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Effects of asparagine mutagenesis of conserved aspartic acids in helix two (D2.50) and three (D3.32) of M₁ – M₄ muscarinic receptors on the irreversible binding of nitrogen mustard analogs of acetylcholine and McN-A-343

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Abstract

We investigated how asparagine mutagenesis of conserved aspartic acids in helix two (D2.50) and three (D3.32) of M₁ – M₄ 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 M₂ – M₄ 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 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 M₁ and M₃ receptors, but not that of the M₄ receptor. BR384 exhibited the converse relationship. The simplest explanation is that acetylcholine mustard and BR384 alkylate at least two residues on M₁ – M₄ 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 on 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

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Supporting Information

Irreversible binding of BR384 to homogenates of rat cerebral cortex depicted in Figure S1, estimates of the dissociation constants (K_D) and alkylation rate constants (k_I) for the interaction of AChM (Table S1) and BR384 (Table S3) with M₁ – M₄ muscarinic receptors and their D3.32N, D2.50N and D2.50N/D3.32N mutants, summary of post hoc comparisons among the former estimates for AChM (Table S2) and BR384 (Table S4), estimates of the dissociation constants of NMS, acetylcholine and McN-A-343 for M₁ – M₄ muscarinic receptors and their D3.32N, D2.50N and D2.50N/D3.32N mutants (Table S5), and summary of post hoc comparisons among the former estimates for NMS (Table S6) and acetylcholine and McN-A-343 (Table S7). This information may be accessed free of charge at <http://pubs.acs.org>.

irreversibly with a highly conserved aspartic acid residue (D3.32, nomenclature scheme of Ballesteros and Weinstein (4)) in helix three 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. After stopping the reaction and washing the receptor preparation, residual unalkylated receptors are estimated using a suitable radioligand, like [³H]N-methylscopolamine ([³H]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 mutation of aspartic acid 3.32 to asparagine in M₁ and M₂ 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). This mutation does not completely prevent receptor alkylation, however, particularly at high concentrations of AChM. We also found that the D3.32N greatly inhibited the alkylation of M₁ and M₂ 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]tri-methylammonium chloride) (Figure 1). The inhibition was due primarily to a reduction in affinity, however, and not to a decreased rate constant for alkylation, suggesting perhaps that BR384 does not alkylate the D3.32 residue of M₁ and M₂ receptors. Nonetheless, the orthosteric muscarinic antagonist, NMS, competitively inhibited alkylation of wild type M₁, wild type M₂ and the D103N mutant of the M₂ receptor by BR384, whereas the known allosteric modulator, gallamine, allosterically prevented alkylation (6, 8, 9). Thus, BR384 probably alkylates another residue within the orthosteric-binding pocket of the M₂ receptor.

McN-A-343 is an important muscarinic agonist because it exhibits a preference for activating M₁ and M₄ muscarinic receptors relative to the other subtypes (10, 11). Intravenous administration of McN-A-343 elicits a pressor response due to activation of M₁ muscarinic receptors in sympathetic ganglia (12, 13). In contrast, nonselective muscarinic agonists activate the M₃ 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 to its lack of contractile action on the guinea pig ileum *in vitro* (16). The latter response is mediated through the M₃ muscarinic receptor (17). Understanding the mode 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 M₃ and M₄ receptors by AChM and BR384. We have also investigated how mutation of a highly conserved residue in helix 2 (D2.50, Figure 2) affects M₁ – M₄ receptor alkylation, by itself and in combination with D3.32N. Our results are consistent with the postulate that AChM and BR384 alkylate D3.32 in addition to another residue within the binding pockets of M₁ – M₄ muscarinic receptors.

Experimental Procedures

Materials

Reagents were obtained from the following sources: Dulbecco's Modified Eagle Medium with high glucose plus 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 $\text{Na}_2\text{S}_2\text{O}_3$ (Sigma-Aldrich, Inc., St. Louis, MO); salts for phosphate buffer and binding buffer, HCl and NaOH (Thermo Fisher Scientific, Waltham, MA); Zippy 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., 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 M₁, M₂, M₃ and M₄ muscarinic receptor cDNAs, cloned into a modified expression vector (pCD-hM₁, pCD-hM₂, pCD-hM₃, pCD-hM₄), were obtained from Dr. Tom Bonner at the National Institute of Mental Health (Bethesda, MD). Mutations were introduced into pCD-hM₁, pCD-hM₂, pCD-hM₃, and pCD-hM₄ using the QuikChange Lightning Site-Directed Mutagenesis kit (Agilent Technologies) and mutagenesis primers. Sequences of mutant receptors were verified by Laragen, Inc. (Culver City, CA). The mutant plasmids were purified using Zippy Plasmid Miniprep Kit or NucleoBond Xtra Midi Plus kit following the manufacturers' protocols.

Cell culture and transfection

Chinese hamster ovary (CHO) cells stably expressing the human M₁, M₂, M₃ and M₄ muscarinic receptors were obtained from Acadia Pharmaceuticals (San Diego, CA) and 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 manufacturer's protocols. 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 (1247g, 10 min) and the supernatant discarded. The pellet was suspended in binding buffer using a Polytron homogenizer (Kinematica, Littau-Lucerne, Switzerland; setting #4, 10 sec). Homogenates of cells expressing M₁ D71N/D105N, M₂ D69N/D103N, M₃ D114N/D148N, or M₄ D78N/D112N receptors were centrifuged once more at high speed (39,400g, 10 min, 4°C) and suspended in fresh binding buffer.

Homogenate was prepared at varying concentrations depending on the assay and receptor construct so that the receptor concentration in the final binding assay would only result in minimal depletion of the free concentration of [³H]NMS (see below).

Treatment of cellular homogenate with cyclized AChM and BR384

The covalent interactions of AChM and BR384 with muscarinic receptors were investigated by first incubating homogenates of cells expressing muscarinic receptors with the aziridinium ions of AChM and BR384, and then measuring residual muscarinic receptors using the radioligand [³H]NMS as described previously (7).

Cellular homogenate (200 µl) was 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 specific times as described under “Results.” An aliquot (0.75 ml) of stopping solution (see below) was added at the end of the incubation, and the mixture was incubated another 20 min to allow inactivation of the aziridinium ions of AChM or BR384.

The reaction tubes were centrifuged (25,000g, 15 min, 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 a volume of 1 ml of binding buffer. Triplicate measurements of [³H]NMS binding were made on each homogenate as described below.

The reactions were stopped in two ways 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 (see equations 4 and 6) were estimated only from 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 to remove residual scopolamine (8). When the concentration of BR384 was increased to 300 µM, however, 28% of wild type M₁ and M₂ muscarinic receptors were alkylated after adding the stopping solution. In contrast, 3 mM AChM only caused 1% inhibition of [³H]NMS binding in the presence of the stopping solution. Thus, measurements of [³H]NMS binding after treatment with 300 µM BR384 were multiplied by a factor (1.39; i.e., 1/(1 – 0.28)) to correct for this continued alkylation after addition of scopolamine and thiosulfate.

[³H]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, [³H]NMS (specific activity, 82 Ci/mmol; PerkinElmer Life and Analytical Sciences, Waltham, MA).

For those experiments involving M₁ D71N/D105N, M₂ D69N/D103N, M₃ D114N/D148N, or M₄ D78N/D112N receptors, a centrifugation assay was used to measure [³H]NMS binding (8) because these mutants exhibited low affinity for [³H]NMS (pK_D, 7.7 – 8.0). We were concerned that [³H]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 [³H]NMS (3.0 nM). The equilibration period was stopped by centrifugation (30,000g, 20 min, 4°C). The supernatant was aspirated and the residual pellet 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 addition of scintillation cocktail (Budget-Solve; Research Products International Corp), radioactivity was measured using a liquid scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA). Nonspecific binding was defined as the residual binding in the presence of 10 µM atropine. All measurements were done in triplicate.

For the experiments involving the other receptor constructs, a filtration assay was used to measure [³H]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 [³H]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 three ml per wash). The filters were placed in scintillation vials, and radioactivity was measured using liquid scintillation spectroscopy as described above.

Ligand/[³H]NMS competition experiments

The competitive inhibition of [³H]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 done as described above except that fresh cellular homogenate was used, and the assay included various concentrations of the nonlabeled competitors.

Analysis of data

The IC_{50} values were estimated by nonlinear regression analysis of the ligand/[³H]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 [³H]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 [³H]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).

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

in which, $[{}^3H]NMS$ denotes the free concentration of [³H]NMS at the IC_{50} point of the competition curve, and K_{NMS} , the dissociation constant of [³H]NMS (units of molarity, M).

The latter was estimated by rearrangement of equation 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/[^3H]NMS competition curves, the Hill coefficient in equation 1 was constrained to one. The concentration of [^3H]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 just described.

For a given experiment, the competitive inhibition of [^3H]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 and SEM of these estimates.

The basis for the estimation of the dissociation constant (K_1) and rate constant for alkylation (k_1) of AChM and BR384 is described by Suga et al. (7). 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 that of the alkylation step (k_1) (see scheme 1).

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 [^3H]NMS at a single concentration (1 or 3 nM).

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

$$\frac{Y_t}{Y_0} = (1 - b)e^{-Ok_1t} + b \quad (4)$$

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

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

in which k_1 , denotes the dissociation constant of the aziridinium ion for the receptor (molar units), and X , the concentration of the aziridinium ion.

A different equation was used to analyze 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}) based on the values of the microscopic constants for formation and decay of the aziridinium ion estimated by Ringdahl et al. (16).

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 [^3H]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 and SEM of these values. We noted 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 \pm SEM values for $\log k_1$, and Figure 7 illustrates these values as well.

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) ($\Delta \log K_{\text{mutant}}$) was estimated using the following equation:

$$\Delta \log K_{\text{mutant}} = \log K_{\text{mutant}} - \log K_{\text{WT}} \quad (7)$$

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

The significance of differences in the estimates of a given parameter among 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 M₄ muscarinic receptor by AChM and BR384

We measured the kinetics of alkylation of the M₄ muscarinic receptor at various concentrations of AChM and BR384 (Figure 3). Homogenates of CHO cells expressing the wild type M₄ 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 [^3H]NMS was measured to estimate residual unalkylated receptors. Figure 3a shows that following treatment with AChM, there is a concentration-dependent loss of [^3H]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 M₄ receptor to form a reversible complex that converts to a covalent complex at a slower rate (Scheme 1). To test this model, we fitted equation 4 to the data to obtain an estimate of the affinity constant of the aziridinium ion of AChM for the M₄ receptor ($\log K_1 = -4.10 \pm .058$), its rate constant for alkylation ($k_1 = 0.44 \pm .038 \text{ min}^{-1}$), and the estimate of the proportion of receptors that can bind [^3H]NMS but not be alkylated by AChM ($b = 17 \pm 2.0\%$). Regression analysis yielded a good fit of equation 4 to the data.

Similar behavior was observed in experiments with BR384 as shown in Figure 3b.

Regression analysis of these data was done using equation 6 to account for the decay in the aziridinium ion of BR384, which is less stable than that of AChM. This analysis yielded estimates of the dissociation constant of aziridinium ion of BR384 for the M₄ receptor ($\log K_1 = -5.05 \pm 0.072$) and its rate constant for alkylation ($k_1 = 0.93 \pm .020 \text{ min}^{-1}$). These estimates of K_1 and k_1 are approximately ten- and two-fold greater, respectively, than those of AChM. The estimate of the proportion of receptors that can bind [³H]NMS but not be alkylated by BR384 was $7.7 \pm 1.8\%$.

Effects of various concentrations of AChM and BR384 on wild type and D2.50N and D3.32N mutants of M₂ and M₄ muscarinic receptors

The conclusion that a component of the M₄ muscarinic receptor population can bind [³H]NMS but not be alkylated by AChM or BR384 (i.e., b values of 17 and 7.7%, respectively; see equations 4 and 6) seems puzzling because both [³H]NMS and the aziridinium ions of AChM and BR384 should have access to the same pool of receptors in cellular homogenates. For example, the charged aziridinium ions might be unable to penetrate ER vesicles containing a high density of wild type or mutant receptor in cellular homogenates, but so would the quaternary ligand [³H]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 starting concentrations of AChM and BR384, respectively). This species of weak base is expected to penetrate lipid barriers and alkylate any receptors inaccessible to [³H]NMS following cyclization within the putative lipid compartment. Perhaps there is cleavage of the covalent AChM- and BR384-receptor bonds or unalkylated receptors trapped within a membrane compartment may be exposed over time with repetitive washing and trituration of homogenate. The rate of recovery of [³H]NMS binding over four hours is negligible in cerebral cortical homogenates having about 85% of their muscarinic receptors alkylated with BR384 indicating that the binding of BR384 to cerebral muscarinic receptors is nearly irreversible over four hours (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 M₂ and M₄ muscarinic receptors for 15 (AChM) and 4 min (BR384), and the reaction was stopped with thiosulfate only and washed once as described under “Experimental Procedures” (Figure 4).

With regard to wild type and the D2.50N mutants of M₂ and M₄ receptors, the data suggest that all of the [³H]NMS binding sites are capable of being alkylated by AChM (Figure 4a and c) and BR384 (Figure 4b and d). The same also applies to the effect of BR384 on the D3.32N mutants (Figure 4b and 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 and c). The same is true with regard to the alkylation of the D2.50N/D3.32N mutant by BR384 (Figure 4b and d). The data also show that the D2.50N mutation increases receptor alkylation, whereas the D3.32N mutation has the opposite effect (Figure 4a, b, c and 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 M₂ receptor (Figure 4a) and to BR384 at both M₂ and M₄ receptors (Figure 4b and d).

To obtain further evidence for the mutually exclusive binding of [³H]NMS and the aziridinium ions of AChM and BR384, we measured the competitive displacement of [³H]NMS binding to the slowly alkylated D2.50N/D3.32N mutants of M₂ and M₄ receptors under conditions where the rate of alkylation of the receptor is negligible or greatly reduced

(60 min at 0°C). Under this condition, the highest concentration AChM tested (3 mM) caused $14 \pm 0.5\%$ and $20 \pm 2.2\%$ alkylation of the double mutants of M₂ and M₄ receptors (Figure 5a and b). The corresponding values for BR384 (0.3 mM) were $48 \pm 4.2\%$ and $44 \pm 0.6\%$, respectively. In the competitive displacement assay, the highest concentration of AChM tested (3 mM) caused $73 \pm 1.7\%$ and $74 \pm 0.4\%$ inhibition of [³H]NMS binding in the double mutants of M₂ and M₄ receptors, with no evidence of a non-zero plateau in the inhibition curve (Figure 5c and d). The corresponding values for BR384 (0.3 mM) were $97 \pm 1.3\%$ and $88 \pm 0.7\%$ 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 near complete displacement of [³H]NMS binding at the highest concentrations of AChM and BR384 used, except for BR384 at M₂ D69N/D103N. The log molar IC_{50} values of AChM at the D2.50N/D3.32N mutants of M₂ and M₄ 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 [³H]NMS in the D2.50N/D3.32N mutants of M₂ and M₄ receptors.

Single time-point kinetic assay for estimation of the affinities and rate constants of AChM and BR384

Data like 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 [³H]NMS binding sites insensitive to the alkylating agent is known.

For example, we analyzed each inhibition curve in Figure 3 by regression analysis using equations 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 $0.36 \pm 0.02 \text{ min}^{-1}$, 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 $0.74 \pm 0.086 \text{ min}^{-1}$) 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 fit of equations 4 (AChM) and 6 (BR384) as long as b is constrained to a value 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 k_1/K_1 is constant. This relationship occurs because incomplete receptor alkylation at 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 M₁ – M₄ muscarinic receptors

We applied the single time-point assay just described to investigate the kinetics of alkylation of M₁ – M₄ muscarinic receptors by AChM and BR384 (Figure 6). In these experiments, we stopped the alkylation step quickly with scopolamine and thiosulfate and washed cellular homogenates three times before measuring 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, and hence, the appearance of new 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 zero, given the length of the incubation of receptor with AChM (15 min) and BR384 (6 min).

Figure 6 shows our data on M₁ (*a* and *b*), M₂ (*c* and *d*), M₃ (*e* and *f*) and M₄ (*g* and *h*) receptors with regard to wild type M₃, the D2.50N mutant of M₂ – M₄ receptors, the D3.32N mutant of M₃ and M₄ receptors and the D2.50N/D3.32N mutant of M₁ – M₄ receptors. Data on the wild type M₄ receptor are only shown in Figure 4, and data for the wild type and D3.32N mutants of M₁ and M₂ receptors are also not shown in Figure 6, but have been published previously (6–9). The data in Figure 6 were analyzed as described above with the parameter b in equations 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_I (*a* and *c*) and k_I (*b* and *d*) for AChM and BR384. Also shown are the parameter estimates for the wild type M₄ receptor from the data in Figure 3 and those for wild type and D3.32N mutants of M₁ and M₂ receptors from our prior studies (6–9). We did not investigate the D2.50N mutant of the M₁ receptor.

With regard to AChM (Figure 7*a* and *b*), the D3.32N mutation (M₁ D105N, M₂ D103N, M₃ D148N and M₄ D112N) reduced the value of the rate constant for alkylation to only one-fiftieth to one-tenth that of the corresponding wild type receptor and had little effect on the affinity of AChM for M₁ – M₃ receptors. In the case of the M₄ receptor, the affinity for AChM was reduced to one-tenth that of wild type.

In contrast, the D2.50N mutation had smaller effects on the rate constant for receptor alkylation by AChM (one-third to three-fourths that of wild type), but increased affinity 8- to 17-fold relative to wild type M₂ – M₄ receptors. The combination of both mutations (D2.50N/D3.32N) reduced the rate constant (one-thirtieth to one-third that of wild type) and affinities (one five-hundredth to one-thirtieth that of wild type) of AChM for M₂ – M₄ receptors. The rate constants for alkylation of double mutant of M₁ and M₃ receptors was greater than those of the corresponding D3.32N mutants, whereas the rate constant for alkylation of M₄ D78N/D112N was smaller than that of M₄ D112N.

These mutations had qualitatively similar effects on the interaction of BR384 with muscarinic receptors (Figure 7*c* and *d*). The D3.32N mutation had little or no inhibitory effect on the rate constant for alkylation, but reduced affinity to only about one-twentieth (M₁ – M₃) and one-fourth (M₄) that of wild type. In contrast, the D2.50N mutation increased affinity about 30-, 5-and 30-fold at M₂ – M₄ receptors while having a modest effect on the rate constant for alkylation of the M₃ receptor. This mutation also reduced the rate constants for alkylation of M₂ and M₄ receptors to values about one-half that of wild type. The combination of both mutations (D2.50N/D3.32N) reduced the rate constant for alkylation to values about one-twentieth (M₂), one-fourth (M₁), one-third (M₄) and one-half (M₃) that of wild type. The associated changes in affinity represented both increases of two-fold (M₁ and M₂) and decreases to about one-fifth of wild type (M₃ and M₄).

The numerical values for the estimates of log K_I and k_I for AChM and BR384 are listed in Tables S1 and S3 of the Supporting Information. Tables S2 and S4 of the Supporting Information summarize post hoc comparisons of the former data, respectively. Analysis of variance showed that the mutations had significant effects on the estimates of log K_I and log k_I for AChM and BR384 at each receptor subtype (Supporting Information, Tables S1 and S3). Post hoc comparisons showed a significant effect of each mutation on the log k_I value for AChM at each receptor subtype. For BR384, all of the mutations had a significant effect

on $\log k_I$ except the D105N mutation of the M₁ receptor, the D103N mutation of the M₂ receptor, the D114N and D148N mutations of the M₃ receptor, and the D112N mutation of the M₄ receptor. With regard to the $\log K_I$ estimates for AChM, all of the mutations had significant effects except the D105N mutation of the M₁ receptor and the D103N and D69N mutations of the M₂ receptor. Finally, with regard to the $\log K_I$ estimates for BR384, the mutations had significant effects except for the D71N/D105N and D69N/D103N mutants of M₁ and M₂ receptors, respectively. Tables S2 and S4 of the Supporting information also list the results of all other possible comparisons among a given receptor subtype.

Because the parameter estimates depend on the value to which b is constrained in equations 4 and 6, we searched parameter space to identify the maximum value of b that would still yield a non-significant increase in the residual sum of squares. This maximum estimate of b was lowest for alkylation of M₃ (0.067) and M₄ (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 M₃ and M₄ receptor constructs when expressed 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_I to K_I is more accurate than either single parameter and independent of the value of b over the range of 0 to a low value (e.g., 10%). The value of $\log k_I/K_I$ represents the combined effect of the mutation on affinity and the rate constant for alkylation. Figure 8 shows a plot of the estimates of the $\log k_I/K_I$ values of AChM and BR384 for wild type M₁, M₂, M₃ and M₄ 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 $\log k_I/K_I$ was significantly different ($P < 0.05$) for every comparison among the various constructs of a given receptor subtype, except for M₁ wild type vs M₁ D2.50N/D3.32N (AChM, $P = 0.062$; BR384, $P = 0.26$) and comparisons between of D3.32N and D2.50N/D3.32N with regard to AChM at M₃ ($P = 0.051$) and M₄ ($P = 0.25$) receptors and BR384 at the M₂ ($P = 0.69$) 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 $\log K_I$ value relative to wild type ($\Delta \log K_I$). These are listed in Table 1 for the single (D2.50N and D3.32N) and double (D2.50N/D3.32N) point mutations of M₂ – M₄ receptors. Also listed is the sum of the $\Delta \log K_I$ values for the two single mutants (D2.50N + D3.32N). At M₂ and M₃ receptors, AChM exhibited a significant increase in the $\Delta \log K_I$ value of the double mutant compared to the sum of the $\Delta \log K_I$ values of the single mutants, indicating an interaction between the mutations. At the M₄ receptor, the sum of the $\Delta \log K_I$ values of AChM for the single mutants was approximately equal to the $\Delta \log K_I$ 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 M₂ and M₃ receptors, but there was for the M₄ receptor.

Interaction of acetylcholine, McN-A-343 and NMS with wild type and D2.50N and D3.32N mutants of M₁ – M₄ 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 [³H]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 [³H]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 one at each receptor mutant, indicating that the K_i value is a good estimate of the dissociation constant (K_I) of the compound. With regard to acetylcholine, its competition curves at M₂ wild type, M₂ D103N and M₃ D114N/D148N receptors had Hill slopes that were substantially less than one (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 M₂ receptor is that a fraction of the receptor population interacts with G_{i/o}, resulting in 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_I).

In most instances, the effects of the mutations on ligand affinity are qualitatively similar to those observed with the corresponding aziridinium ions (compare Figures 7 and 9). Two striking exceptions are the effects of the D3.32N mutation on M₂ and M₃ receptors. This mutation reduced the affinities of acetylcholine to values only one-fiftieth and one-thirtieth those of the wild type receptors, respectively, but had little effect on the aziridinium ion of AChM. This mutation clearly altered how the aziridinium ion interacted with M₂ and M₃ receptors, however, 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 M₃ and M₄ receptors and an increase in affinity for M₁ and M₂ receptors.

Analysis of variance showed that all of the mutations had significant effects on the affinities of NMS, acetylcholine and McN-A-343 for M₁ – M₄ 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 M₁ – M₄ receptors relative to wild type except for the affinities of acetylcholine and NMS for the M₂ 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 $\Delta \log K_i$ 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 at the M₃ receptor, the increase in the $\Delta \log K_i$ value associated with the double mutation (D2.50N/D3.32N) was always significantly greater than the sum of the $\Delta \log K_i$ values of the two single mutations (D2.50N + D3.32N).

Discussion

Our method of estimating the dissociation (K_I) and rate (k_I) constants is based on the assumption that AChM and BR384 reduce the binding capacity of [³H]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 [³H]NMS: 1) direct demonstration that AChM or BR384 treatment reduces the binding capacity of [³H]NMS without affecting affinity for the residual receptors, 2) competitive and allosteric protection from the inhibitory effects of AChM and BR384 by known orthosteric and allosteric ligands, respectively, and 3) complete inhibition of [³H]NMS binding by AChM or BR384.

With regard to the first type of evidence, we have previously shown that prior treatment of M₁ and M₂ receptors with AChM and BR384 causes a reduction in the binding capacity of [³H]NMS without affecting 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 [³H]quinuclidinyl benzilate and [³H]NMS without affecting for the residual receptors affinity (16). These brain regions are abundant in M₁, M₂ and M₄ muscarinic receptors (20–24).

We have also shown that orthosteric ligands, like acetylcholine, NMS and McN-A-343, competitively protect M₁, M₂ and M₂D103N 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 report, we show that treatment with AChM (15 min) or BR384 (4 min) causes a near complete inhibition of [³H]NMS binding to wild type and D2.50N mutants of M₃ and M₄ receptors as well as 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 M₃ and M₄ receptor 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 [³H]NMS, then the requisite negative cooperativity would have to be very great to cause near complete inhibition of [³H]NMS binding and our method of estimating K_I and k_I would be appropriate, nonetheless.

A fourth type of evidence that can be marshaled to support the postulated reduction in 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 M₁ and M₂ muscarinic receptors is consistent with Scheme 1 (6, 7, 9), and in this report, we show the same for the M₄ receptor.

We found, however, that a good fit of the data in Figure 3 to Scheme 1 requires the assumption that a fraction of the sites that bind [³H]NMS do not react covalently with the irreversible ligands. In the case of our prior work on intact cells, recycling of intracellular receptors to the plasma membrane after AChM and BR384 treatment can explain the presence of a fraction of receptors that appear resistant to alkylation. Even in cellular homogenates, there is evidence that the tertiary amine ligand, [³H]quinuclidinyl benzilate, has access to muscarinic receptors in membrane compartments that [³H]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 [³H]NMS, and we described such evidence for M₂ and M₄ receptors under “Results”. As described under “Results”, we suggest that redistribution of receptors during the washing process might account for the small proportion of [³H]NMS sites in Figure 3 that exhibit a so-called resistance to alkylation.

To summarize, our evidence suggests that AChM and BR384 cause a reduction in the binding capacity of [³H]NMS at M₁ – M₄ receptors (Type 1 and 2 evidence), the D2.50 mutants of M₃ and M₄ receptors (Type 3 evidence), and the D3.32N mutant of the M₂ receptor (Type 2 evidence). We also have strong evidence that the D3.32N mutants of M₃ and M₄ 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 [³H]NMS binding in the D2.50N/D3.32N mutants, then 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 [³H]NMS binding in the D2.50N/D3.32N mutants is due, in part, to a change in affinity of [³H]NMS, then this would imply 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 zero.

One might expect 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 (see Figure 1). With regard to acetylcholine and the aziridinium ion of AChM, both had similar affinities for wild type M₁ and M₃ receptors, whereas acetylcholine had about 15- and five-fold higher affinity at wild type M₂ and M₄ receptors, respectively. In the case of the aziridinium ion of BR384, it had four- to seven-fold higher affinity than McN-A-343 at wild type M₁ – M₄ receptors after taking into account 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 to 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-A343 has similar affinities for M₁ through M₄ 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 M₁ and M₄ 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 higher affinity for the active state of M₁ and M₄ receptors relative to those of M₂ and M₃ (11). The combination of equivalent observed affinity for muscarinic receptor subtypes and selectivity for the active states of M₁ and M₄ receptors implies that McN-A-343 has higher efficacy for M₁ and M₄ 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, benzylcholine mustard, bind covalently with D3.32 in the M₁ muscarinic receptor. In the crystal structures of the M₂ and M₃ 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 M₁ – M₄ 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 M₂ and M₃ receptors, D2.50 is located two helical turns beneath D3.32, far from the orthosteric-binding site (see 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 M₁ muscarinic receptors (28). D2.50 is thought to maintain receptor structure by undergoing hydrogen bonding with adjacent asparagine residues in helix 1 (N1.50) and helix 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 central cluster of 10 ordered water molecules (29). S3.39 is conserved across all muscarinic subtypes (human M₂, S110 and rat M₃, S154), and sodium is known to reduce agonist affinity and increase antagonist affinity at the M₂

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 or 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 M₂ 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 MgCl₂ (10 mM). These investigators also observed a large decrease in receptor signaling by M₂ 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 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 thus, how mutations of such a residue alter ligand affinity. We expected, therefore, 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 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 associated with the different mutations. The D2.50N and D3.32N mutations caused small and large increases in the affinity, respectively, of AChM for M₂ and M₃ receptors, whereas the double mutation, D2.50N/D3.32N caused a large reduction in 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 M₄ receptor.

The data with BR384 exhibited the opposite pattern. That is, the mutations had additive effects on the binding affinity of BR384 at M₂ and M₃ receptors, but synergistic effects at the M₄ 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 at the M₃ 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 M₄ receptor and how McN-A-343 and the aziridinium ion of BR384 interact with M₂ and M₃ receptors.

The Hill slopes of the competitive binding curve for acetylcholine (Supporting Information, Table S5) were substantially less than one for M₂ wild type (0.69), M₂ D103N (0.65) and M₃ 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 coupling with G proteins. In these instances, we interpret the $\log K_I$ as a weighted average value of the K_I values of the different receptor populations, and hence, the $\Delta \log K_I$ as being proportional to the weighted average change in the Gibbs free energy of binding for the different populations.

The rate constant for alkylation of wild type M₁ – M₄ muscarinic receptors by AChM was greatly reduced to values equal to or less than one-tenth that of 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_I value of AChM for

alkylation of the M₃ receptor to four-fifths that of wild type and that for alkylation of M₂ and M₄ receptors to values three-fifths and one-third those of the corresponding wild type receptor, respectively. In the double mutants of M₁ and M₃ receptors, however, the D2.50N mutation increased the k_1 value of AChM three-fold relative to that observed in the D3.32 mutant. This result suggests that in the M₁ D71N/D105N and M₃ 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 at M₂ D103N and M₂ D69N/D103N receptors. With regard to the M₄ receptor, the inhibitory effects of the two mutations on the k_1 value of AChM were additive.

To summarize, the D3.32N mutation caused a large reduction (~90%) in the rate constant for alkylation of the M₁ – M₄ subtypes, consistent with the idea that AChM alkylates the free carboxyl group of D3.32 in the wild type receptor. This mutation does not completely prevent receptor alkylation, however, and the D2.50N mutation appears to increase receptor alkylation of another residue by AChM in the D2.50N/D3.32N mutants of M₁ and M₃ receptors. Perhaps the D2.50N mutation reduces alkylation of the same residue in the D2.50N/D3.32N mutant of the M₄ receptor.

In the case of BR384, the D3.32N mutation caused no significant reduction in the rate constant for receptor alkylation. While this might suggest that BR384 does not alkylate D3.32, and hence, the orthosteric site of M₁ – M₄ receptors, we found that irreversible alkylation of M₁ and M₂ receptors was competitively antagonized by NMS and allosterically inhibited by the allosteric modulator, gallamine (6, 9). We observed the same for the M₂ D3.32N mutant (8), suggesting that BR384 can alkylate a residue in the orthosteric binding pocket of M₁ and M₂ 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 one-twentieth that of wild type M₁ - M₃ receptors and to about one-fourth that of the wild type M₄ receptor. The large effect on M₁ – M₃ receptors suggests an important role of D3.32 in the reversible binding of BR384 to these receptors.

At M₁, M₂ and M₄ receptors, BR384 alkylated the double mutant (D2.50N/D3.32N) at a much slower rate than wild type, and the rate constant for alkylation of the D2.50N/D3.32N mutant was substantially and significantly smaller than those of 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 at M₂ and M₃ receptors and synergistic at the M₄. None of the mutations had large effects on the rate constant for alkylation of the M₃ receptor by BR384 although the effect of the D2.50N/D3.32N mutation (reduced k_1 to about three-fifths of wild type) was significant.

One interpretation of the data at M₁, M₂ and M₄ 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 alkylation of M₁, M₂ and M₄ 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 M₂ and M₃ 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 M₁, M₂ and M₄ 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 at the M₂ 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 M₄ receptor might be explained by its ability to alkylate single receptors on either D2.50 or D3.32.

To sum up, BR384 probably alkylates more than one residue on M₁ – M₄ muscarinic receptors. Prior experiments on M₁ and M₂ 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 M₁, M₂ and M₄ 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 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 [³H]NMS from the M₂ 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 muscarinic receptors to which orthosteric ligands bind before shuttling to the primary activation site (34, 35). Recent modeling studies based on the crystal structure of the M₂ receptor are consistent with the prior suggestion by Hulme and coworkers that W157 in the M₁ receptor (W155 in the M₂) is part of a docking site (26). It has been argued that it is difficult to explain the allosteric effect of gallamine on M₂ muscarinic receptor-mediated inhibition of adenylate cyclase assuming a single binding site for orthosteric ligands, but not if allosteric modulation of a docking site is considered (36). Thus, the inhibitory effect of McN-A-343 on the kinetics of [³H]NMS binding might also be attributed to occupancy of a docking site.

A provocative study investigating hemi-ligands 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. With regard to the irreversible inhibitory effect of BR384 on [³H]NMS binding, however, our prior results on M₁ and M₂ muscarinic receptors indicate that this effect is attributed to alkylation of the orthosteric site (6–9).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AChM	acetylcholine mustard
BR384	4-[<i>(2</i> -bromoethyl)methyl-amino]-2-butynyl <i>N</i> -(3-chlorophenyl)carbamate
CHO	Chinese hamster ovary
HEK	Human embryonic kidney

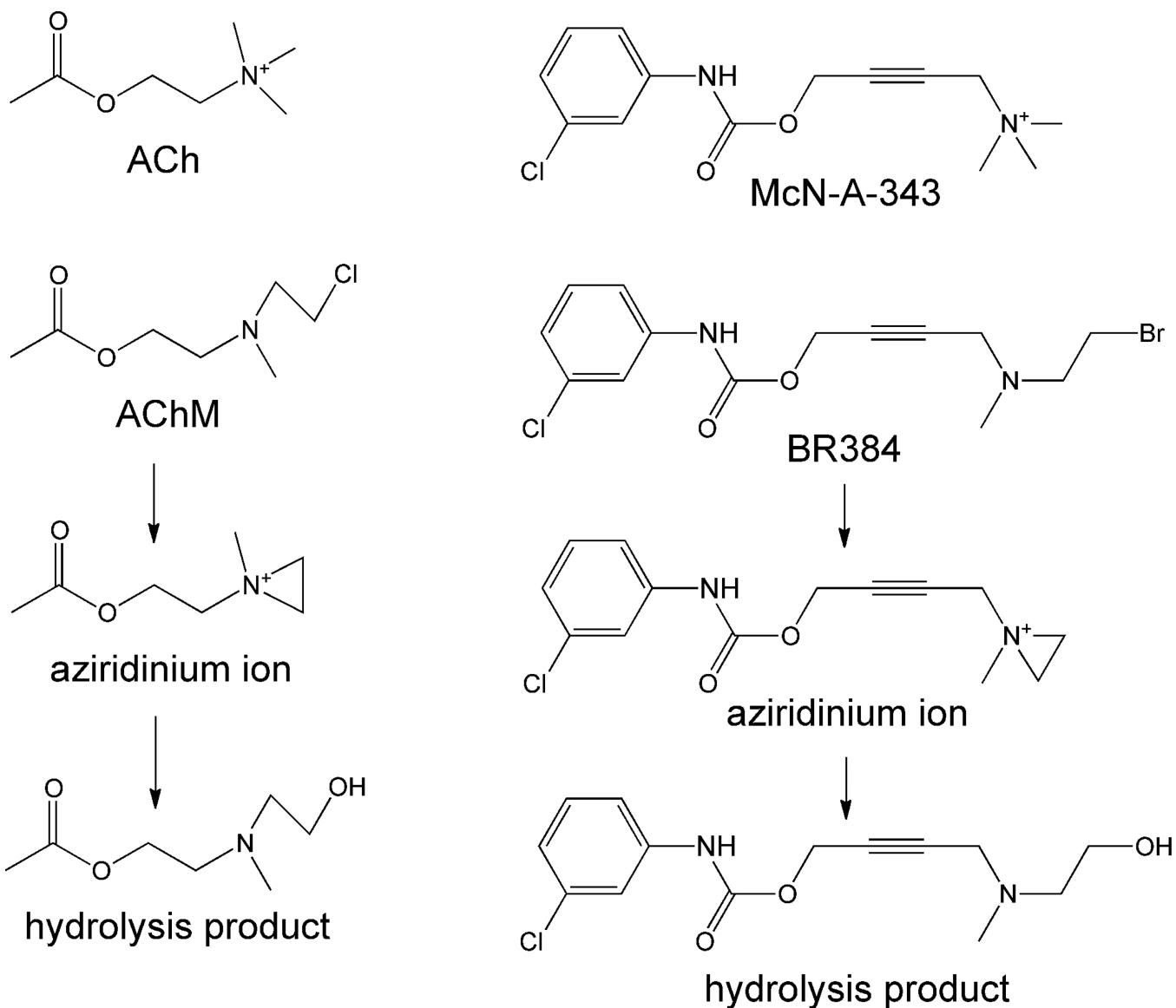
McN-A-343	[4-[[N-(3-chlorophenyl)carbamoyl]oxy]-2-butynyl] trimethylammonium chloride
NMS	<i>N</i> -methylscopolamine

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**Figure 1.**

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

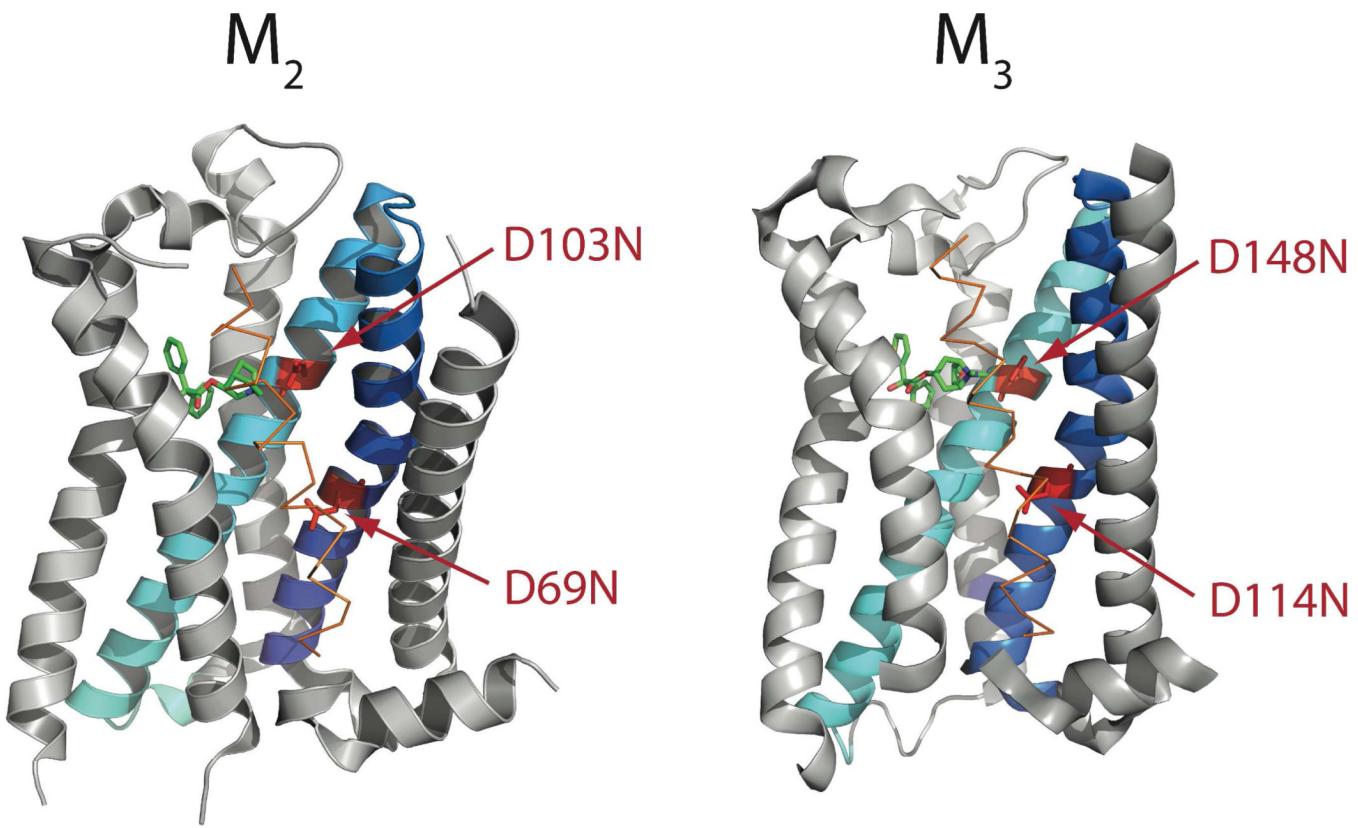
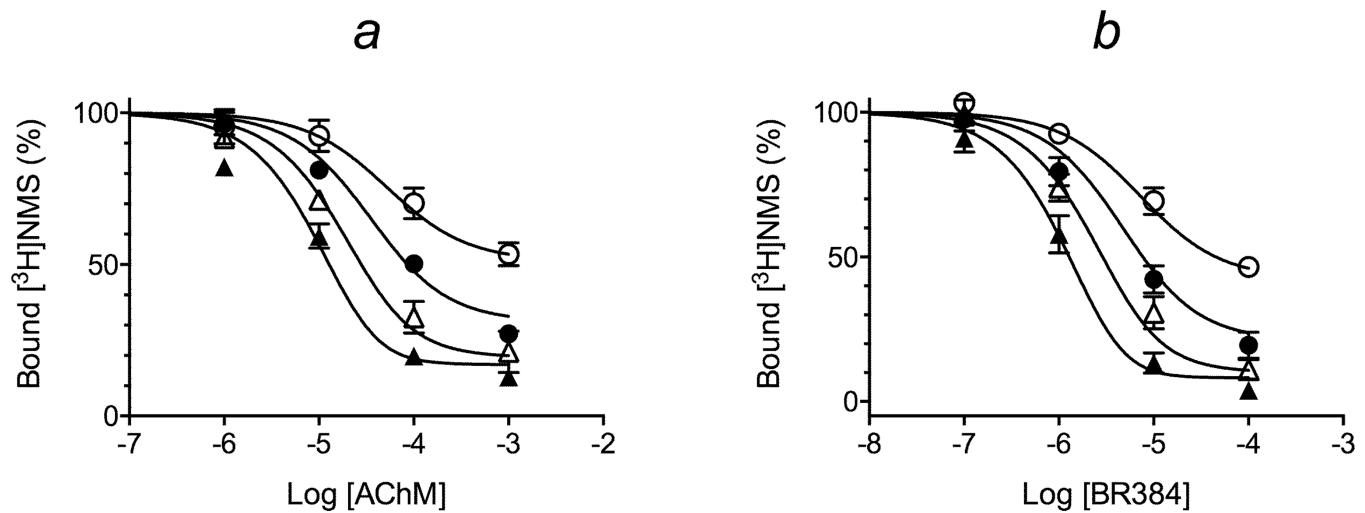
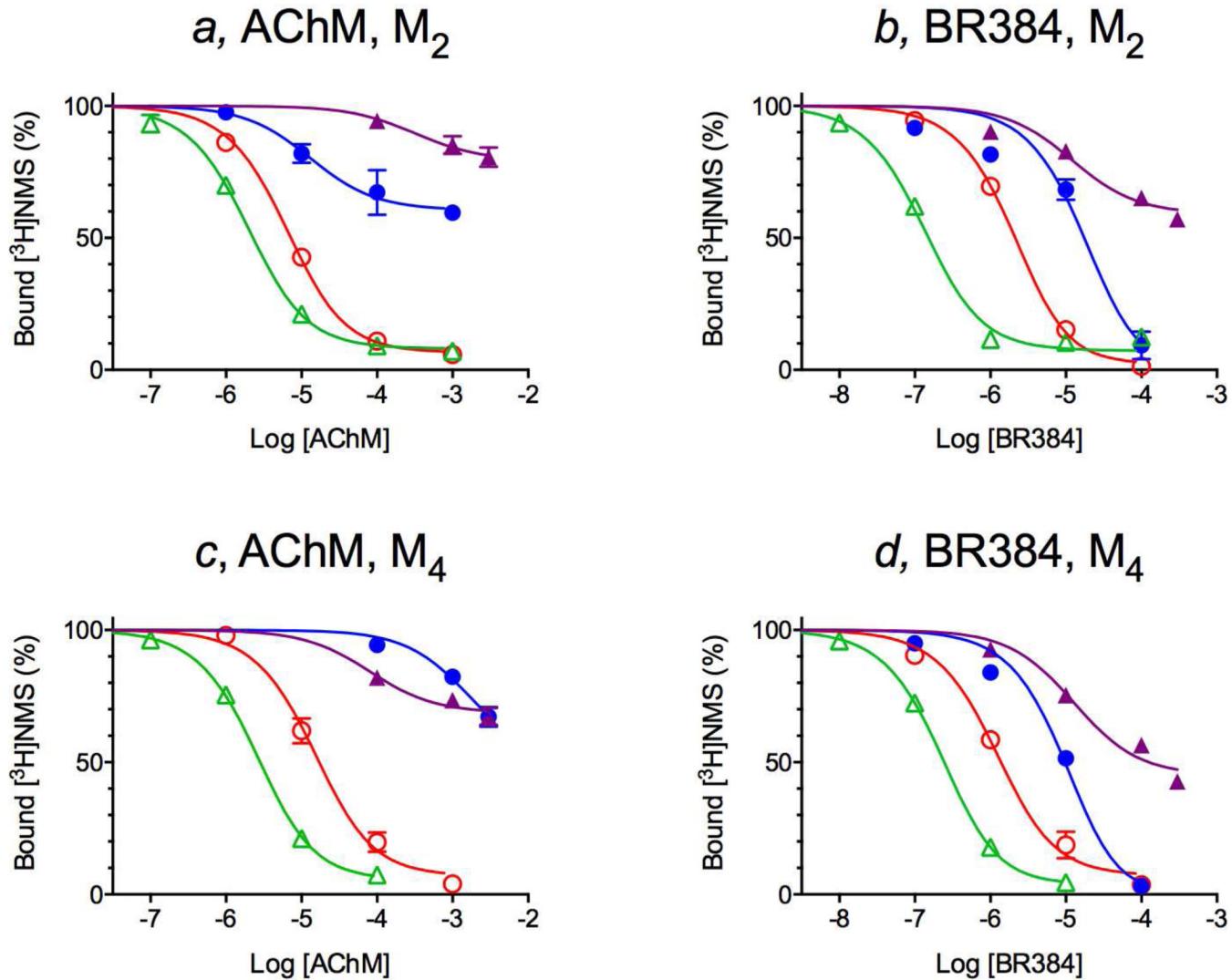


Figure 2.

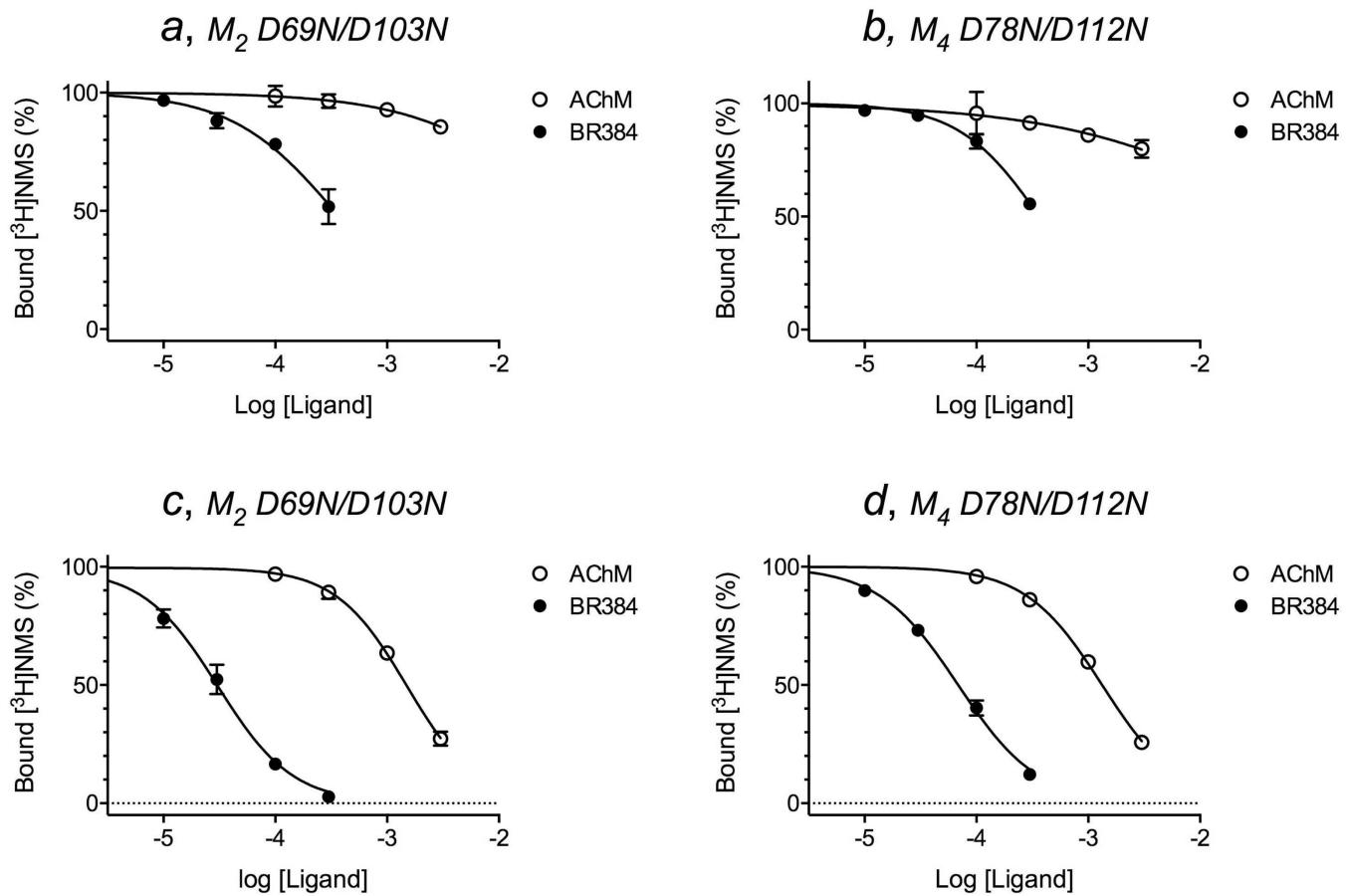
Crystal structures of the helices and extracellular loops of human M_2 and rat M_3 muscarinic receptors bound with 3-quinuclidinyl-benzilate and tiotropium, respectively (26, 27). The secondary structure (helices 1 – 6) and backbone (helix 7) are shown. Aspartic acids 2.50 (M_2 D69 and M_3 D113 (corresponds 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 two and three (PDB ID: 3uon and 4daj for M_2 and M_3 receptors, respectively).

**Figure 3.**

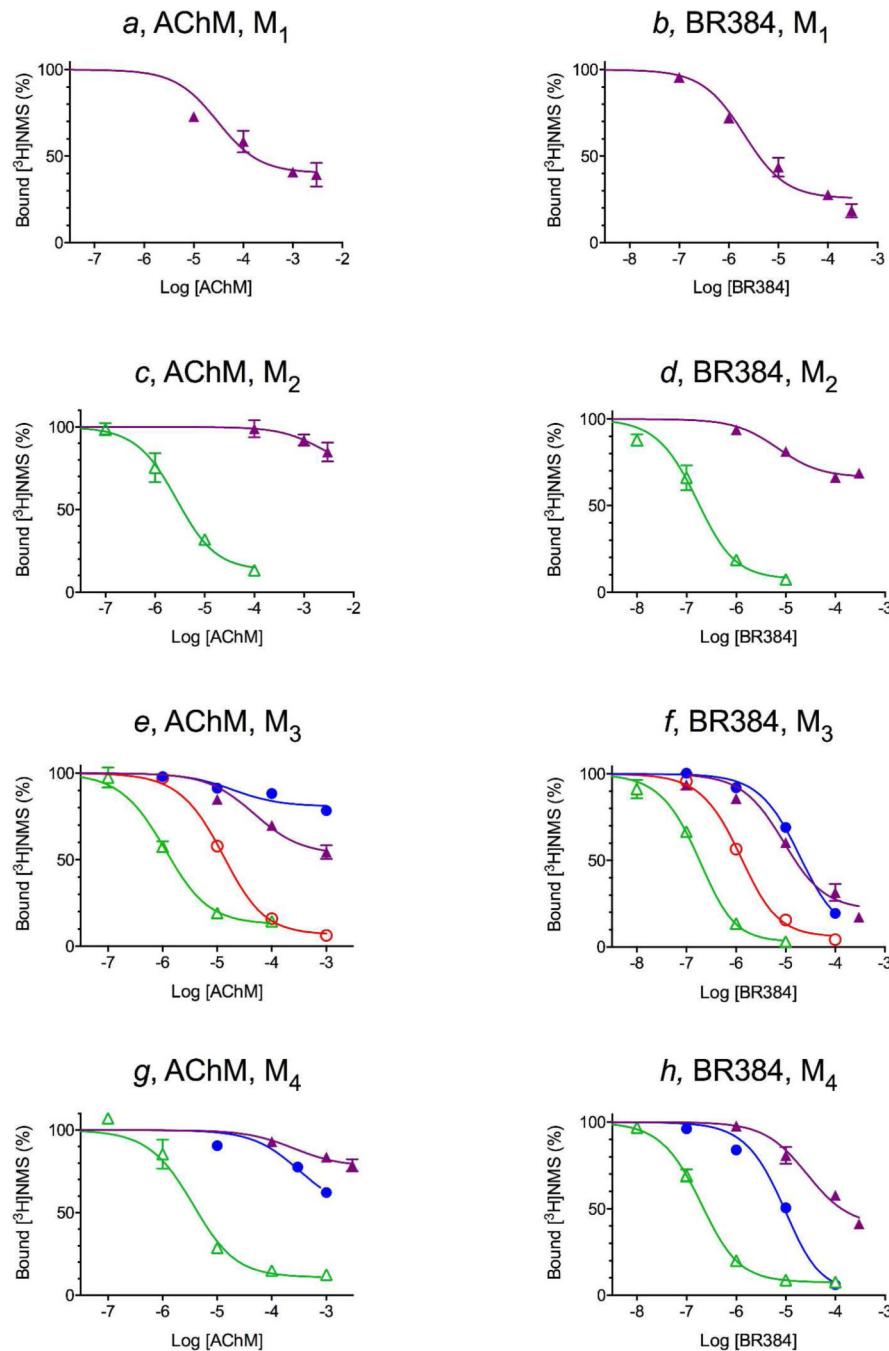
Alkylation of the wild type M_4 muscarinic receptor by AChM and BR384. Homogenates of CHO cells expressing the human M_4 muscarinic receptor were incubated with various concentrations of AChM (a) or BR384 (b) for different times, the reactions were stopped with scopolamine and thiosulfate, the homogenates washed, and the binding of $[^3\text{H}]$ NMS measured at a single concentration of 1.0 nM. The theoretical curves represent the global fit of equations 4 (a) and 6 (b) to the data with the estimates of b , k_1 and k_2 shared. Mean values \pm SEM of three experiments are shown. In a, the incubation times were 2 (○), 4 (●), 8 (△) and 15 (▲) min. In b, the incubation times were 1 (○), 2 (●), 4 (△) and 8 (▲) min.

**Figure 4.**

The interaction of AChM and BR384 with wild type and mutant M₂ (*a* and *b*), and M₄ (*c* and *d*) muscarinic receptors. Homogenates of cells expressing muscarinic receptors were incubated with different concentrations of AChM (*a* and *c*) or BR384 (*b* and *d*) for 15 (AChM, *a* and *c*) or 4 (BR384, *b* and *d*) min. The reactions were stopped with thiosulfate, the homogenates washed, and residual unalkylated muscarinic receptors were estimated by measuring [³H]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 - four experiments are shown.

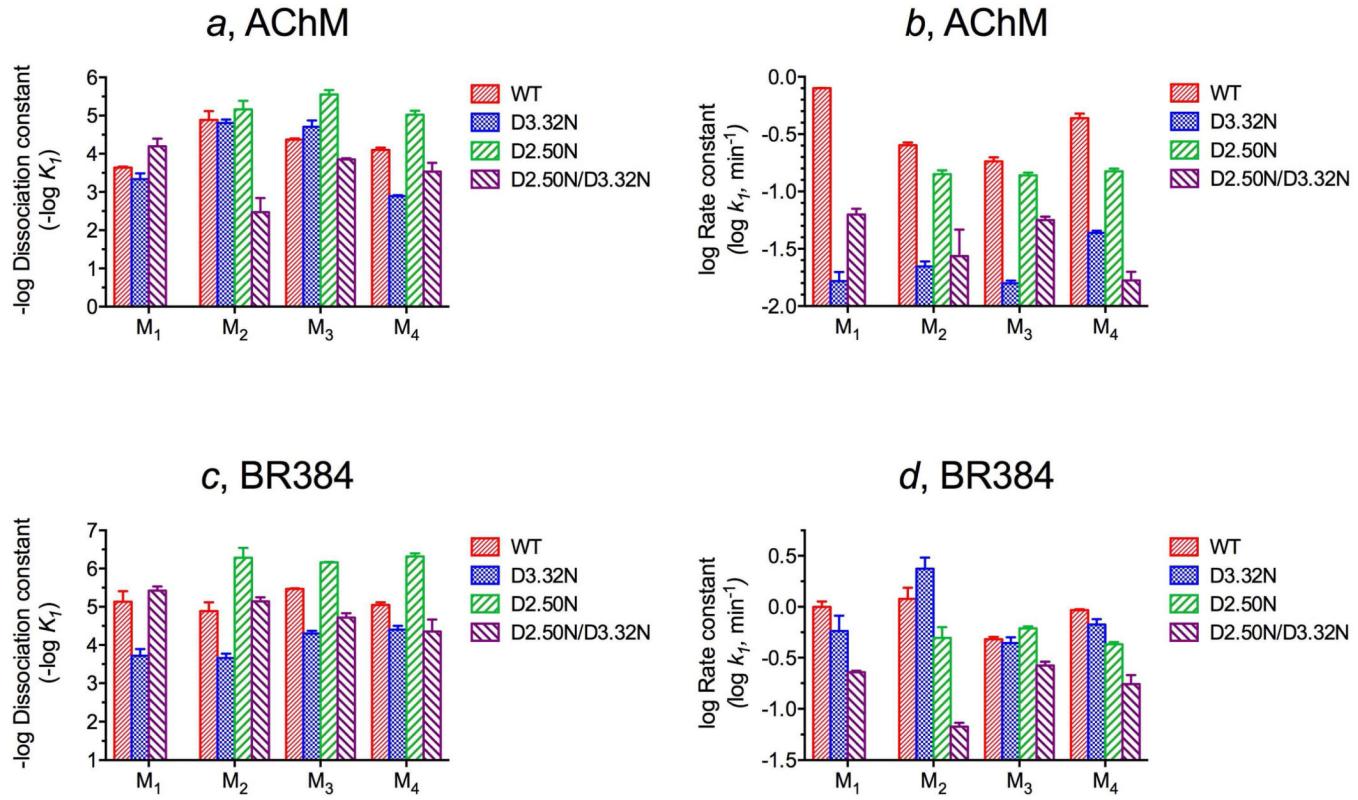
**Figure 5.**

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

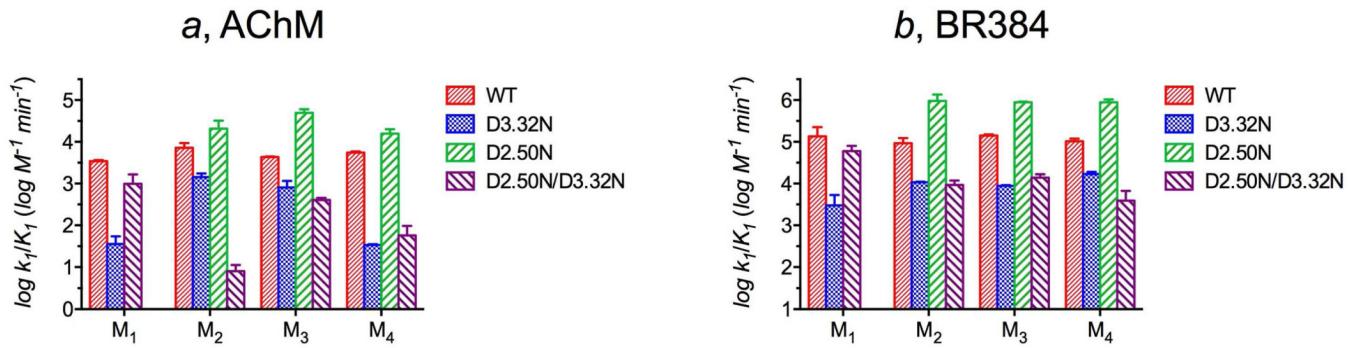
**Figure 6.**

The interaction of AChM and BR384 with wild type and mutant M₁ (*a* and *b*), M₂ (*c* and *d*), M₃ (*e* and *f*) and M₄ (*g* and *h*) muscarinic receptors. Homogenates of 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 washed, and residual unalkylated muscarinic receptors were estimated by measuring [³H]NMS binding at a single concentration (1 or 3 nM). Mean values \pm SEM from three experiments are shown. The different receptors and

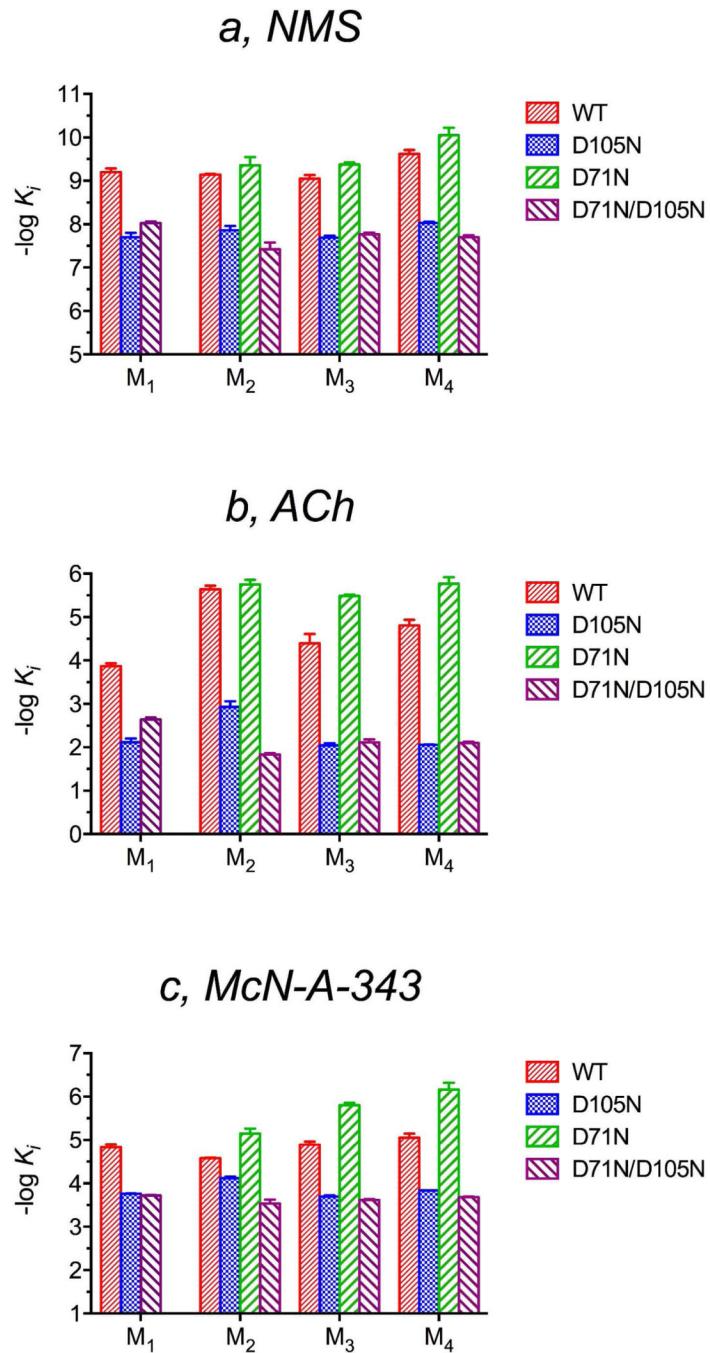
their mutants are indicated as: wild type, ○; D2.50N, △; D3.32N, ● and D2.50N/D3.32N, ▲.

**Figure 7.**

Estimates of the dissociation constants (*a* and *c*) and rate constants for alkylation (*b* and *d*) for AChM (*a* and *b*) and BR384 (*c* and *d*) at wild type and mutant M₁ – M₄ muscarinic receptors. The parameters were estimated from the data in Figures 3 and 6. The parameter estimates for wild type and D3.32N mutants of M₁ and M₂ 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 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.

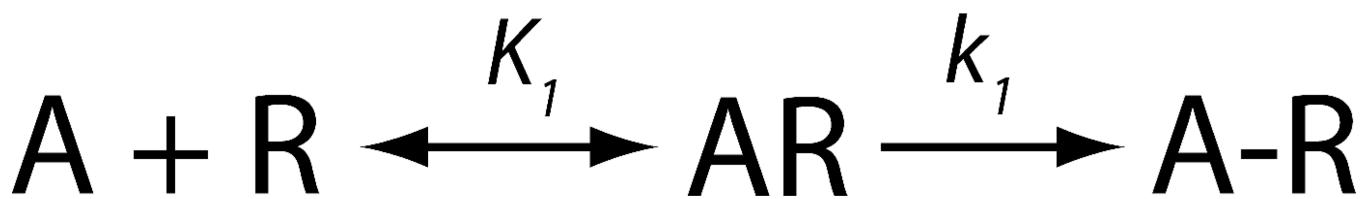
**Figure 8.**

A combined measure of the affinity and reactivity of AChM and BR384 with M₁ – M₄ wild type receptors and their D2.50N, D3.32N and D2.50N/D3.32N mutants. The histograms show the value of the log ratio of the rate constant for alkylation (k_1) and of the affinity constant (K_1) of the aziridinium ion of AChM and BR384. The value of the log of this ratio ($\log k_1/K_1$) is given for wild type and mutant M₁ (a), M₂ (b), M₃ (c) and M₄ (d) muscarinic receptors. Mean values \pm SEM from four experiments are shown. A summary of the numerical values is listed in Tables S1 and S3 of the Supporting Information.

**Figure 9.**

Negative log dissociation constants of NMS (a), acetylcholine (b) and McN-A-343 (c) for wild type and mutant M₁, M₂, M₃ and M₄ muscarinic receptors. The competitive inhibition of the binding of [³H]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 at each receptor subtype (Supporting Information, Table S5). A summary of 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.

**Scheme 1.**

Quasi-equilibrium model for the interaction of the aziridinium ions of AChM and BR384 with the muscarinic receptor. 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 ($A\text{-}R$).

Table 1

Changes in the log K_I of AChM and BR384 associated with single and double point mutations and the sum of the two single mutations (e.g., M₂ D103N + D69N).

	AChM ($\Delta \log K_I$)	BR384 ($\Delta \log K_I$)
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 D1 12N + 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.

Table 2

Changes in the 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., M₂ D103N + D69N).

	Acetylcholine ($\Delta \log K_i$)	McN-A-343 ($\Delta \log K_i$)	NMS ($\Delta \log K_i$)
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 D1 12N + 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 ^d

^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.