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The Binding of a 2-Chloroethylamine Derivative of Oxotremorine (BM 123) to Muscarinic Receptors in the Rat Cerebral Cortex

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SUMMARY

The interaction of a mustard analogue of oxotremorine, *N*-[4-(2-chloroethylmethylamino)-2-butynyl]-2-pyrrolidone (BM 123), with muscarinic receptors in the rat cerebral cortex was investigated using ^{3}H-ligand-binding methods. When cortical homogenates were preincubated with BM 123 (1.0 mM), washed extensively, and then assayed for the binding of the specific muscarinic antagonist, [^{3}H](−)-*N*-methylscopolamine, a decrease in binding capacity was noted without an accompanying change in affinity. The rate at which BM 123 alkylated muscarinic receptors was sensitive to temperature, with little or no receptor alkylation occurring at 0°. Thus, it was possible to estimate the affinity of BM 123 and its transformation products for muscarinic receptors by measuring their ability to competitively inhibit ^{3}H-ligand binding to cortical homogenates at 0°. When measured by competitive inhibition of [^{3}H]oxotremorine-M and [^{3}H](−)-*N*-methylscopolamine binding, the concentrations of the aziridinium ion of BM 123 required to displace 50% of specific ^{3}H-ligand binding were 3.5 nM and 4.5 μM, respectively. In contrast, the parent 2-chloroethylamine and its alcoholic hydrolysis product were much less active. The kinetics of the alkylation of muscarinic receptors by BM 123 were consistent with a model in which the aziridinium ion rapidly forms reversible complexes with superhigh high and low affinity sites which slowly convert to covalent complexes. The rate of alkylation of the superhigh affinity site was slowest whereas the converse was true for the low affinity site. It was possible to alkylate the high and low affinity sites selectively with BM 123 by taking advantage of kinetic differences in the rates of alkylation of these two sites. Atropine, oxotremorine, and oxotremorine-M antagonized the rate of alkylation of muscarinic receptors in a manner that was consistent with competitive inhibition.

INTRODUCTION

Derivatives of oxotremorine which bind covalently with the muscarinic receptor have been developed by substituting a 2-haloalkyl group on the basic nitrogen atom (1). Like nitrogen mustards, these compounds cyclize spontaneously in aqueous solution at neutral pH to form reactive aziridinium ions which can then alkylate suitable nucleophiles (1). One such derivative, BM 123 (*N*-[4-(2-chloroethylmethylamino)-2-butynyl]-2-pyrrolidone), forms an aziridinium ion (BM 123A) structurally similar to the potent muscarinic agonist oxo-M[1] (1) (see Fig. 1). Pharmacologically, BM 123 behaves as a potent muscarinic agonist, and this activity can be attributed almost entirely to its aziridinium ion (1). Prior treatment, both *in vivo* and *in vitro*, of various neuronal and muscular tissues with BM 123 causes a reduction in the binding capacities of the muscarinic antagonists, [^{3}H](−)-NMS and [^{3}H](−)-QNB, that persists after extensive washing and can be prevented by pretreatment with

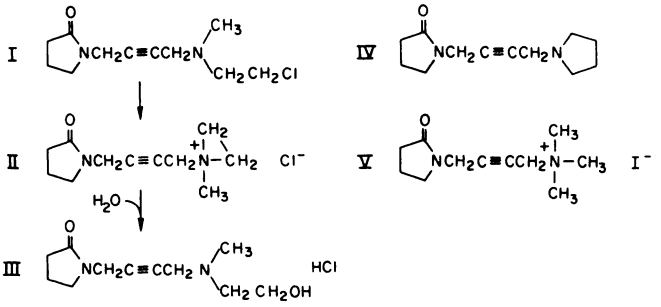

FIG. 1. *The structures of BM 123 (I), its aziridinium ion (II) and alcoholic hydrolysis product (III), oxotremorine (IV), and oxo-M (V)*

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[1] The abbreviations used are: oxo-M, oxotremorine-M; NMS, *N*-methylscopolamine; QNB, quinuclidinyl benzilate; CD, *cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxolane.

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atropine, thereby protecting the muscarinic receptor from irreversible alkylation (1–3). Collectively, these results are consistent with the postulate that BM 123A binds covalently to the recognition site of the muscarinic receptor. In this study we have examined in detail the influence of BM 123 on the binding properties of muscarinic receptors in the rat cerebral cortex.

In principle, the rate of alkylation of a receptor by a reactive ligand should be proportional to receptor occupancy. By measuring the rate of receptor alkylation over a range of ligand concentrations it should be possible to estimate both the rate constant for alkylation and the apparent affinity of the reactive ligand for the receptor. In the present report, the results of such a study are presented and are shown to be consistent with the postulate that BM 123A discriminates kinetically among different agonist subclasses of the muscarinic receptor in rat cerebral cortex. We have also estimated the affinity of the aziridinium ion by competitive inhibition of ^{3}H-ligand binding to the muscarinic receptor at 0°, conditions under which little or no receptor alkylation occurs, and have found general agreement among the estimates of the dissociation constants determined kinetically and at equilibrium.

MATERIALS AND METHODS

Formation of the aziridinium ion. Unless indicated otherwise, solutions of BM 123 were first incubated *in vitro* to allow formation of the aziridinium ion before the solution was used in an experiment. Stock solutions of BM 123 were made up at a concentration of 2.0 mM in 0.05 M phosphate buffer (81 mM Na^+, 9.5 mM K^+, 50 mM PO_4) at pH 7.4 or at a higher concentration of 20 mM in 0.1 M phosphate buffer (188 mM Na^+, 5.8 mM K^+, 100 mM PO_4) at pH 7.8. These solutions were incubated immediately at 37° for 40 min, placed on ice, and used as soon as possible. This incubation procedure represents conditions under which the aziridinium ion reaches its peak concentration of 28% of the original parent mustard concentration (1). BM 123 and its alcoholic hydrolysis product were obtained as described previously (1).

Tissue preparation. In most of the experiments described in this report, homogenates of rat cerebral cortex were incubated with BM 123 for a given length of time, washed extensively, and then assayed for ^{3}H-ligand binding to estimate the amount of receptors alkylated by BM 123. The tissue preparation and incubation conditions for these experiments were as follows. The freshly excised cerebral cortex from male Sprague-Dawley rats (200–250 g) was homogenized in 0.05 M phosphate buffer at pH 7.4 with a Potter-Elvehjem glass homogenizer and Teflon pestle to a concentration of approximately 2.5% (w/v). The homogenate was centrifuged at 30,000 × *g* for 10 min and resuspended in 0.05 M phosphate buffer containing $MgSO_4$ (1.0 mM) to a concentration representing 25 mg of original wet tissue weight/ml of buffer. The homogenate was pipetted (2-ml aliquots) into small plastic tubes and preincubated at 37° for 10 min in a Dubnoff shaking water bath. An aliquot (100 μl) of a solution of BM 123 that had been incubated *in vitro* to allow formation of the aziridinium ion was added, and the tissue was incubated further at 37° for various times. The reaction was stopped immediately by the addition of atropine (0.1 mM) and sodium thiosulfate (1.0 mM). The tissue was washed five times to remove the atropine, thiosulfate, BM 123, and its transformation products. Washing was accomplished by centrifugation at 27,000 × *g* for 10 min followed by resuspension of the pellet in 3 ml of phosphate-buffered saline (181 mM Na^+, 100 mM Cl^-, 9.5 mM K^+, 50 mM PO_4) at pH 7.4. After the first centrifugation, the pellets were resuspended in buffer containing thiosulfate (1.0 mM). After the second, third, and fourth centrifugations, the homogenates were incubated at 37° for 5, 10, and 20 min, respectively, to allow sufficient time for the dissociation of atropine that might have been tighly bound to the muscarinic receptor. After the fifth centrifugation the cortical pellets were frozen immediately. On the next day, when ^{3}H-ligand binding was measured, the pellets were thawed and resuspended to a concentration representing 10 mg of original wet tissue weight/ml of phosphate buffer. The washing procedure described above was sufficient to restore the binding of [^{3}H](−)-QNB to control levels following incubation of cortical homogenates with atropine (0.1 mM) for 1 h.

In other experiments, various nonlabeled drugs including BM 123 and its transformation products were included in the binding assay during the incubation of cortex with ^{3}H-ligand. For these experiments, rat cerebral cortex was homogenized as described above and centrifuged at 30,000 × *g* for 10 min. The pellet was resuspended in 0.05 M phosphate buffer to a concentration representing 10 mg of original wet tissue weight/ml of buffer.

Binding assays. The binding of the specific muscarinic antagonist [^{3}H](−)-QNB (33.4 Ci/mmol, New England Nuclear) was measured using the rapid filtration method of Yamamura and Snyder (4) with the following modifications. Brain homogenate (0.1 ml) was incubated with [^{3}H](−)-QNB in a final volume of 2 ml containing 0.05 M phosphate buffer, at pH 7.4, and 1.0 mM $MgSO_4$. The incubation was always carried out at 37° for 1 hr. Membrane-bound [^{3}H](−)-QNB was trapped subsequently by rapid vacuum filtration of the incubation mixture over glass fiber filters (Whatman, GF/B). The filters were rinsed with three aliquots (4 ml each) of ice-cold saline. All assays were run in triplicate, and nonspecific binding was determined in the presence of 10 μM atropine. Protein was measured by the method of Lowry *et al.* (5) using bovine serum albumin as the standard. The binding of the specific muscarinic antagonist [^{3}H](−)-NMS (84.8 Ci/mmol, New England Nuclear) was measured in the same manner as that described for [^{3}H](−)-QNB except for the following modifications. Since equilibrium is achieved more rapidly with [^{3}H](−)-NMS than with [^{3}H](−)-QNB (6), the incubation of tissue with [^{3}H](−)-NMS can be shortened and run at lower temperatures. In our experiments, incubations with [^{3}H](−)-NMS were done at 30° for 30 min or 0° for 60 min. The incubations were carried out in small plastic scintillation vials (4 ml, Omnivials), and membrane-bound radioactivity was trapped at the end of the incubation by centrifugation at 27,000 × *g* for 10 min. The pellets were washed superficially with two aliquots (3 ml each) of ice-cold saline, and the tubes were inverted and allowed to dry for 1 hr. The insides of the tubes were blotted dry without disturbing the pellet, and scintillation cocktail was added. The tubes were then vortexed immediately to resuspend the membranes in the scintillation fluid.

The procedure for measuring the binding of the specific muscarinic agonists [^{3}H](+)-*cis*-2-methyl-4-dimethylaminomethyl-1,3-dioxolone methiodide ([^{3}H](+)-CD, 38.1 Ci/mmol, New England Nuclear) and [^{3}H]oxo-M (82.5 Ci/mmol, New England Nuclear) was essentially the same as that described for [^{3}H](−)-NMS except that more tissue was used in the incubation (10 mg, based on original wet tissue weight). The incubations were run at 0° for 1 hr.

Kinetic theory. The kinetics of the alkylation of the muscarinic receptor by BM 123A were investigated over a range of BM 123 concentrations so that it would be possible to determine both the affinity of the aziridinium ion for the receptor and its rate constant for alkylation. The kinetic parameters were estimated from the experimental data by nonlinear least squares regression analysis according to the Eqs. 5 and 6, derived below.

The simplest model to describe the interaction of BM 123A (*A*) with the muscarinic receptor (*R*) is:

$$A + R \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} AR \xrightarrow{k_2} A\text{-}R \qquad (1)$$

where k_{+1} and k_{-1} are the rate constants for association and dissociation, respectively, of the reversible BM 123A-receptor complex (*AR*), and k_2 is the rate constant for formation of the alkylated receptor complex (*A-R*). If the values of k_{+1} and k_{-1} are large with respect to k_2, then the

rate of loss of unalkylated receptors can be described by the following differential equation:

$$\frac{dR_f}{dt} = \frac{-k_2 A R_f}{A + K_A} \quad (2)$$

In this equation, R_f is equal to the unalkylated receptors ($R_f = R + AR$) and K_A is equal to the apparent dissociation constant of the AR receptor complex ($K_A = k_{-1}/k_{+1}$). During an incubation of BM 123 with muscarinic receptors at 37°, the concentration of A does not remain constant. In a previous study of the formation and hydrolysis of BM 123A in phosphate buffer at neutral pH, it was shown that the aziridinium ion reaches its peak concentration 40 min after dissolution in aqueous buffer at 37° and that its concentration slowly declines to half of its peak concentration approximately 60 min later (1). Regression analysis of the data published by Ringdahl *et al.* (1) showed that the aziridinium ion decayed from its peak concentration of 28% in a manner well described by the simple exponential equation:

$$A = A_0 e^{-k_0 t} \quad (3)$$

where A_0 is the peak concentration of the aziridinium ion, k_0 is the observed rate constant for decay of the aziridinium ion ($k_0 = 0.0117$ min^{-1}), and t denotes time after the aziridinium ion reaches its peak concentration. As described above, BM 123 was first incubated for 40 min at 37° to allow the aziridinium ion to reach its peak concentration before incubation with tissue homogenate so that the aziridinium ion concentration could be predicted at various times during the incubation by the simple exponential equation (Eq. 3). Substituting Eq. 3 for A in Eq. 2 yields:

$$\frac{dR_f}{dt} = \frac{-k_2 A_0 e^{-k_0 t} R_f}{A_0 e^{-k_0 t} + K_A} \quad (4)$$

Integrating Eq. 4 and evaluating the integral over the time interval beginning with $t = 0$ yields:

$$Y = P\left(\frac{A_0 e^{-k_0 t} + K_A}{A_0 + K_A}\right)^{k_2/k_0} \quad (5)$$

In this equation, Y is the percentage of remaining unalkylated receptors and P is the estimate of 100% binding. The value of Y was estimated by measuring ^{3}H-ligand binding to cortical membranes which had been previously incubated with BM 123 for various times. The kinetics of the loss [^{3}H](−)-QNB-binding sites were adequately described by the two-site version of Eq. 5:

$$Y = P\left\{a\left(\frac{A_0 e^{-k_0 t} + K_H}{A_0 + K_H}\right)^{k_H/k_0} + (1-a)\left(\frac{A_0 e^{-k_0 t} + K_L}{A_0 + K_L}\right)^{k_L/k_0}\right\} \quad (6)$$

where K_H and K_L are the dissociation constants and k_H and k_L are the rate constants of alkylation of the high and low affinity sites, respectively, and a is the proportion of high affinity sites.

Analysis of equilibrium-binding data. The equilibrium-binding parameters of [^{3}H](−)-NMS and nonlabeled drugs were determined by fitting the experimental data to the equations described below using nonlinear least squares regression analysis. The binding data for [^{3}H](−)-NMS were calculated assuming that both enantiomers of racemic [^{3}H](−)-NMS bound nonspecifically to the same extent but that only the (−)-enantiomer contributed to specific binding. The dissociation constant (K_{NMS}) and receptor capacity (B_{max}) of [^{3}H](−)-NMS were determined by regression analysis of the ^{3}H-ligand binding isotherm according to the following equation:

$$B = XB_{max}/(X + K_{NMS}) \quad (7)$$

where B is specifically bound [^{3}H](−)-NMS and X is the free concentration of [^{3}H](−)-NMS. The binding parameters of nonlabeled ligands were determined by measuring their ability to inhibit ^{3}H-ligand binding competitively and by fitting the data to the one- and two-site equations (Eqs. 8 and 9) shown below:

$$Y = P\frac{1}{1 + x/K'} \quad (8)$$

In this one-site equation, Y is the per cent of ^{3}H-ligand bound, P is the estimate of 100% ^{3}H-ligand bound in the absence of the inhibitor, x is the concentration of nonlabeled inhibitor, and K' is the apparent dissociation constant of the nonlabeled inhibitor. In the following two-site equation

$$Y = P\left(\frac{a}{1 + x/K_H'} + \frac{(1-a)}{1 + x/K_L'}\right) \quad (9)$$

a is the proportion of high affinity sites and K_H' and K_L' are the apparent dissociation constants of the high and low affinity sites, respectively. The apparent dissociation constants (K') were corrected to give the true dissociation constants (K) using the following equation:

$$K = K'/(1 + y/K_y) \quad (10)$$

where y is the concentration of the ^{3}H-ligand and K_y is the dissociation constant of the ^{3}H-ligand, which was determined independently. The concentration of nonlabeled inhibitor required for half-maximal occupation of receptors (X_{50}) was calculated from the IC_{50} value (concentration of inhibitor that caused half-maximal inhibition of specific ^{3}H-ligand binding) according to the following equation:

$$X_{50} = IC_{50}/(1 + y/K_y) \quad (11)$$

As described below, low concentrations of [^{3}H]oxo-M and [^{3}H](+)-CD were used to label selectively a subpopulation of agonist-binding sites designated as the superhigh affinity site. The dissociation constants (K_Y) of oxo-M and (+)-CD for the site were determined by running oxo-M/[^{3}H]oxo-M and CD/[^{3}H](+)-CD competition experiments and correcting the respective IC_{50} values according to the following equation which is a special case of Eq. 10:

$$K_Y = IC_{50} - y \quad (12)$$

where y is the concentration of [^{3}H]oxo-M or [^{3}H](+)-CD used in the experiment which was 1.0 or 2.0 nM, respectively.

RESULTS

Alkylation of specific binding sites by BM 123. Treatment of the rat cerebral cortex with BM 123 caused an irreversible inhibition of the binding of [^{3}H](−)-NMS. Homogenates of the rat cerebral cortex were incubated with BM 123 for 20 min and 1 hr and then washed extensively. Control homogenates were treated identically except for exposure to BM 123. Measurements of the specific binding of [^{3}H](−)-NMS were made at various ^{3}H-ligand concentrations, and these results are shown in Fig. 2. Nonlinear regression analysis showed that the control binding measurements (○) were consistent with a single binding site having a dissociation constant of 0.044 nM and a binding capacity of 0.68 pmol/mg of protein. BM 123 treatment caused a selective reduction in the binding capacity of [^{3}H](−)-NMS without significantly affecting the apparent affinity. Cortical homogenates that had been incubated with BM 123 (1.0 mM) for 20 and 60 min showed reduced binding capacities for [^{3}H](−)-NMS of 0.16 and 0.078 pmol/mg of protein, respectively. Analysis of variance showed no significant increase in residual error when the data were fitted by nonlinear regression analysis sharing the control estimate of the dissociation constant among the three binding isotherms ($F_{2,12} = 0.136$, $P = 0.87$).

Experiments were run to determine if a recovery of [^{3}H](−)-NMS binding could be detected in homogenates

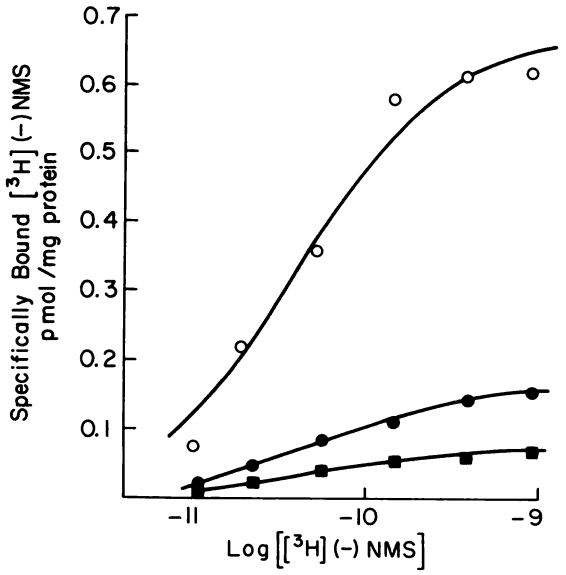

FIG. 2. *The effect of BM 123 treatment on [³H](−)-NMS binding to homogenates of the rat cerebral cortex*

Specific [³H](−)-NMS binding was measured at 30° in control homogenates (○) and homogenates which had been incubated at 37° with BM 123 (1.0 mM) for 20 min (●) and 60 min (■). The data points represents the mean binding values of five experiments, each done in triplicate. The curves represent the least squares fit to the data.

that had been treated with BM 123 and then incubated at 37° for periods up to 4 hr. In these experiments, homogenates of rat cerebral cortex were incubated with BM 123 (10 μM) for 20 min at 37°. The reaction was stopped by a 20-fold dilution with 1.0 mM thiosulfate in 0.05 M phosphate buffer. The homogenate was incubated an additional 20 min at 37° to inactivate the residual aziridinium ion and then washed twice by centrifugation at 30,000 × *g* for 10 min followed by resuspension in fresh phosphate buffer. The homogenate was then incubated at 37° for 4 hr. At the beginning and at various times (0.5, 1, 2, and 4 hr) during the incubation, aliquots of the homogenate were removed and assayed for [³H](−)-NMS binding at 0° using a ³H-ligand concentration of 0.4 nM. In control homogenates, specific [³H](−)-NMS binding declined from an initial value of 0.574 ± 0.031 pmol/mg of protein to 0.485 ± 0.017 pmol/mg of protein after 4 hr at 37°. Specific [³H](−)-NMS binding in the BM 123-treated homogenate was initially 0.113 ± 0.008 pmol/mg of protein and did not change significantly during the 4-hr incubation at 37°. When specific [³H](−)-NMS binding in the BM 123-treated homogenate is expressed as a per cent of control, the binding values increase from an initial value of 20% to a value of 24% after incubation of the homogenate at 37° for 4 hr. Whether this small apparent rate (1%/hr) of recovery of binding is due to a dissociation of the alkylated receptor complex or to proteolysis of receptors in the control homogenate is unclear from the present data.

Experiments were run to determine the effect of tissue concentration on the rate of muscarinic receptor alkylation by BM 123. Homogenates of the rat cerebral cortex were incubated with BM 123 (1 μM) for various times, washed extensively, and then assayed for [³H](−)-QNB binding. The results in Fig. 3 show that the relative rate at which BM 123 caused an irreversible inhibition of [³H](−)-QNB binding, expressed as a per cent of control, was independent of tissue concentration from 5–25 mg of original wet tissue weight/ml. The half-time for the loss of [³H](−)-QNB binding was approximately 40 min in both cases. The remainder of the kinetic experiments was run using a final tissue concentration of 25 mg of original tissue (wet weight)/ml of buffer as described under "Materials and Methods." No significant inhibition of [³H](−)-QNB binding was observed when the incubation of brain homogenate with BM 123 (1.0 μM) was carried out in the presence of sodium thiosulfate (1.0 mM) (see Fig. 3). Also, no significant inhibition of [³H](−)-QNB binding was detected in cortical homogenates which had been treated simultaneously with BM 123 (1.0 mM) and atropine (0.1 mM) and then incubated for 1 hr at 37° (data not shown). These results showed that the addition of atropine (0.1 mM) to an incubation of BM 123 with cortical homogenate immediately stopped the covalent reaction even when BM 123 was present at concentrations as high as 1.0 mM.

The rate at which BM 123 alkylated [³H](−)-QNB-binding sites in cortical homogenates was sensitive to temperature. Reducing the incubation temperature from 37° to 25° caused a 7-fold increase in the half-time for the loss of [³H](−)-QNB-binding sites when cortical homogenates were incubated with BM 123 at a concentration of 10 μM (data not shown). At 0°, no significant alkylation of [³H](−)-QNB-binding sites occurred after incubating homogenates for 1 hr with BM 123 at concentrations from 1.0–100 μM. After incubating cortical homogenates with 1.0 mM BM 123 for 1 hr at 0°, a 10% loss in [³H](−)-QNB binding was measured.

Affinity of BM 123 and its transformation products for binding sites. The slow rate at which BM 123 inactivated [³H](−)-QNB-binding sites at low temperature (0°) en-

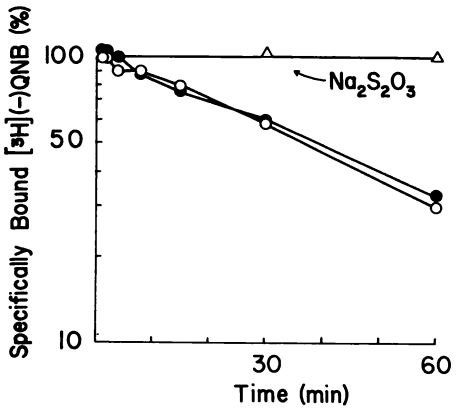

FIG. 3. *The effect of tissue concentration and sodium thiosulfate (1.0 mM) on the rate of alkylation of [³H](−)-QNB-binding sites by BM 123 (1.0 μM)*

Homogenates of the rat cerebral cortex were incubated at 37° with BM 123 for the indicated times, washed extensively, and then assayed for specific [³H](−)-QNB binding at 37° using a ³H-ligand concentration of 0.4 nM. The concentration of the cortical homogenates were 5 mg (○) and 25 mg (●, △) of original wet tissue weight/ml of phosphate buffer. The data points represents the mean binding values of 3 experiments, each done in triplicate.

abled with affinity of BM 123 and its transformation products to be determined in competition experiments with [^{3}H]oxo-M and [^{3}H](−)-NMS. Preliminary binding experiments with [^{3}H]oxo-M and [^{3}H](−)-NMS on the rat cerebral cortex showed that equilibrium was achieved within 1 hr at 0° and that binding measurements after 1–3 hr of incubation were essentially equivalent. To assess the potency of the aziridinium ion, aliquots of a solution of BM 123 that had been incubated *in vitro* at 37° for 40 min to allow formation of the aziridinium ion were added directly into the competitive binding assay at 0°. The data were calculated assuming that the concentration of the aziridinium ion was equivalent to 28% of the parent mustard concentration. To determine the potency of the parent mustard, solutions of BM 123 were made up in dilute HCl at 0° to prevent formation of the aziridinium ion, and aliquots of these solutions were added to the competitive binding assay in the presence of 0.1 mM sodium thiosulfate. The addition of thiosulfate to the assay did not significantly affect the ligand/[^{3}H] oxo-M competition curves. It can be seen in Fig. 4 that BM 123A and oxo-M have nearly equal affinity for the binding site labeled by [^{3}H]oxo-M with their respective IC_{50} values being 3.5 and 2.8 nM. In contrast, the parent 2-chloroethylamine (BM 123) and the alcoholic hydrolysis product were 100–200-fold less potent with IC_{50} values of 0.63 and 2.5 μM, respectively. Previous experiments by Birdsall *et al.* (7) have shown that at a concentration of 1.0 nM, [^{3}H]oxo-M labels almost exclusively a very high affinity agonist subpopulation of muscarinic receptors designated as the superhigh affinity site. The dissociation constant (K_{SH}) of oxo-M, BM 123, and its transformation products for the superhigh affinity site were calculated from their respective IC_{50} values and are given in Table 1.

Fig. 5 shows the competitive inhibition of [^{3}H](−)-NMS binding by oxo-M, BM 123, and its transformation products. It can be seen that oxo-M and the aziridinium ion have similar IC_{50} values of 6.3 and 4.5 μM, respectively, although the shapes of their respective competition curves differed slightly. Both the parent 2-chloroethylamine and the alcoholic hydrolysis product were less active, and both had IC_{50} values of approximately 0.32 mM. Nonlinear regression and analysis of variance showed a significant reduction in residual error when the competition curves of oxo-M and BM 123A were fitted to a two-site model as compared to a one-site model. The dissociation constants of the high and low affinity sites (K_H and K_L) and the relative proportion of high affinity sites are given in Table 1. Analysis of variance showed a significant increase in residual error when the oxo-M and BM 123A competition data were fitted by nonlinear regression analysis sharing the estimate of K_L between the data ($F_{2,13} = 9.603$, $p < 0.01$). The competition curves of the parent 2-chloroethylamine and the alcoholic hydrolysis product were not examined in detail, but their respective IC_{50} values were corrected to give the X_{50} values shown in Table 1.

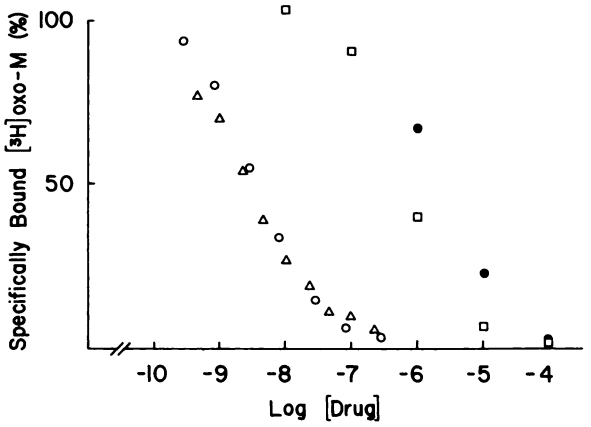

FIG. 4. *The competitive inhibition of [^{3}H]oxo-M binding to the rat cerebral cortex by oxo-M (△), BM 123 (□), its aziridinium ion (○), and alcoholic hydrolysis product (●)*

The specific binding of [^{3}H]oxo-M was measured at 0° in the presence of the indicated concentrations of the various compounds. The concentration of [^{3}H]oxo-M was 1.0 nM. Each data point represents the mean binding value of 3 experiments, each done in triplicate.

TABLE 1

The binding parameters of oxo-M, BM 123, and its transformation products

The binding parameters were estimated from the data shown in Figs. 4 and 5.

Compound	K_{SH}	K_H	K_L	X_{50}	High affinity sites
	nM	*nM*	*μM*	*μM*	%
oxo-M (V)[a]	1.8	19.7	3.06	0.93	31
BM 123A (II)	2.25	12.9	1.23	0.66	25
BM 123 (I)	405			47	
Alcohol (III)	1607			47	

[a] The Roman numerals in parentheses refer to the structures of the compounds shown in Fig. 1.

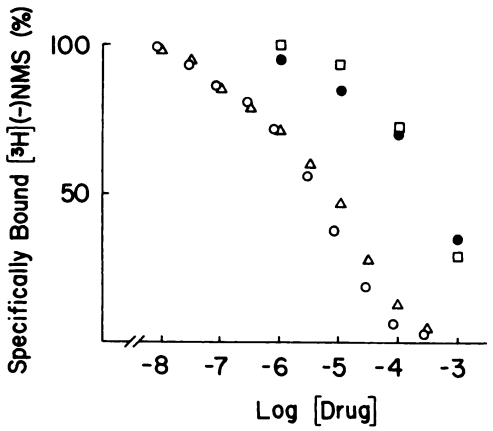

FIG. 5. *The competitive inhibition of [^{3}H](−)-NMS binding to the rat cerebral cortex by oxo-M (△), BM 123 (□), its aziridinium ion (○) and alcoholic hydrolysis product (●)*

The specific binding of [^{3}H](−)-NMS was measured at 0° in the presence of the indicated concentrations of the various compounds. The concentration of [^{3}H](−)-NMS was 0.15 nM. Each data point represents the mean binding value of five experiments, each done in triplicate.

Kinetics of the alkylation of the superhigh affinity site. The kinetics of muscarinic receptor alkylation by BM 123 were investigated by measuring the binding of both [^{3}H](+)-CD and [^{3}H](−)-QNB to cortical homogenates

that had been incubated with BM 123 for various times. The reaction of BM 123 with the muscarinic receptor was stopped immediately by the addition of atropine (0.1 mM) and thiosulfate (1.0 mM), and the tissue was subsequently washed five times as described under "Materials and Methods." Fig. 6 shows the loss of [^{3}H](+)-CD-binding sites following incubation of cortical homogenates with BM 123 (0.001–10.0 μM) for various times. To get a rough estimate of the observed rate constant for alkylation (k_{obs}) at various concentrations of BM 123, the curves shown in Fig. 6 were fitted independently to the simple exponential equation:

$$Y = 100 \times e^{-k_{obs}t} \quad (13)$$

where Y is the per cent of [^{3}H](+)-CD bound and t is time. The estimate of the observed rate constant for alkylation at the various concentrations of BM 123 are plotted in Fig. 7 as the open symbols (○) according to the ordinate scale on the left-hand side of the figure. The concentration of the aziridinium ion causing a half-maximal increase in k_{obs} was estimated to be 2.8 nM. Receptor occupancy for the aziridinium ion was determined independently by measuring the per cent displacement of [^{3}H](+)-CD binding to cortical homogenates by BM 123A at 0°. When the per cent displacement values are scaled to the maximum value of the observed rate constant and corrected for receptor occupancy by [^{3}H](+)-CD the resulting occupancy values (●) (see Fig. 7; right-hand ordinate scale) agree with the observed rate constants for alkylation. Thus, we conclude that the rate of alkylation is proportional to receptor occupancy. The simplest model to explain the interaction of BM 123A with the [^{3}H](+)-CD-binding sites is the scheme in Eq. 1 which is rewritten below in the appropriate terminology for the superhigh affinity site:

$$A + R_{SH} \underset{}{\overset{K_{SH}}{\rightleftharpoons}} AR_{SH} \overset{k_{SH}}{\longrightarrow} A\text{-}R_{SH} \quad (14)$$

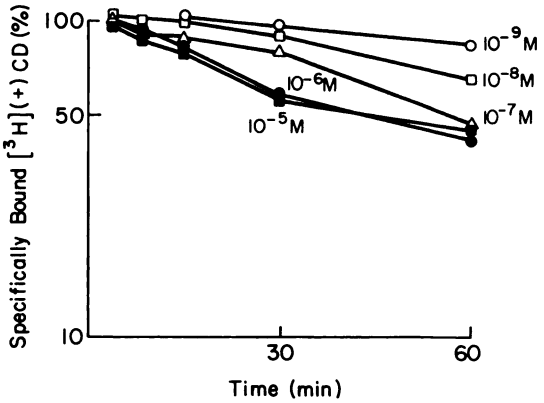

FIG. 6. *The kinetics of the alkylation of [^{3}H](+)-CD-binding sites by BM 123 in the rat cerebral cortex*

Cortical homogenates were incubated at 37° with various concentrations of BM 123 for the indicated times, washed extensively, and then assayed for [^{3}H](+)-CD binding at 0° using a ^{3}H-ligand concentration of 2.0 nM. Specific [^{3}H](+)-CD binding was measured in homogenates that had been previously incubated with BM 123 at concentrations of 0.001 μM (○), 0.01 μM (□), 0.1 μM (△), 1.0 μM (●), and 10.0 μM (■). Each point represents the mean binding value of five experiments, each done in triplicate.

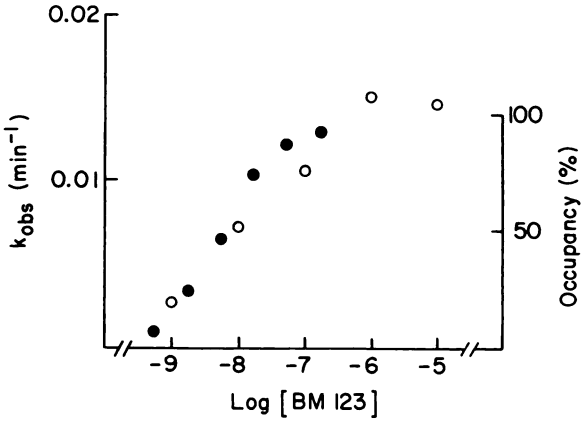

FIG. 7. *A relative comparison of the occupancy and the rate of alkylation of [^{3}H](+)-CD-binding sites by BM 123 at various concentrations*

The observed rate constants for the alkylation of [^{3}H](+)-CD-binding sites were calculated from the data in Fig. 6 as described in the text and are plotted in *open circles* (○) according to the ordinate scale on the left. Receptor occupany was determined by measuring the per cent displacement of [^{3}H](+)-CD binding by the aziridinium ion of BM 123 at 0°, and these estimates are plotted with the *closed circles* (●) according to the ordinate scale on the right. 100% receptor occupany has been scaled to the maximum value of the rate constant. The BM 123/[^{3}H](+)-CD competition curve has been corrected for receptor occupany by [^{3}H](+)-CD. The indicated concentrations of BM 123 refer to the initial concentration of the parent 2-chloroalkylamine.

where R_{SH} is the superhigh affinity site, K_{SH} is the dissociation constant, k_{SH} is the rate constant for alkylation, and AR_{SH} and $A\text{-}R_{SH}$ are the reversible and covalent complexes of the aziridinium ion with the superhigh affinity site, respectively. To test the compatibility of the data with the model shown above, the data in Fig. 6 were fitted simultaneously to Eq. 5 which yielded estimates of K_{SH} and k_{SH} of 2.7 nM and 0.014 min^{-1}, respectively. Analysis of variance showed that the variance estimate based on deviations of the mean binding values from the regression equation was not significantly greater than that estimated by replicate measurements of [^{3}H](+)-CD binding, illustrating that the model adequately described the data ($F_{20,92} = 0.178$, $p > 0.25$).

Kinetics of alkylation of high and low affinity sites. The loss of [^{3}H](−)-QNB-binding sites following incubation of cortical homogenates with various concentrations of BM 123 is shown in Fig. 8. A comparison of the kinetic plots in Figs. 6 and 8 shows that the alkylation of [^{3}H](−)-QNB-binding sites by BM 123 differs from that observed in [^{3}H](+)-CD-binding experiments in the following ways. First, the rate of loss of [^{3}H](−)-QNB sites at high concentrations of BM 123 is much faster than the maximum rate of loss of [^{3}H](+)-CD sites. Moreover, the rate of alkylation of the [^{3}H](−)-QNB sites continues to increase over a broader concentration range and does not plateau until the BM 123 concentration exceeds 0.1 mM. Finally, the kinetic curves for the loss of [^{3}H](−)-QNB sites are more complex, being markedly biphasic at high concentrations of BM 123. This behavior is inconsistent with a simple one-site model, but can be explained by a model in which the aziridinium ion (A) discriminates between high (R_H) and low (R_L) affinity

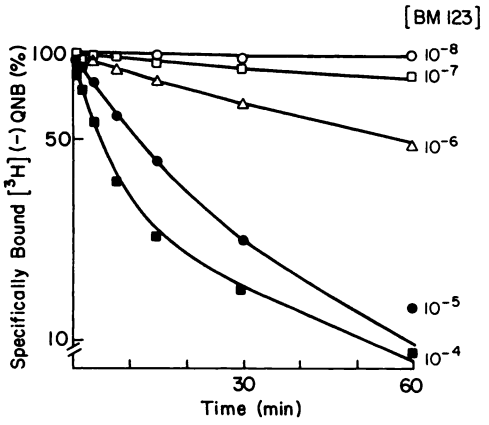

FIG. 8. *The kinetics of the alkylation of [³H](−)-QNB-binding sites in the rat cerebral cortex by BM 123*

Cortical homogenates were incubated at 37° with various concentrations of BM 123 for the indicated times, washed extensively, and then assayed for [³H](−)-QNB binding at 37° using a ³H-ligand concentration of 0.4 nM. Specific [³H](−)-QNB binding was measured in homogenates that had been previously incubated with BM 123 at concentrations of 0.01 μM (○), 0.1 μM (□), 1.0 μM (△), 10.0 μM (●), and 100.0 μM (■). Each point represents the mean binding value of six experiments, each done in triplicate. The theoretical curves represent the least squares fit of the data to Eq. 6.

sites, each having its respective dissociation constant (K_H and K_L) and rate constant for alkylation (k_H and k_L):

$$A + R_H \overset{K_H}{\rightleftharpoons} AR_H \overset{k_H}{\rightarrow} A\text{-}R_H \qquad (15)$$
$$A + R_L \overset{K_L}{\rightleftharpoons} AR_L \overset{k_L}{\rightarrow} A\text{-}R_L$$

In this scheme, the aziridinium ion quickly binds with the receptors forming reversible complexes (AR_H and AR_L) which convert to covalent complexes (A-R_H and A-R_L) at relatively slower rates. To test the model, the kinetic data were fitted simultaneously to Eq. 6, the theoretical basis for which is described under "Materials and Methods." The results of this analysis showed that BM 123A discriminated between high and low affinity sites having dissociation constants of 18.5 nM and 5.3 μM and relative abundances of 30 and 70%, respectively. The rate of alkylation of the high affinity site (k_H = 0.022 min^{-1}) was much slower than that of the low affinity site (k_L = 0.26 min^{-1}). The good agreement between the data and the model can be seen by the theoretical curves in Fig. 8 which represent the least squares fit of the data to Eq. 6. There is some discrepancy between the estimates of the dissociation constants (K_H and K_L) determined kinetically at 37° and those determined at equilibrium by competitive inhibition of [³H](−)-NMS binding at 0° (see Table 1). Part of the discrepancy can be attributed to the difference in the incubation temperature of the two experiments, and this issue is discussed below.

To obtain independent estimates of the rate constants for alkylation of the high and low affinity sites, the kinetics of alkylation were examined at a saturating concentration of BM 123 (1.0 mM) so that both sites would be fully occupied by the aziridinium ion, and the observed rate constant for alkylation of each site ($k_{H(obs)}$, $k_{L(obs)}$) should be equivalent to the actual rate constants (k_H, k_L). Fig. 9 shows the loss of [³H](−)-QNB-binding sites following incubation of cortical homogenates with BM 123 (1.0 mM) for various times. The semi-log plot of measurements of [³H](−)-QNB binding against time (Fig. 9) clearly shows two components. The data in Fig. 9 were fitted to the following biexponential equation to estimate the observed rate constants for each components and the relative proportion (a) of the slowly alkylated component:

$$Y = 100(ae^{-k_{H(obs)}t} + (1 - a)e^{-k_{L(obs)}t}) \qquad (16)$$

In this equation, Y denotes specifically bound [³H](−)-QNB (%). The agreement between the data and Eq. 16 can be seen by the theoretical curve in Fig. 9 which represents the least squares fit to the data. Nonlinear regression analysis showed that 25% of the receptors were alkylated slowly, with an observed rate constant ($k_{H(obs)}$) of 0.016 min^{-1}, whereas the remaining sites were alkylated more rapidly with an observed rate constant ($k_{L(obs)}$) of 0.30 min^{-1}. These estimates of the observed rate constants which were measured at a saturating concentration of BM 123 are in general agreement with the rate constants estimated from the data shown in Fig. 8. Moreover, the estimate (25)% of the proportion of sites which are slowly alkylated by BM 123 at a concentration of 1.0 mM is in reasonable agreement with the estimates of the proportion of high affinity sites determined from the kinetic data in Fig. 8 (30%) and from the equilibrium competition data summarized in Table 1 (25%).

Selective alkylation of high and low affinity sites. Collectively, the foregoing results are consistent with the postulate that the aziridinium ion discriminates between high and low affinity sites and that it alkylates the low affinity site at a much faster rate. To test this postulate further, we examined the agonist-binding properties of the residual unalkylated receptors in cortical homoge-

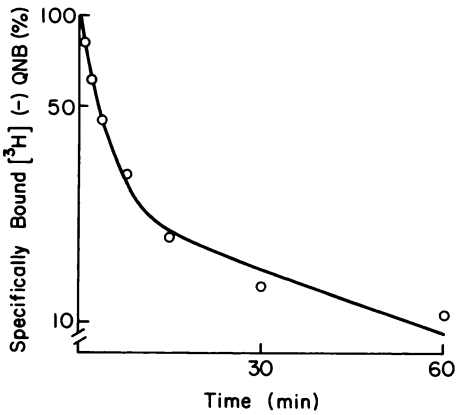

FIG. 9. *The kinetics of the alkylation of [³H](−)-QNB-binding sites in the rat cerebral cortex by BM 123 (1.0 mM)*

Cortical homogenates were incubated at 37° with BM 123 for various times, washed extensively, and then assayed for [³H](−)-QNB binding at 37° using a ³H-ligand concentration of 0.4 nM. Each point represents the mean binding value of four experiments, each done in triplicate. The theoretical curve represents the least squares fit of the data to Eq. 16.

nates which had been treated with BM 123 (1.0 mM) for 10.3 min. This time of incubation with BM 123 corresponds to the bend in the semi-log plot shown in Fig. 9. Using the parameter values estimated above, it can be calculated that, at this point during the incubation, 95% of the low affinity sites had been alkylated with BM 123 whereas only 15% of the high affinity sites had been alkylated. Fig. 10A shows the results of oxo-M/[^{3}H](−)-NMS competition curves run on control tissue and tissue which had been previously incubated with BM 123 (1.0 mM) for 10.3 min and washed extensively. Nonlinear regression analysis showed that the control competition curve was compatible with the existence of high (K_H = 0.028 μM) and low (K_L = 10.6 μM) affinity sites having relative abundances of 30 and 70%, respectively. Following BM 123 treatment, 65% of the receptors were alkylated, and there was a 25-fold reduction in the X_{50} value of oxo-M from 3.0 μM in control homogenates to 0.12 μM in BM 123-treated homogenates. Nonlinear regression analysis showed that this shift in the oxo-M/[^{3}H](−)-NMS competition curve could be attributed to a selective increase in the proportion of high affinity sites to a value of 70%. Similar results were observed when the competitive inhibition of [^{3}H](−)-NMS binding by carbachol was studied in control and BM 123-treated (1.0 mM, 10.3 min) tissue (Fig. 10B). Nonlinear regression showed that the control carbachol/[^{3}H](−)-NMS competition curve was consistent with the presence of high (K_H = 0.21 μM) and low (K_L = 49 μM) affinity sites having relative abundances of 27 and 73%, respectively. Following BM 123 treatment, there was a 25-fold reduction in the X_{50} value of carbachol from 25 μM in controls to 1.0 μM in BM 123-treated homogenates and an increase in the proportion of high affinity sites to a value of 66%.

In contrast, prior treatment of cortical homogenate with BM 123 (1.0 mM) for 10.3 min had no significant influence on the ability of atropine (X_{50} = 0.80 nM) and gallamine to inhibit [^{3}H](−)-NMS binding to the residual receptors (Fig. 11). A high concentration (1.0 mM) of gallamine did not fully displace [^{3}H](−)-NMS binding; consequently, the gallamine inhibition curve plateaued at 80% inhibition of [^{3}H](−)-NMS binding. Stockton *et al.* (8) have shown that gallamine does not directly compete with [^{3}H](−)-NMS for the same site, but rather it binds at an allosteric locus to reduce the affinity of [^{3}H](−)-NMS for the primary site. By fitting the gallamine/[^{3}H](−)-NMS inhibition curve to the appropriate equation given by Stockton *et al.* (8), we estimated that the dissociation constant of gallamine for its site to be 5.1 μM in the absence of [^{3}H](−)-NMS. A small but significant increase in the concentration of pirenzepine required to inhibit [^{3}H](−)-NMS binding by 50% was observed following incubation of cortical homogenates with BM 123 (1.0 mM) for 10.3 min (Fig. 11). The X_{50} value of pirenzepine increased from 80 nM in controls to 142 nM in BM 123-treated homogenate.

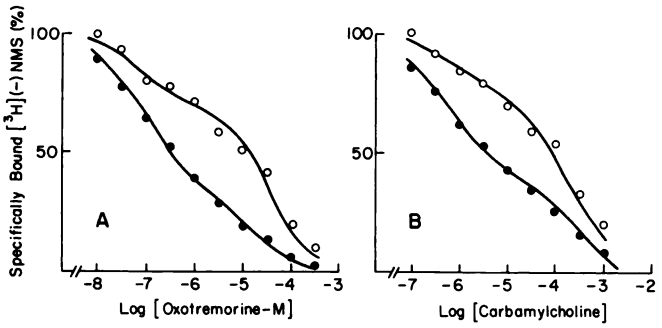

FIG. 10. *The competitive inhibition of [^{3}H](−)-NMS binding by oxo-M (A) and carbamylcholine (B) in control homogenates (open circles) and homogenates which had been incubated at 37° with BM 123 (1.0 mM) for 10.3 min and washed extensively (closed circles)*

The data points represent the mean binding values of 3–4 experiments, each done in triplicate. The incubation of tissue with [^{3}H](−)-NMS (0.15 nM) lasted 30 min at 30°.

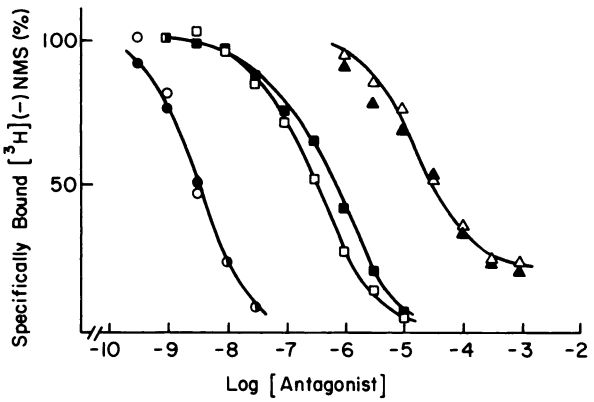

FIG. 11. *The inhibition of [^{3}H](−)-NMS binding by atropine (circles), pirenzepine (squares), and gallamine (triangles) in control homogenates (open symbols) and homogenates which had been at 37° incubated with BM 123 (1.0 mM) for 10.3 min and washed extensively (closed symbols)*

The data points represent the mean binding values of three experiments, each done in triplicate. The incubation of tissue with [^{3}H](−)-NMS (0.15 nM) was 30 min at 30° for the experiments with atropine and pirenzepine and 3 hr at 30° for the experiments with gallamine.

Since the ratio of dissociation constants (K_L/K_H) of the aziridinium ion for the high and low affinity sites was greater than the ratio of alkylation rate constants (k_L/k_H) for the two sites, it seemed likely that we could selectively alkylate the high affinity site by incubating cortical homogenates with a low concentration of BM 123 (0.1 μM) for a relatively long time (1 hr). Fig. 12 shows the results of oxo-M/[^{3}H](−)-NMS competition experiments run of control homogenates and homogenates which had been incubated with BM 123 (0.1 μM) for 1 hr. Nonlinear regression analysis showed that the control competition curve was compatible with the presence of high (K_H = 28 nM) and low (K_L = 4.4 μM) affinity sites having relative abundances of 34 and 66%, respectively. Following treatment with BM 123 (0.1 μM) for 1 hr, 28% of the receptors were alkylated, and there was a small but significant ($p < 0.01$) 1.9-fold increase in the X_{50} of oxo-M from 1.3 μM in controls to 2.5 μM in BM 123-treated homogenates. Regression analysis showed that it was possible to explain this shift as being the result of a small decrease in the proportion of high affinity sites, the proportion of high affinity sites being 23% in the BM 123-treated tissue.

Competitive inhibition of alkylation. Experiments were run to determine if there was agreement between the

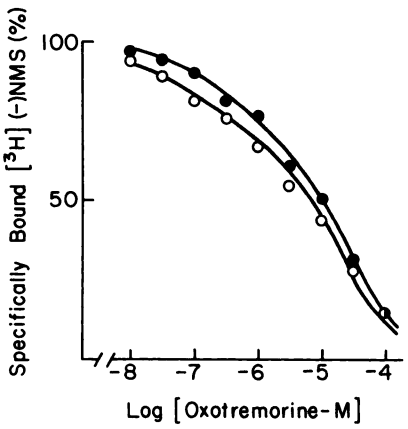

FIG. 12. *The competitive inhibition of [³H](−)-NMS binding by oxo-M in control homogenates (○) and homogenates which had been incubated at 37° with BM 123 (0.1 μM) for 1 hr and washed extensively (●)*

The data points represent the mean binding value of four experiments, each done in triplicate. The incubation of tissue with [³H](−)-NMS (0.15 nM) lasted 30 min at 30°.

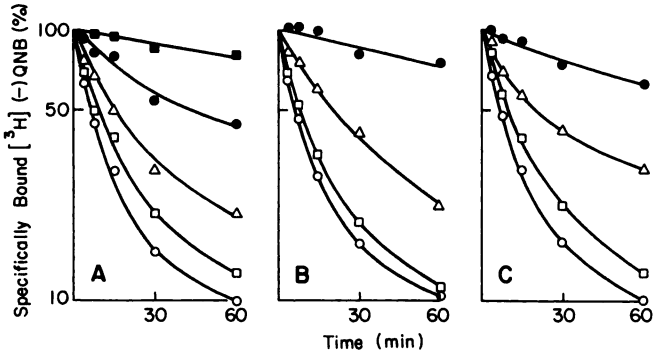

FIG. 13. *The effects of atropine (A), oxotremorine (B), and oxo-M (C) on the rate of alkylation of [³H](−)-QNB-binding sites in rat cerebral cortex by BM 123 (10 μM)*

Cortical homogenates were incubated at 37° with BM 123 for the indicated times in the absence (○) and presence of various concentrations of atropine: 1.0 nM (□), 3.0 nM (△), 10 nM (●) and 100 nM (■); oxotremorine: 0.3 μM (□), 3.0 μM (△), and 30.0 μM (●); and oxo-M: 1.0 μM (□), 10 μM (△), and 100 μM (●). The homogenates were then washed extensively and assayed for [³H](−)-QNB binding at 37° using a ³H-ligand concentration of 0.4 nM. The data points represent the mean binding values of two experiments, each done in triplicate.

potency of various reversible muscarinic agents for competitively slowing the rate of receptor alkylation by BM 123 and their respective potencies for interacting with muscarinic receptors. Fig. 13A shows the results of experiments in which [³H](−)-QNB binding was measured in extensively washed homogenates that had been previously exposed to BM 123 (10 μM) in combination with various concentrations of atropine. It can be seen that nanomolar concentrations of atropine slowed the rate of receptor alkylation by BM 123 and that the rate of receptor alkylation decreased with increasing atropine concentration. The simplest scheme to illustrate the competitive inhibition of receptor alkylation is:

$$\begin{array}{ccc} A + I + R & \overset{K_A}{\rightleftharpoons} & AR \rightarrow A\text{-}R \\ K_I \Big\downarrow\Big\uparrow & & \\ A + IR & & \end{array} \qquad (17)$$

In this scheme, K_A and K_I are the dissociation constants of the aziridinium ion (A) and competitive inhibitor (I), respectively, AR and IR are reversible receptor complexes, and A-R is the covalent receptor complex. If we make the simplifying assumptions that the concentrations of I and A are constant during the reaction and that the rate of the alkylation is slower than that of the reversible interactions, then it can be shown that:

$$R - 1 = I/K_I' \qquad (18)$$

where R is the ratio of the half-times for alkylation of muscarinic receptors in the presence of I divided by that measured in the absence of I, and K_I' is the apparent dissociation constant of the competitive inhibitor ($K_I' = K_I(1 + A/K_A)$). In logarithmic form, Eq. 18 is a straight line having a slope of one and an x intercept equal to log K_I':

$$\log(R - 1) = \log(I) - \log(K_I') \qquad (19)$$

The competition data for atropine (Fig. 13A) and similar data for oxotremorine and oxo-M (Fig. 13, B and C) were plotted according to Eq. 19 above, and the results of this analysis are shown in Fig. 14. The estimates of the apparent dissociation constants for atropine, oxotremorine, and oxo-M were 2.5 nM, 1.0 μM, and 4.0 μM, respectively.

DISCUSSION

In prior studies of the effects of BM 123 on muscarinic receptor-binding properties, we noted a reduction in the binding capacities of [³H](−)-NMS and [³H](−)-QNB in homogenates of the rat cerebral cortex and guinea pig ileum which had been previously incubated with 10 μM BM 123 for 20 min at 37° and then washed extensively (1, 2). These effects on binding capacity were not accompanied by a significant change in the apparent affinity of [³H](−)-NMS or [³H](−)-QNB (1, 2). In the present study, we measured [³H](−)-NMS binding in cortical homogenates which had been previously incubated at 37° with a rather high concentration (1.0 mM) of BM 123 for 20 min and 1 hr, and again observed a reduction in

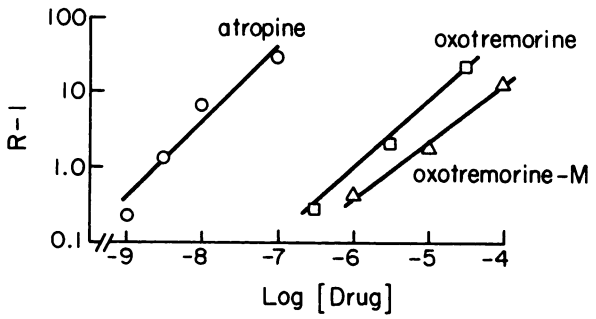

FIG. 14. *The effects of atropine, oxotremorine, and oxo-M on the rate of alkylation of [³H](−)-QNB-binding sites by BM 123 (10 μM)*

R refers to the ratio of half-times for the alkylation of [³H](−)-QNB-binding sites by BM 123 in the presence of the inhibitor divided by that measured in the absence of the inhibitor. The R values were calculated from the data shown in Fig. 13. The straight lines represent the least squares fit to the data for atropine (slope = 1.01), oxotremorine (slope = 0.94), and oxo-M (slope = 0.72).

binding capacity without an effect on affinity. These results illustrate that BM 123, over a wide range of concentrations, reduces the binding capacity of muscarinic receptors without influencing their affinity, presumably by covalently binding to the recognition site of the muscarinic receptor.

Since low temperature inhibits the formation of the aziridinium ion as well as the covalent binding of the aziridinium ion to the muscarinic receptor, it was possible to determine the affinity of BM 123 and its transformation products for muscarinic receptors by measuring their ability to inhibit [^{3}H]oxo-M and [^{3}H](−)-NMS binding competitively. In competition experiments with [^{3}H]oxo-M, the aziridinium ion was 180- and 710-fold more potent than BM 123 and its alcoholic hydrolysis product, respectively. In competition experiments with [^{3}H](−)-NMS, the aziridinium ion was 71-fold more potent than both BM 123 and its alcoholic hydrolysis product. These observations are consistent with the pharmacological experiments of Ringdahl *et al.* (1) who found that the contractile activity of BM 123 on the isolated guinea pig ileum could be attributed almost entirely, though not completely, to the aziridinium ion.

Like other efficacious agonists, BM 123A appears to discriminate among different agonist subclasses of the muscarinic receptor. Birdsall and co-workers (7, 9) have shown that the complex binding properties of muscarinic agonists in rat cerebral cortex can be explained by the existence of 3 classes of binding sites (superhigh, high, and low affinity), each having a different affinity for most agonists and equal affinity for most antagonists. Exceptions to this behavior include the selective ganglionic muscarinic agonist McNA343, the selective muscarinic antagonist pirenzepine (10), and some trichloro derivatives of *cis*-dioxolane having agonistic activity (11). In the present study, we have used low concentrations of [^{3}H]oxo-M and [^{3}H](+)-CD to label the superhigh affinity site selectively so that the binding properties of this site could be studied. Previous experiments with [^{3}H] oxo-M (7) and [^{3}H](+)-CD (12) have shown that it is possible to label the superhigh affinity site selectively with these ^{3}H-ligands. We estimate that the contribution of the superhigh affinity site to ^{3}H-antagonist binding represents less than 3% of the total binding in washed homogenates of rat cerebral cortex. Consequently, we have assumed that the superhigh affinity sites represent a negligible fraction of ^{3}H-antagonist binding and that measurements of [^{3}H](−)-QNB and [^{3}H](−)-NMS binding can be interpreted satisfactorily assuming that the high and low affinity sites account for all of the binding in rat cortex.

It has been shown for a number of agonist analogues that efficacy is correlated with the ratio of dissociation constants for the high and low affinity sites (K_L/K_H) (7, 9). In this respect, the binding properties of BM 123A resemble those of a highly efficacious muscarinic agonist since its ratio of dissociation constants for the high and low affinity sites was relatively large when measured by competitive inhibition of ^{3}H-antagonist binding at 0° ($K_L/K_H = 95$) and by analysis of the kinetics of receptor alkylation at 37° ($K_L/K_H = 286$).

When measured by competitive inhibition of [^{3}H]oxo-M and [^{3}H](−)-NMS binding at 0°, the reversible binding characteristics of BM 123A resembled those of oxo-M. However, there were some significant differences among the binding parameters, the most notable being the value of K_L, which was about 2.5-fold less for the aziridinium ion as compared to oxo-M. In pharmacological experiments, Ringdahl *et al.* (1) noted that BM 123A was approximately 3–4-fold more potent than oxo-M at eliciting contractions of the guinea pig ileum. Thus, the rather minor structural difference between the trimethylammonium group of oxo-M and the aziridinium ring derived from the 2-chloroethylmethylamine group of BM 123, appears to cause small differences in both the pharmacological activity of these compounds as well as their reversible binding characteristics.

The simple one-site model (Eq. 17) used to describe the competitive inhibition of the alkylation of muscarinic receptors by BM 123 is a simplified approximation since the aziridinium ion as well as oxotremorine and oxo-M discriminate among different agonist subtypes of the muscarinic receptor. Nevertheless, this approximation appears to be self-justified since there is good agreement between the kinetically determined K_I values of the reversible inhibitors and the concentration of the reversible drugs required to occupy 50% of the muscarinic receptors in rat cerebral cortex. The concentrations of atropine, oxotremorine, and oxo-M which caused a doubling in the half-time for alkylation of [^{3}H](−)-QNB-binding sites by BM 123 may be considered as an apparent K_I value (K'_I). This apparent K_I value is larger than the true K_I value by a factor of $1 + A/K_A$ (see Eq. 18). If we consider the theoretical X_{50} value (2.2 μM) of the aziridinium ion at 37° as an approximation of K_A, then the kinetically determined K'_I values of atropine, oxotremorine, and oxo-M (2.5 nM, 1.0 μM, and 4.0 μM) can be corrected to their respective true K_I values of 1.1 nM 0.44 μM, and 1.74 μM. These corrected K_I values are in good agreement with the X_{50} values of atropine and oxo-M measured by competitive inhibition of [^{3}H](−)-NMS binding in the present study (0.8 nM and 3 μM, respectively) and with the corrected IC_{50} value of oxotremorine measured by competitive inhibition of [^{3}h]propylbenzilylcholine binding to rat cerebral cortex in previous studies (13). Moreover, atropine, oxotremorine, and oxo-M inhibited the alkylation of muscarinic receptors by BM 123 in a manner that was consistent with competitive antagonism. We conclude that the aziridinium ion alkylates the site on the muscarinic receptor where acetylcholine, atropine, and other directly acting muscarinic agents bind.

Our study of the kinetics of muscarinic receptor alkylation over a range of concentrations of BM 123 enabled us to estimate both the affinity of the aziridinium ion and its rate constant for alkylation of the subclasses of agonist-binding sites. In our analysis of the kinetics, we have assumed a quasi-equilibrium where the aziridinium ion rapidly equilibrates with the receptor, forming a reversible complex which converts to a covalent complex at a relatively slower rate. This model predicts that the rate of loss of unalkylated receptors should be monoex-

ponential so long as the change in the concentration of the aziridinium ion during the incubation is not great. The observed rate constant for alkylation of receptors should increase as a Michaelis-Menten-like function of the aziridinium ion concentration. The kinetics of the alkylation of the superhigh affinity site by BM 123 obeyed the simple one-site model described above as demonstrated by the good agreement between the estimates of the affinity of the aziridinium ion measured kinetically at 37° and that estimated directly by competitive inhibition of [^{3}H]oxo-M and [^{3}H](+)-CD binding at 0°. The agreement between the two independent estimates made at different temperatures suggests that changing the temperature from 0° to 37° had little influence on the affinity of the aziridinium ion for the superhigh affinity site. This conclusion would appear to be reasonable since we found that changing the incubation temperature from 0° to 37° had no significant influence on the potency with which nonlabeled oxo-M inhibited [^{3}H]oxo-M binding to cerebral cortex when assayed at a ^{3}H-ligand concentration of 1.0 nM (data not shown).

Although the concentration of the aziridinium ion fell to approximately half of its starting concentration after an hour of incubation at 37°, this change in concentration did not produce results much different from those to be expected had the concentration of the aziridinium ion remained constant. In other words, there was general agreement between the estimates of the kinetic parameters of the aziridinium ion for alkylation of the superhigh affinity site when the parameters were determined by fitting all of the data simultaneously to Eq. 5 and by fitting the kinetic curves individually to an exponential equation and plotting the observed rate constant as a function of the BM 123 concentration as shown in Fig. 7. Nevertheless, since we could accurately calculate the decline in the concentration of the aziridinium ion during an incubation at 37°, we felt that fitting the data to Eq. 5 and 6 would produce the best estimates of the kinetic parameters of BM 123.

The kinetics of the alkylation of [^{3}H](−)-QNB-binding sites by BM 123 were complex and could not be described by a simple one-site model. Once occupied by the aziridinium ion, 70–75% of the [^{3}H](−)-QNB-binding sites were alkylated with a half-time of 2.7 min whereas the remaining sites were alkylated more slowly with a half-time of 32 min. Apparently, it is primarily the low affinity agonist-binding site which is alkylated at a faster rate since prior incubation of cortical membranes with a saturating concentration of BM 123 (1.0 mM) for a relatively short time (10.3 min) caused a selective loss of low affinity agonist-binding sites as measured by the shift in the oxo-M/[^{3}H](−)-NMS competition curve. When the kinetic data showing the loss of [^{3}H](−)-QNB-binding sites in the presence of various concentrations of BM 123 were analyzed by nonlinear regression analysis, estimates of the dissociation constants of the aziridinium ion for the high and low affinity sites were obtained which were roughly similar to those calculated directly by measuring the competitive inhibition of [^{3}H](−)-NMS binding by the aziridinium ion at 0°. The largest discrepancy was in the value of K_L, which was four times larger when estimated kinetically at 37° than that estimated from the results of the BM 123A/[^{3}H](−)-NMS competition experiment at 0°. This difference in affinity can probably be attributed to temperature since we have found that increasing the temperature from 0° to 37° caused 2- and 4-fold increases, respectively, in the K_H and K_L values of oxo-M, when estimated by nonlinear regression analysis of oxo-M/[^{3}H](−)-NMS competition experiments in rat cerebral cortex (data not shown).

There are some discrepancies between our data for the alkylation of [^{3}H](−)-QNB-binding sites by BM 123 and the consequences of the model for two independent sites (Eq. 6). First, the proportion (75%) of [^{3}H](−)-QNB-binding sites which were alkylated rapidly by BM 123 at a concentration of 1.0 mM was slightly, though significantly, greater than the estimate of the proportion of low affinity sites (70%) determined by nonlinear regression analyses of the kinetic data obtained at lower concentrations of BM 123 (0.01–100 μM). Second, the proportion of residual high affinity sites in cortical homogenate which had been pretreated with 1.0 mM BM 123 for 10.3 min was not as great as that predicted from the kinetic analysis. In control tissue, the ratio of high to low affinity sites was 30:70, and following BM 123 treatment (1.0 mM, 10.3 min), the ratio changed to 70:30. However, using Eq. 6 and the estimates of the kinetic parameters of BM 123, it can be calculated that the ratio of high to low affinity sites should have been 83:17 following exposure to BM 123 (1.0 mM) for 10.3 min. One possible explanation for this discrepancy is that some of the high and low affinity sites in the rat cerebral cortex are interconvertible. Alternatively, it may be that, at high concentrations, BM 123 alkylates nonspecific sites on the muscarinic receptor, causing the affinity of agonists for the primary site to be reduced. However, when we incubated cortical homogenates with BM 123 (1.0 mM) for 1 hr in the presence of atropine (0.1 mM) to protect the primary recognition sites, we were unable to detect any change in the ability of oxo-M to displace [^{3}H](−)-NMS binding to the homogenates competitively after extensive washing. It is possible that the high concentration (0.1 mM) of atropine used in the experiment protected both the primary site and the putative nonspecific site as well. It is unlikely that BM 123 alkylates the allosteric site on the muscarinic receptor to which gallamine binds since we found that incubating cortical homogenates with BM 123 (1.0 mM) for 1 hr had no significant influence on the ability of gallamine to inhibit [^{3}H](−)-NMS binding to the residual receptors (data not shown). Moreover, after incubating cortical homogenates with BM 123 (1.0 mM) for 1 hr, the affinity of [^{3}H](−)-NMS for the residual receptors was unaltered, which might not be expected if BM 123 alkylates the gallamine site.

The relationship between the affinity of the aziridinium ion for the various agonist subtypes of the muscarinic receptor (superhigh, high, and low affinity) and the rate at which the aziridinium ion alkylates these sites is reciprocal in nature. In other words, the aziridinium ion alkylated the highest affinity site at the slowest rate

whereas the converse was true for the lowest affinity site. The probability that such an ordered nonrandom relationship would exist for a group of three distinct and independent binding sites is small. An explanation for this anomaly can be found if we assume that the aziridinium ion, like other muscarinic agonists, induces a conformational change in the receptor when it binds. Accordingly, the aziridinium ion (A) first binds upon the receptor in the ground state (R) and subsequently induces the receptor to assume its activated conformation (R^*), thereby introducing the possibility that the aziridinium ion alkylates the two conformations at different rates as described by the microscopic rate constants (k', k'') shown below:

$$\begin{array}{ccccc} & & K_1 & & K_\alpha \\ A + R & \rightleftharpoons & AR & \rightleftharpoons & AR^* \\ & & \downarrow k' & & \downarrow k'' \\ & & A\text{-}R & & A\text{-}R^* \end{array} \qquad (20)$$

In this scheme, K_1 is the equilibrium dissociation constant describing the binding of the aziridinium ion to the ground state of the receptor, and K_α is the unimolecular equilibrium constant describing the isomerization ($K_\alpha = XR/XR^*$). The overall experimental dissociation constant K_A is given by:

$$K_A = \frac{K_\alpha K_1}{K_\alpha + 1} \qquad (21)$$

During an incubation of the aziridinium ion with receptors, the loss of unalkylated receptors in time is given by:

$$Y = e^{-kpt} \qquad (22)$$

where Y is the proportion of unalkylated receptors, t is time, and p is receptor occupancy by the aziridinium ion ($p = A/(A + K_A)$). The overall experimental rate constant k for alkylation is given by:

$$k = \frac{k'K_\alpha + k''}{K_\alpha + 1} \qquad (23)$$

Birdsall *et al.* (7, 14) have presented evidence that the differences among the dissociation constants of most agonists for the superhigh, high, and low affinity sites are due to differences in the propensity with which agonists isomerize these receptors and not to major differences in the conformations of the ground states of these receptors. According to this postulate, the dissociation constants (K_{SH}, K_H, and K_L) of agonists are given by:

$$K_{SH} = \frac{K_{\alpha SH}K_1}{K_{\alpha SH} + 1} \qquad (24)$$

$$K_H = \frac{K_{\alpha H}K_1}{K_{\alpha H} + 1} \qquad (25)$$

$$K_L = \frac{K_{\alpha L}K_1}{K_{\alpha L} + 1} \qquad (26)$$

where $K_{\alpha SH} < K_{\alpha H} < K_{\alpha L}$. If we assume that the aziridinium ion alkylates the activated conformation at a slower rate than that at which it alkylates the ground conformation ($k'' < k'$), then it follows from Eq. 23 that the magnitudes of the experimental rate constants for alkylation of the superhigh, high, and low affinity sites must have the following rank order: $k_{SH} < k_H < k_L$.

There is no way to calculate explicit values of K_1, $K_{\alpha SH}$, $K_{\alpha H}$, $K_{\alpha L}$, k', and k'' from the data; however, we can make some limiting assumptions. Since the ratios of dissociation constants (K_L/K_H, K_H/K_{SH}) of the aziridinium ion for subtypes of the muscarinic receptor exceed the respective ratios of rate constants (k_L/k_H, k_H/k_{SH}), then it follows that the aziridinium ion alkylates both ground and activated conformations of the receptor at finite rates ($0 < k'' < k'$) and that $K_{\alpha L}/K_{\alpha SH} > k'/k''$. If we speculate that the overall experimental dissociation constant of the aziridinium ion for the low affinity agonist site is similar to K_1 ($K_{\alpha L} \geq 1$), then it follows that $k'' \simeq k_{SH}$ and $k' \simeq k_L$.

The model described above provides a simple explanation for the quantitative relationship between the rate constants and dissociation constants of the aziridinium ion. It has its origins in previous ideas about the significance of agonist receptor heterogeneity in the rat cerebral cortex and states that the tighter binding of the aziridinium ion to the activated conformation of the muscarinic receptor hinders the covalent binding of the aziridinium ion to a nucleophile on the receptor. The question of whether or not the alkylated receptor complex is active or inactive has been addressed in previous reports (1–3). It is hoped that the selectivity of the aziridinium ion for different agonist-binding sites may provide a useful tool for investigating agonist receptor heterogeneity.

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