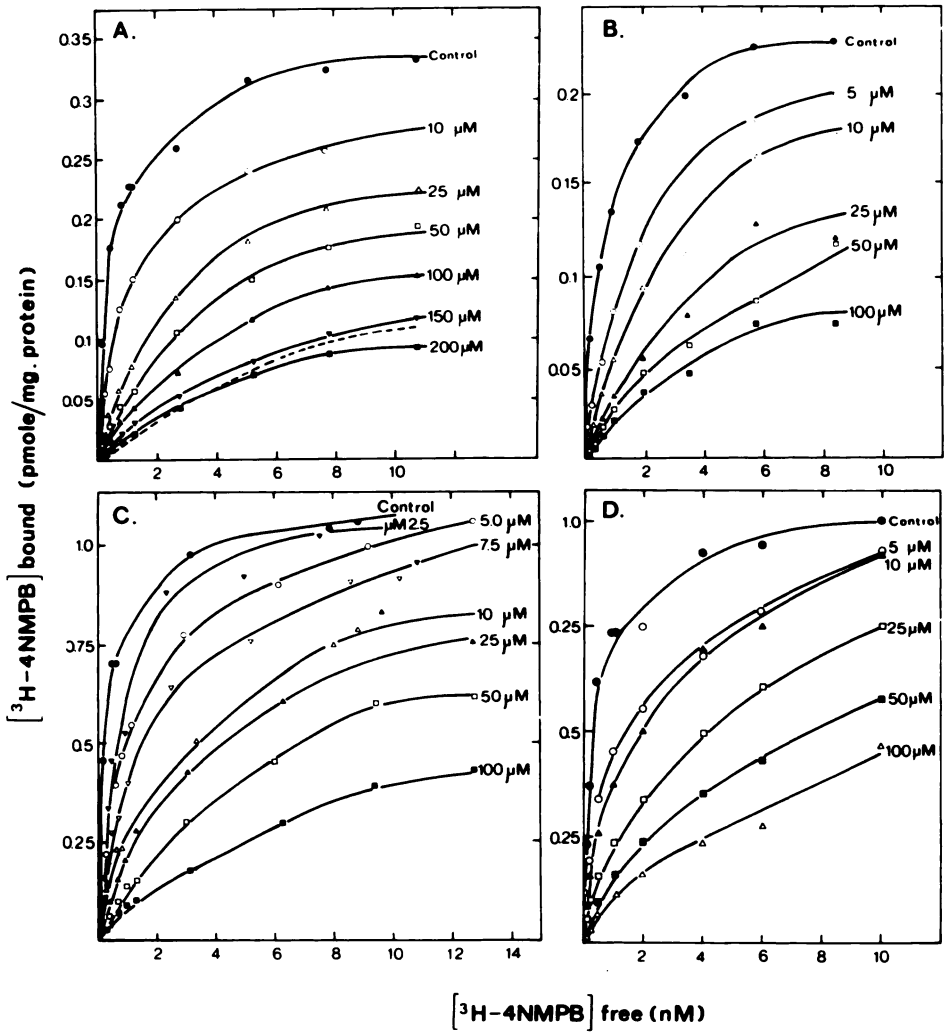

FIG. 3. *Effect of bisquaternary pyridinium oximes on the binding isotherms of [³H]4NMPB*

The data represent specific binding of [³H]4NMPB to muscarinic receptors from either the brain stem (A and B) or the cerebral cortex (C and D), in the absence and in the presence of various concentrations of HGG-42 (A and C) and HGG-12 (B and D). Inhibitor concentrations are noted. Binding was determined as described in Methods, in triplicate samples containing 0.2–0.3 mg of protein (brain stem) and 0.3–0.4 mg of protein (cortex). Lines along experimental data were drawn by hand. The dashed line in A represents a hypothetical curve calculated for [³H]4NMPB binding in the presence of 50 μM HGG-42, assuming mutually exclusive binding and the parameters ([³H]4NMPB) K_d = 0.6 nM and K_I = 1.6 μM).

bind the labeled antagonist. Scatchard plots of [³H]-4NMPB binding to the remaining binding sites were linear, and the apparent binding constant of the ligand was similar to that observed with untreated brain stem muscarinic receptors (Fig. 5). Similar results were obtained in analogous experiments with the muscarinic antagonists [³H]QNB and *N*-[*methyl*-³H]scopolamine; using these ligands, a decrease of 29 and 25%, respectively, was observed in B_{max}.

These results led us to conclude that the apparent decrease in B_{max} observed when brain stem muscarinic receptors were incubated with oximes reflects a true loss of part of the binding sites. In this context, it should be noted that the presence of muscarinic antagonists that compete on the muscarinic sites (e.g., 1 μM atropine) did not protect from the irreversible effects of the oximes. The rest of the sites in this brain region, as well as in the cerebral cortex, do not lose their ability to bind the labeled muscarinic antagonist. Binding of [³H]4NMPB to these sites is inhibited by the oximes as well, as the K_I values for HGG-12 and HGG-42 determined in the brain stem and in the cortex were between 2.5 and 3.5 μM.

Effects of bisquaternary pyridinium oximes on the kinetics of [³H]4NMPB receptor interactions. As pointed out earlier, binding of [³H]4NMPB to muscarinic receptors in the presence of oximes was slower than that observed in their absence. The reduced rate of [³H]-4NMPB association to muscarinic receptors in cortical membranes is apparent in the presence of HGG-42 even at concentrations as low as 10 μM (Fig. 6A). A similar effect on the binding of [³H]4NMPB to brain stem muscarinic receptors was observed (not shown). The dissociation rate of [³H]4NMPB-receptor complexes was also decreased in the presence of HGG-42 (shown in Fig. 6B for brain stem preparation). This effect of the drug

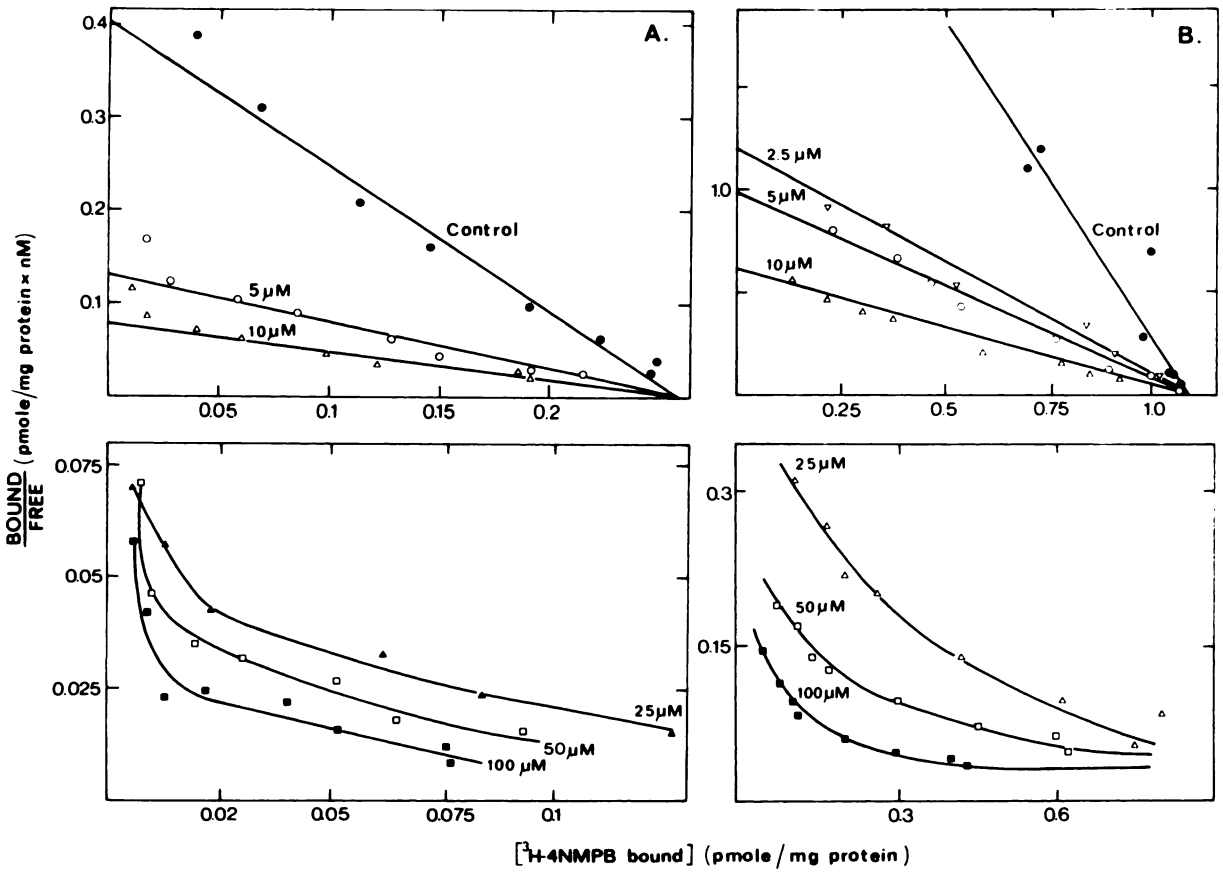

FIG. 4. *Scatchard plots of [³H]4NMPB binding*

The binding data presented in Fig. 3 were replotted according to Scatchard. A, binding of [³H]4NMPB to brain stem muscarinic receptors in the absence (●) and presence of the indicated concentrations of HGG-12. B, binding of [³H]4NMPB to cerebral cortex muscarinic receptors in the absence (●) and presence of the indicated concentrations of HGG-42. Because of the strong inhibition of [³H]4NMPB binding by oximes at a high concentration, the data were plotted on expanded scales (lower parts of A and B), and lines were drawn by hand. Lines in upper parts of A and B were linear as indicated by the correlation coefficient (r) in the linear regression analysis: A: control (r = 0.99), 5 μM (r = 0.98). B: control (r = 0.98), 2.5 μM (r = 0.99), 5.0 μM (r = 0.99).

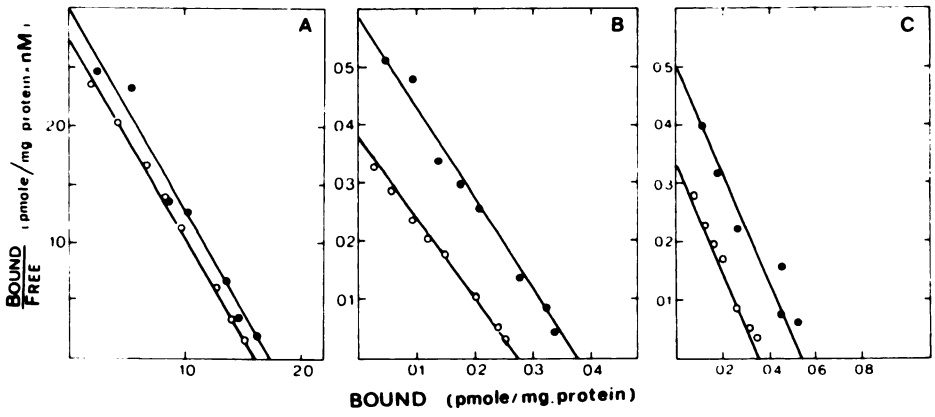

FIG. 5. *Binding of [³H]4NMPB to muscarinic receptors in oxime-treated membranes*

Membranes were incubated for 60 min at 25° in the presence of HGG-12 or HGG-42. The inhibitors were washed out by precipitation of the membranes and washings with inhibition-free buffer, as described in Methods. The washed membranes were used for binding assays under the standard conditions. Control membranes were preincubated and washed under the same conditions, but in the absence of inhibitors. Data are presented in the form of Scatchard plots. A, binding of [³H]4NMPB to cerebral cortex membranes untreated (●) and treated with 200 μM HGG-12 (○). B, binding of [³H]4NMPB to brain stem membranes untreated (●) and treated with 200 μM HGG-12 (○). C, binding of [³H]4NMPB to brain stem membranes untreated (●) and treated with 100 μM HGG-42 (○).

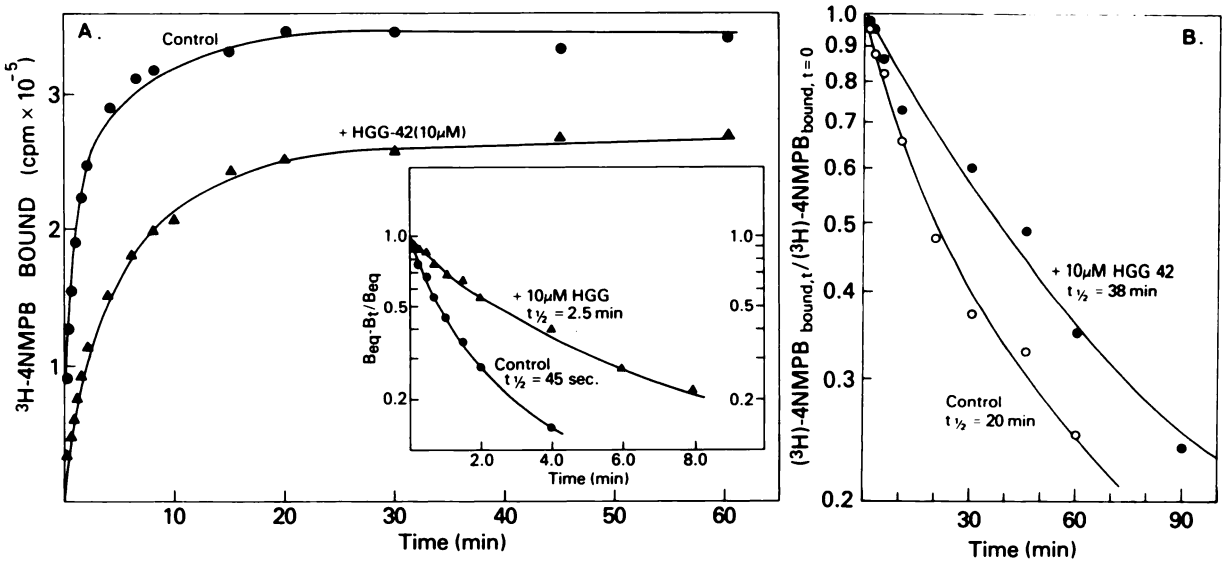

FIG. 6. *Effect of HGG-42 on the time course of [^{3}H]4NMPB association to and dissociation from the muscarinic receptor*

A, kinetics of [^{3}H]4NMPB binding to cortical muscarinic receptors in the absence (control) and presence of 10 μM HGG-42. Binding was determined as described in Methods at the indicated times. Labeled ligand (2.0 nM) and inhibitor were added simultaneously. Data are presented as [^{3}H]4NMPB bound as a function of time. Lines were drawn by hand. Inset: the same data replotted according to Eq. 1 (see Methods). B, time course of dissociation of [^{3}H]4NMPB-receptor complexes measured with brain stem preparation in the absence (control) (○) and presence (●) of 10 μM HGG-42. Membranes were incubated for 30 min in the presence of 5 nM [^{3}H]4NMPB and then precipitated and resuspended in ligand-free buffer or buffer containing 10 μM HGG-42. Samples were filtered immediately ($t = 0$) or at the indicated times (t). Solid lines represent the computerized best fit according to Eq. 2 (see Methods).

was observed in all experiments (six separate experiments) and was independent of the degree of receptor occupancy. If HGG-42 had affected [^{3}H]4NMPB recognition sites directly, the rate of dissociation of [^{3}H]-4NMPB-receptor complexes should have remained unaltered. At most, estimates of the apparent rate might have been inflated due to occupation of free binding sites, thus preventing reassociation of labeled ligand. A decrease in the dissociation rate (Fig. 6B) can be induced only by allosteric interactions between the oxime-binding sites and [^{3}H]4NMPB sites. This conclusion is in agreement with the effects of HGG-42 on the equilibrium binding of [^{3}H]4NMPB as described above.

First order plots of both association and dissociation kinetics of [^{3}H]4NMPB are biphasic (12–16), and remain so even in the presence of oximes (Fig. 6). The biphasic kinetics of muscarinic antagonist binding are thought to reflect ligand-induced isomerization (12–16) according to the scheme:

$$L + R \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} RL \underset{k_{-2}}{\overset{K_2}{\rightleftarrows}} R^*L \qquad (4)$$

where k_1 is the second order rate constant for formation of the ligand-receptor complex (RL), k_{-1} is the first order rate constant for its dissociation, and k_2 and k_{-2} are the first order rate constants for the reaction in which the isomer (R^*L) is formed and is reversed, respectively.

The values of k_{-1} and k_{-2} were estimated by fitting the dissociation kinetics data of each experiment to the sum of two exponential terms, as described by Eq. 3 in Methods. Errors of the asymptotic standard deviations of k_{-1} and k_{-2}, estimated by the computer analysis, were less than 5%. The average values of the constants obtained in these experiments (mean ± standard deviation; n = 6) are $k_{-1} = 0.28 \pm 0.05$ min^{-1} and $k_{-2} = 0.16 \pm 0.01$ min^{-1} for the control and $k_{-1} = 0.31 \pm 0.07$ min^{-1} and $k_{-2} = 0.06 \pm 0.02$ min^{-1} in the presence of oxime. Thus, the most pronounced effect of HGG-42 was on k_{-2}; the rate of [^{3}H]4NMPB-receptor complex isomerization was altered in the presence of the drug. Such an effect would in turn also change the association rate of [^{3}H]4NMPB to its receptor.

In a further set of experiments, we examined whether irreversible effects of bisquaternary pyridinium oximes are also apparent in the kinetics of [^{3}H]4NMPB binding. Brain stem or cortical membranes were treated with 200 μM HGG-12, then washed extensively, and the rate of [^{3}H]4NMPB association to its receptors was measured in the oxime-treated and in control membranes. The results of these experiments (Fig. 7, A and B) indicate that the decrease rate of [^{3}H]4NMPB binding observed in the presence of oxime (Fig. 6A) persists even after extensive washing of the drug. The first order plots were still biphasic, like those of controls (Fig. 7). Since the oxime was washed out in these experiments, we could analyze the association kinetics according to the isomerization model, using the biexponential equation (see Methods, Eq. 2) and on the assumption that the concentration of free oxime was negligible. As in the above case of the data obtained by dissociation kinetics, the k_{-2} values obtained under the conditions of the association experiments (Table 1) were decreased due to the oxime treatments. The forward first order isomerization rate constant (k_2) was increased, while k_1 and k_{-1} were less affected (Table 1). Both in the brain stem and in the cortex, the ratio k_{-2}/k_2 was decreased upon oxime treat-

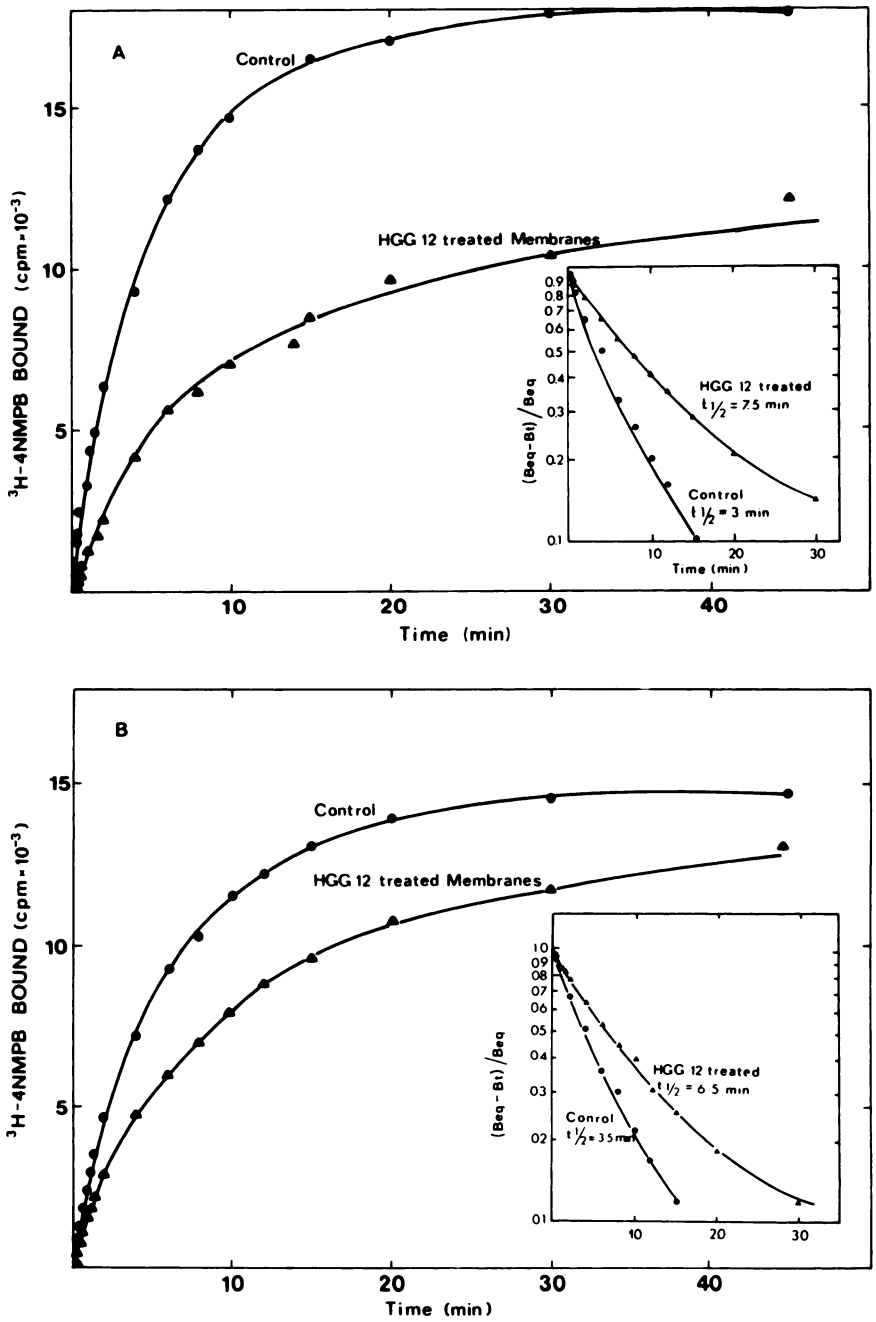

FIG. 7. *Time course of [^{3}H]4NMPB binding to muscarinic receptors pretreated with HGG-12*

Membranes from the brain stem (A) or the cerebral cortex (B) were treated with 200 μM HGG-12 for 60 min and then washed, as described in Methods. Binding to control (●) and oxime-treated (▲) membranes was determined at the indicated times, as described in Methods, in the presence of 0.29 (brain stem) or 0.9 nM (cortex) [^{3}H]4NMPB. Data are presented as [^{3}H]4NMPB bound as a function of time. Solid lines represent the computerized best fit lines according to Eq. 2 (see Methods). The parameters of several such experiments are summarized in Table 1. Insets: the same binding data replotted according to Eq. 1 (see Methods).

ment (from 0.9 to 0.15 in the brain stem and from 0.7 to 0.17 in the cortex). This indicates that the oxime induces an increase in the degree of NMPB-induced isomerization.

Characterization of the oxime-induced loss of brain stem muscarinic receptors. The time course of receptor loss upon incubation of brain stem membranes in the presence of 200 μM HGG-12 is shown in Fig. 8A. The data show that the effect is rapid and develops within 15 min. No further decrease in binding sites was observed after incubation periods of 20 and 60 min. As shown in Fig. 8B, the extent of receptor loss was also dependent on the concentration of HGG-12 (I_{50} ~60 μM). Maximal effect (~30% loss) occurred in the presence of 200 μM HGG-12 and did not increase at higher oxime concentrations. Thus, it seems that only a subpopulation (30%) of the sites is sensitive to inactivation by the oxime. It should be noted that reincubation with 200 μM HGG-12 did not yield a further decrease in the binding capacity of the muscarinic sites. In order to examine whether oxime-

TABLE 1

Rate constants for [^{3}H]4NMPB binding determined in control and HGG-12-treated membranes

Rate constants were derived from kinetic experiments as described in Fig. 7 using Eq. 2 in Methods. Each experiment was repeated four times and each curve was analyzed separately to yield the rate constants. The constants in the table are the mean ± standard error of these values. $\bar{K}_d$ was calculated from the relationships $\bar{K}_d = [k_1/k_{-1}\,(1 + k_2/k_{-2})]^{-1}$ and K_d from equilibrium binding data described in Fig. 5.

	Cortex		Brain stem	
	Control	Treated	Control	Treated
k_1 (M^{-1} min^{-1})	0.15 ± 0.06 × 10^9	0.11 ± 0.02 × 10^9	0.21 ± 0.04 × 10^9	0.13 ± 0.05 × 10^9
k_{-1} (min^{-1})	0.16 ± 0.04	0.20 ± 0.05	0.43 ± 0.12	0.64 ± 0.3
k_2 (min^{-1})	0.15 ± 0.05	0.41 ± 0.1	0.26 ± 0.09	0.8 ± 0.15
k_{-2} (min^{-1})	0.11 ± 0.03	0.07 ± 0.01	0.23 ± 0.06	0.12 ± 0.02
k_{-1}/k_1 (M)	1.1 × 10^{-9}	1.8 × 10^{-9}	2.04 × 10^{-9}	4.9 × 10^{-9}
k_{-2}/k_2	0.73	0.17	0.88	0.15
$\bar{K}_d$ (M)	0.45 × 10^{-9}	0.27 × 10^{-9}	0.96 × 10^{-9}	0.65 × 10^{-9}
K_d (M)	0.39 × 10^{-9}	0.4 × 10^{-9}	0.71 × 10^{-9}	0.61 × 10^{-9}

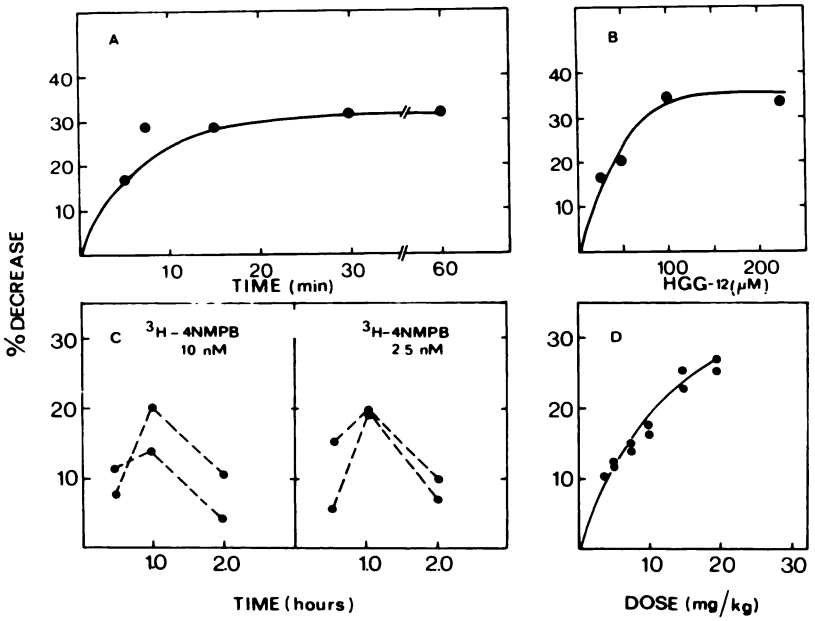

FIG. 8. *Time course and dose dependence of in vitro and in vivo inactivation of brain stem muscarinic receptors by HGG-12*

Data are presented as per cent decrease in binding capacity with respect to control. A, time dependence of inactivation of brain stem muscarinic receptors by 200 μM HGG-12. Binding capacity was determined in control and oxime-treated membranes as described in Fig. 5. Each point represents the average of three separate determinations with 10 nM [^{3}H]4NMPB. The mean value at 5 min differed significantly from controls ($p < 0.01$, $n = 3$), and was significantly lower than that recorded at 15 min ($p < 0.025$, $n = 4$) (Student's t test). B, concentration dependence of the inactivation of brain stem muscarinic receptors pretreated for 60 min with oxime. Binding was determined in control and oxime-treated membranes as described in Fig. 5, using 10 nM [^{3}H]4NMPB. Each point represents the average of three separate determinations. C, time course of the effect of HGG-12 on brain stem muscarinic receptors after subcutaneous injection of the drug. Rats were injected with HGG-12 (10 mg/kg) and sacrificed at the indicated times. Brain stems were removed and homogenized, and the membranes were washed three times with buffer and then tested for their binding capacity in triplicate samples with 2.5 and 10.0 nM [^{3}H]4NMPB. Data from two separate experiments are presented. The mean value at 1.0 hr was significantly higher than that recorded at 30 min and at 2 hr ($p < 0.05$, $n = 4$ and $p < 0.025$, $n = 4$, respectively; Student's t test). D, dose dependence of oxime-induced decrease in brain stem muscarinic receptors following subcutaneous injection of the drug. Rats were injected with the indicated dose of HGG-12 and sacrificed after 1 hr. Muscarinic receptors in the brain stem were determined in triplicate samples with 10 nM [^{3}H]4NMPB in washed membranes prepared as described in Methods. Data from two separate experiments are presented.

sensitive subpopulations of muscarinic sites also exist *in situ*, we injected HGG-12 into rats and measured [^{3}H]-4NMPB binding in membranes prepared from cerebral cortex and from brain stem. The membranes were prepared and washed as in our *in vitro* studies, with the omission of the preincubation period. The results of these experiments are summarized in Fig. 8, C and D. Loss of muscarinic receptors was clearly observed in the brain stem but not in the cortex. In the brain stem, receptor loss was maximal about 1 hr after injection of HGG-12 (10 mg/kg). Unlike the receptor loss *in vitro*, the *in vivo* effect was reversible as evidenced by the recovery of binding sites 2 hr after the injection (Fig. 8C). Nevertheless, the maximal decrease in binding sites observed *in vivo*, like that *in vitro* in the presence of the oxime, was about 30% (Fig. 8D).

Among the functional groups possessed by biquaternary pyridinium oximes, the most obvious candidate for irreversibly affecting muscarinic binding sites is the oxime group. In a previous report, it was shown that the bisquaternary pyridinium analog SAD-128, which does not possess an oxime group (Fig. 1), is a potent inhibitor of [^{3}H]4NMPB binding (9). We therefore examined the reversibility of the effects of SAD-128 on [^{3}H]4NMPB binding to brain stem muscarinic receptors. The membranes were incubated in the presence of 200 μM SAD-128 for 60 min and then washed as described above. As shown in Fig. 9, [^{3}H]4NMPB binding was strongly inhibited in the presence of SAD-128, but the effects of the drug were completely reversible, unlike the situation with HGG-12 or HGG-42. These results strongly suggest that the oxime group is necessary for the induced loss of muscarinic receptors.

DISCUSSION

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 both in brain stem 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 (28). 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 brain stem 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 (29, 30), pancuronium (29), and quinidine (31). The Scatchard plots of antagonist binding to the muscarinic receptor became nonlinear in the presence of, for instance, gallamine and pancuronium (29) and both binding inhibition and decreased dissocation rates from the muscarinic sites were observed in the presence of gallamine (29, 30) and quinidine (31). Another type of allosteric interactions with the muscarinic sites was observed with clomiphene (32), where the data were interpreted in terms of positive cooperativity. Dunlap and Brown (29) 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 brain stem 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 (33). Stockton *et al.* (30) 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 the effects of guanyl nucleotides. The bisquaternary pyridinium oximes do, however, interfere with the effects of guanyl nucleotides.[2]

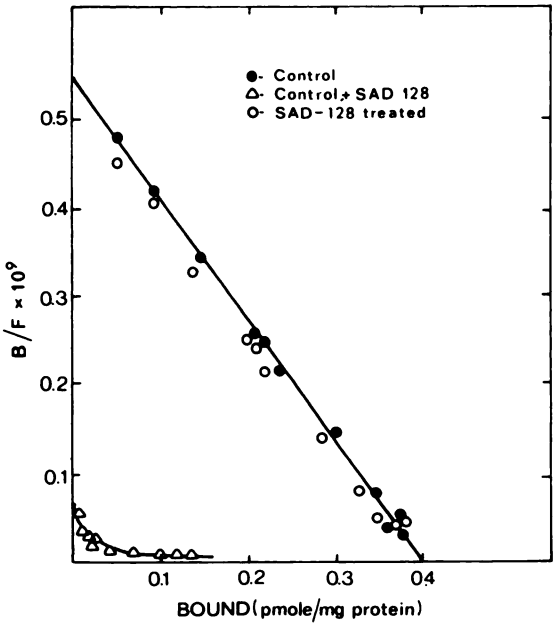

FIG. 9. *Scatchard plots of [^{3}H]4NMPB binding to brain stem muscarinic receptors in control and SAD-128-treated membranes*

Membranes were incubated in the presence of 200 μM SAD-128 for 60 min and then washed as described in Methods. The data represent binding of [^{3}H]4NMPB to control (●) and SAD-128-treated membranes (○). Biding of [^{3}H]4NMPB in the presence of 200 μM SAD-128 is strongly inhibited, as shown (△). *B/F*, bound over free ligand in picomoles/mg of protein × molar concentration.

The time course of the binding of muscarinic antagonists to their receptors proceeds according to a two-step isomerization pattern (12–16, 19) (shown in Eq. 4 of Results). We have therefore investigated the effects of the oximes on the isomerization step. Our results (Fig. 7, Table 1) demonstrate that HGG-12 alters the rates of isomerization of [^{3}H]4NMPB-receptor complexes both in cortical and in brain stem preparations. The kinetic parameters (Table 1) are increased 3- to 5-fold (k_2) and 2-fold (k_{-2}) according to the scheme:

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

The ratio of k_{-2}/k_2 is therefore decreased by roughly 4- to 6-fold due to the oximes, resulting in a higher proportion of slowly dissociating [^{3}H]4NMPB-receptor com-

[2] Y. Kloog and M. Sokolovsky, manuscript in preparation.

plexes. 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 (1, 2), 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 (1, 2). 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 nucleophilic 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 brain stem 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 (~30%) in the brain stem to the significantly smaller loss (~10%) in the cortex. This difference as well as the fixed and final number of receptors lost in the brain stem 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 brain stem 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 brain stem 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 (30) and brain stem (30, 34) muscarinic receptors, as opposed to pirenzepine, which selectively blocks muscarinic receptors in peripheral ganglia and cerebral cortex (34–36).

In conclusion, the results presented here point to the possible existence on the muscarinic receptor of a second site/component through which bisquaternary oximes act. These drugs could thus be employed as valuable tools in the identification of possible site-site interactions among muscarinic receptors[2] as well as in the characterization of the receptors' structure.

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