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Effects of Asparagine Mutagenesis of Conserved Aspartic Acids in Helix 2 (D2.50) and 3 (D3.32) of M_1−M_4 Muscarinic Receptors on the Irreversible Binding of Nitrogen Mustard Analogs of Acetylcholine and McN-A-343

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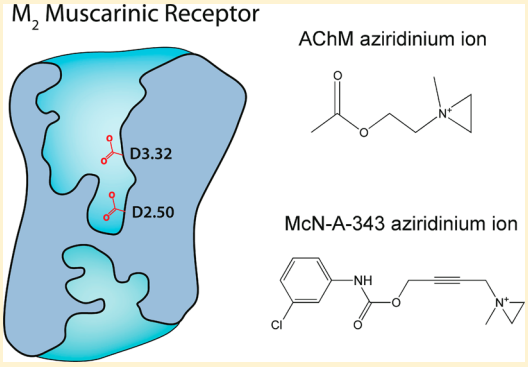
Supporting Information

ABSTRACT: We investigated how asparagine mutagenesis of conserved aspartic acids in helix 2 (D2.50) and 3 (D3.32) of M_1−M_4 muscarinic receptors alters the irreversible binding of acetylcholine mustard and BR384 (4-[(2-bromoethyl)methyl-amino]-2-butynyl *N*-(3-chlorophenyl)-carbamate), a nitrogen mustard derivative of McN-A-343 ([4-[[*N*-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl] trimethylammonium chloride). The D2.50N mutation moderately increased the affinity of the aziridinium ions of acetylcholine mustard and BR384 for M_2−M_4 receptors and had little effect on the rate constant for receptor alkylation. The D3.32N mutation greatly reduced the rate constant for receptor alkylation by acetylcholine mustard but not by BR384, although the affinity of BR384 was reduced. The combination of both mutations (D2.50N/D3.32N) substantially reduced the rate constant for receptor alkylation by BR384 relative to that of wild type and mutant D2.50N and D3.32N receptors. The change in binding affinity caused by the mutations suggests that the D2.50N mutation alters the interaction of acetylcholine mustard with D3.32 of the M_1 and M_3 receptors but not that of the M_4 receptor. BR384 exhibited the converse relationship. The simplest explanation is that acetylcholine mustard and BR384 alkylate at least two residues on M_1−M_4 receptors and that the D2.50N mutation alters the rate of alkylation of D3.32 relative to another residue, perhaps D2.50 itself.



Irreversible ligands are often useful in studies of drug−receptor interactions. One approach for synthesizing an irreversible ligand is to incorporate a nitrogen mustard group into its structure.[1] This strategy has yielded several anticancer agents that react covalently with DNA to form guanine adducts.[2,3] Muscarinic receptor ligands seem ideally suited for this modification because the reactive aziridinium ion derived from a nitrogen mustard group resembles the quaternary ammonium group of acetylcholine. Indeed, the nitrogen mustard analog of acetylcholine, acetylcholine mustard (AChM), has been shown to bind irreversibly to a highly conserved aspartic acid residue (D3.32, nomenclature scheme of Ballesteros and Weinstein[4]) in helix 3 of muscarinic receptors.[5] Thus, 2-haloethylamine muscarinic ligands may have the capacity to react covalently with the receptor at D3.32.

We have used acetylcholine mustard (AChM) (Figure 1) as a probe to investigate whether a test ligand interacts competitively or allosterically with the orthosteric site alkylated by AChM.[6,7] The method involves first incubating the muscarinic receptor with AChM and different concentrations of the test ligand. Next, the reactions are stopped, receptor preparations are washed, and residual unalkylated receptors are estimated using a suitable radioligand such as [^{3}H]*N*-methylscopolamine ([^{3}H]NMS). Competitive and allosteric modulators exhibit a difference in how they affect receptor alkylation. This approach has advantages over equilibrium and kinetic binding methods for analyzing allosteric interactions.[6,7]

We have shown that mutating aspartic acid 3.32 to asparagine in M_1 and M_2 muscarinic receptors causes a large decrease in the rate constant for receptor alkylation by AChM, which is consistent with the postulate that AChM alkylates D3.32.[8] However, this mutation does not completely prevent receptor alkylation (particularly at high concentrations of AChM). We also found that D3.32N greatly inhibited the alkylation of M_1 and M_2 receptors by a nitrogen mustard analog (BR384; 4-[(2-bromoethyl)methyl-amino]-2-butynyl *N*-(3-chlorophenyl)carbamate) of the functionally selective muscarinic agonist McN-A-343 ([4-[[*N*-(3-chlorophenyl)carbamoyl]-oxy]-2-butynyl]trimethylammonium chloride) (Figure 1). However, the inhibition was primarily due to a reduction in affinity and not to a decreased rate constant for alkylation, suggesting perhaps that BR384 does not alkylate the D3.32 residue of the M_1 and M_2 receptors. Nonetheless, the orthosteric muscarinic antagonist NMS competitively inhibited

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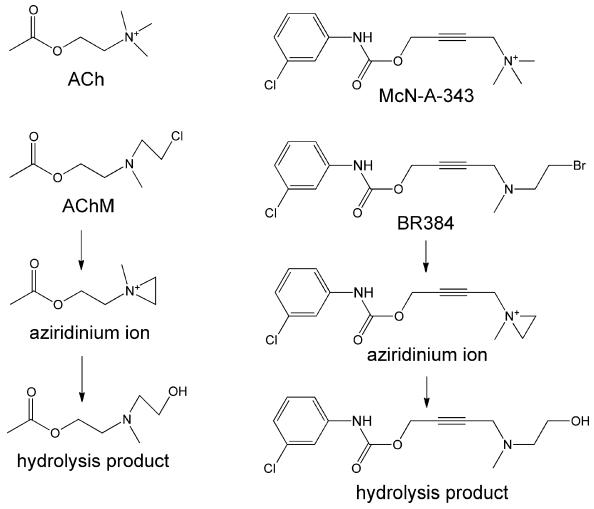

Figure 1. Structures of acetylcholine, McN-A-343, their nitrogen-mustard derivatives, and their transformation products in aqueous solution at neutral pH.

alkylation of wild-type M_1, wild-type M_2, and the D103N mutant of the M_2 receptor by BR384, whereas known allosteric modulator gallamine allosterically prevented alkylation.[6,8,9] Thus, BR384 probably alkylates another residue within the orthosteric binding pocket of the M_2 receptor.

McN-A-343 is an important muscarinic agonist because it exhibits a preference for activating M_1 and M_4 muscarinic receptors relative to the other subtypes.[10,11] Intravenous administration of McN-A-343 elicits a pressor response due to the activation of M_1 muscarinic receptors in sympathetic ganglia.[12,13] In contrast, nonselective muscarinic agonists activate the M_3 muscarinic receptor on peripheral blood vessels causing vasodilatation and a reduction in blood pressure.[14,15] The aziridinium ion of BR384 behaves like McN-A-343 with regard to its pressor response in vivo and its lack of contractile action on the guinea pig ileum in vitro.[16] The latter response is mediated through the M_3 muscarinic receptor.[17] Understanding the mode of interaction of BR384 with muscarinic receptors may shed light on the development of novel selective orthosteric ligands.

In this study, we have investigated how the D3.32N mutation (Figure 2) affects the alkylation of the M_3 and M_4 receptors by AChM and BR384. We have also investigated how the mutation of a highly conserved residue in helix 2 by itself (D2.50, Figure 2) and in combination with D3.32N affects M_1–M_4 receptor alkylation. Our results are consistent with the postulate that AChM and BR384 alkylate D3.32 in addition to another residue within the binding pockets of the M_1–M_4 muscarinic receptors.

EXPERIMENTAL PROCEDURES

Materials. Reagents were obtained from the following sources: Dulbecco's Modified Eagle's Medium with high glucose and L-glutamine, Luria–Bertani broth, trypsin–EDTA, and penicillin–streptomycin (Invitrogen, Carlsbad, CA); fetal calf serum (HyClone Laboratories Inc., South Logan, UT); G418 (InvivoGen, San Diego, CA); NMS, atropine, acetylcholine perchlorate, HEPES, EDTA, scopolamine, and $Na_2S_2O_3$ (Sigma-Aldrich, Inc., St. Louis, MO); salts for phosphate buffer and binding buffer, HCl, and NaOH (Thermo Fisher Scientific, Waltham, MA); Zyppy Plasmid Miniprep Kit (Zymo Research, Irvine, CA); NucleoBond Xtra Midi Plus (Clontech Laboratories, Inc., Mountain View, CA); GeneJammer (Agilent Technologies, Cedar Creek, TX); and oligonucleotide primers (Integrated DNA Technologies, Inc.,

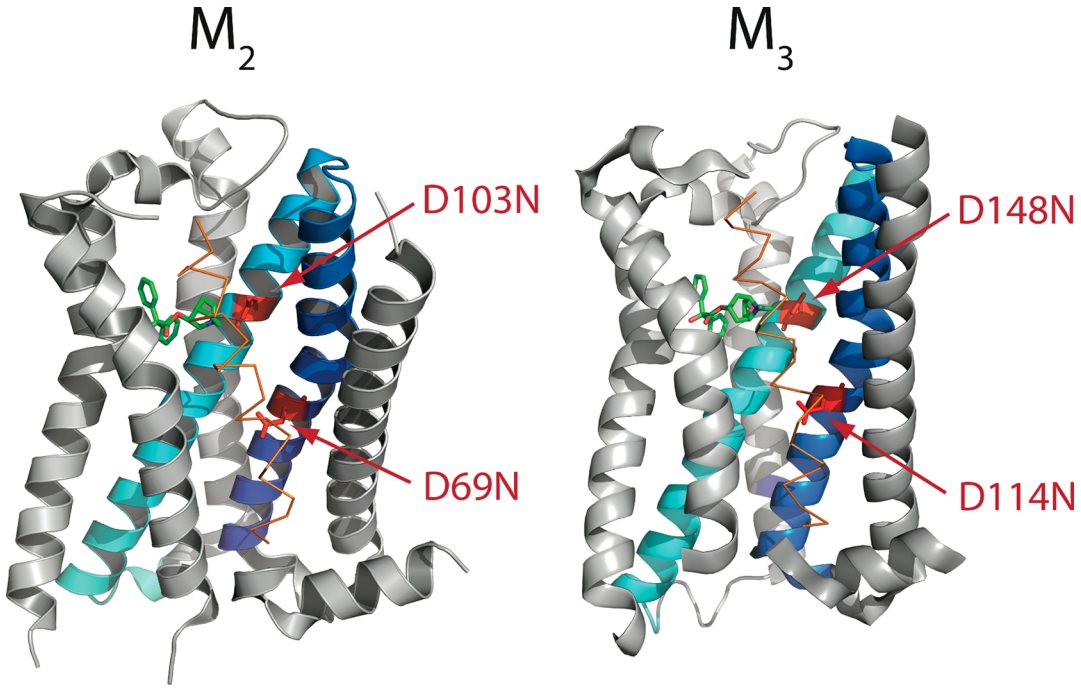

Figure 2. Crystal structures of the helices and extracellular loops of the human M_2 and rat M_3 muscarinic receptors bound with 3-quinuclidinyl-benzilate and tiotropium, respectively.[26,27] The secondary structure (helices 1–6) and backbone (helix 7) are shown. Aspartic acids 2.50 (M_2 D69 and M_3 D113 (corresponds to human M_3 D114)) and 3.32 (M_2 D103 and M_3 D147 (corresponds to human M_3 D148)) are indicated on helices 2 and 3 (PDB ID 3uon and 4daj for M_2 and M_3 receptors, respectively).

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San Diego, CA). AChM and McN-A-343 were synthesized as described previously.[7] BR384 was synthesized by the method of Ringdahl et al.[16]

Both AChM and BR384 were first cyclized to their reactive aziridinium ions as described previously[7,9] before being used in the assays described below.

Site-Directed Mutagenesis. The human M_1, M_2, M_3, and M_4 muscarinic receptor cDNAs, cloned into a modified expression vector (pCD-hM_1, pCD-hM_2, pCD-hM_3, pCD-hM_4), were obtained from Dr. Tom Bonner at the National Institute of Mental Health (Bethesda, MD). Mutations were introduced into pCD-hM_1, pCD-hM_2, pCD-hM_3, and pCD-hM_4 using the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) with mutagenesis primers. The sequences of the mutant receptors were verified by Laragen, Inc. (Culver City, CA). The mutant plasmids were purified using the Zyppy Plasmid Miniprep Kit or NucleoBond Xtra Midi Plus Kit following the manufacturers' protocol.

Cell Culture and Transfection. Chinese hamster ovary (CHO) cells stably expressing the human M_1, M_2, M_3, and M_4 muscarinic receptors were obtained from Acadia Pharmaceuticals (San Diego, CA) and were cultured as described previously.[7] Human embryonic kidney (HEK) 293 cells were cultured as described previously [8] and transfected with plasmids encoding mutated muscarinic receptors using GeneJammer following the manufacturers' protocol. After transfection, the cells were incubated for 48 h and harvested for assays.

Preparation of Cellular Homogenates. CHO or HEK 293 cells expressing muscarinic receptors were grown to confluence in 100 mm dishes (Corning Life Sciences, Acton, MA) and scraped into binding buffer (20 mM sodium-HEPES, pH 7.4, 100 mM NaCl, and 10 mM EDTA) using a Teflon spatula. The mixture was centrifuged at low speed (1247*g*, 10 min), and the supernatant was discarded. The pellet was suspended in binding buffer using a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland; setting no. 4, 10 s). The homogenates of cells expressing M_1 D71N/D105N, M_2 D69N/D103N, M_3 D114N/D148N, or M_4 D78N/D112N receptors were centrifuged once more at high speed (39 400*g*, 10 min, and 4 °C) and suspended in fresh binding buffer.

The homogenates were prepared at varying concentrations, depending on the assay and receptor construct, so that the receptor concentration in the final binding assay would result only in a minimal depletion of the free concentration of [^{3}H]NMS (see below).

Treatment of Cellular Homogenates with Cyclized AChM and BR384. The covalent interactions of AChM and BR384 with muscarinic receptors were investigated by first incubating the homogenates of cells expressing muscarinic receptors with the aziridinium ions of AChM and BR384 followed by measuring the residual muscarinic receptors using the radioligand [^{3}H]NMS as described previously.[7]

Cellular homogenates (200 μL) were incubated at 37 °C in a shaking water bath, and an aliquot (50 μL) of cyclized AChM, BR384, or binding buffer (control) was added to yield a final volume of 0.25 mL. The reaction was allowed to proceed for a specified amount of time, as described in the Results section. An aliquot (0.75 mL) of stopping solution (see below) was added at the end of the incubation, and the mixture was incubated for another 20 min to allow for the inactivation of the aziridinium ions of AChM or BR384.

The reaction tubes were centrifuged (25 000*g*, 15 min, and 4 °C), and the pellets were suspended in fresh buffer to remove the transformation products of AChM and BR384. If the stopping solution contained scopolamine (see below), the centrifugation step was repeated two more times. Ultimately, the final pellets were suspended in 1 mL of binding buffer. Triplicate measurements of [^{3}H]NMS binding were made on each homogenate as described below.

The reactions were stopped by two methods, depending on the goal of the experiment. In the first method, the stopping solution contained sodium thiosulfate (1.33 mM) in binding buffer. Thiosulfate forms a covalent adduct with the remaining aziridinium ion derived from AChM and BR384, and this first-order process is complete in about 15 min. During this time, additional alkylation of the receptor can occur. In the second method, the stopping solution contained both scopolamine (10 μM) and sodium thiosulfate (1.33 mM). The scopolamine immediately stops the reaction, and the thiosulfate slowly inactivates the aziridinium ion. The kinetic constants of AChM and BR384 (eqs 4 and 6) were estimated only from the reactions that were stopped using the latter method.

We previously showed that our stopping procedure with scopolamine immediately prevents receptor alkylation by BR384 (100 μM) and that the associated washing step is adequate for removing residual scopolamine.[8] When the concentration of BR384 was increased to 300 μM, however, 28% of the wild-type M_1 and M_2 muscarinic receptors were alkylated after adding the stopping solution. In contrast, 3 mM AChM caused only a 1% inhibition of [^{3}H]NMS binding in the presence of the stopping solution. Thus, the measurements of [^{3}H]NMS binding after treatment with 300 μM BR384 were multiplied by a factor of 1.39 (i.e., $1/(1 - 0.28)$) to correct for the continued alkylation that occurs after the addition of scopolamine and thiosulfate.

[^{3}H]NMS Binding Assays. The residual amount of free muscarinic receptors in cellular homogenates treated with AChM and BR384 was estimated using a binding assay with the muscarinic antagonist radioligand [^{3}H]NMS (specific activity 82 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA).

For the experiments involving M_1 D71N/D105N, M_2 D69N/D103N, M_3 D114N/D148N, or M_4 D78N/D112N receptors, a centrifugation assay was used to measure [^{3}H]NMS binding [8] because these mutants exhibited a low affinity for [^{3}H]NMS (pK_D 7.7−8.0). We were concerned that [^{3}H]NMS−receptor complexes might dissociate during the washing phase of the filtration assay that was used for the other receptors (see below). An aliquot (0.3 mL) of cellular homogenate was incubated in a microcentrifuge tube (G-tube; Thermo Fisher Scientific) for 30 min at 37 °C in a final volume of 0.5 mL containing binding buffer and [^{3}H]NMS (3.0 nM). The equilibration period was stopped by centrifugation (30 000*g*, 20 min, and 4 °C). The supernatant was aspirated, and the residual pellet was washed twice with 0.6 mL of ice-cold binding buffer. An aliquot (0.2 mL) of 1 M NaOH was added to dissolve the pellet. Following an overnight incubation, the solubilized material was acidified with 1 M HCl (0.25 mL) and transferred to a scintillation vial (Research Products International Corp., Mount Prospect, IL). Following the addition of the scintillation cocktail (Budget-Solve; Research Products International Corp.), radioactivity was measured using a liquid scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA). The nonspecific binding was defined as the residual binding in the presence of 10 μM atropine. All measurements were recorded in triplicate.

For the experiments involving the other receptor constructs, a filtration assay was used to measure [^{3}H]NMS binding[7] in homogenates previously treated with AChM or BR384. An aliquot (0.3 mL) of cellular homogenate was incubated for 30 min at 37 °C in a final volume of 1 mL containing binding buffer and 1.0 nM of [^{3}H]NMS. The equilibration was stopped by rapid filtration over glass-fiber filters (Whatman GFB) using a cell harvester (Brandel Inc., Gaithersburg, MD). The filters were washed three times with ice-cold 0.9% saline (approximately 3 mL per wash). The filters were placed in scintillation vials, and radioactivity was measured using liquid scintillation spectroscopy as described above.

Ligand/[^{3}H]NMS Competition Experiments. The competitive inhibition of [^{3}H]NMS binding to muscarinic receptors by acetylcholine, McN-A-343, and NMS was measured in cellular homogenates using the centrifugation or filtration assay, depending on the receptor construct (double mutant or all others, respectively). The assay was performed as described above, with the exception that fresh cellular homogenate was used, and the assay included various concentrations of the nonlabeled competitors.

Analysis of the Data. The IC_{50} values were estimated by nonlinear regression analysis of the ligand/[^{3}H]NMS competition curves using Prism 6.0 (GraphPad Software Inc., San Diego, CA) and the following equation:

$$B = P\left(1 - \frac{I^n}{I^n + IC_{50}^n}\right) \quad (1)$$

In this equation, P and B denote the specific binding of [^{3}H]NMS in the absence and presence of nonlabeled inhibitor, respectively, IC_{50}, the concentration of inhibitor causing half-maximal inhibition of specific binding, and n, the Hill coefficient.

The concentration of homogenate was such that less than 2% of the total concentration of [^{3}H]NMS was depleted at the IC_{50} point of the competition curve. Equilibrium dissociation constants (K_i, units of molarity) were estimated from the IC_{50} values (concentration of competitor causing half-maximal displacement of specific binding) using the standard competitive-inhibition relationship.[18]

$$K_i = \frac{IC_{50}}{1 + \frac{[[^3H]NMS]}{K_{NMS}}} \quad (2)$$

where [[^{3}H]NMS] denotes the free concentration of [^{3}H]NMS at the IC_{50} point of the competition curve and K_{NMS}, the dissociation constant of [^{3}H]NMS (units of molarity, M). The latter was estimated by rearrangement of eq 2 for the case where the competitor and radioligand are the same:

$$K_{NMS} = IC_{50} - [[^3H]NMS] \quad (3)$$

In the analysis of the NMS/[^{3}H]NMS competition curves, the Hill coefficient in eq 1 was constrained to 1. The concentration of [^{3}H]NMS was established with accuracy by first making a concentrated stock solution and calculating its concentration from the amount of radioactivity associated with a small aliquot of it. This solution was diluted to a working stock solution that was used in the competition experiment, and its concentration was subsequently determined for each experiment as was just described.

For a given experiment, the competitive inhibition of [^{3}H]NMS binding by acetylcholine, McN-A-343, and nonlabeled NMS was measured simultaneously so that individual values of log K_i or log K_{NMS} could be estimated for each nonlabeled ligand. The text, figures, and tables report the mean ± SEM of these estimates.

The basis for the estimation of the dissociation constant (K_1) and rate constant for the alkylation (k_1) of AChM and BR384 is described by Suga and Ehlert.[9] Our analysis rests on the assumption that the rate constants describing the reversible interaction of the aziridinium ion with the receptor are much faster than those of the alkylation step (k_1) (Scheme 1).

Scheme 1. Quasi-Equilibrium Model for the Interaction of the Aziridinium Ions of AChM and BR384 with the Muscarinic Receptor[a]

$$A + R \underset{}{\overset{K_1}{\longleftrightarrow}} AR \xrightarrow{k_1} A\text{-}R$$

[a] The dissociation constant K_1 (units of M) describes the reversible interaction of the aziridinium ion (A) with the receptor (R) to yield the reversible receptor complex (AR). The rate constant k_1 (units of inverse time, min^{-1}) describes the instantaneous rate of receptor alkylation (k_1AR) to yield the irreversible receptor complex (A–R).

The data needed for this kinetic analysis comes from an experiment in which aliquots of a given receptor preparation are incubated with different concentrations of the nitrogen mustard (AChM or BR384) for single or multiple incubation times. The amount of unalkylated receptors is estimated subsequently by measuring the binding of [^{3}H]NMS at a single concentration (1 or 3 nM).

The kinetic analysis for AChM involves fitting the following regression equation to the measurements of [^{3}H]NMS binding:

$$\frac{Y_t}{Y_0} = (1 - b)\, e^{-Ok_1 t} + b \quad (4)$$

In this equation, Y_t denotes [^{3}H]NMS binding after incubation with the irreversible ligand, Y_0, the estimate of [^{3}H]NMS binding in the absence of irreversible ligand, k_1, the rate constant for alkylation, t, the time of incubation, O, the receptor occupancy by the aziridinium ion, and b, the fraction of receptors that can bind [^{3}H]NMS but are resistant to receptor alkylation. The variable O is given by

$$O = \frac{X}{X + K_1} \quad (5)$$

where K_1 denotes the dissociation constant of the aziridinium ion for the receptor (units of M) and X, the concentration of the aziridinium ion.

A different equation was used to analyze the data obtained with BR384 because the concentration of the aziridinium ion decays substantially during the incubation. The basis of this equation is described by Ehlert and Jenden:[19]

$$\frac{Y_t}{Y_0} = (1 - b)\left(\frac{X_0\, e^{-t/\tau} + K_1}{X_0 + K_1}\right)^{\tau k_1} + b \quad (6)$$

In this equation, τ denotes the macroscopic time constant for the decay in the concentration of the aziridinium ion from its peak concentration. The parameter τ was constrained to a constant (0.07 min^{-1}) on the basis of the values of the microscopic constants for the formation and decay of the aziridinium ion estimated by Ringdahl et al.[16]

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For all of the kinetic experiments, the homogenate concentration during the subsequent binding assay was adjusted so that the maximal depletion of the free concentration of [^{3}H]NMS was less than 2.5%.

For each kinetic experiment, individual estimates of log K_1 and k_1 were made, and the text and tables report the mean ± SEM of these values. We note that the error in the estimate of k_1 tended to be proportional to the measurement such that a log transformation yielded a more uniform variance. Consequently, all statistical analyses were done using the log k_1 values. The tables in the Supporting Information also include the mean ± SEM values for log k_1, and Figure 7 illustrates these values.

The mutation-induced change in the log K_1 values of the nitrogen mustards (AChM and BR384) or the log K_i values of the nonlabeled competitors (NMS, acetylcholine, and McN-A-343) ($\Delta \log K_{mutant}$) was estimated using the following equation

$$\Delta \log K_{mutan\ t} = \log K_{mutan\ t} - \log K_{WT} \quad (7)$$

where the subscripts "mutant" and "WT" denote whether the dissociation constant (K) is associated with a mutant or wild-type receptor, respectively. The variance of $\Delta \log K_{mutant}$ is equal to the sum of the variances of the individual log K_{mutant} and log K_{WT} estimates.

The significance of the differences in the estimates of a given parameter among the wild type and mutants of a given receptor subtype were determined by one-way analysis of variance with post hoc comparisons (Holm-Sidak's multiple comparisons test) using Prism 6.0.

RESULTS

Kinetics of Alkylation of the M_4 Muscarinic Receptor by AChM and BR384. We measured the kinetics of alkylation of the M_4 muscarinic receptor at various concentrations of AChM and BR384 (Figure 3). Homogenates of CHO cells expressing the wild-type M_4 receptor were incubated with different concentrations of cyclized AChM or BR384 for different times, and the reaction was subsequently stopped with scopolamine and thiosulfate. The homogenates were washed, and the specific binding of [^{3}H]NMS was measured to estimate the residual unalkylated receptors. Figure 3a shows that following treatment with AChM there is a concentration-dependent loss of [^{3}H]NMS binding and that the magnitude of the loss increases with an increase in time. This behavior suggests that the aziridinium ion binds rapidly to the M_4 receptor to form a reversible complex that converts to a covalent complex at a slower rate (Scheme 1). To test this model, we fitted eq 4 to the data to obtain an estimate of the affinity constant of the aziridinium ion of AChM for the M_4 receptor (log K_1 = −4.10 ± 0.058), its rate constant for alkylation (k_1 = 0.44 ± 0.038 min^{-1}), and the estimate of the proportion of receptors that can bind [^{3}H]NMS but not be alkylated by AChM (b = 17 ± 2.0%). Regression analysis yielded a good fit of eq 4 to the data.

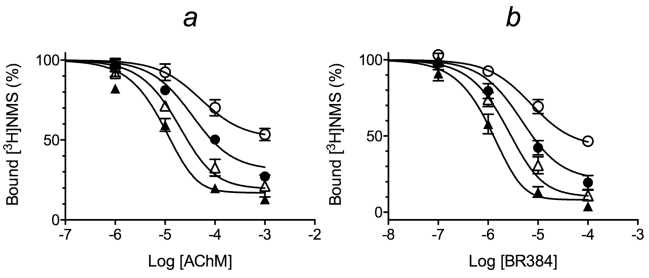

Figure 3. Alkylation of the wild-type M_4 muscarinic receptor by AChM and BR384. Homogenates of CHO cells expressing the human M_4 muscarinic receptor were incubated with various concentrations of AChM (a) or BR384 (b) for different times, the reactions were stopped with scopolamine and thiosulfate, the homogenates were washed, and the binding of [^{3}H]NMS was measured at a single concentration of 1.0 nM. The theoretical curves represent the global fit of eqs 4 (a) and 6 (b) to the data with the estimates of b, k_1, and K_1 shared. Mean values ± SEM of three experiments are shown. In panel a, the incubation times were 2 (○), 4 (●), 8 (△), and 15 (▲) min. In panel b, the incubation times were 1 (○), 2 (●), 4 (△), and 8 (▲) min.

Similar behavior was observed in experiments with BR384, as shown in Figure 3b. Regression analysis of these data was done using eq 6 to account for the decay in the aziridinium ion of BR384, which is less stable than AChM. This analysis yielded estimates of the dissociation constant for the aziridinium ion of BR384 with the M_4 receptor (log K_1 = −5.05 ± 0.072) and its rate constant for alkylation (k_1 = 0.93 ± 0.020 min^{-1}). The estimates of K_1 and k_1 are approximately 10- and 2-fold greater than those of AChM, respectively. The estimate of the proportion of receptors that can bind [^{3}H]NMS but not be alkylated by BR384 is 7.7 ± 1.8%.

Effects of Various Concentrations of AChM and BR384 on Wild Type and D2.50N and D3.32N Mutants of the M_2 and M_4 Muscarinic Receptors. The conclusion that a component of the M_4 muscarinic receptor population can bind [^{3}H]NMS but not be alkylated by AChM or BR384 (i.e., b values of 17 and 7.7%, respectively; eqs 4 and 6) seems puzzling because both [^{3}H]NMS and the aziridinium ions of AChM and BR384 should have access to the same pool of receptors in the cellular homogenates. For example, the charged aziridinium ions might be unable to penetrate ER vesicles containing a high density of wild-type or mutant receptors in the cellular homogenates; however, this would also be the case for the quaternary ligand [^{3}H]NMS, particularly at the low concentration used in the binding assay. In addition, when solutions of AChM and BR384 are cyclized to yield their maximal concentrations of aziridinium ion there is still a modest amount of the parent mustard in solution (2 and 30% of the starting concentrations of AChM and BR384, respectively). This species of weak base is expected to penetrate lipid barriers and alkylate any receptors inaccessible to [^{3}H]NMS following its cyclization within the putative lipid compartment. Perhaps there is a cleavage of the covalent AChM− and BR384−receptor bonds or there are unalkylated receptors trapped within a membrane compartment that may be exposed over time with repetitive washing and trituration of the homogenate. The rate of recovery of [^{3}H]NMS binding over 4 hr is negligible in cerebral cortical homogenates, which have about 85% of their muscarinic receptors alkylated with BR384, indicating that the binding of BR384 to cerebral muscarinic receptors is nearly irreversible over 4 hr (Supporting Information, Figure S1).

Because of this complication, we investigated a more rapid experiment employing a single incubation time and washing step. In these experiments, various concentrations of the irreversible agonists were incubated with the M_2 and M_4 muscarinic receptors for 15 (AChM) and 4 min (BR384), and the reaction was stopped with thiosulfate only and washed once as described in the Experimental Procedures (Figure 4).

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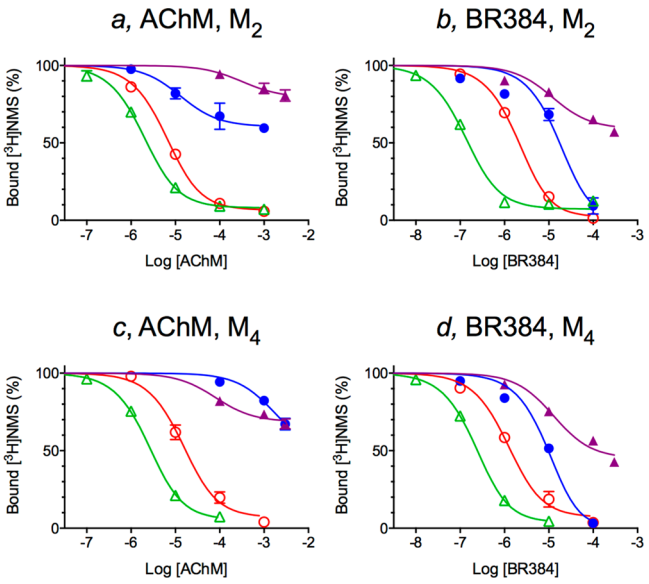

Figure 4. Interaction of AChM and BR384 with wild-type and mutant M_2 (a, b) and M_4 (c, d) muscarinic receptors. Homogenates of cells expressing muscarinic receptors were incubated with different concentrations of AChM (a, c) or BR384 (b, d) for 15 (AChM; a, c) or 4 (BR384; b, d) min. The reactions were stopped with thiosulfate, the homogenates were washed, and the residual unalkylated muscarinic receptors were estimated by measuring [^{3}H]NMS binding at a concentration of 3 nM (D2.50N/D3.32N) or 1 nM (wild type, D2.50N, and D3.32N). The different receptors and their mutants are indicated as wild type (○), D2.50N (△), D3.32N (●), and D2.50N/D3.32N (▲). Mean values ± SEM from three to four experiments are shown.

With regard to the wild-type and D2.50N mutants of the M_2 and M_4 receptors, the data suggest that all of the [^{3}H]NMS binding sites are capable of being alkylated by AChM (Figure 4a,c) and BR384 (Figure 4b,d). The same also applies to the effect of BR384 on the D3.32N mutants (Figure 4b,d). Presumably, AChM alkylates the D3.32N and D2.50N/D3.32N mutants too slowly for near-complete alkylation to occur during the incubation period (Figure 4a,c). The same is true with regard to the alkylation of the D2.50N/D3.32N mutant by BR384 (Figure 4b,d). The data also show that the D2.50N mutation increases receptor alkylation, whereas the D3.32N mutation has the opposite effect (Figure 4a−d). In contrast, the D2.50N mutation clearly inhibited receptor alkylation when added in combination with the D3.32N mutation with regard to AChM at the M_2 receptor (Figure 4a) and to BR384 at both the M_2 and M_4 receptors (Figure 4b,d).

To obtain further evidence for the mutually exclusive binding of [^{3}H]NMS and the aziridinium ions of AChM and BR384, we measured the competitive displacement of [^{3}H]NMS binding to the slowly alkylated D2.50N/D3.32N mutants of the M_2 and M_4 receptors under conditions where the rate of alkylation of the receptor is negligible or greatly reduced (60 min at 0 °C). Under these conditions, the highest concentration of AChM tested (3 mM) caused 14 ± 0.5 and 20 ± 2.2% alkylation of the double mutants of the M_2 and M_4 receptors, respectively (Figure 5a,b). The corresponding values for BR384 (0.3 mM) were 48 ± 4.2 and 44 ± 0.6%, respectively. In the competitive displacement assay, the highest concentration of AChM tested (3 mM) caused 73 ± 1.7 and 74 ± 0.4% inhibition of [^{3}H]NMS binding in the double mutants of the M_2 and M_4 receptors, with no evidence of a nonzero plateau in the inhibition curve (Figure 5c,d). The corresponding values for BR384 (0.3 mM) were 97 ± 1.3 and 88 ± 0.7%, respectively. All of the competition curves are consistent with a model of competitive inhibition (i.e., 100% displacement of specific binding). The affinity was too low, however, for the near-complete displacement of [^{3}H]NMS binding at the highest concentrations of AChM and BR384 used, with the exception of BR384 on M_2 D69N/D103N. The log molar IC_{50} values for AChM on the D2.50N/D3.32N mutants of the M_2 and M_4 receptors were −2.80 ± 0.32 and −2.86 ± 0.32 (N = 3 each), respectively. The corresponding values for BR384 were −4.54 ± 0.32 and −4.16 ± 0.04 (N = 3 each). Thus, the data are consistent with the postulate that the aziridinium ions of AChM and BR3884 have access to all of the sites labeled by [^{3}H]NMS in the D2.50N/D3.32N mutants of the M_2 and M_4 receptors.

Single-Time-Point Kinetic Assay for the Estimation of the Affinities and Rate Constants of AChM and BR384. Data such as those shown in Figure 4 are sufficient for estimating the dissociation (K_1) and rate (k_1) constants of the irreversible ligand provided that the covalent reaction is stopped quickly and the fraction of [^{3}H]NMS binding sites insensitive to the alkylating agent is known.

For example, we analyzed each inhibition curve in Figure 3 by regression analysis using eqs 4 (AChM, panel a) and 6 (BR384, panel b) with the value of the unreactive sites (b) constrained to that obtained in the global analysis in Figure 3 (17% for AChM; 7.7% for BR384). When applied to the data in panel a, this analysis yielded independent estimates of the log dissociation constant of AChM for the 2, 4, 8, and 15 min time points (−4.04 ± 0.11, −4.01 ± 0.025, −4.25 ± 0.10, and −4.20 ± 0.052). The corresponding estimates of the rate constant were 0.45 ± 0.030, 0.51 ± 0.020, 0.38 ± 0.090, and 0.36 ± 0.02 min^{-1}, respectively. Analysis of the data in panel b yielded estimates of the log dissociation constants (−4.91 ± 0.11, −5.05 ± 0.044, −5.10 ± 0.045, and −5.17 ± 0.043) and rate constants (0.99 ± 0.045, 1.05 ± 0.10, 0.75 ± 0.06, and 0.74 ± 0.086 min^{-1}) of BR384 at incubation times of 1, 2, 4, and 8 min, respectively. The average estimates of log K_1 (AChM, −4.13; BR384, −5.06) and k_1 (AChM, 0.42 min^{-1}; BR384, 0.82 min^{-1}) are nearly the same as those described above in connection with Figure 3.

For a given curve, it is possible to obtain good fits of eqs 4 (AChM) and 6 (BR384) as long as b is constrained to a value that is less than the plateau level of the curve. Over this domain, the estimate of k_1 is correlated with that of b and K_1 such that the ratio of k_1/K_1 is constant. This relationship occurs because the incomplete receptor alkylation under receptor-saturating concentrations of AChM or BR384 could be attributed to a significant fraction of receptors resistant to alkylation (significant b value) or a rate constant for alkylation of insufficient magnitude for complete alkylation during the incubation period.

Kinetics of the Interaction of AChM and BR384 with Wild-Type and D2.50N and D3.32N Mutants of M_1−M_4 Muscarinic Receptors. We applied the single-time-point assay to investigate the kinetics of the alkylation of the M_1−M_4 muscarinic receptors by AChM and BR384 (Figure 6). In these experiments, we stopped the alkylation step quickly with scopolamine and thiosulfate, and we washed the cellular homogenates three times before measuring the residual muscarinic receptors. Our rationale was that by reducing the number of data points the assay for a given receptor could be completed more quickly; hence, the appearance of new

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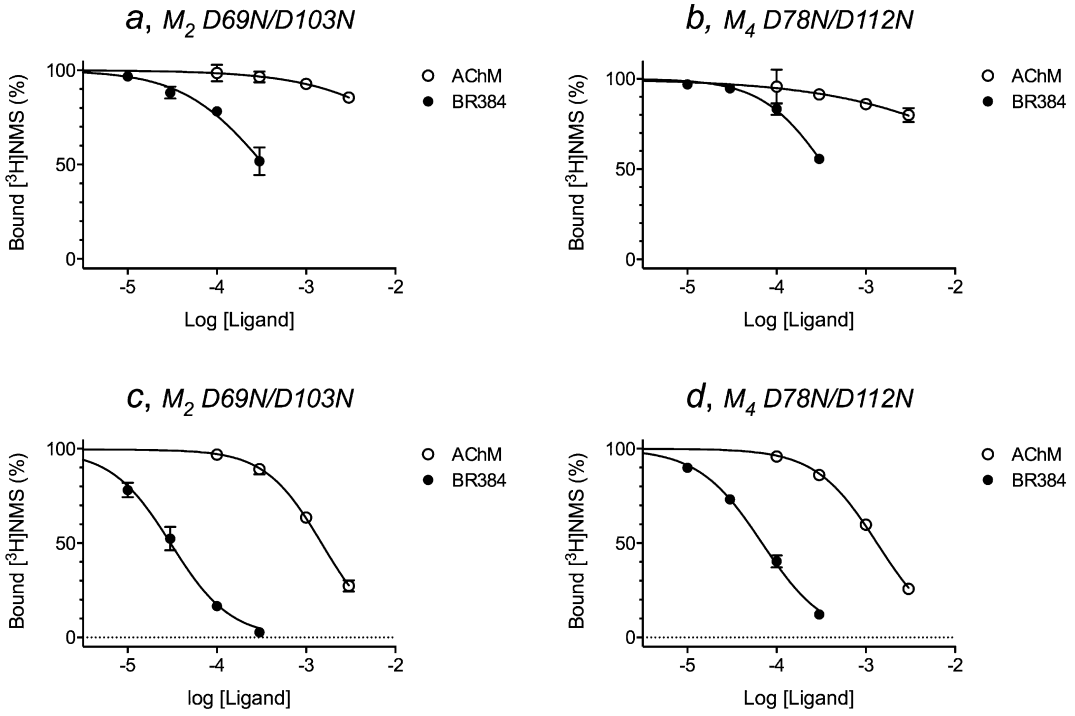

Figure 5. Effects of pretreatment (a, b) or coincubation (c, d) with AChM (○) and BR384 (●) (60 min at 0 °C) on the specific binding of [^{3}H]NMS to the M_2 D69N/D103N (a, c) and M_4 D78N/D112N receptors (b, d). (a) Various concentrations of AChM or BR384 were incubated with the homogenates of HEK 293 cells expressing the M_2 D69N/D103N receptor for 60 min at 0 °C. The reaction was stopped immediately with scopolamine and thiosulfate and washed. Residual binding was measured with [^{3}H]NMS (3 nM). (b) The same as panel a except that the receptor preparation was M_4 D78N/D112N. (c) The specific binding of [^{3}H]NMS (3 nM) to the M_2 D69N/D103N receptor was measured in the presence of various concentrations of AChM or BR384. The incubation lasted for 60 min at 0 °C. (d) The same as panel c except that the receptor preparation was M_4 D78N/D112N. The data represent mean values ± SEM from three experiments, with each measurement recorded in triplicate. The theoretical curve represents the least-squares fit of eq 1 to the data in panels c and d.

unalkylated receptors (i.e., significant *b* value) would be greatly reduced or eliminated. The results obtained with the more rapidly alkylated receptors (wild type and D2.50N mutants) suggest a *b* value of 0 for the given length of the incubation of the receptor with AChM (15 min) and BR384 (6 min).

Figure 6 shows our data for the M_1 (a,b), M_2 (c,d), M_3 (e,f), and M_4 (g,h) receptors with regard to the wild-type M_3, the D2.50N mutant of the M_2−M_4 receptors, the D3.32N mutant of M_3 and M_4 receptors, and the D2.50N/D3.32N mutant of the M_1−M_4 receptors. The data for the wild-type M_4 receptor are shown only in Figure 4, and the data for the wild-type and D3.32N mutants of the M_1 and M_2 receptors are not shown in Figure 6 because they have been published previously.[6−9] The data in Figure 6 were analyzed as described above with parameter *b* in eqs 4 (AChM) and 6 (BR384) constrained to 0. The results of this analysis are illustrated in Figure 7, which shows the estimates of −log K_1 (a,c) and k_1 (b,d) for AChM and BR384. Also shown are the parameter estimates for the wild-type M_4 receptor from the data in Figure 3 and those for wild-type and D3.32N mutants of the M_1 and M_2 receptors from our prior studies.[6−9] We did not investigate the D2.50N mutant of the M_1 receptor.

With regard to AChM (Figure 7a,b), the D3.32N mutation (M_1 D105N, M_2 D103N, M_3 D148N, and M_4 D112N) reduced the value of the rate constant for alkylation to between only $^1/_{50}$ and $^1/_{10}$ of the corresponding wild-type receptor and had little effect on the affinity of AChM for the M_1−M_3 receptors. In the case of the M_4 receptor, the affinity for AChM was reduced to $^1/_{10}$ of the wild-type receptor.

In contrast, the D2.50N mutation had smaller effects on the rate constant for receptor alkylation by AChM ($^1/_3$ to $^3/_4$ that of the wild type) but showed increased affinity (8- to 17-fold relative to that of wild-type M_2−M_4 receptors). The combination of both mutations (D2.50N/D3.32N) reduced the rate constant ($^1/_{30}$ to $^1/_3$ that of the wild type) and affinities ($^1/_{500}$ to $^1/_{30}$ that of the wild type) of AChM for the M_2−M_4 receptors. The rate constants for alkylation of the double mutant of the M_1 and M_3 receptors was greater than those of the corresponding D3.32N mutants, whereas the rate constant for alkylation of M_4 D78N/D112N was smaller than that of M_4 D112N.

These mutations had qualitatively similar effects on the interaction of BR384 with muscarinic receptors (Figure 7c,d). The D3.32N mutation had little or no inhibitory effect on the rate constant for alkylation, but it reduced the affinity to only about $^1/_{20}$ (M_1−M_3) and $^1/_4$ (M_4) that of the wild type. In contrast, the D2.50N mutation increased the affinity about 30-, 5-, and 30-fold for the M_2−M_4 receptors, whereas it had a modest effect on the rate constant for alkylation of the M_3 receptor. This mutation also reduced the rate constants for alkylation of the M_2 and M_4 receptors to values that are about $^1/_2$ that of the wild type. The combination of both mutations (D2.50N/D3.32N) reduced the rate constant for alkylation to values about $^1/_{20}$ (M_2), $^1/_4$ (M_1), $^1/_3$ (M_4), and $^1/_2$ (M_3) that of the wild type. The associated changes in the affinity represented both increases of 2-fold (M_1 and M_2) and decreases to about $^1/_5$ of the wild type (M_3 and M_4).

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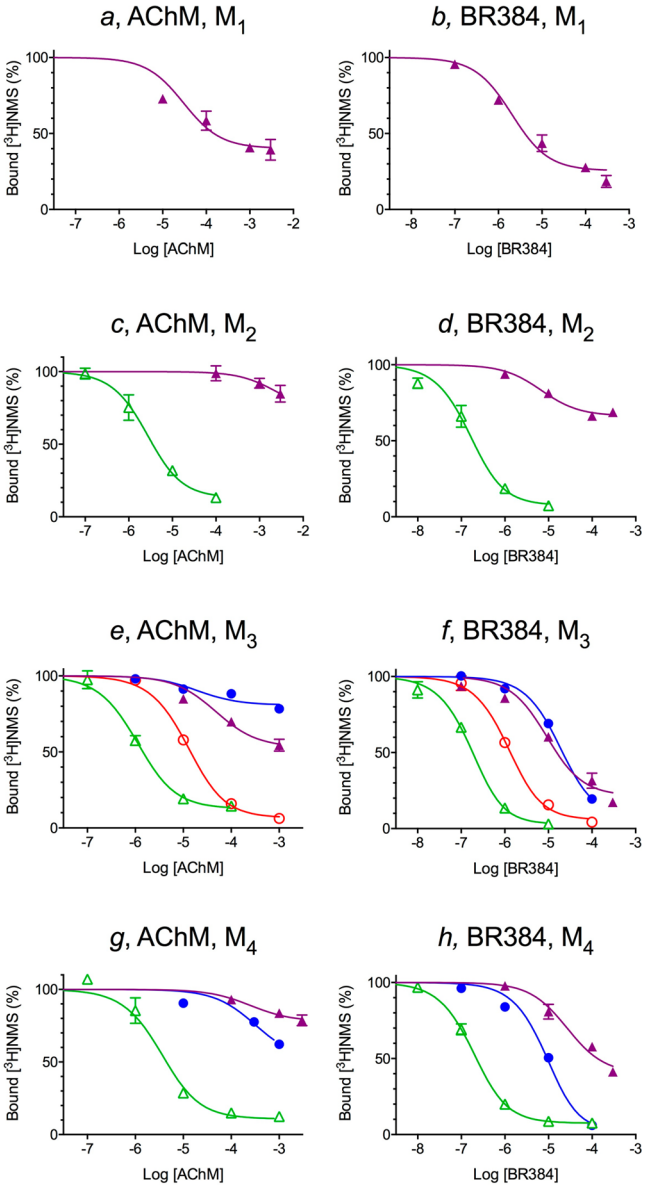

Figure 6. Interaction of AChM and BR384 with the wild-type and mutant M_1 (a, b), M_2 (c, d), M_3 (e, f), and M_4 (g, h) muscarinic receptors. Homogenates of the cells expressing muscarinic receptors were incubated with different concentrations of AChM (a, c, e, and g) or BR384 (b, d, f, and h) for 15 (AChM) or 6 (BR384) min. The reactions were stopped with thiosulfate and scopolamine, the homogenates were washed, and the residual unalkylated muscarinic receptors were estimated by measuring [^{3}H]NMS binding at a single concentration (1 or 3 nM). Mean values ± SEM from three experiments are shown. The different receptors and their mutants are indicated as wild type (○), D2.50N (△), D3.32N (●), and D2.50N/D3.32N (▲).

The numerical values for the estimates of log K_1 and k_1 for AChM and BR384 are listed in Tables S1 and S3 of the Supporting Information. Tables S2 and S4 of the Supporting Information summarize the post hoc comparisons of the former data, respectively. Analysis of variance showed that the mutations had significant effects on the estimates of log K_1 and log k_1 for AChM and BR384 for each receptor subtype (Supporting Information, Tables S1 and S3). Post hoc comparisons showed a significant effect of each mutation on the log k_1 value of AChM for each receptor subtype. For BR384, all of the mutations had a significant effect on log k_1 except for the D105N mutation of the M_1 receptor, the D103N mutation of the M_2 receptor, the D114N and D148N mutations of the M_3 receptor, and the D112N mutation of the M_4 receptor. With regard to the log K_1 estimates for AChM, all of the mutations had significant effects except for the D105N mutation of the M_1 receptor and the D103N and D69N mutations of the M_2 receptor. Finally, with regard to the log K_1 estimates for BR384, the mutations had significant effects except for the D71N/D105N and D69N/D103N mutants of the M_1 and M_2 receptors, respectively. Tables S2 and S4 of the Supporting Information also list the results of all other possible comparisons for a given receptor subtype.

Because the parameter estimates depend on the value to which b is constrained in eqs 4 and 6, we searched the parameter space to identify the maximum value of b that would still yield a nonsignificant increase in the residual sum of squares. This maximum estimate of b was lowest for the alkylation of the M_3 (0.067) and M_4 (0.085) receptors by BR384. These low values suggest that constraining b to 0 during regression analysis is reasonable. Constraining b to 0.065, for example, had little or no effect on parameter estimates for the M_3 and M_4 receptor constructs relative to that of another receptor construct examined in the same analysis (e.g., wild type or D2.50N).

The estimate of the log ratio of k_1 to K_1 is more accurate than either single parameter and is independent of the value of b over the range of 0 to a low value (e.g., 10%). The value of log k_1/K_1 represents the combined effect of the mutation on the affinity and rate constant for alkylation. Figure 8 shows a plot of the estimates of the log k_1/K_1 values of AChM and BR384 for the wild-type M_1, M_2, M_3, and M_4 receptors and the mutants thereof. The numerical values of these estimates are listed in Tables S1 and S3 of the Supporting Information. One-way analysis of variance showed a highly significant effect of the mutations on each receptor subtype. Post hoc comparisons showed that the estimate of log k_1/K_1 was significantly different ($P < 0.05$) for every comparison among the various constructs of a given receptor subtype except for M_1 wild type versus M_1 D2.50N/D3.32N (AChM, $P = 0.062$; BR384, $P = 0.26$) and comparisons between D3.32N and D2.50N/D3.32N with regard to AChM at the M_3 ($P = 0.051$) and M_4 ($P = 0.25$) receptors and BR384 at the M_2 ($P = 0.69$) receptor.

To quantify the effect of single and double point mutations on the binding affinities of AChM and BR384, we calculated the corresponding change in the log K_1 value relative to that of the wild type (Δ log K_1). These values are listed in Table 1 for the single (D2.50N and D3.32N) and double (D2.50N/D3.32N) point mutations of the M_2−M_4 receptors. Also listed is the sum of the Δ log K_1 values for the two single mutants (D2.50N + D3.32N). For the M_2 and M_3 receptors, AChM exhibited a significant increase in the Δ log K_1 value of the double mutant compared to the sum of the Δ log K_1 values of the single mutants, indicating an interaction between the mutations. For the M_4 receptor, the sum of the Δ log K_1 values of AChM for the single mutants was approximately equal to the Δ log K_1 value of the double mutant. In contrast, there was no evidence of an interaction between the mutations with regard to the binding affinity of BR384 for the M_2 and M_3 receptors, but there was evidence of an interaction between the mutations for the M_4 receptor.

Interaction of Acetylcholine, McN-A-343, and NMS with Wild Type and D2.50N and D3.32N Mutants of the

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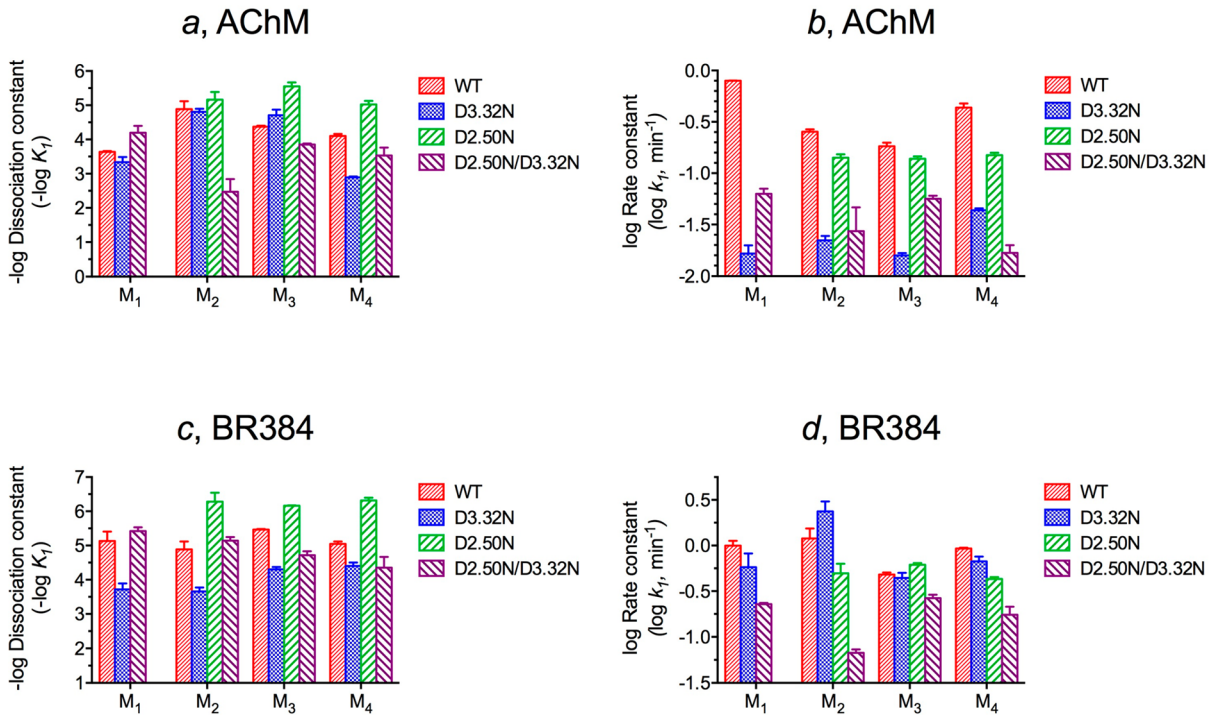

Figure 7. Estimates of the dissociation constants (a,c) and rate constants for alkylation (b,d) by AChM (a,b) and BR384 (c,d) of the wild-type and mutant M_1–M_4 muscarinic receptors. The parameters were estimated from the data in Figures 3 and 6. The parameter estimates for the wild type and D3.32N mutants of the M_1 and M_2 receptors are from our prior studies.[6−9] Analysis of variance showed that for both AChM and BR384 there were significant differences among the parameter estimates for the different mutants at each receptor subtype (Supporting Information, Tables S1 and S3). A summary of the post hoc comparisons of the parameter estimates is given in Tables S2 and S4 of the Supporting Information, and the numerical values of the parameters are listed in Tables S1 and S3 of the Supporting Information.

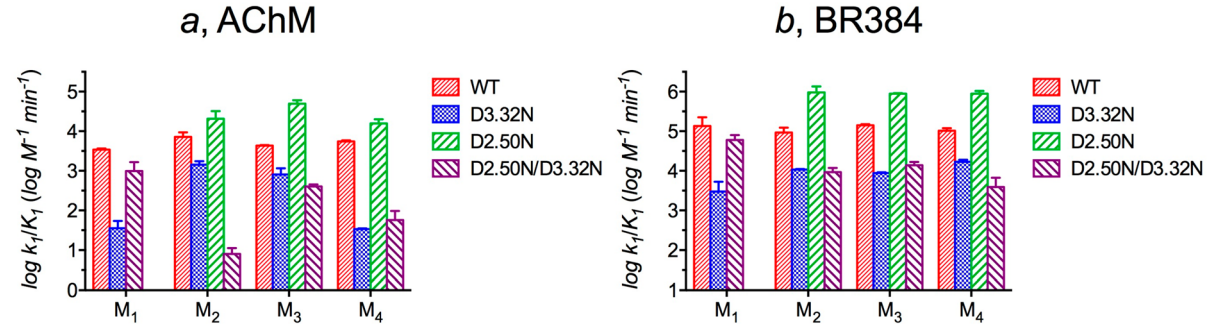

Figure 8. Combined measure of the affinity and reactivity of AChM and BR384 with M_1–M_4 wild-type receptors and their D2.50N, D3.32N, and D2.50N/D3.32N mutants. The graphs show the value of the log ratio of the rate constant for alkylation (k_1) and the affinity constant (K_1) of the aziridinium ion of AChM (a) and BR384 (b). The value of the log of this ratio (log k_1/K_1) is given for the wild-type and mutant M_1, M_2, M_3, and M_4 muscarinic receptors. Mean values ± SEM from four experiments are shown. A summary of the numerical values is listed in Tables S1 and S3 of the Supporting Information.

M_1–M_4 Muscarinic Receptors. We initially expected that the dissociation constants of acetylcholine and McN-A-343 for the different receptor mutants might be similar to the aziridinium ions of AChM and BR384 because of the close structural similarity of the compounds (Figure 1). To explore this question, we measured the competitive inhibition of [^{3}H]NMS binding by acetylcholine and McN-A-343 as well as nonlabeled NMS. For each competition curve, the IC_{50} value was estimated and corrected for the competitive effect of [^{3}H]NMS to yield the K_i value of the competitor. These estimates are illustrated in Figure 9, and the numerical values are listed in Table S5 of the Supporting Information together with the Hill slopes of the competition curves for acetylcholine and McN-A-343. The Hill coefficients of the competition curves for McN-A-343 were approximately equal to 1 for each receptor mutant, indicating that the K_i value is a good estimate of the dissociation constant (K_1) of the compound. With regard to acetylcholine, its competition curves for the M_2 wild type, M_2 D103N, and M_3 D114N/D148N receptors had Hill slopes that were substantially less than 1 (0.69, 0.60, and 0.56, respectively), indicating behavior consistent with at least two types of binding sites. A likely explanation in the case of the M_2 receptor is that a fraction of the receptor population interacts with $G_{i/o}$, resulting in the higher observed affinity. Thus, in most but not all cases, the −log K_i values of acetylcholine adequately represent the −log dissociation constant for the receptor (−log K_1).

In most instances, the effects of the mutations on the ligand affinity are qualitatively similar to those observed with the

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Table 1. Changes in log K_1 of AChM and BR384 Associated with Single and Double Point Mutations and the Sum of the Two Single Mutations (e.g., M_2 D103N + M_2 D69N)

	AChM (Δ log K_1)	BR384 (Δ log K_1)
M_2 D103N	−0.36 ± 0.16	1.23 ± 0.26
M_2 D69N	−0.71 ± 0.26	−1.39 ± 0.35
M_2 D103N + M_2 D69N	−1.07 ± 0.31	−0.17 ± 0.43
M_2 D69N/D103N	1.98 ± 0.40[a]	−0.25 ± 0.25
M_3 D148N	−0.34 ± 0.16	1.17 ± 0.07
M_3 D114N	−1.18 ± 0.13	−0.69 ± 0.02
M_3 D148N + M_3 D114N	−1.51 ± 0.20	0.48 ± 0.08
M_3 D114N/D148N	0.52 ± 0.04[b]	0.75 ± 0.12
M_4 D112N	1.21 ± 0.06	0.65 ± 0.13
M_4 D78N	−0.92 ± 0.12	−1.27 ± 0.11
M_4 D112N + M_4 D78N	0.29 ± 0.14	−0.62 ± 0.17
M_4 D78N/D112N	0.57 ± 0.24	0.69 ± 0.32[c]

[a]Significantly different from D2.50N + D3.32N, $P < 10^{-4}$. [b]Significantly different from D2.50N + D3.32N, $P < 10^{-6}$. [c]Significantly different from D2.50N + D3.32N, $P < 0.01$.

corresponding aziridinium ions (compare Figures 7 and 9). Two striking exceptions are the effects of the D3.32N mutation on the M_2 and M_3 receptors. This mutation reduced the affinities of acetylcholine to values of only $^1/_{50}$ and $^1/_{30}$ those of the wild-type receptors, respectively, but had little effect on the aziridinium ion of AChM. However, this mutation clearly altered how the aziridinium ion interacted with the M_2 and M_3 receptors because its rate constant for alkylation was substantially reduced. A more moderate difference was noted in the effect of the D2.50N/D3.32N mutation. This mutation always greatly reduced the affinities of acetylcholine and McN-A-343 but caused less of a decrease in the affinities of their analogous aziridinium ions for the M_3 and M_4 receptors and an increase in the affinity for the M_1 and M_2 receptors.

Analysis of variance showed that all of the mutations had significant effects on the affinities of NMS, acetylcholine, and McN-A-343 for the M_1−M_4 receptors (Supporting Information, footnotes to Table S5). Post hoc comparisons showed that all of the mutations had significant effects on the affinity of NMS, acetylcholine, and McN-A-343 for the M_1−M_4 receptors relative to wild type except for the affinities of acetylcholine and NMS for the M_2 D69N mutant. A summary of these and all other post hoc comparisons is given in Tables S6 (NMS) and S7 (acetylcholine and McN-A-343) of the Supporting Information.

We also calculated the Δ log K_i values for the effects of the single and double point mutations on the binding of acetylcholine, McN-A-343, and NMS, and these values are listed in Table 2. With the exception of NMS on the M_3 receptor, the increase in the Δ log K_i value associated with the double mutation (D2.50N/D3.32N) was always significantly greater than the sum of the Δ log K_i values of the two single mutations (D2.50N + D3.32N).

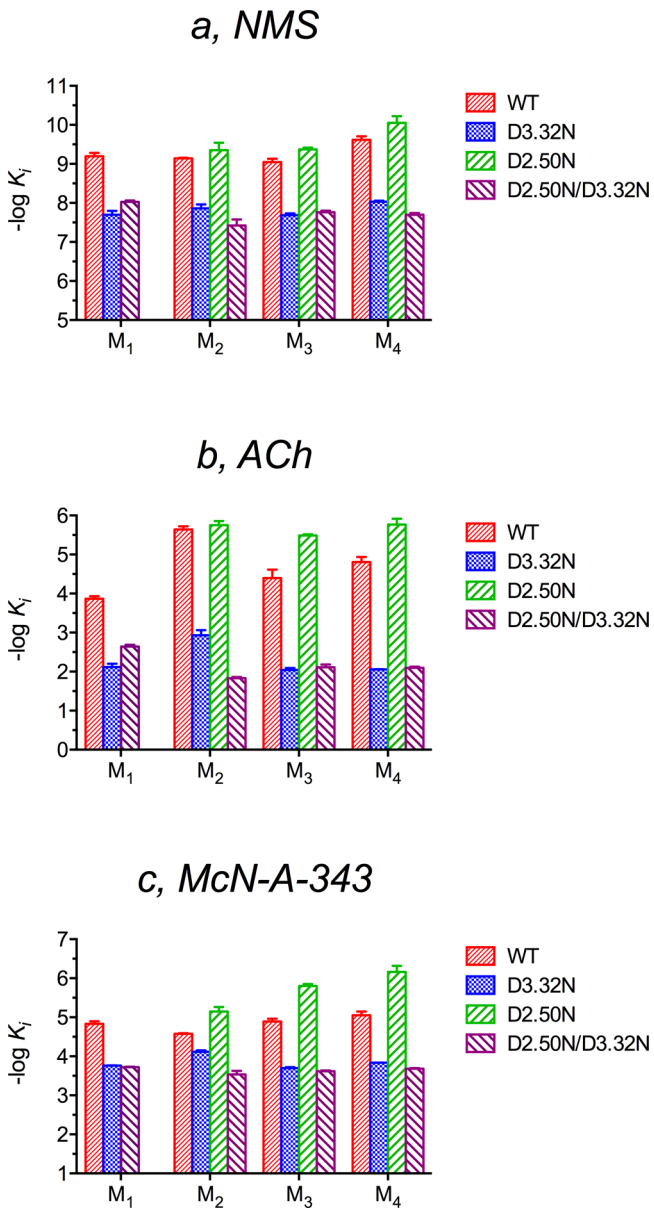

Figure 9. Negative log dissociation constants of NMS (a), acetylcholine (b), and McN-A-343 (c) for the wild-type and mutant M_1, M_2, M_3, and M_4 muscarinic receptors. The competitive inhibition of the binding of [^{3}H]NMS to the different wild-type and mutant receptors was measured, and the data were analyzed to estimate the dissociation constant of each ligand. Mean values ± SEM from three experiments are shown. Analysis of variance showed that for each ligand there were significant differences among the estimates of pK_i for the different mutants of each receptor subtype (Supporting Information, Table S5). A summary of the post hoc comparisons of the pK_i estimates is given in Tables S6 (NMS) and S7 (acetylcholine and McN-A-343) of the Supporting Information, and a list of the numerical values of the parameter estimates is given in Table S5 of the Supporting Information.

DISCUSSION

Our method for estimating the dissociation (K_1) and rate (k_1) constants is based on the assumption that AChM and BR384 reduce the binding capacity of [^{3}H]NMS for a given population of receptors without affecting its affinity for the unalkylated receptors. We begin by explaining the evidence for this assumption and then describe how our conclusions would be modified if our assumption is incorrect.

Three types of experimental evidence can be used to demonstrate that prior treatment of muscarinic receptors with AChM or BR384 causes a reduction in the binding capacity of [^{3}H]NMS: (1) A direct demonstration that AChM or BR384 treatment reduces the binding capacity of [^{3}H]NMS without affecting the affinity for the residual receptors, (2) a competitive and allosteric protection from the inhibitory effects of AChM

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Table 2. Changes in log K_i of Acetylcholine, McN-A-343, and NMS Associated with Single and Double Point Mutations and the Sum of the Two Single Mutations (e.g., M_2 D103N + M_2 D69N)

	acetylcholine (Δ log K_i)	McN-A-343 (Δ log K_i)	NMS (Δ log K_i)
M_2 D103N	2.71 ± 0.15	0.46 ± 0.040	1.28 ± 0.11
M_2 D69N	−0.11 ± 0.13	−0.57 ± 0.12	−0.21 ± 0.19
M_2 D103N + M_2 D69N	2.60 ± 0.20	−0.12 ± 0.12	1.07 ± 0.21
M_2 D69N/ D103N	3.81 ± 0.089[c]	1.04 ± 0.16[d]	1.72 ± 0.16[a]
M_3 D148N	2.36 ± 0.22	1.19 ± 0.075	1.36 ± 0.096
M_3 D114N	−1.09 ± 0.22	−0.91 ± 0.086	−0.33 ± 0.095
M_3 D148N + M_3 D114N	1.27 ± 0.31	0.28 ± 0.11	1.03 ± 0.14
M_3 D114N/ D148N	2.29 ± 0.090[a]	1.27 ± 0.073[d]	1.28 ± 0.092
M_4 D112N	2.75 ± 0.13	1.22 ± 0.095	1.59 ± 0.093
M_4 D78N	−0.96 ± 0.20	−1.11 ± 0.18	−0.43 ± 0.19
M_4 D112N + M_4 D78N	1.80 ± 0.23	0.11 ± 0.21	1.15 ± 0.21
M_4 D78N/ D112N	2.71 ± 0.13[b]	1.37 ± 0.096[c]	1.92 ± 0.10[b]

[a]Significantly different from D2.50N + D3.32N, $P < 0.05$. [b]Significantly different from D2.50N + D3.32N, $P < 10^{-2}$. [c]Significantly different from D2.50N + D3.32N, $P < 10^{-3}$. [d]Significantly different from D2.50N + D3.32N, $P < 10^{-5}$.

and BR384 by known orthosteric and allosteric ligands, respectively, and (3) a complete inhibition of [^{3}H]NMS binding by AChM or BR384.

With regard to the first type of evidence, we have previously shown that prior treatment of the M_1 and M_2 receptors with AChM and BR384 causes a reduction in the binding capacity of [^{3}H]NMS without affecting their affinity for the residual receptors.[6,7,9] It has also been shown that prior treatment of rodent forebrain or cerebral cortex with BR384 causes a reduction in the binding capacity of [^{3}H]quinuclidinyl benzilate and [^{3}H]NMS without affecting its affinity for the residual receptors.[16] These brain regions have abundant M_1, M_2, and M_4 muscarinic receptors.[20−24]

We have also shown that orthosteric ligands, such as acetylcholine, NMS, and McN-A-343, competitively protect M_1, M_2, and M_2 D103N receptors from alkylation by AChM and BR384, whereas gallamine allosterically inhibits alkylation[6−9] (type 2 evidence). Similarly, NMS and atropine competitively protect cerebral cortical muscarinic receptors from alkylation by BR384[6,16] (type 2 evidence).

In this Article, we show that treatment with AChM (15 min) or BR384 (4 min) causes a near complete inhibition of [^{3}H]NMS binding to the wild-type and D2.50N mutants of the M_2 and M_4 receptors as does BR384 treatment of the corresponding D3.32N mutants (Figure 4). Given the brief time of incubation and the concentration of the alkylating agents, the data are consistent with the postulate that populations of the former M_2 and M_4 receptors are potentially sensitive to complete inactivation by AChM and BR384 (type 3 evidence). If the irreversible ligands acted at an allosteric site to alter the affinity of [^{3}H]NMS, then the requisite negative cooperativity would have to be very great to cause a near-complete inhibition of [^{3}H]NMS binding, and our method of estimating K_1 and k_1 would be appropriate nonetheless.

A fourth type of evidence that can be marshaled to support the postulated reduction in the binding capacity is a consistency of the alkylation process with Scheme 1 provided that orthosteric ligands competitively prevent alkylation and complete alkylation is possible under the appropriate conditions (lengthy incubation and receptor saturating concentration of irreversible ligand). We have previously shown that alkylation of the M_1 and M_2 muscarinic receptors is consistent with Scheme 1,[6,7,9] and in this Article we show the same for the M_4 receptor.

However, we found that a good fit of the data in Figure 3 to Scheme 1 requires the assumption that a fraction of the sites that bind [^{3}H]NMS do not react covalently with the irreversible ligands. In the case of our prior work on intact cells, the recycling of intracellular receptors to the plasma membrane after AChM and BR384 treatment can explain the presence of a fraction of the receptors that appear resistant to alkylation. Even in the cellular homogenates there is evidence that the tertiary amine ligand [^{3}H]quinuclidinyl benzilate has access to muscarinic receptors in membrane compartments that [^{3}H]NMS does not.[22] Nonetheless, we would expect that the aziridinium ions of AChM and BR384 would have access to all of the sites labeled by [^{3}H]NMS, and we described such evidence for the M_2 and M_4 receptors in the Results section. As described in the Results section, we suggest that a redistribution of the receptors during the washing process might account for the small proportion of [^{3}H]NMS sites in Figure 3 that exhibit a so-called resistance to alkylation.

Our evidence suggests that AChM and BR384 cause a reduction in the binding capacity of [^{3}H]NMS on the M_1−M_4 receptors (type 1 and 2 evidence), the D2.50 mutants of the M_2 and M_4 receptors (type 3 evidence), and the D3.32N mutant of the M_2 receptor (type 2 evidence). We also have strong evidence that the D3.32N mutants of the M_2 and M_4 receptors undergo a reduction in binding capacity following treatment with BR384 (type 3 evidence). Because we still observe a small-to-moderate inhibitory effect of AChM and BR384 on [^{3}H]NMS binding in the D2.50N/D3.32N mutants, a residue or residues other than D2.50 and D3.32 must be involved in the covalent binding. If the inhibitory effect of AChM and BR384 on [^{3}H]NMS binding in the D2.50N/D3.32N mutants is due to, in part, a change in the affinity of [^{3}H]NMS, then this would imply the alkylation of an allosteric site. Such an effect would not invalidate our conclusions regarding the participation of D3.32 or D2.50 in receptor alkylation of the wild-type receptor (see below). It would also imply that the rate of alkylation of the orthosteric site in the double mutant might very well be essentially 0.

One might expect there to be little difference in the reversible binding properties of acetylcholine and McN-A-343 and their respective aziridinium ions derived from AChM and BR384 because the latter atomic structures differ by only two hydrogens (Figure 1). With regard to acetylcholine and the aziridinium ion of AChM, both had similar affinities for the wild-type M_1 and M_3 receptors, whereas acetylcholine had about 15- and 5-fold higher affinities for the wild-type M_2 and M_4 receptors, respectively. In the case of the aziridinium ion of BR384, it had 4- to 7-fold higher affinity than McN-A-343 for the wild-type M_1−M_4 receptors after taking into consideration that the aziridinium ion only accounts for 54% of the initial amount of BR384.[16] It seems likely that the differences in affinity can be attributed to the smaller bond angles (~60°) in the aziridinium rings of cyclized AChM and BR384 compared

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to those in the tetrahedral structure of the trimethylammonium head groups of acetylcholine and McN-A-343 (bond angles, ~110°). In contrast, there is little difference in the affinity of the aziridinium ion of BM123 and its corresponding stable analog (oxotremorine-M) for rat cerebral cortical muscarinic receptors.[19]

Our competitive binding data indicate that McN-A-343 has similar affinities for the M_1−M_4 muscarinic receptors (Figure 9 and Supporting Information, Table S5). These data are consistent with the idea that the functional selectivity of McN-A-343 for the M_1 and M_4 muscarinic receptors[10] is based on its ability to activate these receptors subtypes selectively. It has been demonstrated that relative to carbachol, McN-A-343 exhibits a higher affinity for the active state of the M_1 and M_4 receptors relative to those of the M_2 and M_3 receptors.[11] The combination of the equivalent observed affinities for the muscarinic receptor subtypes and the selectivity for the active states of the M_1 and M_4 receptors implies that McN-A-343 has a higher efficacy for the M_1 and M_4 receptors.

Using a peptide mapping strategy, Spalding et al.[5] and Curtis et al.[25] showed that the aziridinium ions of AChM and the muscarinic antagonist benzilylcholine mustard bind covalently with D3.32 of the M_1 muscarinic receptor. In the crystal structures of the M_2 and M_3 muscarinic receptors bound with the antagonists 3-quinuclidinyl benzilate and tiotropium, respectively, the basic amino group of each antagonist is coordinated with D3.32.[26,27] Given the large reduction in the alkylation rate constant of AChM caused by the D3.32N mutation of the M_1−M_4 receptors, it seems likely that AChM primarily alkylates D3.32N. A slower alkylation process occurs at high concentrations of AChM, which presumably represents an interaction with another residue.

In the crystal structures of M_2 and M_3 receptors, D2.50 is located two helical turns beneath D3.32, which is far from the orthosteric binding site (Figure 2).[26,27] This residue is highly conserved among GPCRs, and its mutation to alanine causes a large reduction in receptor expression and ligand binding affinity to M_1 muscarinic receptors.[28] D2.50 is thought to maintain the receptor structure by undergoing hydrogen bonding with adjacent asparagine residues in helices 1 (N1.50) and 7 (N7.49).[26−28] In the crystal structure of the inactive state of the human A_{2A} adenosine receptor, a sodium ion is coordinated by D2.50, S3.39, and three water molecules within a central cluster of 10 ordered water molecules.[29] S3.39 is conserved across all muscarinic subtypes (human M_2 S110 and rat M_3 S154), and sodium is known to reduce agonist affinity and increase antagonist affinity for the M_2 muscarinic receptor.[30] These results are consistent with the stabilization of the inactive structure of the A_{2A} adenosine receptor by sodium.

The hydrogen donating and accepting functions of D2.50 should be maintained with asparagine. Accordingly, we observed little-to-no loss in the binding affinities of the ligands for the D2.50N mutants, and usually a moderate increase in affinity was observed. In a study on the human M_2 muscarinic receptor, Vogel et al.[31] observed a large reduction in ligand affinity with the D2.50N mutation when binding was measured in hypotonic Na/Hepes (20 mM) containing $MgCl_2$ (10 mM). These investigators also observed a large decrease in receptor signaling by M_2 D2.50N.

If two residues are located within the binding pocket of a receptor, then an additive contribution to the Gibbs free energy of binding is expected. If a residue is located far from the binding pocket, then any effect on the affinity can be attributed to a change in the conformation of the receptor. This change could alter how a given residue in the binding pocket interacts with the ligand and therefore how mutations of such a residue alter ligand affinity. Therefore, we expected that the D2.50N mutation would alter how the D3.32N mutation affected the interaction of AChM and BR384 with the orthosteric binding site of the muscarinic receptors.

The change in the Gibbs free energy of binding is proportional to the log dissociation constant. Consequently, we estimated the $\Delta \log K_I$ values of AChM and BR384 that are associated with the different mutations. The D2.50N and D3.32N mutations caused small and large increases in the affinity of AChM for the M_2 and M_3 receptors, respectively, whereas the D2.50N/D3.32N double mutation caused a large reduction in the affinity, indicating a strong synergistic effect of the mutations on the binding affinity of AChM. In contrast, the mutations had near-additive effects on the binding affinity of AChM for the M_4 receptor.

The data with BR384 exhibited the opposite pattern. That is, the mutations had additive effects on the binding affinity of BR384 for the M_2 and M_3 receptors but synergistic effects for the M_4 receptor.

With regard to the reversible ligands (i.e., acetylcholine, McN-A-343, and NMS), the mutations always had large synergistic inhibitory effects on affinity except in the case of NMS for the M_3 receptor. With the exception of the latter result, the data are consistent with the postulate that the D2.50N mutation acts at a distance to modify how these ligands interact with the orthosteric binding pocket of the receptor. These results illustrate differences in how acetylcholine and the aziridinium ion of AChM interact with the M_4 receptor and how McN-A-343 and the aziridinium ion of BR384 interact with the M_2 and M_3 receptors.

The Hill slopes of the competitive binding curve for acetylcholine (Supporting Information, Table S5) were substantially less than 1 for the M_2 wild-type (0.69), M_2 D103N (0.60), and M_3 D114N/D148N (0.56) receptors. This behavior most likely represents the contribution of at least two receptor populations exhibiting a difference in affinity for acetylcholine because of differences in their coupling with G proteins. In these instances, we interpret $\log K_i$ as a weighted average value of the K_i values of the different receptor populations; hence, we interpret $\Delta \log K_i$ as being proportional to the weighted average change in the Gibbs free energy of binding for the different populations.

The rate constant for the alkylation of the wild-type M_1−M_4 muscarinic receptors by AChM was greatly reduced to values equal to or less than $^1/_{10}$ that of the wild type by the introduction of the D3.32N mutation, suggesting that this residue participates in the covalent reaction with AChM. In contrast, the D2.50N mutation only reduced the k_1 value of AChM for alkylation of the M_3 receptor to $^4/_5$ that of the wild type and for alkylation of the M_2 and M_4 receptors to values $^3/_5$ and $^1/_3$ of those for the corresponding wild-type receptor, respectively. However, in the double mutants of the M_1 and M_3 receptors, the D2.50N mutation increased the k_1 value of AChM 3-fold relative to that observed in the D3.32 mutant. This result suggests that in the M_1 D71N/D105N and M_3 D114N/D148N mutants, the D2.50N mutation alters the conformation of the double mutant to increase the rate of alkylation of a residue other than D3.32. There was no significant difference between the k_1 values of AChM for the M_2 D103N and M_2 D69N/D103N receptors. With regard to the

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M_4 receptor, the inhibitory effects of the two mutations on the k_1 value of AChM were additive.

The D3.32N mutation caused a large reduction (≥90%) in the rate constant for the alkylation of the M_1−M_4 subtypes, which is consistent with the idea that AChM alkylates the free carboxyl group of D3.32 in the wild-type receptor. However, this mutation does not completely prevent receptor alkylation, and the D2.50N mutation appears to increase receptor alkylation of another residue by AChM in the D2.50N/D3.32N mutants of the M_1 and M_3 receptors. Perhaps the D2.50N mutation reduces the alkylation of the same residue in the D2.50N/D3.32N mutant of the M_4 receptor.

In the case of BR384, the D3.32N mutation caused no significant reduction in the rate constant for receptor alkylation. Although this might suggest that BR384 does not alkylate D3.32 and hence the orthosteric site of the M_1−M_4 receptors, we found that the irreversible alkylation of the M_1 and M_2 receptors was competitively antagonized by NMS and allosterically inhibited by the allosteric modulator gallamine.[6,9] We observed the same for the M_2 D3.32N mutant,[8] suggesting that BR384 can alkylate a residue in the orthosteric binding pocket of the M_1 and M_2 receptors other than D3.32. Although the D3.32N mutation lacked an inhibitory effect on the rate constant for alkylation, it did reduce the affinity of the aziridinium ion to about $^1/_{20}$ of that for the wild-type M_1−M_3 receptors and to about $^1/_4$ of that for the wild-type M_4 receptor. The large effect on M_1−M_3 receptors suggests an important role of D3.32 in the reversible binding of BR384 to these receptors.

For the M_1, M_2, and M_4 receptors, BR384 alkylated the double mutant (D2.50N/D3.32N) at a much slower rate than the wild type, and the rate constant for the alkylation of the D2.50N/D3.32N mutant was substantially and significantly smaller than those of the wild type, D2.50N, and D3.32N. With regard to the binding affinity of BR384, the effects of the D2.50N and D3.32N mutations were additive for the M_2 and M_3 receptors and synergistic for the M_4. None of the mutations had large effects on the rate constant for the alkylation of the M_3 receptor by BR384, although the effect of the D2.50N/D3.32N mutation, which reduced k_1 to about $^3/_5$ of wild type, was significant.

One interpretation of the data for the M_1, M_2, and M_4 receptors is that the remote D2.50N mutation alters the orientation of the aziridinium ion of BR384 with D3.32 to increase its alkylation of this residue while interfering with its alkylation of other nucleophiles. This hypothesis would explain the lack of substantial effects of the single mutations on the rate constant for the alkylation of the M_1, M_2, and M_4 receptors despite the large inhibitory effects of the double mutation on the values of k_1 (77, 95, and 80% inhibition, respectively).

Another more speculative possibility is based on the crystal structures of the M_2 and M_3 muscarinic receptors, which show that the aqueous binding pocket extends down to a level one helical turn below D2.50.[26,27] Perhaps BR384 is capable of alkylating either D3.32 or D2.50 in the wild-type M_1, M_2, and M_4 receptors, and that it is necessary to mutate both residues to cause a substantial reduction in the rate constant for alkylation. This interpretation is consistent with the additive contribution of D2.50 and D3.32 to the binding affinity of the aziridinium ion for the M_2 receptor, although the latter observation does not prove that BR384 interacts with either residue. Perhaps the additive effects of the mutations on the affinity of AChM for the M_4 receptor might be explained by its ability to alkylate single receptors at either D2.50 or D3.32.

BR384 probably alkylates more than one residue on the M_1−M_4 muscarinic receptors. Prior experiments on the M_1 and M_2 receptors mentioned above indicate that BR384 alkylates the orthosteric binding pocket. The large decrease in the rate constant for alkylation in the D2.50N/D3.32N mutant of the M_1, M_2, and M_4 receptors suggests that D3.32 is alkylated by BR384. The D2.50N mutation may reduce the rate of alkylation of a non-D3.32 residue or perhaps it may prevent the alkylation of D2.50. Although it seems unlikely that more than one residue is alkylated in the orthosteric binding pocket of a given receptor, within a population of receptors different receptors may be alkylated on different residues.

Moderate-to-high concentrations of McN-A-343 and other orthosteric ligands inhibit the dissociation of [^{3}H]NMS from the M_2 muscarinic receptor, suggesting that McN-A-343 interacts with an allosteric site.[32,33] Other kinetic and mutagenesis studies implicate a peripheral docking site on the muscarinic receptors to which orthosteric ligands bind before shuttling to the primary activation site.[34,35] Recent modeling studies[26] based on the crystal structure of the M_2 receptor are consistent with the prior suggestion by Hulme and coworkers[34] that W157 in the M_1 receptor (W155 in the M_2) is part of a docking site. It has been argued that it is difficult to explain the allosteric effect of gallamine on the M_2 muscarinic receptor-mediated inhibition of adenylate cyclase when assuming a single binding site for orthosteric ligands; however, this is not the case if the allosteric modulation of a docking site is considered.[36] Thus, the inhibitory effect of McN-A-343 on the kinetics of [^{3}H]NMS binding might also be attributed to the occupancy of a docking site.

A provocative study investigating hemiligands of McN-A-343 is consistent with the postulate that McN-A-343 interacts simultaneously with allosteric and orthosteric sites[37] causing competition between McN-A-343 and orthosteric ligands.

Given the structural resemblance of BR384 and McN-A-343, the studies mentioned in the prior two paragraphs suggest that BR384 might also alkylate the allosteric site of the muscarinic receptor. Our studies do not rule out this possibility. However, with regard to the irreversible inhibitory effect of BR384 on [^{3}H]NMS binding, our prior results on the M_1 and M_2 muscarinic receptors indicate that this effect is attributed to the alkylation of the orthosteric site.[6−9]

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Irreversible binding of BR384 to homogenates of rat cerebral cortex; estimates of the dissociation constants (K_1) and alkylation rate constants (k_1) for the interaction of AChM and BR384 with M_1−M_4 muscarinic receptors and their D3.32N, D2.50N, and D2.50N/D3.32N mutants; summary of the post hoc comparisons among the former estimates for AChM and BR384, estimates of the dissociation constants of NMS, acetylcholine, and McN-A-343 for M_1−M_4 muscarinic receptors and their D3.32N, D2.50N, and D2.50N/D3.32N mutants; and summary of the post hoc comparisons among the former estimates for NMS, acetylcholine, and McN-A-343. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

AChM, acetylcholine mustard; BR384, 4-[(2-bromoethyl)-methyl-amino]-2-butynyl *N*-(3-chlorophenyl)carbamate; CHO, Chinese hamster ovary; HEK, human embryonic kidney; McN-A-343, [4-[[*N*-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl] trimethylammonium chloride; NMS, *N*-methylscopolamine

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