Mutagenesis of Nucleophilic Residues Near the Orthosteric Binding Pocket of M₁ and M₂ Muscarinic receptors: Effect on the Binding of Nitrogen Mustard Analogs of Acetylcholine and McN-A-343

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Abbreviations: ACh, acetylcholine; AChM, acetylcholine mustard; BR384, 4-[(2-bromoethyl)methyl-amino]-2-butynyl N-(3-chlorophenyl)carbamate; CHO, Chinese hamster ovary; HEK, Human embryonic kidney; KRB, Krebs Ringer bicarbonate; McN-A-343, [4-[[N-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl] trimethylammonium chloride; NMS, N-methylscopolamine; WIN 51,708, 17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-β]pyrimido[1,2-α]benzimidazole.
ABSTRACT

Investigating how a test drug alters the reaction of a site-directed electrophile with a receptor is a powerful method for determining if the drug acts competitively or allosterically, provided that the binding site of the electrophile is known. In this study, therefore, we mutated nucleophilic residues near and within the orthosteric pockets of M1 and M2 muscarinic receptors to identify where the nitrogen mustard analogs of McN-A-343 (BR384) and acetylcholine bind covalently. The latter are useful for characterizing allosterism at muscarinic receptors. Mutation of the highly conserved aspartic acid in M1 (D105) and M2 (D103) receptors to asparagine largely prevented receptor alkylation by acetylcholine mustard, although modest alkylation still occurred at M2 D103N at high concentrations of the mustard. Receptor alkylation by BR384 was also greatly inhibited in the M1 D105N mutant, but some alkylation still occurred at high concentrations of the compound. In contrast, BR384 rapidly alkylated the M2 D103N mutant. Its affinity was reduced to one-tenth, however. The alkylation of M2 D103N by BR384 was competitively inhibited by N-methylscopolamine and allosterically inhibited by gallamine. Mutation of a variety of other nucleophilic residues, some in combination with D105N, had little effect on M2 receptor alkylation by BR384. Our results suggest that BR384 alkylates at least one residue other than the conserved aspartic acid at the ligand-binding site of M1 and M2 receptors. This additional residue appears to be located within or near the orthosteric-binding pocket, and is not part of the allosteric site for gallamine.
INTRODUCTION

Allosteric interactions at G protein coupled receptors are often studied by measuring the effect of the putative modulator on the binding of an orthosteric radioligand at equilibrium (Christopoulos, 2000). This approach has many advantages, but is limited in discriminating between competitive inhibition and high negative cooperativity. This requires the use of a radioligand concentration in proportion to the negative cooperativity, which may be unfeasible because of high cost and nonspecific binding.

Kinetic analysis is an alternative when the negative cooperativity is great and the modulator acts only at the allosteric site (Lazareno and Birdsall, 1995). For in most cases, only an allosteric modulator should affect the dissociation kinetics of the radioligand. In situations where the modulator acts at both the allosteric and orthosteric sites, it may be impossible to determine kinetically to which site the modulator binds with higher affinity (Birdsall and Lazareno, 2005). At muscarinic receptors, the problem is confounded because occupancy of the allosteric site often prevents the transit of radioligands to the orthosteric site (Proska and Tucek, 1994). Thus, the effect of an allosteric modulator on kinetic rate constants often includes two components – an inhibition of radioligand transit to and from the binding pocket and a selection of a receptor conformation with altered kinetics. Only the latter is allosteric because it represents a change in the equilibration of receptor conformations. Using kinetic and equilibrium methods, it would be impossible to discriminate a high affinity, highly negatively cooperative modulator from a ligand with no cooperativity, but capable of binding to the orthosteric and allosteric sites with high and low affinity, respectively (Birdsall and Lazareno, 2005).

An alternative approach involves dividing the assay into two phases (Ehlert and Jenden, 1985; Suga et al., 2008). In the first phase, the putative modulator equilibrates with the receptor and a site-directed electrophile (primary probe) for the orthosteric site. If the latter is small and has an affinity constant not much higher than $10^7$ M$^{-1}$, it should equilibrate rapidly with the receptor and modulator if its rate constant for alkylation is much slower. In the second phase,
the extent of receptor alkylation by the primary probe is determined using a secondary probe (radioligand) for the orthosteric site. Competitive and allosteric inhibitors have distinct profiles for the concentration-dependence of their receptor protection. The working concentration range of the primary probe can be much greater than that of a radioligand in the conventional equilibrium analysis of allosterism. Also, because the interacting ligands are likely to be in equilibrium during the first phase of the experiment, potential effects of the allosteric ligand on the access and egress of the primary probe to the orthosteric site are eliminated. Thus, the method represents a quasi-equilibrium approach with an extended concentration range for the primary orthosteric probe.

We have used this approach to determine how allosteric and orthosteric ligands modify M₁ and M₂ muscarinic receptor alkylation by acetylcholine mustard (AChM) and a nitrogen mustard derivative of McN-A-343 (BR384; 4-[(2-bromoethyl)methyl-amino]-2-butynyl N-(3-chlorophenyl)carbamate) (Suga et al., 2008; Suga and Ehlert, 2010; Figueroa et al., 2010). We found that the orthosteric ligands, N-methylscopolamine (NMS) and acetylcholine (ACh) competitively inhibit receptor alkylation, whereas the allosteric ligands, gallamine and WIN 51,708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-β]pyrimido[1,2-α]benzimidazole), had partial or no effect, which is consistent with an allosteric action. We also found that the M₁ and M₄ selective agonist, McN-A-343 ([4-[[N-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl] trimethylammonium), competitively protected M₁ and M₂ receptors from alkylation. These results are consistent with a recent modeling study suggesting that the binding site for McN-A-343 overlaps with the orthosteric site (Valant et al., 2008).

In this report, we used mutagenesis to investigate which amino acid side chains in M₁ and M₂ muscarinic receptors are involved in the covalent binding of AChM and BR384. Our results suggest that the two nitrogen mustard analogs alkylate at least one residue in addition to aspartic acid 3.32 (numbering scheme of Ballesteros and Weinstein (1995)) in M₁ and M₂ receptors and that the additional site is located near or within the orthosteric-binding pocket.
MATERIALS AND METHODS

Materials: Reagents were obtained from the following sources. Dulbecco’s Modified Eagle Medium with high glucose plus L-glutamine, Dulbecco's phosphate buffered saline (+), LB broth and penicillin-streptomycin were from Invitrogen Corporation, Grand Island, NY. Fetal calf serum was from HyClone Laboratories Inc., South Logan, UT. G418 disulfate salt, NMS, atropine, acetylcholine perchlorate, gallamine, Hepes, EDTA, scopolamine, McN-A-343 and Na₂S₂O₃ were from Sigma-Aldrich, Inc., St. Louis, MO. Salts for phosphate buffer, sodium bicarbonate, HCl and NaOH were obtained from Fisher Scientific, Fair Lawn, NJ. Poly-D-lysine coated cell culture dishes were from BD Biosciences, Bedford, MA. NucleoBond Xtra Midi Plus was from Clontech Laboratories Inc., Mountain View, CA. GeneJammer was from Agilent Technologies, Cedar Creek, TX.

The reagents for organic synthesis were obtained from Sigma-Aldrich. AChM was synthesized as described previously (Suga, et al., 2008). The boiling point (65 – 67°, 0.5 mm) of the free base of AChM was consistent with that reported by Jackson and Hirst (1972) (65 – 66°, 0.5 mm). BR384 was synthesized by the method of Ringdahl et al. (1990). The melting point (120 – 122°) of the oxalate salt of the immediate precursor to BR384 (4-[(2-Hydroxyethyl)methylamino]-2-butylnyl N-(3-Chloropheny1)carbamate was similar to that described previously (Ringdahl et al., 1990) (121 – 122°). Upon dissolution in 10 mM sodium phosphate buffer, pH 7.4, the product liberated an equivalent of halide and formed a maximal aziridinium ion concentration (52.2%) similar to that reported previously (Ringdahl et al., 1990) (54%). Bromide and aziridiridinium ions were estimated by titrations with silver chloride and thiosulfate, respectively (Skoog and West, 1965).

Site-Directed Mutagenesis: The human M₁ and M₂ muscarinic receptor cDNAs, cloned into a modified Okayma-Berg expression vector (pCD-hM₁ and pCD-hM₂), were provided by Dr. Tom Bonner at the National Institute of Mental Health (Bethesda, MD). Point mutations were
introduced into pCD-hM1 and pCD-hM2 using the QuikChange II Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and various mutagenesis primers. All of the sequences of mutant receptors were verified at the Oklahoma State University core DNA sequencing facility.

**Cell Culture and Transfection:** Chinese hamster ovary (CHO) cells stably expressing the human M1 muscarinic receptor (CHO hM1 cells) and human M2 muscarinic receptor (CHO hM2 cells) were obtained from Acadia Pharmaceuticals (San Diego, CA) and cultured as described previously (Suga et al., 2008). Human embryonic kidney 293 (HEK 293) cells were 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 and penicillin-streptomycin (100 units/ml and 100 µg/ml, respectively) at 37°C in a humidified atmosphere with 5% CO2/95% air. The plasmids encoding mutated muscarinic receptors were transfected into HEK 293 cells using GeneJammer following the manufacturer's protocols. After transfection, the cells were incubated for 48 h and harvested for assays.

**Preparation of cellular homogenates:** CHO hM1, CHO hM2 or HEK 293 cells expressing mutant muscarinic receptors grown to confluence in 100 mm dishes (Corning Incorporated, Corning, NY) were scraped into binding buffer (20 mM Na Hepes, pH 7.4, 100 mM NaCl and 10 mM EDTA) using a Teflon spatula. The mixture of buffer and cells was centrifuged (1,247 x g, 10 min) and the supernatant discarded. The pellet was homogenized in binding buffer using the Polytron (Brinkmann Instruments, Westbury, NY) at setting #4 for approximately 10 sec.

The homogenates of HEK 293 cells expressing hM1 D105N, hM2 D97N/D103N, hM2 D97N/D103N/E172Q/D173N/E175Q, hM2 D103N/Y104F, hM2 D103N/E172Q/D173N/E175Q or hM2 D103N/C429A receptors were centrifuged (39,400 x g, 10 min, 4°C), and the supernatant discarded. The pellet was suspended in fresh binding buffer and homogenized using the Polytron (Brinkmann Instruments, Westbury, NY) at setting #4 for approximately 10 sec.
Cyclization of AChM and BR384: Solutions of AChM and BR384 were incubated in 50 and 10 mM phosphate buffer at pH 7.4 (pH after dissolution) for 15 and 5 min, respectively, to allow formation of the reactive aziridinium ion as described previously (Suga et al., 2008; Suga and Ehlert, 2010). The cyclized solutions were placed on ice and used as soon as possible. The peak concentrations of the aziridinium ions of AChM and BR384 form after 21 and 6.3 min of incubation at 37°C at neutral pH and represent 92 and 54% of the initial amount of the respective parent mustard derivatives. These decay to 90 and 5.3% 30 min after the time of peak concentration, respectively. All experiments reported here were done with cyclized AChM and BR384, and the concentrations of the aziridinium ion are indicated under “Results” with respect to the starting concentration of the parent mustard derivative.

Treatment of cellular homogenate with cyclized AChM and BR384: In most experiments, homogenates of cells expressing wild type and mutant muscarinic receptors were incubated with cyclized AChM or BR384 for a period of time and then treated with sodium thiosulfate (1 mM) to inactivate the aziridinium ion. Sodium thiosulfate and the transformation products of the mustard derivatives were removed by centrifugation and aspiration of the supernatant. The pellets were suspended in binding buffer for estimation of the residual, unalkylated receptors using an [3H]NMS binding assay as described previously (Suga et al., 2008).

Except for those mutants described under “Centrifugation assay for [3H]NMS binding”, all of the binding assays were done using the filtration method. An aliquot (0.3 ml) of cellular homogenate was incubated in a final volume of 1 ml of binding buffer containing 1 nM [3H]NMS (specific activity, 82 Ci/mmol; PerkinElmer, Boston, MA) for 25 – 30 min at 37°C. The specific binding of [3H]NMS was measured using a filtration assay as described previously (Suga et al., 2008). Residual binding in the presence of atropine (10 µM) was defined as nonspecific.
Kinetic analysis of the alkylation of wild type and mutant (D103N) M2 muscarinic receptors by AChM and BR384: For a single kinetic experiment involving approximately 20 incubation tubes, the pooled cells from four dishes were suspended to a volume of approximately 3.5 – 4.5 ml. An aliquot of this homogenate (0.15 ml) was added to each assay tube (microfuge tube, Corning Incorporated, Corning, NY), and additional buffer or test drug (gallamine or NMS) was added in a volume of 0.05 ml. The tubes were incubated at 37°C in a shaking water bath. The reaction was started by the addition of an aliquot (0.05 ml) of cyclized BR384 to yield a final assay volume of 0.25 ml. After various incubation times, the reaction was stopped by the addition of an aliquot (0.75 ml) of binding buffer containing Na₂S₂O₃ and scopolamine to yield final concentrations of 1 mM and 7.5 μM, respectively. Control homogenates were also treated with the stopping solution. The tubes were allowed to incubate for 20 min at 37°C to inactivate the aziridinium ion. Thiosulfate, scopolamine and the transformation products of AChM or BR384 were removed by centrifugation (25,000 x g, 15 min, 4°C) and aspiration of the supernatant. The pellets were suspended in fresh binding buffer (1 ml), and the washing step repeated two times. The final pellets were suspended in 1 ml of binding buffer.

An aliquot (0.3 ml) of this homogenate was incubated in a final volume of 1 ml of binding buffer containing 1 nM [³H]NMS (specific activity, 82 Ci/mmol; PerkinElmer, Boston, MA) for 25 – 30 min at 37°C. Specific [³H]NMS binding was measured using a filtration assay as described previously (Suga et al., 2008).

Treatment of control homogenate from CHO hM₁ or hM₂ cells with the stopping solution followed by the washing procedure caused no subsequent inhibition of [³H]NMS binding, indicating that the washing step was sufficient to remove the scopolamine from the stopping solution. Treatment of the homogenate with the stopping solution followed by 100 μM BR384 caused no inhibition of [³H]NMS binding, indicating that the stopping solution was effective in preventing receptor alkylation by BR384. When the concentration of BR384 was increased to 300 μM, however, BR384 caused 28% inhibition of [³H]NMS binding in the presence of the stopping solution.
**Centrifugation assay for [3H]NMS binding:** For HEK 293 cells expressing hM1 D105N, hM2 D97N/D103N, hM2 D97N/D103N/E172Q/D173N/E175Q, hM2 D103N (Figure 3a only), hM2 D103N/Y104F, hM2 D103N/E172Q/D173N/E175Q and hM2 D103N/C429A, the specific binding of [3H]NMS was measured using a centrifugation assay. An aliquot of cellular homogenate in binding buffer (0.3 ml) was added to each microfuge tube (G-tube; Fisher Scientific) and incubated in a final volume of 0.5 ml of binding buffer containing 3 nM [3H]NMS for 25 – 30 min at 37°C. In some instances, nonlabeled competitive inhibitors were also added. Following this incubation, the tubes were centrifuged (30,000 x g, 20 min, 4°C), and the supernatants were removed by aspiration. The pellets were rapidly rinsed twice with 0.6 ml of ice-cold binding buffer, and an aliquot (0.2 ml) of 1 M NaOH was added. After overnight incubation at room temperature, the solubilized material was acidified by the addition of 0.25 ml of 1 M HCl. The solution was transferred to a scintillation vial (Research Products International Corp., Mount Prospect, IL) and mixed with scintillation cocktail (Budget-Solve; Research Products International Corp., Mount Prospect, IL). Radioactivity was measured with a Beckman Liquid scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA). Non-specific binding was defined as the residual binding measured in the presence of 10 µM atropine. All measurements were made in triplicate.

**Analysis of data:** The data for the inhibition of [3H]NMS binding by nonlabeled drugs were analyzed by nonlinear regression analysis using GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA).

We developed a model to analyze the kinetics of receptor alkylation by AChM and BR384 as shown in scheme 1. The model illustrates the interaction of the aziridinium ion of either AChM or BR384 (X) with the M2 muscarinic receptor (R) in the presence of an allosteric modulator (A). $K_1$ and $K_2$ represent the affinity constants of the aziridinium ion and allosteric modulator, respectively, and $\alpha$ denotes the cooperativity constant for their interaction. The
aziridinium ion first forms a reversible complex with the receptor (XR or XRA), which converts to a covalent complex (X-R or X-RA) at a relatively slower rate as characterized by the rate constant, $k_1$. We developed equations that describe this model under the condition where the concentration of the aziridinium ion is constant during the incubation and the rate of receptor alkylation is slow relative to the reversible binding step (Suga et al., 2008). In the absence of the allosteric modulator, the loss of receptor binding as a function of time is described by

$$\frac{Y_t}{Y_o} = (1 - b) \times e^{-\left(\frac{X}{1 + K_1}\right) k_{1t}} + b$$  \hspace{1cm} 1$$

in which $Y_t$ denotes the free, unalkylated receptor at time $t$, $Y_o$, the total amount for receptors at the start of the incubation, and $b$, the proportion of the receptor population that is insensitive to aziridinium ion. In the presence of the allosteric modulator, the loss of receptor binding as a function of time is described by:

$$\frac{Y_t}{Y_o} = (1 - b) \times e^{-\left(\frac{X}{1 + AAK_2}\right) k_{1t}} + b$$  \hspace{1cm} 2$$

In the presence of a competitive inhibitor, the loss of receptor binding as a function of time is described by:

$$\frac{Y_t}{Y_o} = (1 - b) \times e^{-\left(\frac{X}{1 + IK_i}\right) K_1} + b$$  \hspace{1cm} 3$$

in which $I$ denotes the concentration of competitive inhibitor and $K_i$, its affinity constant. The appropriate kinetic data with AChM were fitted to equation 1 – 3 because the aziridinium ion of AChM is stable.

We also developed an analogous series of equations (4 – 6) to account for the condition where the aziridinium ion decays during the reaction with the receptor (Suga and Ehlert, 2010). The corresponding equation for the loss of receptor binding as a function of time is
\[ \frac{y}{y_0} = (1 - b) \left( \frac{X_0 e^{-t/\tau_x} K_i + 1}{X_0 K_i + 1} \right)^{k_i \tau_x} + b \]

in which \( \tau_x \) denotes the time constant for the decay of the aziridinium ion and \( X_0 \) denotes the initial concentration of the cyclized mustard analog. In the presence of the allosteric modulator, the loss of receptor binding as a function of time is described by:

\[ \frac{y}{y_0} = (1 - b) \left( \frac{X_0 e^{-t/\tau_x} K_i \left( \frac{1 + A \alpha K_2}{1 + AK_2} \right)}{X_0 K_i \left( \frac{1 + A \alpha K_2}{1 + AK_2} \right) + 1} \right)^{k_i \tau_x} + b \]

In the presence of a competitive inhibitor, the loss of receptor binding as a function of time is described by:

\[ \frac{y}{y_0} = (1 - b) \left( \frac{X_0 e^{-t/\tau_x} K_i \left( \frac{1}{1 + IK_i} \right)}{X_0 K_i \left( \frac{1}{1 + IK_i} \right) + 1} \right)^{k_i \tau_x} + b \]

The appropriate kinetic data with BR384 were fitted to equation 4 – 6 setting the time constant for the decay of the aziridinium ion to \((0.07 \text{ min}^{-1})\) as described previously.

We also used a more empirical approach to display the effects of inhibitors on the observed rate constant for alkylation (Suga et al., 2008). First, the observed rate constant for receptor alkylation \((k_{obs})\) by the mustard analog was estimated in the absence and presence of the various concentrations of the allosteric modulator or competitive inhibitor using an exponential decay equation. In the case of BR384, only the data obtained during the first eight minutes of alkylation were used to avoid error associated with the decay of the aziridinium ion. In the presence of a competitive inhibitor, the time constant for receptor alkylation is described by:

\[ \text{Log}(R) = \text{Log} \left( \frac{\tau}{\tau'} \right) = \text{Log} \left( \frac{1 + X K_i + IK_i}{1 + X K_i} \right) \]

in which \( R \) denotes the ratio of the time constants for receptor alkylation in the absence \((\tau)\) and presence \((\tau')\) of the competitive inhibitor, respectively. The time constant is defined as the
reciprocal of the observed rate constant for alkylation (i.e., $\tau = 1/k_{obs}$). In the presence of an allosteric modulator, the $R$ value is described by:

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

The log $R$ values for BR384 measured in the presence of increasing concentrations of either NMS or gallamine were plotted against the concentration of the inhibitor, and equations 8 or 9 were fitted to the data to illustrate the nature of the interaction.

The data for the inhibition of $[^3H]$NMS binding to wild type and D103N M2 receptors by gallamine was analyzed to estimate the affinity constant ($K_{NMS}$) and cooperativity factor ($\alpha$) of gallamine. The cooperativity factor was estimated as described previously (Ehlert, 1988):

$$\alpha = \frac{Y'}{1 + \left([^3H]NMS\right)K_{NMS}(1 - Y')}$$

in which $Y'$ denotes the estimate of the fractional $[^3H]$NMS binding in the presence of saturating concentrations of gallamine. The affinity constant of gallamine was also estimated as described previously (Ehlert, 1988):

$$K = \frac{[^3H]NMS\cdot K_{NMS} + 1}{IC_{50} \left(\left[^3H\right]NMS\alpha K_{NMS} + 1\right)}$$

in which $K$ denotes the affinity constant of gallamine and $IC_{50}$, the concentration of gallamine that causing 50% of its maximal inhibitory effect on $[^3H]$NMS binding.
RESULTS

*Screening hM1 and hM2 receptor mutants for alkylation by AChM and BR384:* We replaced various nucleophilic residues in M1 and M2 muscarinic receptors and measured the consequences on the alkylation of the mutants by AChM and BR384. The mutations were designed to eliminate nucleophilic groups (e.g., mutation of S or C to A removes OH or SH) or to replace them with non-nucleophilic ones (e.g., mutation of D to N and E to Q replaces COOH with CONH₂) so that the electrophilic aziridinium ions would be unable to react covalently with the replaced amino acid.

A candidate residue is D 3.32 (M1 D105 and M2 D103), because it is known to be alkylated by [³H]AChM in the M₁ receptor (Spalding et al., 1994). We also investigated D 3.26 (M₁ D99 and M₂ D97) because this acidic residue may be part of a peripheral ligand-docking site (Jakubik et al., 2000). The E2 loop of muscarinic receptors is thought to be linked to the top of TM3 through a disulfide linkage (Curtis et al., 1989), which tethers a triad of acidic residues in the E2 loop of the M₂ receptor (E172, D173 and E175) near the top of the orthosteric binding pocket. Hence, these residues were investigated. In the β₂-adrenoceptor structure (Cherezov et al., 2007), the residue cognate to M₁ C407 and M₂ C429 lies close to D 3.32. Given the reactivity of the thiol group of cysteine, we investigated the corresponding M₁ C407A and M₂ C429A mutants. We also investigated M₂ C413A and M₂ C416A because of their reactivity even though they are in E₃ and presumably not part of the orthosteric binding pocket.

While tryptophan and the hydroxyl groups of serine and tyrosine are not expected to react with the aziridinium ions of the mustard analogs, it is conceivable that other nearby residues in the receptor might enhance their reactivity. Thus, we investigated serine, tyrosine and tryptophan at positions in the M₂ sequence that are expected, based on the β₂-adrenoceptor structure, to be close to D103 or to line the central aqueous binding pocket. We also investigated additional residues in the M₂ receptor that may be part of the allosteric site or the peripheral docking site. Figure 2 illustrates the residues in the M₂ receptor that were investigated.
The mutant receptors were expressed HEK 293 cells, and homogenates of these cells were incubated at 37°C with cyclized AChM (1 mM) or BR384 (0.1 mM) for four and two min, respectively. Thiosulfate (1 mM) was added, and after 20 min, the preparation was washed and [³H]NMS binding measured. It requires about 15 – 20 min for thiosulfate to react with the aziridinium ion. The time that the receptors were exposed to the mustard analogs, therefore, was longer than the initial two or four min incubation. The results of these experiments are shown in Figure 3, which illustrates [³H]NMS binding to wild type and receptor mutants following treatment with AChM or BR384. Binding is expressed as a percent of that measured before treatment. Thus, high binding reflects a prevention of irreversible alkylation by the mutation.

Under these conditions, AChM and BR384 caused about 95% alkylation of the wild type M₁ and M₂ muscarinic receptors. Alkylation of the M₁ receptor by BR384 was greatly inhibited by the D3.32N (D105N) mutation (Figure 3a). The single mutations of D99N and C407A did not inhibit M₁ receptor alkylation by BR384. As described below, alkylation of the M₁ receptor by AChM was also greatly inhibited in M₁ D105N.

Alkylation of the M₂ receptor by AChM was reduced to only 12 – 15% in M₂ D103N, M₂ D97N/D103N (double mutant), and M₂ D97N/D103N/E172Q/D173N/E175Q (quintuple mutant). The alkylation was also greatly reduced in the D103A mutant, but not to the same extent. In contrast, all of these M₂ receptor mutants were readily alkylated by BR384 (Figure 3a).

We also tested BR384 at M₂ receptors harboring other combinations of the M₂ mutations shown in Figure 3a as well as single, double, triple or quadruple point mutants at the other loci mentioned above and illustrated in Figure 2. None of these prevented M₂ receptor alkylation by BR384 (Figure 3b).

Alkylation of M₁ D105N and M₂ D103N by various concentrations of AChM and BR384: We investigated the concentration dependence of the alkylation of both M₁ and M₂ receptors containing the D3.32N mutation (Figure 4). These experiments were done like those of the prior
section except that, the concentration of the mustard analogs was varied, and the incubation time with AChM (15 min) or BR384 (4 min) was increased.

As the concentration of cyclized AChM increased over the range of 1 – 100 µM, alkylation of the M₁ receptor increased to about 90%, and half-maximal alkylation occurred at a concentration of about 10 µM (Figure 4a). Alkylation of the M₁ D105N mutant was nearly prevented over the same concentration range (Figure 4b), but maximal 22% receptor alkylation occurred when the concentration of AChM increased to 3 mM.

BR384 readily alkylated the wild type M₁ receptor as the concentration of a cyclized solution of the compound increased from 0.1 – 10 µM (Figure 4a). Half-maximal and near maximal (90%) receptor alkylation occurred at 1.5 and 20 µM concentrations of cyclized BR384, respectively. The potency of BR384 for alkylating M₁ D105N was reduced to about 3% that of wild type (i.e., 30-fold rightward shift in the inhibition curve) (Figure 4b). The data do not allow an estimate of the maximal effect of BR384 on the M₁ D105N mutant, although about 80% receptor alkylation was measured at the highest concentration of cyclized BR384 tested (300 µM). Thus, at high concentrations, BR384 readily alkylates the M₁ D105N mutant.

Alkylation of the wild type M₂ receptor steadily increased as the concentration of cyclized AChM increased from about 1 – 100 µM (Figure 4c). The 50 and 90% levels of receptor alkylation occurred at cyclized AChM concentrations of 8 and 100 µM, respectively. Receptor alkylation by AChM was greatly reduced in the M₂ D103N mutant (Figure 4d). This was associated with a modest increase in the concentration of cyclized AChM required for half maximal alkylation (20 µM). In contrast, the maximal effect of AChM was reduced to about 40% receptor alkylation, reflecting a large reduction in the rate constant for alkylation. The data suggest that AChM may alkylate at least one residue other than D105N, but with a much slower rate constant.

Alkylation of the wild type M₂ receptor by BR384 steadily increased as the concentration of cyclized mustard increased from about 0.1 – 10 µM (Figure 4c). The 50 and 90% levels of receptor alkylation occurred at cyclized BR384 concentrations of 2 and 20 µM, respectively.
BR384 also readily alkylated the M$_2$ D103N mutant although its potency was about one-tenth that observed at the wild type receptor (Figure 4d).

**Kinetic analysis of the alkylation of M$_1$ D105N and M$_2$ D103N:** We compared wild type and D3.32N mutants of M$_1$ and M$_2$ receptors with respect to their kinetics of alkylation by AChM and BR384. Homogenates of cells expressing the wild type receptor or the D3.32N mutant were incubated with various concentrations of the cyclized mustard derivatives for various times. The reactions were stopped immediately with a solution of scopolamine and thiosulfate, and the homogenates were washed prior to measuring the binding of $[^3]$H]NMS. Regression analysis of the data with equation 1 (AChM) or 4 (BR384) yielded estimates of the affinity and rate constant for alkylation.

AChM caused a time- and concentration-dependent alkylation of wild type M$_1$ muscarinic receptors (Figure 5a). Global nonlinear regression analysis of the data with equation 1 yielded a log affinity constant of 3.61 ± 0.05 and a rate constant for alkylation of 0.80 ± 0.07 min$^{-1}$. The data from the M$_1$ D105N mutant showed limited alkylation, making it difficult to obtain reliable parameter estimates (Figure 5b). BR384 readily alkylated the wild type M$_1$ receptor, and its kinetics were characterized by a log affinity constant of 5.14 ± 0.08 and a rate constant for alkylation of 0.95 ± 0.13 min$^{-1}$ (Figure 5c). At the M$_1$ D105N mutant, the log affinity constant of BR384 was less (3.44 ± 0.47), and its rate constant for alkylation was 0.88 ± 0.29 min$^{-1}$ (Figure 5d). The estimates of the residual unalkylated receptor populations for AChM at M$_1$ and BR384 at M$_1$ and M$_1$ D105N were 10.8 ± 1.3%, 12 ± 2.9% and 10.1 ± 4.7%, respectively.

AChM readily alkylated the wild type M$_2$ receptor, and the analysis of the kinetic curves yielded a log affinity constant of 4.41 ± 0.05 and a rate constant for alkylation of 0.24 ± 0.05 min$^{-1}$ (Figure 6a). Alkylation of the M$_2$ receptor was greatly inhibited in the D103N mutant (Figure 6b), and it was difficult to obtain accurate parameters for AChM. BR384 also readily alkylated the wild type M$_2$ receptor, and analysis of its kinetics yielded an affinity constant of
4.88 ± 0.08 and a rate constant of 1.17 ± 0.16 min⁻¹ (Figure 6c). The M₂ D₁₀₃N mutant was alkylated by BR384 with a greater rate constant 3.30 ± 0.80 min⁻¹), but the log affinity constant of was reduced to 3.58 ± 0.13 (Figure 6d).

The data suggest that both M₁ D₁₀₅N and M₂ D₁₀₃N are alkylated by AChM and BR384 but with reduced affinity and, in the case of AChM, with a greatly reduced rate constant.

Estimation of affinity using equilibrium binding: To obtain an independent estimate of the affinity constants of the aziridinium ions of AChM and BR384 for wild type and D₃.₃₂N mutant receptors, we measured the competitive inhibition of the binding of [³⁵S]NMS by ACh and McN-A-343. While the structures of the latter compounds differ from those of their respective aziridinium ions by the addition of two hydrogens (Figure 1), the approach should provide at least a reasonable estimate of the change in affinity cause by the mutation.

The results of these experiments are shown in Figure 7 together with data for nonlabeled NMS. At both M₁ and M₂ receptors, the affinity constant of ACh was greatly reduced in the D₃.₃₂N mutant (Figure 7b and d). Similar behavior was observed with McN-A-343 and NMS, although the reduction in affinity was not as great. These competition experiments were carried out using [³⁵S]NMS concentrations of 1 (wild type and M₂ D₁₀₃N) and 3 nM (M₁ D₁₀₅N). Analysis of the data showed that the reduction in the affinity constant of [³⁵S]NMS for the mutant receptor was substantial. This change means that the mutation-induced shift in the IC₅₀ value of a given ligand underestimates the true loss in affinity. Consequently, the Kᵢ values were calculated from the IC₅₀ values to correct for the competitive effect of [³⁵S]NMS. These values are listed in Table 1 together with the kinetic estimates of affinity. There is general agreement between the two estimates.

We also measured -log Kᵢ values for some of the other mutants described above. For ACh, McN-A-343 and NMS at M₂ D₉₇N the values are 3.65 ± 0.02, 4.63 ± 0.04 and 8.86 ± 0.01, for McN-A-343 and NMS at M₂ W₉₉F, the values are 4.79± 0.03 and 8.94 ± 0.03, and for McN-A-343 and NMS at M₂ S₁₀₇A/S₁₁₀A, the values are 4.41 ± 0.04 and 8.92 ± 0.04. The binding
affinities of McN-A-343 and NMS were little affected by these mutations whereas that of ACh for the M₂ D97N mutation was reduced to one-hundredth of wild type.

**Effects of NMS and gallamine on the alkylation of the D3.32N mutants:** So far, our data indicate that BR384 alkylates a residue other than D103 in the M₂ receptor, although it seems likely that it may also alkylate D3.32 in M₁ and M₂ receptors. AChM might also alkylate the non-D 3.32 residue, but with a much slower rate constant. To determine whether the non-D103 residue in the M₂ receptor is part of the orthosteric binding pocket, we investigated how the orthosteric ligand, NMS, and the allosteric ligand, gallamine, interfered with its alkylation. In these experiments, the rate of alkylation of M₂ D103N by cyclized BR384 (0.1 mM) was measured in the absence and presence of various concentrations of either NMS or gallamine.

NMS caused a concentration-dependent slowing and near cessation of the alkylation of M₂ D103N by BR384 (Figure 8a). Increasing the concentration of gallamine also slowed the rate of alkylation, but the effect of gallamine approached a limit, which is consistent with an allosteric mechanism (Figure 8b). Global nonlinear regression analysis yielded a good fit of the competitive (equation 6) and allosteric (equation 5) models to the data with NMS and gallamine, respectively. The competitive effect of NMS is apparent in Figure 8c where the increase in the time constant for alkylation increases proportionately with the NMS concentration. In contrast, gallamine’s effect reaches a plateau at high concentrations (Figure 8d). Regression analysis of the data yielded an estimate of the log affinity constant of NMS to be 7.62 ± 0.04. This value agrees with that obtained in the NMS/[³H]NMS competition experiment summarized in Table 1. The estimates of the log affinity (log K₂) and cooperativity constant (log α) for gallamine were 5.50 ± 0.06 and -1.31 ± 0.05, respectively.

To obtain an independent estimate of the affinity constant for gallamine, we measured the inhibition of [³H]NMS binding to wild type and D103N M₂ receptors by gallamine (Figure 9). Gallamine did not cause a complete displacement of [³H]NMS binding at high concentrations, which is consistent with an allosteric mechanism. Analysis of the data using equations 10 and 11
yielded estimates of 6.44 ± 0.02 and 5.15 ± 0.05 for the affinity constants in wild type and D103N receptors, respectively. The corresponding log cooperativity values were -1.63 ± 0.06 and -0.88 ± 0.09. There is agreement between the affinity constant of gallamine for M2 D103N as estimated by inhibition of [3H]NMS binding and by the kinetic experiments.
DISCUSSION

One can never be certain in this type of mutagenesis study that the mutation prevents alkylation by eliminating the nucleophilic side chain involved in covalent bond formation with the mustard derivative. Rather, it could cause a structural change in the receptor that greatly reduces the reversible binding characteristics of the aziridinium ion so that it is unable to bind to the receptor in the first place. We attempted to reduce this potential problem by making conservative mutations.

We assume that if the aziridinium ions of AChM or BR384 alkylate muscarinic receptors then they must occupy the receptor reversibly before alkylation it. If so, then the rate of receptor alkylation should be proportional to receptor occupancy. This hypothesis is consistent with our prior observations on the alkylation of M₁ and M₂ receptors by various concentrations of AChM and BR384 in intact cells (Suga et al., 2008; Suga and Ehlert, 2010; Figueroa et al., 2010). The rate of receptor alkylation was proportional to receptor occupancy as judged by the ability of the aziridinium ions to inhibit [³H]NMS binding under conditions where the covalent reaction is greatly inhibited (0°C). Alkylation reduced the binding capacity, but not the affinity, of [³H]NMS in washed AChM- and BR384-treated receptor preparations. The orthosteric ligands NMS, ACh and McN-A-343 inhibited the alkylation competitively while the allosteric ligand gallamine did so by means of negative cooperativity. The allosteric ligand WIN 51,708 had no effect. These prior results suggest that AChM and BR384 alkylate the orthosteric site of M₁ and M₂ muscarinic receptors.

In this study, we examined the alkylation of wild type M₁ and M₂ muscarinic receptors by various concentrations of BR384 and AChM in homogenates of CHO cells. For AChM at the M₁ receptor and BR384 at M₁ and M₂, there was general agreement between the affinity constants measured kinetically and those estimated for the corresponding reversible ligands (ACh and McN-A-343) in competitive binding experiments with [³H]NMS (Table 1). The agreement supports the hypothesis concerning the proportionality of occupancy and the
alkylation rate. With ACh at the M2 receptor, however, the binding affinity measured in ACh/[3H]NMS competition experiments in cellular homogenate was 13-fold higher than that measured for the aziridinium ion of AChM in alkylation experiments (Table 1), assuming that the aziridinium ion represents 70% of the starting concentration of AChM. This difference may be due, in part, to the possible lower affinity of the aziridinium ion of AChM for the M2 receptor. It has a potency about one-fourth that of ACh in the guinea pig ileum when measured in the presence of physostigmine to prevent cholinesterase activity (Robinson et al., 1975). Another explanation is the presence of a population of a high affinity receptor-G protein complex in M2 CHO cell homogenates. We have previously argued that this complex is alkylated at a slower rate (Suga et al., 2008). This site would be more difficult to detect in alkylation experiments, where the data are determined mainly by the more predominant, rapidly alkylated receptor population. If alkylation were measured at earlier time points (< 1 min), it might have been possible to resolve two components to the alkylation process. Alternatively, if the alkylation reaction with BR384 and the competitive ACh/[3H]NMS binding experiment were carried out in the presence of a G protein-saturating concentration of GTP or one of its analogs, there might have been closer agreement between the two estimates under the present assay conditions.

Our experiments with AChM on M1 D105N and M2 D103N are consistent with the postulate that AChM primarily alkylates D3.32. Both mutants showed only minor, slow alkylation at high concentrations of AChM, the effect being a little greater at M2 D103N compared to M1 D105N. The mutated aspartic acid (D3.32) is conserved among amine GPCRs, and it is thought to act as a counter ion for the cationic neurotransmitter (Hulme et al., 2003). In addition, [3H]AChM is known to alkylate D105 in the M1 receptor (Spalding et al., 1994). The results suggest that the free carboxyl group of D3.32 is the site of covalent alkylation by AChM at both M1 and M2 receptors in addition to another site that is alkylated at a much slower rate with lower affinity.

In contrast, our experiments with BR384 showed marked alkylation of M1 D105N and M2 D103N, although the affinity of the aziridinium ion for these mutant receptors was only one-
thirtieth and one-tenth that of wild type, respectively. Two explanations can account for these data: 1) BR384 alkylates a residue other than D3.32, but exhibits low affinity for M1 and M2 D3.32N or 2) BR384 alkylates D3.32 as well as at least one other residue on both M1 and M2 receptors. The tendency for AChM to alkylate both D3.32 and an additional residue on the M1 and particularly the M2 receptor suggests, perhaps, that BR384 may do the same. Regardless, the non-D103 residue that reacts with the aziridinium ion of BR384 during its covalent binding to the M2 receptor appears to be located within or near the covalent binding pocket because alkylation of M2 D103N is inhibited competitively by NMS and allosterically by gallamine. We cannot rule out the possibility that BR384 interacts with an allosteric site on M2 D103N and exhibits high negative cooperativity with [3H]NMS. Nonetheless, the site of covalent attachment cannot be part of the allosteric site for gallamine.

The rate of alkylation of M2 D103N by BR384 appears proportional to receptor occupancy because the affinity constant of BR384 determined from the kinetics of receptor alkylation is in general agreement with that determined for McN-A-343 in competitive binding experiments with [3H]NMS (Table 1). In addition, the affinity constants of NMS and gallamine for antagonizing the alkylation of M2 D103N by BR384 generally agree with those estimated in equilibrium binding experiments with [3H]NMS (Table 1).

Using hemi-ligands of McN-A-343, Valant et al. (2008) showed that 3-chlorophenylcarbamate acts as a negative allosteric modulator of [3H]NMS at the M2 receptor, whereas tretramethylammonium acts as an agonist, presumably by interacting with D103. A butyne chain connects these groups in McN-A-343, suggesting that the latter interacts with both D103 via its trimethylammonium group and with the allosteric site via its 3-chlorophenyl group. Molecular modeling showed that the M2 receptor could accommodate McN-A-343 in this orientation (Valant et al., 2008), suggesting that BR384 alkylates D103 in addition to at least one other residue.

While the D3.32N mutation decreased the alkylation rate for AChM substantially at both M1 and M2 receptors, it had less of an effect on alkylation by BR384, particularly at the M2
receptor. Given that all highly nucleophilic residues within the binding pocket of M₁ and M₂ receptors are conserved, these results might suggest that there is greater movement of the aziridinium of BR384 in the binding pocket of the M₂ receptor, relative to that of the M₁, enabling it to react with an additional nucleophile. If the same applies to McN-A-343, the greater stability of the McN-A-343-M₁ receptor interaction might explain the selectivity of McN-A-343 for eliciting M₁-G₄ signaling vs. M₂-G₁ signaling (Lazareno et al., 1993; Figueroa et al., 2008).

Several investigators have reported how the mutations investigated in this study influence the binding of NMS, ACh and McN-A343 to M₁ and M₂ receptors (Leppik et al., 1994; Schwarz et al., 1995; Vogel et al., 1999). Our results are consistent with these prior findings. The pKₐs of the hydroxyl groups of serine and tyrosine are sufficiently high that it seems unlikely that these residues are alkylated unless they are coordinated by proton withdrawing residues. Regardless, we cannot rule out the possibility that one of these residues is alkylated because alkylation could be rescued by alkylation of D 3.32 or another residue. To prevent M₂ receptor alkylation by BR384 completely, it is probably necessary to mutate more than one residue.

Determining where a drug binds on a receptor based on how it interferes with the binding of a site-directed electrophile is a powerful approach for discriminating between competitive and highly negatively cooperative interactions. Our results show that AChM and BR384 react covalently with the orthosteric site of the M₂ muscarinic receptor, and hence, that these reactive probes can be used as site-directed electrophiles for the orthosteric site. This useful approach for investigating drug-receptor interactions can be enhanced through the design of other higher affinity electrophiles, and it can be applied to study allosteric interactions at numerous other receptors.
ACKNOWLEDGEMENTS

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REFERENCES


FOOTNOTES

This work was supported by the National Institutes of Health [GM 69829 and NS 057742].
LEGENDS TO FIGURES

**Scheme 1:** Model for the allosteric modulation of the alkylation of M₁ and M₂ muscarinic receptors by the aziridinium ions of AChM and BR384: The aziridinium ion (X) and allosteric modulator (A) bind to their respective sites on the receptor (R) with characteristic affinity constants of $K_1$ (XR) and $K_2$ (RA), respectively, and a cooperativity factor of $\alpha$ for their interaction. Once occupied by the aziridinium ion, the two possible receptor complexes (XR and XRA) undergo first order alkylation characterized by the rate constant, $k_1$, to yield the covalent complexes, X-R and X-RA.

**Figure 1:** Structures of ACh, McN-A-343, AChM, BR384 and the transformation products of the nitrogen mustard derivatives in aqueous solution at neutral pH.

**Figure 2:** Primary sequence of the human M₂ muscarinic receptor indicating the residues that were mutated in this study. Black disks, while lettering, nucleophilic residues lining the central aqueous binding pocket; gray disks, white lettering, nucleophilic residues that are though to be part of a peripheral docking site; light-gray disks, black lettering, acidic residues tethered near the top of the aqueous binding pocket by the disulfide bond linking E2 with the top of TM3; and white disks with black halo, reactive cysteine residues in E3.

**Figure 3:** Effects of cyclized AChM and BR384 on the binding of $[^3]H$NMS to homogenates of cells expressing wild type and mutant M₁ and M₂ muscarinic receptors. Cellular homogenate was incubated at 37°C with cyclized AChM (1 mM) or BR384 (0.1 mM) for 2 and 4 min, respectively. Thiosulfate was added, and the incubation was allowed to proceed for another 20 min. The homogenate was washed, and $[^3]H$NMS binding was subsequently measured. Control homogenate was treated similarly except for exposure to the nitrogen mustard derivative. The binding values in the figure are normalized relative to the binding observed in control.
homogenate.  

Effects of AChM on M₂ receptor mutants and of BR384 on M₁ and M₂ receptor mutants. These mutations focus on conserved nucleophilic residues at the orthosteric site D3.32 (M₁ D105, M₂ D103), the postulated peripheral docking site D3.26 (M₁ D99, M₂ D97) and a cysteine residue in TM7 close to D3.32 (M₁ C407, M₂ C429). Also included are mutations in a triad of acidic residues in E2 of the M₂ receptor that are close to the opening of the central binding pocket (E172, D173 and E175). 

Effects of BR384 on M₂ receptor mutants. Additional combinations of the mutations described in a are shown. Mutations in several weakly acidic nucleophilic residues are shown that either 1) line the aqueous binding pocket in TM3 (W99, Y104, S107 and S110), TM6 (W400, Y403) or TM7 (Y426, W427, Y430, S433 and T434), 2) are close to D3.32 in TM2 (S76), 3) are thought to constitute residues of the allosteric site (W422, T423) or 4) peripheral docking site (W155F), or 5) are reactive residues in E3 (C413 and C416). The concentration of [³H]NMS was either 3 nM (mutations involving D3.32) or 1 nM (all others). The data represent the mean binding values of two experiments, each done in triplicate. The two binding estimates are given in parentheses beside each mutant in the following list: M₁, BR384, WT (22, 21%); D99N (6.1, 7.4%); D105N (75, 73.7%); C407A (4.0, 1.6%); M₂, AChM, WT (5.82, 7.79%); D97N/D103N (90, 86%); D97N/D103N/E172Q/D173N/E175Q (77, 87%); D103A (62, 70%); D103N (85, 79%); M₂, BR384, WT (1.33, 1.29%); D103A (21, 15%); D103N (2, 3%); C429A (2.3, -0.5%); D97N/D103N (0.7, 1.0%); D97N/D103N/E172Q/D173N/E175Q, (-1.1, 0.5%); S76A, (1.33, 1.29%); D97N, (13, 3%); W99F, (0.14, 3.3%); D103N/Y104F, (2.4, 1.3%); D103N/E172Q/D173N/E175Q, (1.9, 3.5%); D103C/C429A, (-0.9, -0.03%); S107A/S110A, (0.34, 0.29%); W155F, (0.7, 7.3%); W400F/Y403F, (0.7, 7.3%); C413A/C416A, (0.6, 8.5%); W422F/T423A/Y426FW427F, (3.7, 8.2%); and Y430F/S433A/T434A, (0.3, 2.1%).

Figure 4: Effects of treatment with various concentrations of cyclized AChM and BR384 on the binding of [³H]NMS to wild type and D3.32N mutant M₁ and M₂ muscarinic receptors. The experiments were carried out as described in the text and legend to Figure 3 except that the
incubation time with AChM and BR384 was increased to 15 and 4 min, respectively. The concentrations on the abscissa refer to the starting concentration of the cyclized mustard derivative. The binding values in the figure are normalized relative to the binding measured in control (untreated) homogenate. The experiments were done on wild type and mutant M1 (a and b, respectively) and M2 (c and d, respectively) receptors. The data represent the mean binding values ± SEM of three experiments, each done in triplicate.

**Figure 5:** The kinetics of alkylation of wild type (a and c) and D3.32N mutant (b and d) M1 muscarinic receptors by cyclized AChM (a and b) and BR384 (c and d). Homogenates of cell expressing the indicated receptor were incubated with various concentration of the cyclized mustard analogs. The reaction was stopped at the times indicated on the abscissa. [3H]NMS binding was measured in the washed homogenates as described under “Materials and Methods”. The binding values in the figure are normalized relative to the binding measured in control (untreated) homogenate. The concentration of [3H]NMS was either 1 (wild type) or 3 (mutant) nM. The concentrations of AChM and BR384 refer to the starting concentration of the cyclized mustard derivative. The data represent the mean values ± SEM of three experiments, each done in triplicate. The theoretical curves represent the global least squares fit of equation 1 (AChM) and 4 (BR384) to the data.

**Figure 6:** The kinetics of alkylation of wild type (a and c) and D3.32N mutant (b and d) M2 muscarinic receptors by cyclized AChM (a and b) and BR384 (c and d). Homogenates of cell expressing the indicated receptor were incubated with various concentration of the cyclized mustard analogs. The reaction was stopped at the times indicated on the abscissa. [3H]NMS binding was measured in the washed homogenates as described under “Materials and Methods”. The binding values in the figure are normalized relative to the binding measured in control (untreated) homogenate. The concentration of [3H]NMS was 1 nM. The concentrations of AChM and BR384 refer to the starting concentration of the cyclized mustard analog. The data
represent the mean values ± SEM of three to seven experiments, each done in triplicate. The theoretical curves represent the global least squares fit of equation 1 (AChM) and 4 (BR384) to the data.

**Figure 7:** The competitive inhibition of the binding of $[^3$H]NMS to wild type and D3.32N mutant $M_1$ (a and b) and $M_2$ (c and d) muscarinic receptors by ACh, McN-A-343 and NMS. Homogenates of cells expressing the indicated receptor were incubated at 37°C in the absence and presence of the indicated concentrations of the inhibitors. The specific binding of $[^3$H]NMS was measured at a concentration of 1 (wild type and $M_2$ D103N) or 3 (M1 D105N) nM as described under “Materials and Methods”. The binding values in the figure are normalized relative to the binding measured in control (untreated) homogenate. The data represent the mean binding values ± SEM of three experiments, each done in triplicate.

**Figure 8:** Effects of NMS (a and c) and gallamine (b and d) on the kinetics of alkylation of $M_2$ D103N by BR384. $a$ and $b$, Homogenates of HEK 293 cells expressing $M_2$ D103N were incubated with BR384 (0.1 mM) in the presence of various concentrations of either NMS (a) or gallamine (b). The reaction was stopped immediately at the times indicated on the abscissa. The binding of $[^3$H]NMS (1 nM) to the washed homogenates was subsequently measured. The data represent the mean binding values ± SEM of three (gallamine or NMS) or seven (control) experiments, each done in triplicate. The binding values in the figure are normalized relative to the binding measured in untreated homogenate. The theoretical curves represent the least squares fit of equation 6 (NMS) or 5 (gallamine) to the data. $c$ and $d$, Each decay curve in $a$ and $b$ was fitted to an exponential decay equation to estimate the time constant ($\tau$) for decay. This parameter represents the reciprocal of the observed rate constant for decay. The log of the ratio $(R)$ of the time constant measured in the presence of NMS (c) or gallamine (d), divided by that measured in their absence, is plotted against the log concentration of the inhibitor. The
theoretical curves represent the least squares fit of equations 8 (NMS) or 9 (gallamine) to the data.

**Figure 9:** *Inhibition of [3H]NMS binding to wild type and mutant (D103N) M₂ muscarinic receptors by gallamine.* The binding of [3H]NMS (1 nM) to homogenates of cells expressing wild type and mutant (D103N) M₂ muscarinic receptors was measured at 37°C in the presence of increasing concentrations of gallamine. The data represent the mean binding values ± SEM of three experiments, each done in triplicate. The theoretical curve represents the least squares fit of a four-parameter-logistic equation to the data. The plateau (Y’) and IC₅₀ values were then corrected using equations 10 and 11 to estimate the cooperativity of the gallamine-NMS interaction (α) and dissociation constant of gallamine for the free receptor (K).
Table 1: Comparison of the affinity constants of the aziridinium ions of AChM and BR384 determined from the kinetics of receptor alkylation with those estimated for ACh and McN-A-343 in competition experiments with [³H]NMS.

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<th>McN-A-343</th>
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<th>BR384</th>
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<tr>
<td>Wild type</td>
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<td>3.58 ± 0.13</td>
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* The parameter values ± SEM were estimated from the [³H]NMS competition curves shown in Figure 7.

* The parameter values ± SEM were estimated by global nonlinear regression analysis of the data in Figures 5 and 6 using equations 1 (AChM) or 4 (BR384).
Scheme 1

\[
X + R + A \quad \xleftrightarrow{K_1} \quad XR + A \quad \xrightarrow{k_1} \quad X-R + A
\]

\[
X + RA \quad \xleftrightarrow{\alpha K_1} \quad XRA \quad \xrightarrow{k_1} \quad X-RA
\]

\[
X + R + A \quad \xleftrightarrow{K_2} \quad XR + A \quad \xrightarrow{\alpha K_2} \quad X-R + A
\]
Figure 1

ACh

\[
\begin{array}{c}
\text{O} \\
\text{H}_3\text{C} \quad \text{C} \quad \text{O} \\
\text{CH}_2\text{CH}_2\text{N} \quad \text{CH}_3 \\
\text{CH}_3 \\
\end{array}
\]

AChM

\[
\begin{array}{c}
\text{O} \\
\text{H}_3\text{C} \quad \text{C} \quad \text{O} \\
\text{CH}_2\text{CH}_2\text{N} \quad \text{CH}_2\text{CH}_2\text{Cl} \\
\text{Cl} \\
\end{array}
\]

\[
\text{aziridinium ion}
\]

\[
\begin{array}{c}
\text{O} \\
\text{H}_3\text{C} \quad \text{C} \quad \text{O} \\
\text{CH}_2\text{CH}_2\text{N} \quad \text{CH}_2 \\
\text{CH}_2 \\
\end{array}
\]

\[
\text{hydrolysis product}
\]

McN-A-343

\[
\begin{array}{c}
\text{O} \\
\text{Cl} \\
\end{array}
\]

\[
\text{O} \\
\text{Cl} \\
\end{array}
\]

\[
\text{aziridinium ion}
\]

\[
\begin{array}{c}
\text{O} \\
\text{Cl} \\
\end{array}
\]

\[
\text{hydrolysis product}
\]

BR384

\[
\begin{array}{c}
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\text{Cl} \\
\end{array}
\]

\[
\begin{array}{c}
\text{O} \\
\text{Cl} \\
\end{array}
\]

\[
\text{hydrolysis product}
\]
**Figure 3**

**a**

<table>
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<th>M2, AChM</th>
<th>M2, BR384</th>
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<td>WT</td>
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<tr>
<td>D7N</td>
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<td>D7N/D103N</td>
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<td>D7N/D103N/E172Q</td>
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</tbody>
</table>

**b**

- **M2, BR384**
Figure 4

(a, WT M₁) [Graph showing the binding of [³H]NMS to AChM and BR384 in the presence of Mustard with a logarithmic scale for concentrations.]

(b, M₁ D105N) [Graph showing the binding of [³H]NMS to AChM and BR384 in the presence of Mustard with a logarithmic scale for concentrations.]

(c, WT M₂) [Graph showing the binding of [³H]NMS to AChM and BR384 in the presence of Mustard with a logarithmic scale for concentrations.]

(d, M₂ D103N) [Graph showing the binding of [³H]NMS to AChM and BR384 in the presence of Mustard with a logarithmic scale for concentrations.]
Figure 5

(a, WT M₁) [Data representation of WT M₁ with 
log AChM values: ○ -6, ● -5, △ -4, ▲ -3]

(b, M₁ D105N) [Data representation of M₁ D105N with 
log AChM values: ○ -3, ● -2.5]

(c, WT M₁) [Data representation of WT M₁ with 
log BR384 values: ○ -7, ● -6, △ -5, ▲ -4]

(d, M₁ D105N) [Data representation of M₁ D105N with 
log BR384 values: ○ -5, ● -4, △ -3.5]
**Figure 6**

*a, WT $M_2$*  
$b, M_2 D103N$  
$c, WT $M_2$*  
$d, M_2 D103N$
Figure 7

a, WT M₁

b, M₁ D105N

c, WT M₂

d, M₂ D103N

[³H]NMS bound (%) vs Log[Inhibitor] (M)
Figure 8

(a) 

[3H]NMS bound (%) over time (min) for different concentrations of NMS:
- Control
- 0.03 μM
- 0.3 μM
- 3 μM

(b) 

[3H]NMS bound (%) over time (min) for different concentrations of Gallamine:
- Control
- 0.01 mM
- 0.1 mM
- 1 mM

(c) 

Log R vs. Log [NMS] for NMS concentration ranging from 10^-9 to 10^-5.

(d) 

Figure 9

$[^3]H\text{NMS}$ bound (%) vs. Log [Gallamine] for $M_2$ WT and $M_2$ D103N.