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Investigating the interaction of McN-A-343 with the M_2 muscarinic receptor using its nitrogen mustard derivative

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ABSTRACT

We investigated whether the aziridinium ion formed from a nitrogen mustard derivative (4-[(2-bromoethyl)methyl-amino]-2-butynyl N-(3-chlorophenyl)carbamate; BR384) structurally related to McN-A-343 (4-(trimethyl-amino)-2-butynyl N-(3-chlorophenyl)carbamate) interacts allosterically or orthosterically with the M_2 muscarinic receptor. Chinese hamster ovary cells expressing the human M_2 muscarinic receptor were incubated with the aziridinium ion of BR384 in combination with McN-A-343 or other known orthosteric and allosteric ligands for various incubation times. After removing unreacted ligands, we measured the binding of [^{3}H]N-methylscopolamine to residual unalkylated receptors. Affinity constants, rate constants for alkylation, and cooperativity constants were estimated for the interacting ligands using a mathematical model. Receptor alkylation by BR384 was consistent with a two-step process. After rapidly equilibrating with the receptor (step one), the aziridinium ion–receptor complex became covalently linked with a first order rate constant of about 0.95 min^{-1} (step two). McN-A-343, acetylcholine and N-methylscopolamine competitively protected the M_2 receptor from irreversible alkylation by BR384. In contrast, the allosteric modulators, gallamine and WIN 51,708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-β]pyrimido[1,2-α]benzimidazole), allosterically inhibited or had no effect on, respectively, receptor alkylation by BR384. There was good agreement between affinity constants estimated from the kinetics of receptor alkylation and by displacement of [^{3}H]N-methylscopolamine binding. Our results suggest that BR384 covalently binds to the orthosteric site of the M_2 receptor and that McN-A-343 binds reversibly at the same locus. Our method of analyzing allosteric interactions does not suffer from the limitations of more conventional approaches and can be adapted to detect allosteric interactions at receptors other than the muscarinic subtypes.

1. Introduction

The compound 4-(trimethyl-amino)-2-butynyl N-(3-chlorophenyl)carbamate (McN-A-343) is a muscarinic ganglionic stimulant that causes a selective increase in blood pressure and heart rate *in vivo* [1]. It has little effect on the brain when administered peripherally because of its quaternary ammonium structure. In contrast, most quaternary muscarinic agonists elicit bradycardia and hypotension as well as salivation due to activation of the M_2 receptor in the sinoatrial node and M_3 receptors on the endothelium of blood vessels and in the salivary glands, respectively. The selectivity of McN-A-343 *in vivo* can be attributed to its greater agonist activity at M_1 and M_4 relative to the M_2 and M_3 receptor subtypes [2–4].

The mode of interaction of McN-A-343 with muscarinic receptor subtypes differs from that of related oxotremorine analogs because of the large 3-chlorophenylcarbamate moiety in McN-A-343 in place of the small pyrrolidino ring of oxotremorine (see Fig. 1). In binding experiments on cerebral cortex, high concentrations of McN-A-343 caused a complete displacement of the binding of the muscarinic antagonist [^{3}H]N-methylscopolamine ([^{3}H]NMS), whereas in heart McN-A-343 caused only partial inhibition [5]. These results are consistent with competitive and allosteric behavior, respectively, in the two tissues. This difference reflects a discrimination between muscarinic receptor subtypes, because the heart expresses M_2 receptors and the cerebral cortex mainly M_1 and M_4.

In a study on hemi-ligands based on the McN-A-343 structure, it was shown that the ethyl and methyl esters of 3-chlorophenylcarbamate behave as allosteric modulators, whereas trimethylammonium acts as a muscarinic agonist, presumably through interaction with aspartic acid 103 in the M_2 receptor [6] (D 3.32 using the numbering scheme of Ballesteros and Weinstein [7]). These two hemi-ligands are connected through a butyne chain in

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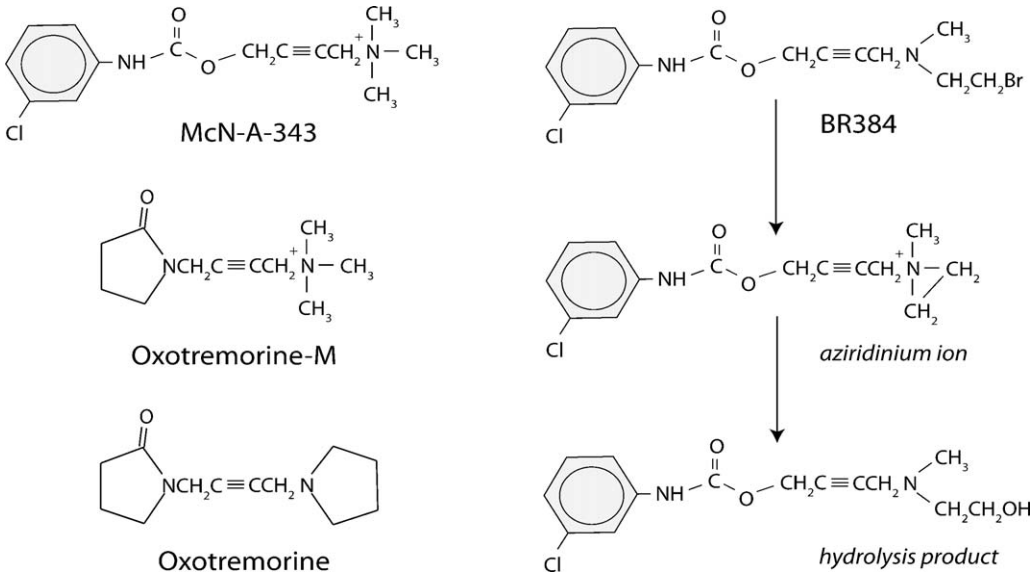

Fig. 1. Structures of McN-A-343, oxotremorine-M, oxotremorine, and BR384 and its transformation products in aqueous solution at neutral pH.

McN-A-343, suggesting that the intact molecule interacts simultaneously with allosteric and orthosteric sites.

Several investigators have shown that high concentrations of McN-A343 slow the dissociation of [^{3}H]NMS from the M_2 receptor [8,9]. This phenomenon is consistent with the trapping of [^{3}H]NMS by McN-A-343 when it occupies the allosteric site, because the allosteric site is located superficially to the orthosteric site in the normal cellular context. It has been suggested that McN-A-343 can bind independently to both sites with different affinities, but the symmetry of this model precludes the identification of which site it interacts with higher affinity in conventional kinetic experiments [10].

Mutagenesis of some residues in the M_2 receptor that are critical for orthosteric agonist activity have little effect on the activity of McN-A-343, whereas mutation of specific residues affecting the binding of allosteric antagonists enhance the activity of McN-A-343 [11]. These results also illustrate differences in how McN-A-343 and prototypic, acetylcholine-like orthosteric agonists interact with the M_2 receptor.

Irreversible ligands have advantages in identifying the mode of interaction of another ligand with a receptor. Their interaction with the receptor is consistent with a two-step process in which the reactive ligand first forms a reversible complex with the receptor (step one) followed by a subsequent alkylation step (step two) [12–14]. If a small agonist ligand with rapid binding kinetics is used as the alkylating agent, then it can achieve equilibrium quickly in the presence of an allosteric modulator or a competitive inhibitor during step one. The subsequent concentration-inhibition pattern of the modulator for inhibiting receptor alkylation (step two) reflects the nature of the reversible interactions during step one. Competitive inhibitors cause a concentration-dependent inhibition of alkylation and are capable of completely preventing alkylation at high concentrations. In contrast, any effect of an allosteric modulator reaches a limit at high concentrations [13]. We have found that McN-A-343 causes a competitive inhibition of receptor alkylation by acetylcholine mustard (AChM), indicating that the two ligands interact at the orthosteric site of the M_2 muscarinic receptor [13].

While these prior studies indicate that McN-A-343 binds to the orthosteric site of the muscarinic receptor, they do not rule out the possibility that it might also interact with other potential sites on the M_2 receptor linked allosterically to the orthosteric site. Having an irreversible analog of McN-A-343 would be useful for addressing this question in radioligand binding assays using both orthosteric and allosteric radioligands. Mutagenesis could also be used to identify the nucleophilic residue or residues that it alkylates.

To begin to address some of these questions, we have investigated the interaction of a nitrogen mustard derivative of McN-A-343 (4-[(2-bromoethyl)methyl-amino]-2-butynyl N-(3-chlorophenyl)carbamate; BR384) with the M_2 receptor. This compound has been shown to elicit immediate sympathetic effects on the cardiovascular system *in vivo* and bind irreversibly with muscarinic receptors, causing a long lasting inhibition of muscarinic responses [15]. In the present report, we describe a characterization of the interaction of BR384 with the human M_2 muscarinic receptor. We find that NMS, acetylcholine (ACh) and McN-A-343 competitively protect the M_2 receptor from irreversible alkylation by BR384, whereas gallamine and WIN 51,708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-β]pyrimido[1,2-α]benzimidazole) have partial or no protection consistent with an allosteric mechanism. Our results show that BR384 binds covalently to the orthosteric site of the M_2 muscarinic receptors, but do not rule out the possibility that it binds covalently or reversibly to additional sites.

2. Materials and methods

2.1. Cell culture

Chinese hamster ovary (CHO) cells stably expressing the human M_2 muscarinic receptor (CHO hM_2 cells) were obtained from Acadia Pharmaceuticals (San Diego, CA) and cultured in Dulbecco's Modified Eagle Medium with high glucose plus L-glutamine supplemented with 10% fetal calf serum, 3.7 g/l sodium bicarbonate, penicillin–streptomycin (100 units/ml and 100 μg/ml, respectively) and 0.4 mg/ml G418 disulfate salt at 37 °C in a humidified atmosphere with 5% CO_2/95% air.

2.2. Animals

Male Sprague–Dawley rats (200–250 g) were used as a source of tissue for the assay on homogenates of the rat heart.

2.3. Kinetics of M_2 muscarinic receptor alkylation by cyclized BR384

Our experiments for measuring the kinetics of receptor alkylation by BR384 involved three phases: (1) incubation of the

receptor preparation with BR384 followed by stopping the reaction with a competitive inhibitor (scopolamine or N-methylamitriptyline) and thiosulfate, (2) removing BR384, its transformation products, and the components of the stopping solution by washing, and (3) estimating unalkylated M_2 muscarinic receptors with [^{3}H]NMS.

Experiments on intact cells were carried out as described previously [13]. The medium from CHO hM_2 cells grown in 24-well culture plates (Corning Incorporated, Corning, NY) was replaced with Krebs Ringer Bicarbonate (KRB) buffer (26 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 124 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1.3 mM $MgCl_2$, 10 mM glucose, pH 7.4, gassed with O_2/CO_2 (19:1)), and the cells were allowed to incubate at 37 °C for 10 min. Test drugs (competitive inhibitors or allosteric modulators) were added, and the cells were incubated for an additional 15 min. The reaction was started with the addition of an aliquot of cyclized BR384 (see Section 2.7), and the cells were allowed to incubate for various times at 37 °C in a final volume of 0.6 ml of KRB buffer. The reaction was stopped by the addition of thiosulfate and N-methylamitriptyline at final concentrations of 10 mM and 10 μM, respectively, and the cells were incubated for 15 min at 37 °C to allow thiosulfate to inactivate the aziridinium ion of BR384. The cells were washed a total of five times, and unalkylated M_2 muscarinic receptors were measured in intact cell binding assays using [^{3}H]NMS as described below.

Experiments on homogenates of CHO hM_2 cells were also carried out as described by previously [13]. Confluent cells from eight 100 mm dishes (Corning Incorporated, Corning, NY) were collected and ultimately homogenized in 5.5 ml of assay buffer (20 mM Na HEPES, pH 7.4, 100 mM NaCl and 10 mM EDTA). An aliquot (0.15 ml) of this homogenate was added to microfuge tubes with an additional aliquot (0.05 ml) of assay buffer or buffer plus test drug. The tubes were pre-incubated at 37 °C in a shaking water bath for approximately 10 min, and then the reaction was started by adding an aliquot (0.05 ml) of cyclized BR384. After incubating the tubes for various times, the reaction was stopped by the addition of an aliquot (0.75 ml) of assay buffer containing thiosulfate (1.3 mM) and scopolamine (1.3 or 10 μM, depending upon whether the concentration of BR384 was 0.001 or 0.01–0.1 mM, respectively). Control homogenates were also treated with the stopping solution. The tubes were incubated at 37 °C for 20 min to inactivate the aziridinium ion. Thiosulfate, scopolamine and the transformation products of BR384 (see Fig. 1) were removed by washing (centrifugation at 25,000 × g for 15 min followed by aspiration of the supernatant) a total of two or three times, depending upon whether the concentration of scopolamine was 1.3 or 10 μM, respectively. The final pellets were suspended in 1 ml of 20 mM Na CHES, pH 9.3, 100 mM NaCl and 10 mM EDTA. Unalkylated muscarinic receptors were measured using [^{3}H]NMS as described below.

2.4. [^{3}H]NMS binding assay, intact cells

The specific binding of [^{3}H]NMS (specific activity, 82 Ci/mmol; PerkinElmer, Boston, MA) to intact CHO hM_2 cell monolayers was measured as described previously [13,16]. The incubation medium was 0.6 ml of KRB buffer. The incubation conditions varied depending on the purpose of the experiment. When the kinetics of receptor alkylation by BR384 were investigated at 37 °C, the subsequent incubation with [^{3}H]NMS (1 nM) was 1 h at room temperature. When receptor alkylation by BR384 was investigated at 0 °C, the subsequent incubation with [^{3}H]NMS (1 nM) was for 1 h at 0 °C. The competition experiments with McN-A-343, BR384 and its transformation products at 0 °C were run with a 1-h incubation at 0 °C using 1 nM [^{3}H]NMS. Additional McN-A-343/[^{3}H]NMS competition experiments were run at 37 °C for 1 h using 1 nM [^{3}H]NMS. [^{3}H]NMS saturation experiments at 0 °C were carried out at 0 °C for 3 h, whereas those at 37 °C were run with a 30-min incubation. Following incubation with [^{3}H]NMS, the cells were washed quickly with ice-cold KRB buffer, lysed with NaOH, and [^{3}H]NMS measured as previously described [13]. Non-specific binding was defined as residual binding in the presence of 10 μM atropine.

2.5. [^{3}H]NMS binding assay, cellular homogenates

The specific binding of [^{3}H]NMS to homogenates of CHO hM_2 cells that had been previously reacted with BR384 was measured as described previously [13]. If the homogenate had been exposed to the stopping solution containing scopolamine, the incubation was carried out at pH 9.3 in 20 mM Na CHES, 100 mM NaCl and 10 mM EDTA. Otherwise, pH 7.4 assay buffer was used (20 mM Na HEPES, pH 7.4, 100 mM NaCl and 10 mM EDTA). The high pH buffer was used because the affinity of [^{3}H]NMS for the M_2 receptor is nearly maintained at pH 9.3, whereas that of scopolamine is reduced to one-fiftieth [17]. Aliquots of homogenate were incubated at 37 °C for about 30 min in a final volume of 1.0 ml of assay buffer containing [^{3}H]NMS. The concentration of [^{3}H]NMS was 1 nM for those experiments investigating the kinetics of receptor alkylation by BR384. Specifically bound [^{3}H]NMS was trapped by rapid filtration using a cell harvester (Brandel, Gaithersburg, MD) as described previously [17]. Non-specific binding was defined as the residual binding in the presence of 10 μM atropine (pH 7.4) or 10 μM NMS (pH 9.3). All measurements were made in triplicate. Experiments on the kinetics of muscarinic receptor alkylation in homogenates of the rat heart were done as described previously [14].

2.6. Analysis of data

The binding data were analyzed by nonlinear regression analysis using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA) to estimate first order decay rate constants and binding affinities from competitive inhibition and saturation experiments. The calculation of the parameters was as described by Griffin et al. [16] and Ehlert and Griffin [18].

In our analysis of the kinetics of M_2 receptor alkylation by BR384 in the presence of an allosteric modulator (A), we have assumed that the aziridinium ion (X) rapidly forms a reversible complex with the M_2 receptor (R), which converts to a covalent complex (X–R) at a slower rate characterized by the rate constant, k_1, as shown in Fig. 2. In this scheme, K_1 and K_2 denote the affinity constants (inverse molar units; reciprocal of dissociation constant) of the aziridinium ion of BR384 and the allosteric modulator for the free receptor, respectively, and α denotes the cooperativity factor for their interaction. Our derivation of the solution to this model follows the strategy described previously [13], but with the correction for the decay in the aziridinium ion described by Ehlert and Jenden [14]. The rate of loss of unalkylated receptors is described by the differential equation:

$$\frac{dy}{dt} = -[XR + XRA]k_1 \quad (1)$$

in which y denotes the total amount of receptors not alkylated by the aziridinium ion ($R + XR + XRA$). Replacing the term [$XR + XRA$] with a function for receptor occupancy by the aziridinium ion in the presence of an allosteric modulator yields [19]:

$$\frac{dy}{dt} = -\frac{Xy}{X + (1/K_1((1 + A\alpha K_2)/(1 + AK_2)))}k_1 \quad (2)$$

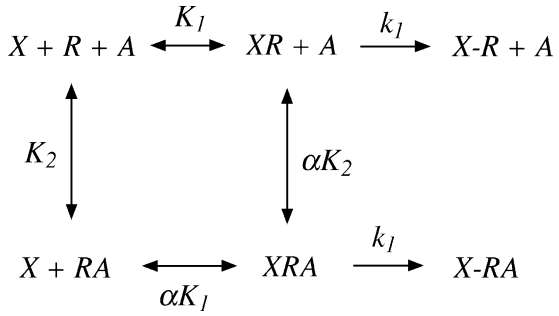

Fig. 2. Model for the interaction of the aziridinium ion of BR384 (X) with the M_2 muscarinic receptor (R) in the presence of an allosteric modulator (A). The affinity constants of the aziridinium ion and allosteric modulator are denoted by K_1 and K_2, respectively. The cooperativity between the binding of the two ligands is denoted by α.

in which, K_1 and K_2 denote the affinity constants of X and A, and α denotes the cooperativity factor for their allosteric interaction. Dividing both sides by y and integrating over the time interval $t = 0$ to t yields:

$$\ln\frac{y}{y_0} = -k_1\int_0^t \frac{X}{X + (1/(K_1(1 + A\alpha K_2)/(1 + AK_2)))}dt \tag{3}$$

in which, y_0 denotes the total receptor density at the start of the reaction, and y denotes the residual, unalkylated receptors at time t. The concentration of the aziridinium ion is not constant during the incubation, but decays exponentially from its peak concentration according to the following equation:

$$X = X_0 e^{-t/\tau_x} \tag{4}$$

in which X_0 denotes the concentration of the aziridinium ion at the start of the incubation and t_x denotes the time constant for the decay of the aziridinium ion. The value of τ_x was estimated at 14.3 min by nonlinear regression analysis of the data reported by Ringdahl et al. [15] for the solvolysis of the aziridinium ion in aqueous solution at pH 7.4. A time constant is used to describe the observed decay of the aziridinium ion from its peak concentration to distinguish it from the two fundamental rate constants for the formation (k_f) and decay (k_d) of the aziridinium ion. Substituting Eq. (4) for X in Eq. (3) yields:

$$\ln\frac{y}{y_0} = -k_1\int_0^t \frac{X_0 e^{-t/\tau_x}}{X_0 e^{-t/\tau_x} + (1/(K_1(1 + A\alpha K_2)/(1 + AK_2)))}dt \tag{5}$$

Evaluating the integral over the interval $t = 0$ to t yields:

$$\ln\frac{y}{y_0} = k_1\tau_x\left[\ln\left(X_0 e^{-t/\tau_x} + \frac{1}{K_1((1 + A\alpha K_2)/(1 + AK_2))}\right) - \ln\left(X_0 + \frac{1}{K_1((1 + A\alpha K_2)/(1 + AK_2))}\right)\right] \tag{6}$$

This equation reduces to:

$$\frac{y}{y_0} = \left(\frac{X_0 e^{-t/t_x}K_1((1 + A\alpha K_2)/(1 + AK_2)) + 1}{X_0 K_1((1 + A\alpha K_2)/(1 + AK_2)) + 1}\right)^{k_1\tau_x} \tag{7}$$

Eq. (7) was modified to included a BR384 insensitive component of receptors (b), because a small proportion (b) of M_2 receptors was not alkylated by BR384:

$$\frac{y}{y_0} = (1 - b)\left(\frac{X_0 e^{-t/\tau_x}K_1((1 + A\alpha K_2)/(1 + AK_2)) + 1}{X_0 K_1((1 + A\alpha K_2)/(1 + AK_2)) + 1}\right)^{k_1\tau_x} + b \tag{8}$$

This equation describes the loss of [^{3}H]NMS binding sites caused by incubation with BR384 in the presence of an allosteric modulator. If the negative cooperativity is great (i.e., $\log\alpha \ll 0$), then A behaves like a competitive inhibitor, and Eq. (8) reduces to:

$$\frac{y}{y_0} = (1 - b)\left(\frac{X_0 e^{-t/\tau_x}K_1(1/(1 + IK_i)) + 1}{X_0 K_1(1/(1 + IK_i)) + 1}\right)^{k_1\tau_x} + b \tag{9}$$

Here, A and K_2 have been replaced with I and K_i, respectively, to denote the concentration and affinity constant of the competitive inhibitor. In the absence of A, Eq. (8) reduces to:

$$\frac{y}{y_0} = (1 - b)\left(\frac{X_0 e^{-t/\tau_x}K_1 + 1}{X_0 K_1 + 1}\right)^{k_1\tau_x} + b \tag{10}$$

In assessing the inhibitory effect of ACh on the alkylation of M_2 receptors by BR384 in CHO hM_2 homogenates, we introduced a Hill slope (n) into Eqs. (8) and (9) because it is known that highly efficacious agonists exhibit shallow occupancy curves in agonist/[^{3}H]NMS competition binding experiments. The corresponding equations are:

$$\frac{y}{y_0} = (1 - b)\left(\frac{X_0 e^{-t/\tau_x}K_1((1 + (A\alpha K_2)^n)/(1 + (AK_2)^n)) + 1}{X_0 K_1((1 + (A\alpha K_2)^n)/(1 + (AK_2)^n)) + 1}\right)^{k_1\tau_x} + b \tag{11}$$

$$\frac{y}{y_0} = (1 - b)\left(\frac{X_0 e^{-t/\tau_x}K_1(1/(1 + (IK_i)^n)) + 1}{X_0 K_1(1/(1 + (IK_i)^n)) + 1}\right)^{k_1\tau_x} + b \tag{12}$$

We also used an empirical approach to assess the effects of allosteric modulators and competitive inhibitors on the kinetics of M_2 receptor alkylation by BR384. During an incubation with BR384, unalkylated M_2 receptors undergo a first order decay characterized by an observed rate constant (k_{obs}). This constant was estimated from the binding data using nonlinear regression analysis with a first order decay equation. Only the data obtained over the first 8 min of incubation with BR384 were used to avoid error associated with the decline of the aziridinium ion concentration. We define the time constant (τ) for alkylation as:

$$k_{obs} = \frac{1}{\tau} \tag{13}$$

As described previously [13], the ratio (R) of the time constant for receptor alkylation in the presence of an allosteric modulator (τ'') divided by that measured in its absence (τ) is described by:

$$\log(R) = \log\left(\frac{\tau''}{\tau}\right) = \log\left(\frac{XK_1 + ((1 + AK_2)/(1 + A\alpha K_2))}{1 + XK_1}\right) \tag{14}$$

The corresponding ratio for a competitive inhibition (τ'/τ) is [13]:

$$\log(R) = \log\left(\frac{\tau'}{\tau}\right) = \log\left(\frac{1 + XK_1 + IK_i}{1 + XK_1}\right) \tag{15}$$

If $\log(R - 1)$ is plotted against $\log I$, a competitive interaction will yield a straight line with a slope of 1 and an x-interceptor of $-\log K_i + \log(1 + XK_1)$ as described previously for the analogous relationship using dissociation constants instead of affinity constants [14]. We did not transform the data in this manner, however, because this transformation tends to magnify error in the estimation of low R values. For reasons described above in the case of Eqs. (11) and (12), a Hill slope was added to Eqs. (14) and (15) when the behavior of ACh was analyzed:

$$\log(R) = \log\left(\frac{\tau''}{\tau}\right) = \log\left(\frac{XK_1 + ((1 + (AK_2)^n)/(1 + (A\alpha K_2)^n))}{1 + XK_1}\right) \tag{16}$$

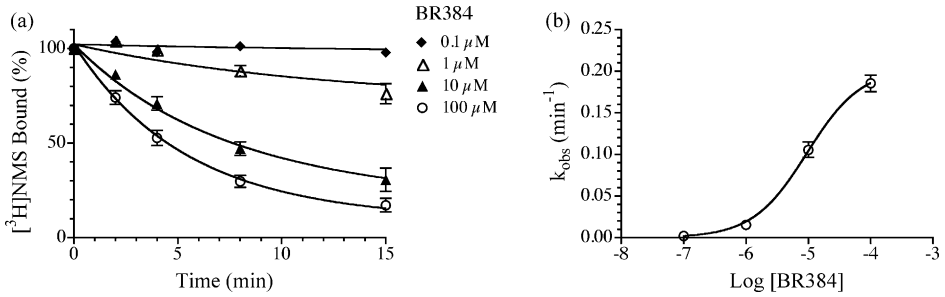

Fig. 3. The kinetics of the alkylation of the M_2 muscarinic receptor by BR384. (a) Intact CHO hM_2 cells were incubated at 37 °C with various concentrations of cyclized BR384 for the indicated times, washed and assayed for [^{3}H]NMS binding. The data are plotted with respect to the initial concentration of the parent mustard. Mean binding values ± SEM of two experiments, each done in triplicate, are plotted. The theoretical curve represents the least-squares fit of Eq. (10) to the data. The concentration of [^{3}H]NMS in the binding assay was 1 nM. (b) The observed rate constants for the loss of [^{3}H]NMS binding at each concentration of BR384 were calculated from the data in Fig. 2a and are plotted against the log of the initial concentration of BR384. A simple one-site binding model was fitted to the data.

$$\log(R) = \log\left(\frac{\tau'}{\tau}\right) = \log\left(\frac{1 + XK_1 + (IK_i)^n}{1 + XK_1}\right) \quad (17)$$

To test whether the allosteric models provided a better fit to the data than the competitive models, we computed the statistic F:

$$F = \frac{(SS_1 - SS_2)/(df_1 - df_2)}{SS_2/df_2} \quad (18)$$

in which SS_1 and SS_2 denote the residual sum of squares for the best fit of Eqs. (9) and (8) to the data, respectively, and df_1 and df_2, denote the corresponding degrees of freedom. The statistic F is distributed according to an F distribution with the degrees of freedom in the numerator and denominator being $df_1 - df_2$ and df_2, respectively.

2.7. Cyclization of BR384

A solution of BR384 (5 mM) was routinely stored at −20 °C in acetone. On the day of the experiment, an aliquot of this solution was diluted in 4 volumes of 10 mM phosphate buffer (Na_2HPO_4/KH_2PO_4), pH 7.4, and immediately incubated at 37 °C for 5 min. Then the solution was immediately transferred to a small flask suspended in air, and the acetone removed *in vacuo* in about 5–10 min using a rotary evaporator. The actual temperature of the solution quickly dropped to about 10 °C because of loss of the heat of vaporization. The solution was immediately placed on ice and used as soon as possible. The formation of the aziridinium ion was measured previously by ^{1}H NMR, and the rate constants for formation (k_f, 0.24 ± 0.01 min^{-1}) and decay (k_d, 0.099 ± 0.012 min^{-1}) of the aziridinium reported [15]. The peak concentration of the aziridinium ion forms at approximately 6.3 min at 37 °C and represents 54% of the initial parent mustard concentration. The aziridinium ion decays to approximately 5.3% after 30 more min of incubation.

2.8. Materials

Dulbecco's Modified Eagle Medium with high glucose plus L-glutamine, fetal calf serum and penicillin–streptomycin, were obtained from Invitrogen Corporation, Grand Island, NY. Amitriptyline, G418 disulfate salt, NMS, WIN 51,708, atropine, acetylcholine perchlorate gallamine, HEPES, CHES, EDTA, scopolamine, sodium thiosulfate and our initial supply of McN-A-343 were from Sigma–Aldrich, St. Louis, MO. Additional McN-A-343 was synthesized as described previously [20], and N-methylamitriptyline, BR384 and its hydrolysis product were synthesized as described by Suga et al. [13] and Ringdahl et al. [15], respectively. The reactants for organic synthesis were obtained from Sigma–Aldrich. Salts for KRB buffer and phosphate buffer were obtained from Fisher Scientific, Fair Lawn, NJ.

3. Results

3.1. Alkylation of the M_2 muscarinic receptor by cyclized BR384

Incubation of homogenates of CHO hM_2 cells with cyclized BR384 (1 μM) at 37 °C for 2 min caused a decrease in the binding capacity of the muscarinic antagonist [^{3}H]NMS when binding measurements were made at pH 7.4 and 0 °C after washing the homogenates to remove unreacted mustard and its transformation products. In control cells, the estimate of the logarithm of the affinity constant ($\log K_{NMS}$) of [^{3}H]NMS was 9.32 ± 0.057. After treatment with BR384 the binding capacity was reduced to 67 ± 2.1% of control while $\log K_{NMS}$ was unaffected (9.38 ± 0.041). Similar results were obtained when the binding assay carried out at pH 9.3 instead of 7.4. The data are consistent with the postulate that BR384 binds irreversibly to the M_2 muscarinic receptor.

3.2. Kinetics of the alkylation of the M_2 muscarinic receptor by BR384

We investigated the kinetics of the loss of [^{3}H]NMS binding caused by the aziridinium ion of BR384. Intact CHO hM_2 cells were incubated with cyclized BR384 for various periods of time, and the cells were washed prior to measuring [^{3}H]NMS binding at a single concentration (Fig. 3a). BR384 caused a first order loss of binding with the time constant for the loss being dependent on the concentration of BR384. The data appear to be consistent with scheme shown in Fig. 2, in which the rate of alkylation is proportional to receptor occupancy by the aziridinium ion. To test this postulate we fitted Eq. (10) to the data sharing the estimate of the affinity constant of the aziridinium ion (K_1) and the rate constant for alkylation (k_1) among the curves. Regression analysis yielded estimates ± SEM for $\log K_1$ (5.20 ± 0.066) and k_1 (0.20 ± 0.023). Approximately 9% of the receptor population behaved as resistant to alkylation by BR384, perhaps because of recycling of receptors to the membrane. We also fitted the initial portion of each curve (0–8 min) in Fig. 3a independently to a decreasing exponential equation (with the plateau value fixed at 9%) and plotted the estimate of the observed rate constant for alkylation (k_{obs}) against the concentration of BR384 (see Fig. 3b). Only the initial part of the decay curves was used to avoid error in the estimate of k_{obs} due to decay of the aziridinium ion. The data show that the observed rate for alkylation increases as a mass-action-like function of the BR384 concentration. A half-maximal increase in rate occurred at a negative log BR384 concentration (pEC_{50} = 5.04 ± 0.08) nearly equivalent to

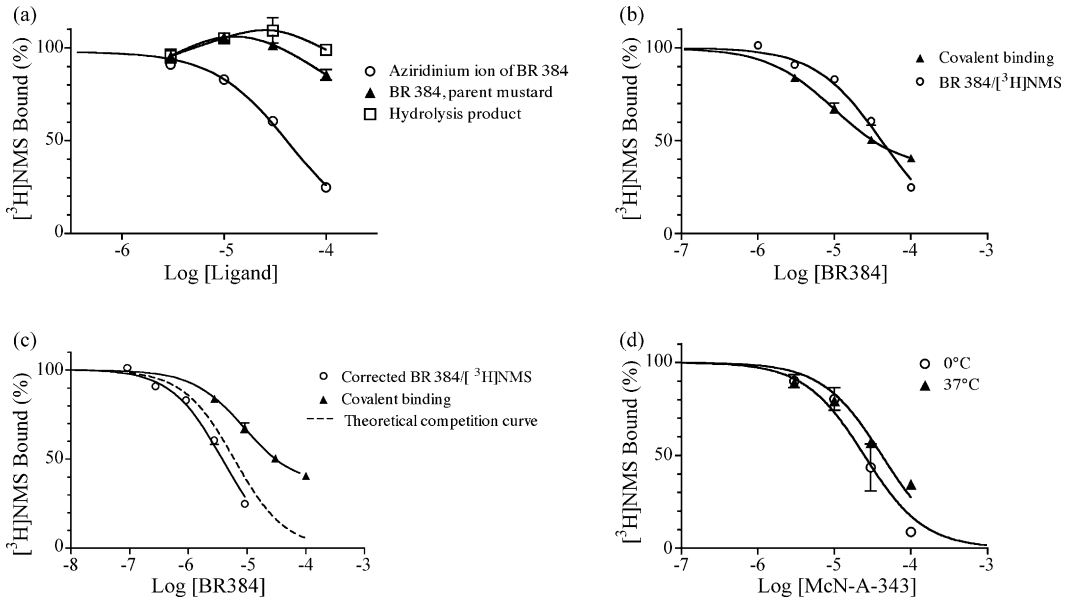

Fig. 4. The binding of McN-A-343, BR384 and its transformation products to intact CHO hM_2 cells at 0 °C. (a) The specific binding of [^{3}H]NMS (1 nM) was measured in intact CHO hM_2 cells in the presence of various concentrations of BR384 in the form of parent mustard, aziridinium ion and alcoholic hydrolysis product. The incubation for the binding assay lasted 1 h at 0 °C. Mean binding values ± SEM from nine (aziridinium ion) and two (parent mustard and alcoholoic hydrolysis product) experiments, each done in triplicate, are shown. (b) The data indicated as "covalent binding" refer to experiments in which CHO hM_2 cells were first incubated with the indicated concentrations of the aziridinium ion of BR384 for 1 h and then washed. The binding of [^{3}H]NMS (1 nM) to the residual, unalkylated receptors was then measured. The BR384/[^{3}H]NMS competition curve from panel 'a' is also shown for comparison. Mean binding values ± SEM from nine experiments, each done in triplicate, are shown. (c) The data indicated as "corrected BR384/[^{3}H]NMS" represent the competitive binding curve for the aziridinium ion of BR384 shown in panel 'a', shifted to the left by a factor of 11 to correct for the competitive effect of 1 nM [^{3}H]NMS. The data indicated as "covalent binding" represent the covalent binding of the aziridinium ion of BR384 from panel *b*. The dashed line represents the corrected BR384/[^{3}H]NMS competition curve normalized to the covalent binding curve. (d) The competitive inhibition of [^{3}H]NMS binding (1 nM) by various concentration of McN-A-343 was measured at 0 and 37 °C to intact CHO hM_2 cells. Mean binding values ± SEM from two experiments, each done in triplicate, are shown.

the estimate of log K_1 determined from the analysis shown in Fig. 3a. Also, the estimate of the maximal value of the observed rate constant (0.20 min^{-1}) is nearly identical to that of k_1. These results indicate that BR384 equilibrates rapidly with the M_2 muscarinic receptor to form a reversible receptor complex (log K_1 = 5.20) that converts to a covalent complex at a relatively slower rate (0.20 min^{-1}).

3.3. Competitive [^{3}H]NMS binding experiments with BR384 and its transformation products

We measured the inhibition of [^{3}H]NMS binding to intact CHO hM_2 cells by BR384 and its transformation products at 0 °C. Lowering the temperature to 0 °C slows the rate of alkylation of the M_2 receptor by the aziridinium ion of BR384. Fig. 4a shows that at 0 °C, the aziridinium ion inhibits [^{3}H]NMS binding with a pIC_{50} value of 4.39, whereas the parent mustard and the alcoholic hydrolysis product were much less active. When BR384 was tested in its parent form, it was dissolved in ice-cold dilute HCl to prevent formation of the aziridinium ion, and then aliquots of this solution were added to the binding assay at pH 7.4 in the presence of thiosulfate (1 mM). At 0 °C, the formation of the aziridinium ion is negligible over an hour at pH 7.4.

The inhibitory effect of the aziridinium ion shown in Fig. 4a could be caused by a combination of covalent and competitive effects. To determine the amount of covalent binding, we incubated intact CHO cells with various concentration of the aziridinium ion of BR384 at 0 °C for 1 h, washed the cells, and then measured [^{3}H]NMS binding after a 1-h incubation at 0 °C. These results are plotted in Fig. 4b together with the BR384/[^{3}H]NMS inhibition curve from panel 'a' for comparison. The results show that a substantial wash-resistant inhibition of [^{3}H]NMS binding occurs after 1 h at 0 °C and that this covalent inhibition of [^{3}H]NMS binding by BR384 is actually greater than that shown for the BR384/[^{3}H]NMS inhibition curve over the concentration range of 3–30 μM BR384. In the case of the BR384/[^{3}H]NMS inhibition curve, however, [^{3}H]NMS (1 nM) competitively reduces both the reversible and irreversible binding of BR384 to the receptor. To determine the theoretical composite binding curve (reversible and irreversible binding) for BR384 in the absence of [^{3}H]NMS at 0 °C, we corrected the BR384/[^{3}H]NMS inhibition curve for the competitive effect of [^{3}H]NMS at 1 nM (11-fold shift), and plotted the corrected curve in Fig. 4c together with the covalent binding data for comparison. Next, we expressed the binding values of the corrected BR384 composite binding curve relative to the covalent binding curve to generate the theoretical reversible component of the binding curve for the aziridinium ion of BR384. This theoretical curve is shown with the dashed line in Fig. 4c. The log affinity constant for the aziridinium ion was estimated from this curve as 5.22. This estimate is similar to that calculated from the data shown in Fig. 3 (log K_1 = 5.2–5.0). We also measured the competitive inhibition of [^{3}H]NMS binding by McN-A-343 at 0 and 37 °C (Fig. 4d), and the log affinity constants were 4.65 ± 0.16 and 4.38 ± 0.02, respectively.

3.4. Effect of muscarinic ligands on the kinetics M_2 receptor alkylation by BR384

We measured the influence of NMS on the alkylation of the M_2 muscarinic receptor by cyclized BR384 in intact CHO hM_2 cells. Increasing the concentration of NMS progressively slowed the rate of alkylation caused by BR384 (0.1 mM) (Fig. 5a). The data were consistent with a competitive model (Eq. (9)), and there was no significant improvement in residual error when the allosteric model was used. Regression analysis was done setting the affinity constant of the aziridinium ion (K_1) equal to that estimated from the data in Fig. 2 (5.2). The estimate of the mean log affinity constant ± SEM of NMS was 10.67 ± 0.06. The consistency of the data

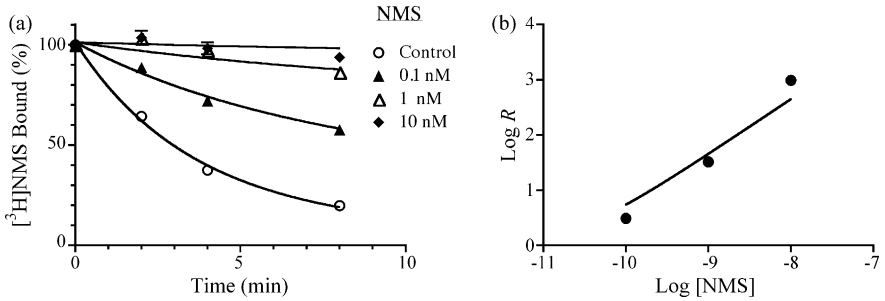

Fig. 5. The effect of NMS on the rate of alkylation of the M_2 muscarinic receptor by BR384. (a) CHO hM_2 cells were incubated at 37 °C with 0.1 mM BR384 for the indicated times in the absence and presence of various concentrations of NMS. The cells were washed and assayed for [^{3}H]NMS binding (1 nM). The data represent the mean binding values ± SEM of two experiments, each done in triplicate. (b) The ratio (R) of the time constant for receptor alkylation by BR384 in the presence of NMS divided by that measured in its absence is plotted against the concentration of NMS. The theoretical curve represents the least-squares fit of Eq. (15) to the data, which yielded an estimate of log K_i(10.8). The R values have been calculated from the data shown in panel 'a'.

with competitive behavior is apparent in Fig. 5b, which shows that the relative increase in the time constant for alkylation (R) is proportional to the NMS concentration.

A similar type of experiment was done with the allosteric modulator, gallamine [8,21], in intact CHO hM_2 cells. Although gallamine slowed the rate of receptor alkylation caused by BR384 (0.01 mM), its effect reached a limit at high concentrations (0.01–0.1 mM) (Fig. 6a). At a higher concentration of BR384 (0.1 mM), gallamine had little effect on the kinetics of receptor alkylation (Fig. 6b). The data were well described by the allosteric model (Eq. (8)), and regression analysis yielded log mean estimates ± SEM for the affinity constant of gallamine (6.12 ± 0.14) and its cooperativity with BR384 (−0.90 ± 0.06). The deviation from competitive behavior is clearly shown in Fig. 6c which shows that the time constant for alkylation tends to reach a plateau at a high concentration of gallamine and is unaffected by gallamine when the concentration of BR384 is high (0.1 mM).

We also investigated the effects of WIN 51,708, which is thought to interact at an allosteric site distinct from that of gallamine and to cause a modest reduction in the affinity (about 2-fold) of acetylcholine for the M_2 muscarinic receptor [22]. When intact CHO hM_2 cells were incubated with BR384 at concentration of 0.01 and 0.1 mM, WIN 51,708 had little or no effect on receptor alkylation (Fig. 7b and c, respectively). We repeated this experiment using a lower concentration of BR384 (1 μM) in homogenates of CHO hM_2 cells, because we found that the rate of receptor alkylation was faster in homogenates. This condition would enable us to detect a slowing effect of WIN 51,708 on receptor alkylation by lower concentrations of BR384 more easily. We were still unable to detect an effect of WIN 51,708 on the rate of receptor alkylation (Fig. 7a).

The effects of ACh and McN-A-343 on M_2 receptor alkylation by BR384 (0.01 and 0.1 mM) in homogenates of CHO hM_2 cells are shown in Figs. 8a and b and 9a and b, respectively. Both compounds caused a competitive-like inhibition of the rate of alkylation of the M_2 receptor by BR384 as illustrated by the linear increase in the relative time constant for alkylation (R) as the concentration of each competitor increased (Figs. 8c and 9c). There was no significant increase in residual error when the competitive model was fitted to the data as compared to the allosteric model. In the case of ACh, regression analysis (Eq. (12)) yielded mean estimates of the log affinity constant (K_i) and Hill slope (n) of 6.12 ± 0.09 and 0.78 ± 0.034, respectively. Control experiments showed that the stopping solution used in our kinetic experiments immediately prevented receptor alkylation by BR384 (0.1 mM). When McN-A-343 was present at the highest concentration (10 mM), however, the stopping solution did not stop the reaction of BR384 with the receptor immediately, but allowed a further 10–20% alkylation. As a result, the kinetic curve obtained in the presence

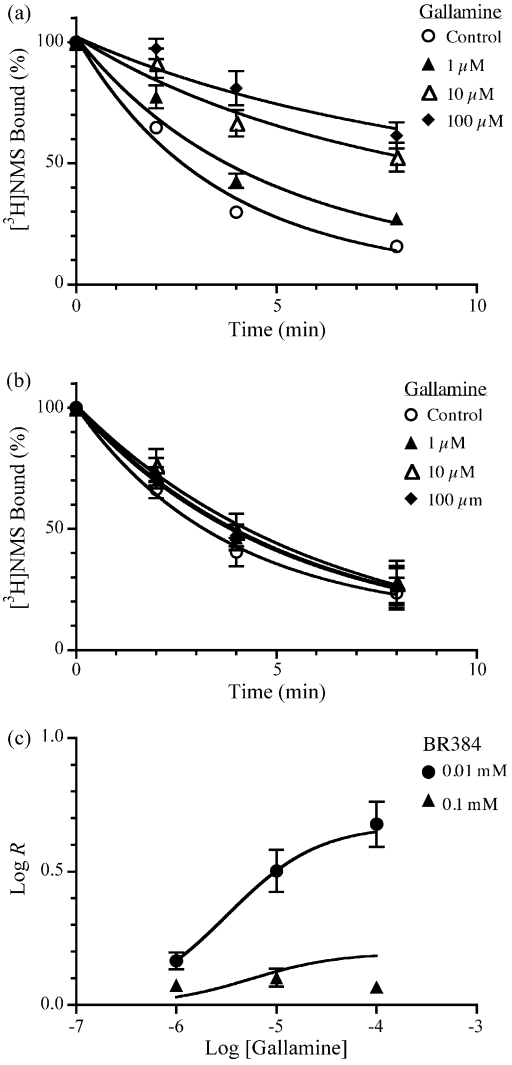

Fig. 6. The effect of gallamine on the rate of alkylation of the M_2 muscarinic receptor by BR384. CHO hM_2 cells were incubated at 37 °C with BR384 at concentrations of 0.01 mM (a) or 0.1 mM (b) for the indicated times in the absence and presence of the indicated concentrations of gallamine, washed and assayed for [^{3}H]NMS binding. The data represent the mean binding values ± SEM of four (a) and two (b) experiments, each done in triplicate. The concentration of [^{3}H]NMS in the binding assay was 1 nM. (c) The ratio (R) of the time constant for receptor alkylation by BR384 in the presence of gallamine divided by that measured in its absence is plotted against the concentration of gallamine. The theoretical curves represent the global, least-squares fit of Eq. (14) to the data, which yielded estimates of log K_2(6.18) and log α(−1.02). The R values have been calculated from the data shown in panels 'a' and 'b'.

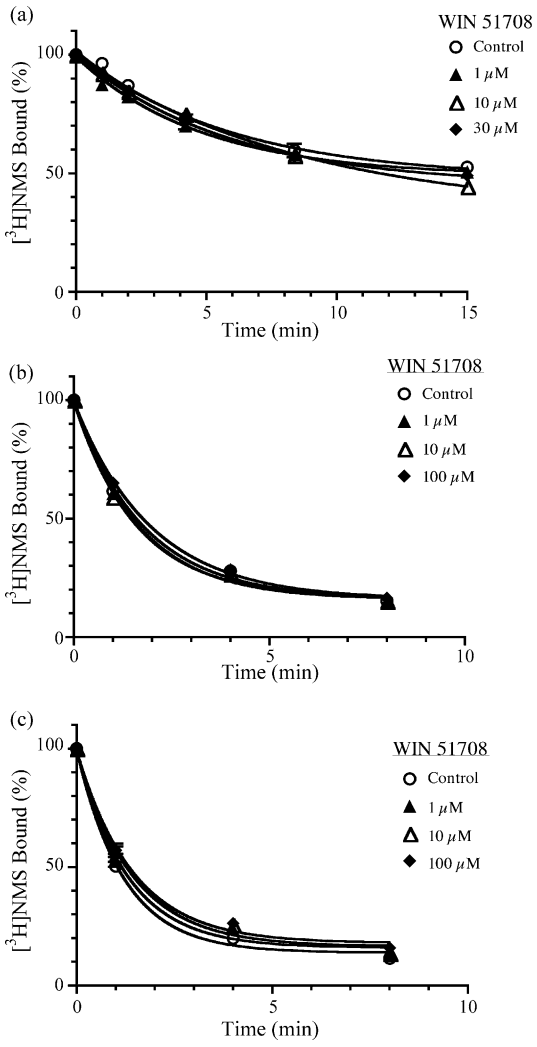

Fig. 7. The effect of WIN 51,708 on the rate of alkylation of the M_2 muscarinic receptor by BR384. (a) Homogenates of CHO hM_2 cells were incubated at 37 °C with BR384 (1 μM) for the indicated times in the absence and presence of various concentrations of WIN 51,708, washed and assayed for [^{3}H]NMS binding. (b) The experiment was the same as that shown in 'a' except that intact CHO hM_2 cells were used and the concentration of BR384 was 0.01 mM. (c) Same as *b* except that the concentration of BR384 was 0.1 mM. The data represent the mean binding values ± SEM of two experiments, each done in triplicate. The concentration of [^{3}H]NMS in the binding assay was 1 nM.

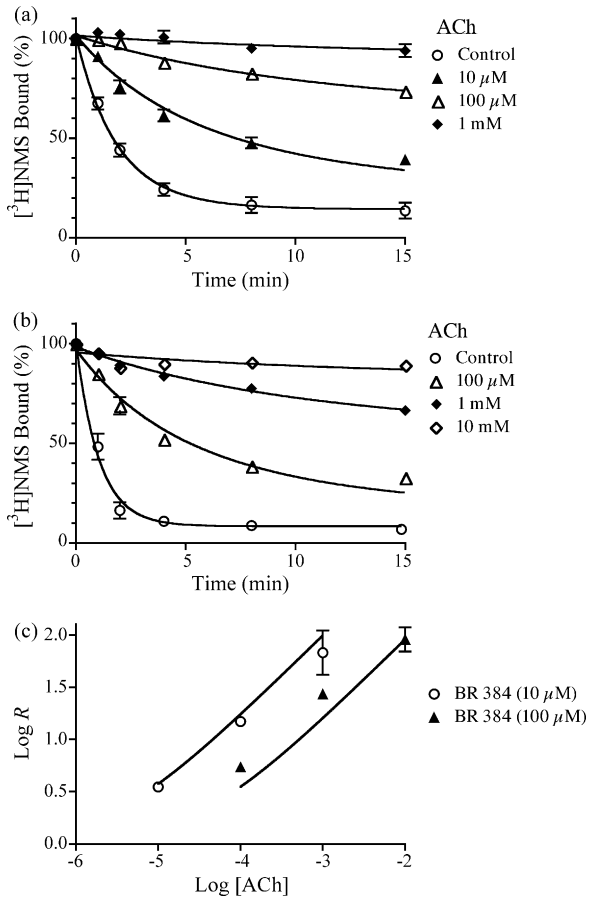

Fig. 8. The effect of ACh on the rate of alkylation of the M_2 muscarinic receptor by BR384. Homogenates of CHO hM_2 cells were incubated at 37 °C with BR384 at concentrations of 0.01 mM (a) or 0.1 mM (b) for the indicated times in the absence and presence of various concentrations of ACh, washed and assayed for [^{3}H]NMS binding. The data represent the mean binding values ± SEM of three (a) or two (b) separate experiments, each done in triplicate. The concentration of [^{3}H]NMS was 1 nM. (c) The ratio (R) of the time constant for receptor alkylation by BR384 in the presence of ACh divided by that measured in its absence is plotted against the concentration of ACh. The theoretical curves represent the global, least-squares fit of Eq. (17) to the data, which yielded estimates of log K_2(6.10) and n(0.78). The R values have been calculated from the data shown in panels 'a' and 'b'.

of 0.1 mM BR384 and 10 mM McN-A-343 intersected the ordinate (time = 0) at approximately 80% of control binding. When analyzing these data according to Eqs. (8) and (9), we normalized the data obtained in the presence of 10 mM McN-A-343 relative to its own maximum (about 80%). Global regression analysis of the data in Fig. 9a and b yielded an estimate of 4.91 ± 0.07 for the affinity constant of McN-A-343. We repeated experiments with McN-A-343 on intact cells and obtained an estimate of the log affinity constant of McN-A-343 of 4.78 ± 0.06 (data not shown).

We also investigated the effects of NMS and gallamine on the kinetics of muscarinic receptor alkylation by BR384 (0.1 mM) in homogenates of the rat heart. The rat heart is known to express a relatively homogenous population of M_2 muscarinic receptors [23]. The data with NMS (Fig. 10a) were consistent with a competitive mechanism (Eq. (9)), and regression analysis yielded an estimate of 9.36 ± 0.09 for the affinity constant of NMS. The data with gallamine (Fig. 10b) are similar to those obtained in CHO hM_2 cells with 0.1 mM BR384 (Fig. 6b), and are not inconsistent with an allosteric mechanism, although they are insufficient to estimate the affinity constant of gallamine.

4. Discussion

Treatment of intact CHO hM_2 cells with BR384 followed by washing caused a reduction in the binding capacity of [^{3}H]NMS without a significant change in affinity. Similar results were observed in homogenates of the rat cerebral cortex that had been previously treated with cyclized BR384 and washed [15]. Our results show that BR384 binds covalently with the M_2 muscarinic receptor presumably through the reaction of the aziridinium ion with a nucleophile on the receptor.

The mode of binding of BR384 was consistent with a two-step mechanism in which the aziridinium ion rapidly equilibrates with the M_2 receptor in the form of a reversible complex that converts to a covalent complex at a slower rate. The observed rate constant for the conversion of the reversible complex to the covalent complex appeared to be proportional to the amount of reversible receptor complex. The good agreement between the log affinity constant of BR384 estimated from the kinetics of alkylation (5.20, Fig. 3) and that measured by competitive inhibition of [^{3}H]NMS binding (5.22, Fig. 4c) supports this model.

Once occupied by the aziridinium ion of BR384, M_2 receptors in intact CHO cells are alkylated with an average rate constant of

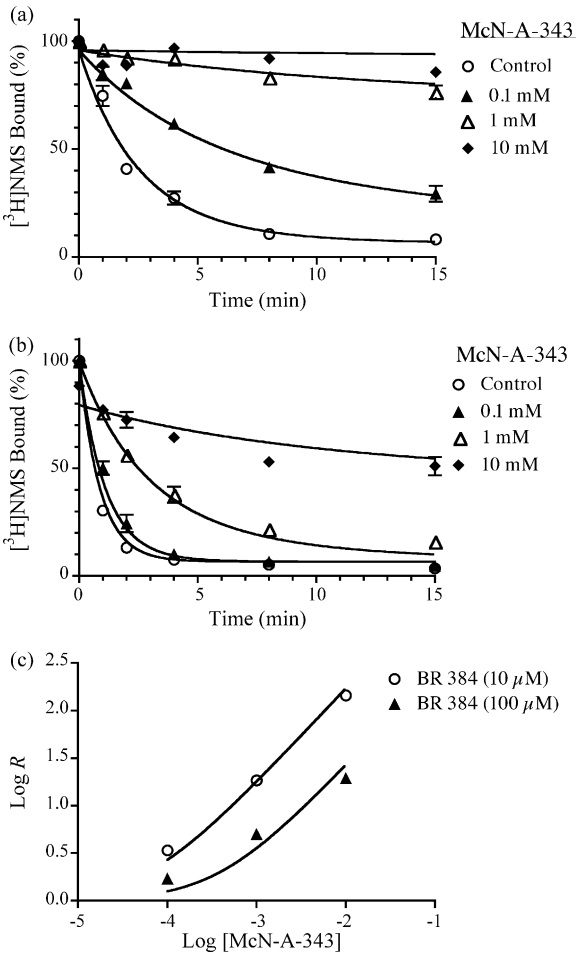

Fig. 9. The effect of McN-A-343 on the rate of alkylation of the M_2 muscarinic receptor by BR384. Homogenates of CHO hM_2 cells were incubated at 37 °C with BR384 at concentrations of 0.01 mM (a) and 0.1 mM (b) for the indicated times in the absence and presence of various concentrations of McN-A-343, washed and assayed for [^{3}H]NMS binding. The data represent the mean binding values ± SEM of three experiments, each done in triplicate. The concentration of [^{3}H]NMS was 1 nM. (c) The ratio (R) of the time constant for receptor alkylation by BR384 in the presence of McN-A-343 divided by that measured in its absence is plotted against the concentration of McN-A-343. The theoretical curves represent the global, least-squares fit of Eq. (15) to the data, which yielded an estimate of log K_2(4.70). The R values have been calculated from the data shown in panels 'a' and 'b'.

0.46 ± 0.09 min^{-1} (pooled estimate from all experiments on intact cells), which corresponds to a half-time of about 1.5 min. In homogenates, the average rate constant was approximately 2-fold larger (0.95 ± 0.03 min^{-1}, half-time = 44 s). This difference may be related to recycling of receptor in intact cells. We have reported a similar finding with AChM, although the overall rates were slower [13].

The results of our experiments with ACh, McN-A-343 and NMS showed that these ligands inhibited receptor alkylation in a manner proportional to their concentration, which is consistent with the postulate that BR384 and the three drugs bind in a mutually exclusive manner to the M_2 receptor (i.e., competitively). Because of their considerably larger size, McN-A-343 and NMS obviously derive some affinity by interacting with additional residues in and around the orthosteric binding pocket that ACh does not interact with. Our results, therefore, are not inconsistent with the postulate of Valant et al. [6], that McN-A-343 is a bitopic ligand that interacts simultaneously with allosteric and orthosteric binding sites.

In contrast, the inhibition of alkylation caused by gallamine reached a plateau at high concentrations, which is consistent with an allosteric mechanism. Also, WIN 51,708 had little or no influence on the alkylation of the M_2 receptor by BR384. Thus, the irreversible BR384-mediated reduction in [^{3}H]NMS binding is not caused by alkylation of the allosteric sites for gallamine and WIN 51,708.

If BR384 (0.1 mM), scopolamine (10 μM) and thiosulfate (1 mM) are added to homogenates of CHO M_2 cells simultaneously, there is no detectable alkylation of M_2 receptors. The reaction of thiosulfate (1 mM) with the aziridinium ion of BR384 (as well as other nitrogen mustards) requires about 15–20 min for completion so that substantial receptor alkylation can occur after the addition of thiosulfate unless a competitive inhibitor (e.g., scopolamine) is present to stop the alkylation. We did find that at a very high concentration (10 mM), McN-A-343 inhibited the ability of scopolamine (10 μM) to stop the reaction of BR384 (0.1 mM) with the M_2 receptor. This observation suggests that McN-A-343 binds to the allosteric site at high concentrations and prevents access of scopolamine to the orthosteric site. Previous investigators have concluded that Mc-A-343 interferes with the access and egress of [^{3}H]NMS to the orthosteric site [8,9].

There is general agreement between estimates of the affinity constants of ACh, McN-A-343 and gallamine that we measured by inhibition of the kinetics of receptor alkylation and those estimated in equilibrium binding experiments with [^{3}H]NMS. This concurrence supports the two-step model for receptor alkylation. The estimate of the log cooperativity between the binding of gallamine and the aziridinium ion of BR384 was −0.9, which corresponds to a reduction in binding affinity to one-eighth of control. Previously, we found that the log cooperativity between gallamine and the aziridinium ion of AChM was approximately −2.1, corresponding to a reduction in affinity of about one hundredth of control. These differences in the negative cooperativity between the binding of gallamine and the two nitrogen mustard analogs are entirely consistent with an allosteric mechanism. The estimate of the affinity constant of gallamine for its allosteric site was approximately the same whether

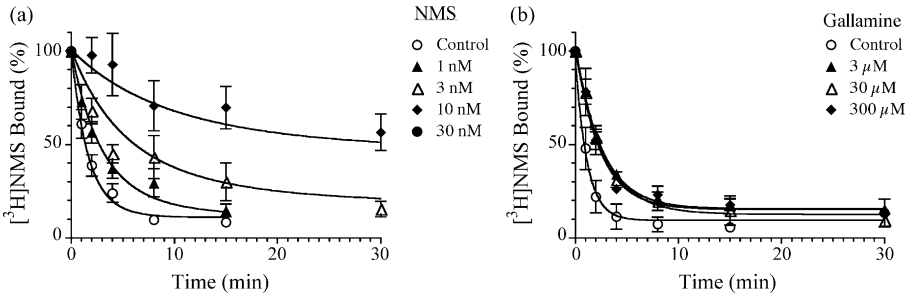

Fig. 10. The Effect of NMS (a) and gallamine (b) on the rate of alkylation of muscarinic receptors in rat heart homogenates by BR384 (0.1 mM). Cardiac homogenates were incubated with BR384 and the indicated concentrations of NMS or gallamine for various times and washed. The specific binding of [^{3}H]NMS (1 nM) was measured to the residual, unalkylated receptors. The data represent the mean binding values ± SEM of four (NMS) and two (gallamine) experiments, each done in triplicate.

measured by the inhibition of receptor alkylation by BR384 or AChM, which is consistent with the allosteric model.

When expressed relative to the initial concentration of the parent mustard, the log affinity constant of the aziridinium ion of BR384 was estimated at 5.20 in experiments on both the kinetics of receptor alkylation and the competitive inhibition of [^{3}H]NMS binding at 0 °C. The true estimate of the log affinity constant of the aziridinium ion should be about 5.45, however, because the initial concentration of the aziridinium ion in our experiments was at most about 54% that of the initial BR384 concentration. This value is approximately 3.5- to 6.3-fold greater than that determined for McN-A-343 in experiments on the inhibition of receptor alkylation ($\log K_1 = 4.91$, Fig. 9) and of [^{3}H]NMS binding at 0 °C ($\log K_1 = 4.65$, Fig. 4d), respectively. The replacement of the trimethylammonium group of McN-A-343 with the N-methylaziridinium ring of cyclized BR384, therefore, enhances binding affinity by as much as 6-fold, which is remarkable given the structural similarity of the two ammonium groups.

From the perspective of a single site of action, it is unlikely that BR384 binds reversibly to an allosteric site on the M_2 receptor but then reacts covalently with a nucleophile within the nearby orthosteric site because receptor alkylation was not competitively inhibited by gallamine or WIN 51,708. The simplest model to explain our data is that BR384 alkylates the orthosteric binding site on the M_2 muscarinic receptor. Given the close structural similarity of the aziridinium ion of BR384 with McN-A-343, our data strongly indicate that McN-A-343 binds to the orthosteric site of the M_2 muscarinic receptor. It is possible, nonetheless, that BR384 also alkylates the allosteric site in addition to the orthosteric site. Mutagenesis studies identifying the residue or residues in the M_2 sequence alkylated by BR384 should resolve this question. BR384 might also bind reversibly to the allosteric site, and our results do not rule out the possibility that McN-A-343 may as well.

Conventional methods for investigating drug interactions in binding assays include measuring the effect of a test compound on (1) the equilibrium binding of a radioligand and (2) the kinetics of radioligand binding. The first method is limited in discriminating between a competitive inhibitor and an allosteric modulator with high negative cooperativity, because there is often a practical limit to how high the radioligand concentration can be raised [5]. Kinetics are often viewed as a solution to this problem, but at muscarinic receptors, occupancy of the allosteric site by many modulators probably blocks the access and egress of [^{3}H]NMS from the orthosteric binding pocket. One can never be certain, therefore, whether the test drug acts on the allosteric or orthosteric site with higher affinity [10]. Another caveat is that muscarinic receptors may have a peripheral ligand-docking site composed of highly conserved residues including $W^{4.57}$ and $D^{3.26}$ [24,25]. It follows that high concentrations of many orthosteric ligands may inhibit the dissociation of [^{3}H]NMS simply by occupying the peripheral docking site. For these reasons, investigating the effects of putative modulators on the kinetics of radioligand binding is not an unequivocal method for detecting allosterism.

Investigating how a test drug affects the kinetics of receptor alkylation by a reactive orthosteric ligand does not suffer from these disadvantages. The primary interaction is between the orthosteric alkylating agent and the test drug, both of which can be used over a wide range of concentrations, unlike a radioligand. One can remove the interacting ligands and estimate the residual unalkylated receptors at a later time using a feasible concentration of radioligand, because the nature of the ligand interaction is preserved through the covalent bond with the receptor. As shown by our model, competitive inhibitors and allosteric modulators differ in their pattern of receptor protection. In addition, the problem of slow equilibration and radioligand trapping can be eliminated because the primary interaction is between rapidly equilibrating ligands (site directed electrophile and a competitive or allosteric ligand) and does not involve a slowly equilibrating radioligand, like [^{3}H]NMS. The method can be adapted to study allosterism at other receptors for which moderately potent, irreversible, orthosteric ligands are available. It should be possible to introduce a reactive electrophilic moiety into the structure of many orthosteric ligands to enable them to bind covalently to their wild type receptor. In addition, mutagenesis could be used to introduce an accessible nucleophilic residue (e.g., D or C) into the binding pocket to enable the electrophilic ligand to bind covalently. These methods should have widespread application in drug discovery.

Acknowledgements

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