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Use of acetylcholine mustard to study allosteric interactions at the M₂ muscarinic receptor

Hinako Suga, Katherine W. Figueroa, and Frederick J. Ehlert

Department of Pharmacology, School of Medicine, University of California, Irvine, Irvine, California 92697-4625

Abstract

We explored the interaction of a nitrogen mustard derivative of acetylcholine with the human M₂ muscarinic receptor expressed in CHO cells using the muscarinic radioligand, [³H]N-methylscopolamine. Acetylcholine mustard caused a concentration-dependent, first order loss of [³H]N-methylscopolamine binding at 37°C, with the half maximal rate constant occurring at 24 μM and a maximal rate constant of 0.16 min⁻¹. We examined the effects of various ligands on the rate of alkylation of M₂ receptors by acetylcholine mustard. N-methylscopolamine and McN-A-343 (4-(trimethylamino)-2-butynyl-(3-chlorophenyl)carbamate) competitively slowed the rate of alkylation, whereas the inhibition by gallamine reached a plateau at high concentrations, indicating allosteric inhibition. In contrast, WIN 51708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-b]pyrimido[1,2-a]benzimidazole) had no effect. We also measured the inhibition of [³H]NMS binding by acetylcholine mustard at 0°C, conditions under which there is little or no detectable covalent binding. In these experiments, the dissociation constant of the aziridinium ion of acetylcholine mustard was estimated to be 12.3 μM. In contrast, the parent mustard and alcoholic hydrolysis product of acetylcholine mustard were without effect. Our results show that measurement of the effects of ligands on the rate of inactivation of the orthosteric site by a small site-directed electrophile is a powerful method for discriminating competitive inhibition from allosterism.

INTRODUCTION

A variety of drugs have been shown to interact allosterically with the M₂ muscarinic receptor to modulate the binding of orthosteric ligands at equilibrium (Birdsall and Lazareno, 2005). Binding measurements of this type can be analyzed with the allosteric ternary complex model to ascertain whether the data are consistent with allosterism (Stockton et al., 1983; Ehlert, 1988). If a modulator exhibits high negative cooperativity, however, it may be difficult, if not impossible, to make the requisite binding measurements at high concentrations of the radioligand to discriminate allosterism from simple competition.

An alternative approach is to investigate the influence of the putative modulator on the kinetics of radioligand binding. This strategy is often useful because the greater the negative cooperativity, the more likely the modulator is to accelerate the dissociation kinetics of the radioligand. For example, the rate of dissociation of [³H]oxotremorine-M from the M₂ muscarinic receptor is greatly enhanced in the presence of guanine nucleotides, which bind to G_{i/o} to exert negative cooperativity (Waelbroeck et al., 1982). In contrast, many, but not all, ligands that bind to the allosteric site of the M₂ receptor slow the dissociation of the orthosteric

To whom correspondence should be addressed: Frederick J. Ehlert, Ph.D., Department of Pharmacology, University of California, Irvine, Irvine, California 92697-4625, Tel: 949-824-6208, FAX: 949-824-4855, e-mail: E-mail: fjehlert@uci.edu.
Send print requests to: Frederick J. Ehlert, Ph.D., Department of Pharmacology, School of Medicine, University of California, Irvine, Irvine, California 92697-4625

antagonist, [³H]NMS ([³H]N-methylscopolamine), while reducing its observed affinity, presumably because of a greater net reduction in association relative to dissociation (Birdsall and Lazareno, 2005). It has been suggested that the negative allosteric effect of gallamine involves an outward rectification of access to the activation site of the M₂ receptor through modulation of a relay site located superficially to the activation site (Ehlert and Griffin, 2008). Regardless of the nature of the allosteric mechanism, it is also possible that when an allosteric modulator is bound to its site, it could physically prevent access to and egress from the orthosteric site by [³H]NMS (Proska and Tucek, 1994). By itself, this action would have no effect on [³H]NMS binding at equilibrium, but rather, cause an increase in the time required to reach equilibrium. Thus, the effect of an allosteric modulator, like gallamine, on the kinetics of the orthosteric radioligand could include a large component unrelated to an allosteric change in the conformation of the receptor, but rather, to a simple competitive effect on access to and egress from the binding pocket. Alternatively, it is conceivable, though unlikely, that gallamine induces a conformational change in the receptor so that the orthosteric binding pocket engulfs [³H]NMS, thereby preventing association and dissociation of [³H]NMS. Regardless, this situation makes it difficult to use kinetics as the basis for establishing allosteric interactions at the muscarinic receptor. It has been pointed out that a high affinity orthosteric ligand could exhibit low affinity for the allosteric site, and hence, it could bind to the allosteric site at high concentrations and retard the dissociation kinetics of [³H]NMS (Birdsall and Lazareno, 2005). This kinetic behavior would be indistinguishable from that of a highly potent, highly negatively cooperative allosteric mechanism, because such a modulator would exhibit low affinity for the allosteric site when [³H]NMS is bound to the orthosteric site, and hence it would exhibit low potency for influencing the dissociation kinetics of [³H]NMS.

To avoid these problems, we developed a novel approach to investigate allosteric interactions at the muscarinic receptor. The primary interaction is between the modulator and a small, site-directed electrophile for the orthosteric binding site. Using a small, lower potency ligand avoids potential equilibration problems associated with inhibiting access to and egress from the orthosteric site. Moreover, since the primary interaction is between an irreversible agent and the putative modulator, both of which are non-radioactive, it is possible to study the interaction over a broad range of concentrations of each agent. After stopping the reaction, the extent of receptor inactivation can be measured by washing the preparation and measuring the residual receptors with a suitable radioligand, like [³H]NMS. Competitive inhibitors and allosteric modulators exhibit different profiles for affecting the rate of receptor alkylation over a range of concentrations of the interacting ligands.

In this study, we use acetylcholine mustard (AChM) to investigate the interaction of ligands with the M₂ muscarinic receptor. AChM has been previously shown to bind covalently with muscarinic receptors (Robinson et al., 1975). We found that NMS and McN-A-343 (4-(trimethylamino)-2-butynyl-(3-chlorophenyl)carbamate) competitively protect the receptor from irreversible alkylation by AChM, whereas gallamine and WIN 51708 (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-b]pyrimido[1,2-a]benzimidazole) exhibit partial or no protection, consistent with their interaction at allosteric sites.

METHODS

Materials

Dulbecco's Modified Eagle Medium with high glucose plus L-glutamine, fetal calf serum and penicillin-streptomycin, were obtained from Invitrogen Corporation, Grand Island, NY. Amitriptyline, G418 disulfate salt, NMS, WIN 51708, atropine, gallamine, Hepes, CHES, EDTA, NMS, scopolamine, Na₂S₂O₃ and our initial supply of McN-A-343 were from Sigma-Aldrich, St. Louis, MO. Additional McN-A-343 as well as other compounds were synthesized as described below. Unless indicated otherwise, the reactants for organic synthesis were

obtained from Sigma-Aldrich. Salts for Krebs Ringer bicarbonate (KRB) buffer and phosphate buffer, sodium bicarbonate, HCl and NaOH were obtained from Fisher Scientific, Fair Lawn, NJ.

Synthesis of acetylcholine mustard

AChM was synthesized by the method of (Jackson and Hirst, 1972) with modification. Following acetylation of N-methyldiethanolamine using acetic anhydride, the product (N-methyl-N-(2-ethanol)-2-aminoethylacetate) was first purified by vacuum distillation (72 – 74 °C at 0.5 mm Hg) and then by chromatography on silica gel (methanol). After chlorinating this product using SOCl_2 in CHCl_3 , the volatiles were removed under reduced pressure. The residue was dissolved in ice-cold H_2O and quickly extracted into ice-cold ether by alkalizing the aqueous phase (approximately pH 9.0) with aliquots of cold 10 M NaOH and 1 M Na_2CO_3 . The ether extract was dried (anhydrous MgSO_4) and filtered into an ethereal solution of oxalic acid yielding a crude precipitate of the oxalate salt of AChM. The precipitate was collected the next day by filtration and was recrystallized in acetonitrile and ether to yield the oxalate salt of AChM.

Synthesis of N-methylamitriptyline

N-methylamitriptyline was synthesized from amitriptyline hydrochloride by first converting the salt to a free base by extraction with chloroform under basic conditions and reacting the latter with iodomethane in acetone. The product was collected by filtration, and it was recrystallized in acetonitrile.

Synthesis of McN-A-343

2-Propynyl-N-(3-chlorophenyl)carbamate was synthesized from 3-chlorophenyl isocyanate and propargyl alcohol as described by Mellin et al. (1989). The desmethyl derivative of McN-A-343, 4-(Dimethylamino)-2-butynyl-(3-chlorophenyl)carbamate, was synthesized by a condensation between 2-propynyl-N-(3-chlorophenyl)carbamate and bis(dimethylamino) methane using a procedure analogous to that described by Mellin et al. (1989) for the synthesis of a bromo-analog of McN-A-343. The oxalate salt was recrystallized in a mixture of acetone/methanol (8:1). The oxalate salt of desmethyl McN-A-343 was converted to the free base by first dissolving the salt in a mixture of acetone/water (approximately 1/1) and extracting the base into dichloromethane by alkalizing the aqueous phase with 1 M Na_2CO_3 . The organic extract was dried (anhydrous MgSO_4), and the dichloromethane removed under reduced pressure. The residue was dissolved in acetone and a two-fold excess of iodomethane was added. After the solution was allowed to stand for two days, the iodide salt of McN-A-343 was collected by filtration. This salt has a maximum solubility of approximately 15 mM in aqueous solution. The chloride salt was made by ion exchange chromatography of a 15 mM solution of the iodide salt on Dowex AG 1 X8 (chloride form, 100 – 200 mesh, Bio-Rad Laboratories, Hercules, CA). The eluate from the column was collected, and the water removed by heating (80°C) under reduced pressure. The residue was dried further *in vacuo*.

Cyclization of acetylcholine mustard

The formation of the aziridinium ion from a 2 mM solution of AChM at 37°C was measured by its reaction with excess thiosulfate and estimating residual thiosulfate by titration with potassium triiodide as described in Skoog and West (1965). The rate constants for formation ($0.18 \pm .028 \text{ min}^{-1}$) and decay ($0.0045 \pm .0021 \text{ min}^{-1}$) of the aziridinium ion were estimated by nonlinear regression analysis of the data as described previously (Thomas et al., 1992). The peak concentration of the aziridinium ion was formed at approximately 21 min. The concentration of the aziridinium ion reached 98% of the peak value after 15 min of incubation at 37°C, and only declined to 92% and 86% of the peak after 45 and 60 min, respectively. A

solution of AChM was routinely stored at -20°C in ethanol. On the day of the experiment, an aliquot of this solution was diluted in 9 volumes of 50 mM phosphate buffer ($\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$), pH 7.4, and incubated at 37°C for 15 min. The solution was placed on ice and used as soon as possible. For preparation of alcoholic hydrolysis product of AChM, the incubation was carried out overnight at 37°C .

Cell culture

Chinese hamster ovary (CHO) cells stably expressing the human M_2 muscarinic receptor (CHO h M_2 cells) were obtained from Acadia Pharmaceuticals (San Diego, CA) and cultured in Dulbecco's Modified Eagle Medium with high glucose plus L-glutamine supplemented with 10% fetal calf serum, 3.7 g/l sodium bicarbonate, penicillin-streptomycin (100 units/ml and 100 $\mu\text{g}/\text{ml}$, respectively) and 0.4 mg/ml G418 disulfate salt at 37°C in a humidified atmosphere with 5% $\text{CO}_2/95\%$ air.

Kinetics of M_2 muscarinic receptor alkylation by cyclized AChM

The general strategy for measuring the alkylation of M_2 receptors by AChM involved 1) incubating the receptor preparation with AChM and test ligand for various periods of time, 2) stopping the reaction with a competitive inhibitor and thiosulfate, 3) washing the receptor preparation, and 4) measuring the binding of [^3H]NMS to estimate the residual unalkylated receptors. Initial experiments were carried out on intact CHO h M_2 cells grown in 24-well culture plates. This approach enabled the attached cells to be easily washed with medium to remove AChM, its transformation products and other drugs. For these experiments, confluent CHO h M_2 cells cultured in 24-well plates (Corning Incorporated, Corning, NY) were washed with 0.6 ml of KRB buffer (26 mM NaHCO_3 , 1.2 mM KH_2PO_4 , 124 mM NaCl , 5 mM KCl , 1.8 mM CaCl_2 , 1.3 mM MgCl_2 , 10 mM glucose, pH 7.4) and incubated for 10 min at 37°C . Cells were incubated for another 15 min in the absence or presence of other test ligands at 37°C . The reaction was initiated by adding cyclized AChM, and the cells were incubated for the indicated times at 37°C . In some experiments, these incubations were carried out at 0°C to determine the extent to which AChM inactivates muscarinic receptors at this low temperature. Subsequently, the reaction was stopped by adding 10 mM $\text{Na}_2\text{S}_2\text{O}_3$ and 10 μM N-methylamitriptyline, and the cells were incubated for 15 min at 37°C . The cells were washed quickly three times with KRB buffer, and then two more times with a five-min incubation at 37°C preceding these last two washes. Afterwards, residual muscarinic receptors were measured using 1 nM [^3H]NMS as described below. N-methylamitriptyline was used as the stopping agent because it is a potent inhibitor of muscarinic binding ($pK_D = 8.0$; Ehler et al., 1990), yet its potency is not so great that it would be difficult to wash out readily.

In other experiments, the alkylation of M_2 receptors by AChM was investigated in homogenates of CHO h M_2 cells. Cells grown to confluence in 75 cm^2 flasks or 100 mm dishes (Corning Incorporated, Corning, NY) were scraped into binding buffer (20 mM Na/HEPES, pH 7.4, 100 mM NaCl and 10 mM EDTA) using a Teflon spatula or cell scraper. The mixture of buffer and cells was centrifuged ($1,247 \times g$, 10 min) and the supernatant discarded. The pellet was homogenized in binding buffer using the Polytron (Brinkmann Instruments, Westbury, NY) at setting #4 for approximately 10 sec. For a single kinetic experiment involving approximately 36 incubation tubes, the pooled cells from six flasks or eight dishes were suspended to a volume of approximately 5.5 ml. An aliquot of cellular homogenate (0.15 ml) was added to each microfuge tube followed by an additional aliquot (0.05 ml) of binding buffer or buffer plus test drug. The tubes were incubated at 37°C in a shaking water bath, and the reaction was started by the addition of an aliquot (0.05 ml) of cyclized AChM. After various incubation times, the reaction was stopped by the addition of an aliquot (0.75 ml) of binding buffer containing thiosulfate (1.3 mM) and scopolamine (10 or 1.3 μM , depending upon whether the concentration of AChM was 1.0 mM or 0.1 and 0.01 mM, respectively). Control

homogenates were also treated with the stopping solution. The tubes were allowed to incubate 20 min at 37°C to inactivate the aziridinium ion. Thiosulfate, scopolamine and the transformation products of AChM were removed by centrifugation (25,000 × g; 15 min) and aspiration of the supernatant. The pellets were suspended in fresh binding buffer (1 ml) and the washing step repeated one or two times, depending upon whether the concentration of scopolamine was 1.3 or 10 μM, respectively. The final pellets were suspended in 1 ml of 20 mM Na/CHES, pH 9.3, 100 mM NaCl and 10 mM EDTA. An aliquot (0.3 ml) of this homogenate was incubated in a final volume of 1 ml of binding buffer, pH 9.3, containing 1 nM [³H]NMS (specific activity, 82 Ci/mmol; PerkinElmer, Boston, MA) for 25 min at 37°C. Additional details of the binding assay are given below. Ligand binding was measured at pH 9.3 because the affinity of the competitive inhibitor used to stop the reaction, scopolamine, is greatly reduced to 1/80th at this pH compared to pH 7.4. In contrast, the affinity of [³H]NMS is little affected by increasing the pH from 7.4 to 9.3 (Ehlert and Delen, 1990). A pH sensitive stopping reagent can be removed in fewer washes compared to that required for the stopping reagent used in intact cells (N-methylamitriptyline). Since additional washing tends to increase variability in tissue homogenates, we settled on two or three washes and measured binding at pH 9.3. Using a stopping solution containing scopolamine (10 μM) had no effect on [³H]NMS binding when measured subsequently at pH 9.3 after three washes.

[³H]NMS binding assay, intact cells

The specific binding of [³H]NMS to intact CHO hM₂ cell monolayers was measured as described previously (Griffin et al., 2003). The incubation medium was KRB buffer, and incubation times and temperatures varied depending on the nature of the experiment. Following incubation with [³H]NMS, the cells were rapidly washed twice with ice-cold KRB buffer by quickly aspirating the media and replacing it with fresh buffer (0.6 ml). The entire washing process was accomplished in approximately 5 sec. The cells were lysed with an aliquot (0.2 ml) of 1 M NaOH. After at least 20 min at room temperature, the lysates were acidified by the addition of 0.3 ml of 1M HCl. The solution was transferred to scintillation vials (Research Products International Corp., Mount Prospect, IL) and mixed with scintillation cocktail (Budget-Solve; Research Products International Corp., Mount Prospect, IL), and the radioactivity counted with Beckman Liquid scintillation counter (LS 6500; Beckman Coulter, Fullerton, CA). Nonspecific binding was defined as the residual binding in the presence of 10 μM atropine. In experiments where the residual unalkylated receptors were measured after incubation with AChM at 37°C, [³H]NMS binding was measured after a one-hour incubation at room temperature. When the receptor inactivation step was carried out at 0°C, the subsequent binding assay incorporated a one-hour incubation at 0°C with 1 nM [³H]NMS. When the competitive inhibition of [³H]NMS (1 nM) binding by AChM and its transformation products was measured, the incubation lasted one hour at 0°C to minimize receptor alkylation by the aziridinium ion or the formation of the aziridinium ion from AChM. In these experiments, parent AChM was dissolved in distilled water just before use and kept on ice to avoid cyclization. To estimate the affinity of [³H]NMS, saturation experiments were carried out with a three-hour incubation at 0°C. The competitive inhibition of [³H]NMS by gallamine or McN-A-343 was measured using a four-hour incubation at 0°C.

[³H]NMS binding assay, cellular homogenates

The specific binding of [³H]NMS was measured by the rapid filtration technique using a cell harvester (Brandel, Gaithersburg, MD) as described previously (Griffin et al., 2003). Cellular homogenates were prepared in pH 7.4 binding buffer (20 mM Na/HEPES, pH 7.4, 10 mM EDTA and 100 mM NaCl), and were submitted to the various treatment conditions followed by washing steps involving centrifugation and suspension in fresh buffer. The final pellets were suspended in either pH 7.4 or pH 9.3 (20 mM Na/CHES, pH 9.3, 100 mM NaCl and 10 mM EDTA) binding buffer. Aliquots of homogenate were incubated at 37°C for 25 – 30 min in a

final volume of 1.0 ml of binding buffer containing [^3H]NMS. Specifically bound [^3H]NMS was trapped by rapid filtration as described previously. Non-specific binding was defined as the residual binding in the presence of 10 μM atropine (at pH 7.4) or 10 μM NMS (at pH 9.3). All measurements were made in triplicate.

Analysis of data

The data from individual experiments were analyzed by nonlinear regression analysis using the GraphPad Prism 4.0 (GraphPad Software Inc., San Diego, CA). Time course, competitive binding and saturation binding data were fitted to a one phase exponential decay, a one-site competition and a one site binding function, respectively. The calculation of the parameters was done according to Griffin et al. (2003) and Ehlert and Griffin (2008).

Our analysis of the interaction of the aziridinium ion of AChM (X) with the M_2 muscarinic receptor (R) in the presence of an allosteric modulator (A) is based on the model shown in Scheme 1 in which the aziridinium ion rapidly forms a reversible complex with the receptor (XR), which converts to a covalent complex ($X-R$) at a relatively slower rate. In this scheme, K_1 and K_2 denote the affinity constants (inverse molar units; reciprocal of dissociation constant) of the aziridinium ion of AChM and the allosteric modulator for the free receptor, respectively, α denotes the cooperativity factor for their interaction, and k_1 denotes the rate constant for alkylation of the receptor by the aziridinium ion. In this analysis, it is assumed that the rate of conversion of the XR complex to the covalent complex is that same as that of the XRA complex. The consequence of a difference in these rate constants is described under "Discussion." If the rate constants describing the reversible binding of X and A are fast relative to k_1 and if the concentration of the aziridinium ion is nearly constant during the incubation, then the rate of loss of unalkylated receptors (R_f) can be described by the following differential equation:

$$\frac{dR_f}{dt} = -pR_fk_1 \quad 1$$

in which p denotes fractional receptor occupancy by the aziridinium ion in the form a reversible complex in the presence of the allosteric modulator. Occupancy of the receptor by the aziridinium ion in the presence of the allosteric modulator is described by the following equation (Ehlert, 1988):

$$p = \frac{X}{X + \frac{1}{K_1 \left(\frac{1 + \alpha \alpha K_2}{1 + \alpha K_2} \right)}} \quad 2$$

Substituting in equation 2 for occupancy in equation 1 yields a differential equation for the inactivation of muscarinic receptors by the aziridinium ion in the presence of the allosteric modulator:

$$\frac{dR_f}{dt} = - \frac{XR_f}{X + \frac{1}{K_1 \left(\frac{1 + \alpha \alpha K_2}{1 + \alpha K_2} \right)}} k_1 \quad 3$$

Integrating this equation with respect to time and evaluating the integral over the time interval from $t = 0$ to time t yields:

$$\frac{Y_t}{Y_0} = e^{-\left(\frac{X}{X + \frac{1}{K_1(1 + A\alpha K_2)}}\right) k_1 t} \quad 4$$

in which Y_t denotes the free receptor at time t and Y_0 denotes the total amount for receptors at the start of the incubation. We found that most, but not all, of the M_2 receptor population was alkylated by the aziridinium ion of AChM in kinetic experiments. Hence, we modified equation 4 to include AChM-sensitive ($1-b$) and insensitive (b) components:

$$\frac{Y_t}{Y_0} = (1-b) \times e^{-\left(\frac{X}{X + \frac{1}{K_1(1 + A\alpha K_2)}}\right) k_1 t} + b \quad 5$$

In the absence of allosteric modulator ($A = 0$), equation 5 reduces to:

$$\frac{Y_t}{Y_0} = (1-b) \times e^{-\left(\frac{X}{X + \frac{1}{K_1}}\right) k_1 t} + b \quad 6$$

When there is an infinite amount of negative cooperativity ($\alpha = 0$), then there is no formation of the ternary complex (XRA), and A behaves like a competitive inhibitor at equilibrium. Under this condition, equation 5 reduces to equation 7 in which K_2 has been replaced with K_i and A has been replaced with I to indicate competitive binding at the same site to which X binds:

$$\frac{Y_t}{Y_0} = (1-b) \times e^{-\left(\frac{X}{X + \frac{1}{K_1(1 + IK_i)}}\right) k_1 t} + b \quad 7$$

Equation 5–Equation 7 were utilized to analyze the binding of [3H]NMS to CHO hM₂ cells that had been previously incubated with AChM at various concentrations for various times in the absence (equation 6) and presence of various concentrations of allosteric (equation 5) and competitive (equation 7) ligands. To test whether there was a significant improvement in residual error when the allosteric model (equation 5) was fitted to the data instead of the competitive model (equation 7), the following statistic (F) was calculated as described previously (Draper and Smith, 1998):

$$F = \frac{\frac{SS_1 - SS_2}{df_1 - df_2}}{\frac{SS_2}{df_2}} \quad 8$$

in which SS_1 and SS_2 denote the residual sum of squares for the best fit of equation 7 and equation 5 to the data, respectively, and df_1 and df_2 , denote the corresponding degrees of freedom. The latter were calculated by subtracting the number of parameters in each model from the total number of data points. The statistic F exhibits an F distribution with the degrees of freedom in the numerator and denominator being $df_1 - df_2$ and df_2 , respectively.

We also used a more empirical approach to analyze the effects of inhibitors on the observed rate constant for alkylation. It can be shown that the observed rate constant for alkylation by the aziridinium ion of AChM (k_{obs}) is given by:

$$k_{obs} = \frac{1}{\tau} = \frac{XK_1}{XK_1 + 1} \times k_1 \quad 9$$

in which τ denotes the time constant. In the presence of a competitive inhibitor (I), the k_{obs}' of AChM is given by:

$$k_{obs}' = \frac{1}{\tau'} = \frac{XK_1}{1 + XK_1 + IK_i} \times k_1 \quad 10$$

In the presence of allosteric modulator (A), the k_{obs}'' of AChM is given by:

$$k_{obs}'' = \frac{1}{\tau''} = \frac{XK_1}{XK_1 + \frac{1 + AK_2}{1 + A\alpha K_2}} \times k_1 \quad 11$$

The effect of a competitive inhibitor on the rate of receptor alkylation by AChM can be defined as the ratio (R) of the time constant (τ') measured in the presence of the inhibitor divided by that measured in its absence (τ). Dividing equation 9 by 10 and taking the logarithm of both sides yields:

$$\text{Log}(R) = \text{Log}\left(\frac{\tau'}{\tau}\right) = \text{Log}\left(\frac{1 + XK_1 + IK_i}{1 + XK_1}\right) \quad 12$$

To derive an equation describing the effect of an allosteric modulator on $\text{Log } R$, equation 9 is divided by 11, and the result is converted to logarithms:

$$\text{Log}(R) = \text{Log}\left(\frac{\tau''}{\tau}\right) = \text{Log}\left(\frac{XK_1 + \frac{1 + AK_2}{1 + A\alpha K_2}}{1 + XK_1}\right) \quad 13$$

Equation 12 and Equation 13 were fitted to the plot of $\text{Log } R$ against the inhibitor concentration by nonlinear regression analysis to determine if the data were consistent with competitive inhibition (equation 12) or allosteric inhibition (equation 13). The F statistic (equation 8) was used to determine if there was a significant improvement in residual error with the more complex allosteric model.

RESULTS

Alkylation of the M_2 muscarinic receptor by cyclized acetylcholine mustard

Treatment of homogenates of CHO hM₂ cells with AChM (10 μ M) at 37°C for four min caused a decrease in the binding capacity of the muscarinic antagonist [³H]NMS when binding measurements were made at pH 7.4 after washing the homogenates to remove unreacted mustard (Figure 1). In control cells, the logarithm of the affinity constant ($\text{Log } K_{NMS} \pm \text{SEM}$) of [³H]NMS was 9.32 ± 0.057 . Following treatment with AChM the binding capacity was reduced to 50 ± 2.4 % of control while the $\text{Log } K_{NMS}$ was unaffected (9.37 ± 0.061). Similar

results were obtained when the homogenate was treated in the same manner, but the binding assay carried out at pH 9.3 instead of 7.4 (data not shown). In this experiment, treatment with AChM caused a 52.8 % reduction in binding capacity while having little or no effect on the affinity of [³H]NMS (control, Log K_{NMS} = -9.02; AChM treated, Log K_{NMS} = -9.07). At pH 9.3, the binding affinity of [³H]NMS was reduced about two-fold compared to that measured at pH 7.4. The data show that AChM binds irreversibly to the M₂ muscarinic receptor.

Kinetics of the alkylation of M₂ muscarinic receptors

We investigated the ability of AChM to alkylate the M₂ muscarinic receptor by first incubating CHO hM₂ cells with different concentrations of cyclized AChM for various periods of time and then washing the cells and measuring the residual, unalkylated receptor population using a single concentration of [³H]NMS (Figure 2a). AChM caused a time-dependent loss in specific [³H]NMS binding with the rate of loss being dependent on the concentration of AChM. The data are generally consistent with the postulate that the rate of alkylation is proportional to receptor occupancy by the aziridinium ion. To test this postulate we fitted equation 6 to the data sharing the estimate of the affinity constant of the aziridinium ion (K_I) and the rate constant for alkylation (k_I) among the curves. Regression analysis provided a satisfactory fit as shown by the agreement between the data points and the theoretical curves in Figure 2a. The best fit yielded parameters estimates $\pm$ SEM of 4.62 ± 0.048 for Log K_I and $0.16 \pm 0.012 \text{ min}^{-1}$ for k_I . Approximately 19 % of the receptor population behaved as resistant to alkylation by AChM, perhaps because of recycling of receptors to the membrane during the washing steps and binding assay. We also fitted each curve in Figure 2a independently to a decreasing exponential equation and plotted the observed rate constant for alkylation (k_{obs}) against the concentration of AChM (see Figure 2b). The data show that the observed rate for alkylation increases as a mass-action-like function of the AChM concentration with a half maximal increase in rate occurring at a concentration whose negative logarithm ($pEC_{50} = 4.57$) is nearly equivalent to the logarithm of the affinity constant (K_I) of AChM. Also, the estimate of the maximal value of the observed rate constant (0.16 min^{-1}) is identical to the estimate of k_I . These results are consistent with the postulate that AChM equilibrates rapidly with the M₂ muscarinic receptor to form a reversible receptor complex (Log $K_I = 4.62$) that converts to a covalent complex at a relatively slower rate (0.16 min^{-1}).

Effect of muscarinic ligands on M₂ receptor alkylation by acetylcholine mustard

The results in Figure 2 show that the rate of receptor alkylation by AChM is proportional to receptor occupancy. It follows that it should be possible to determine how a reversible ligand affects receptor occupancy by the aziridinium ion of AChM simply by measuring its effect on the rate of receptor alkylation. This approach should provide a means of discriminating between competitive and allosteric modification of receptor occupancy by the aziridinium ion of AChM.

To explore this issue, we measured the influence of NMS on the alkylation of the M₂ muscarinic receptor by cyclized AChM in intact CHO hM₂ cells. Increasing the concentration of NMS progressively slowed the rate of alkylation caused by AChM at concentrations of 0.1 mM (Figure 3a) and 1.0 mM (Figure 3b). The data are generally consistent with the idea that NMS competitively protects the receptor from irreversible alkylation by AChM. To test this postulate, we fitted equation 7 to the data in Figure 3a and b, sharing the estimate of the affinity constant of NMS (K_i) among the data and setting the affinity constant of AChM (K_I) to a constant value equivalent to that estimated from the data in Figure 2 (i.e., Log $K_I = 4.62$). Regression analysis of the data showed that equation 7 provided a reasonable fit to the data with the estimate of Log $K_{NMS} \pm$ SEM being 10.61 ± 0.08 . There was no significant improvement in residual error when the allosteric model (equation 5) was fitted to the data ($F_{1,76} = 2.69$; $P = 0.104$). The higher apparent affinity of NMS in these kinetic experiments compared to those shown in Figure 1a for [³H]NMS can probably be attributed to our method

of first incubating the cells with NMS for 15 min prior to adding AChM in the kinetic assay. The rate of dissociation of NMS from the M₂ receptor is slow relative to the rate of receptor alkylation by AChM. Under such conditions, occupancy by NMS would be greater than that expected at equilibrium, and hence, its affinity overestimated.

A similar type of experiment was done with the neuromuscular blocking agent gallamine, which is known to be an allosteric modulator of the M₂ muscarinic receptor (Clark and Mitchelson, 1976; Stockton et al., 1983). Gallamine slowed the rate of receptor alkylation caused by AChM (0.1 mM) in a manner that resembled a competitive mechanism, with the inhibitory effect increasing with the gallamine concentration (Figure 4a). When a higher concentration of AChM was used (1.0 mM) however, the inhibitory effect of gallamine reached a plateau at 0.1 mM and little or no further inhibition was observed at 1 mM gallamine (Figure 4b). This limiting effect of gallamine is consistent with an allosteric mechanism mediated through the formation of a ternary complex consisting of gallamine, the aziridinium ion of AChM and the M₂ muscarinic receptor (scheme 1). To test this postulate, equation 5 was fitted to the data in Figure 4a and b sharing the estimate of the cooperativity (α) and the affinity constant of gallamine for the allosteric site (K_2) among the curves. Regression analysis showed that equation 5 provided a good fit to the data. The estimates $\pm$ SEM of the log affinity constant (Log K_2) and cooperativity constant (Log α) of gallamine were 6.35 ± 0.035 and -2.15 ± 0.048 , respectively. In contrast, there was a highly significant increase in residual error when the data were fitted to the simpler competitive model (equation 7) ($F_{1,76} = 172$; $P = 3.2 \times 10^{-21}$).

The effect of WIN 51708 on the rate of receptor inactivation by AChM in intact cells is shown in Figure 5. WIN 51708 is a novel allosteric modulator of the M₂ receptor that is thought to interact at an allosteric site distinct from that of gallamine and to cause a modest reduction in the affinity (about five-fold) of acetylcholine for the M₂ muscarinic receptor (Lazareno et al., 2002). We were unable to measure any effect of WIN 51708 on the alkylation of M₂ receptor by AChM (0.1 mM; see Figure 5b). We repeated this experiment using a lower concentration of AChM (0.01 mM) in homogenates of CHO hM₂ cells, and we were still unable to detect an effect of WIN 51708 on the rate of receptor alkylation (Figure 5a). These results are consistent with the postulate that WIN 51708 and the aziridinium ion of AChM bind to distinct sites on the muscarinic receptor and exhibit little or no cooperativity between them.

We also investigated the effects of McN-A-343 on the kinetics of receptor alkylation by AChM in intact CHO hM₂ cells. McN-A-343 is a novel muscarinic agonist (Roszkowski, 1961) that activates M₁ and M₄ muscarinic subtypes selectively over the M₂ and M₃ subtypes (Lazareno et al., 1993). McN-A-343 caused a concentration-dependent slowing in the rate of receptor alkylation by AChM when the latter was used at a concentration of 1.0 mM (Figure 6a). The inhibitory effect continued to increase with the concentration of McN-A-343 and did not reach a plateau at the highest concentration tested (10 mM). The data are not inconsistent with a competitive mechanism, and regression analysis of the data according to equation 7 yielded an estimate $\pm$ SEM of 4.79 ± 0.063 for the affinity constant of McN-A-343. There was no significant improvement in residual error when the allosteric model was fitted to the data ($F_{1,45} = 2.327$; $P = 0.13$).

We repeated the experiments with McN-A-343 on cellular homogenates because the rate of receptor alkylation by AChM was faster in homogenates, and hence, this preparation provided a larger “window” for measuring a slowing in the rate of alkylation. The faster rate in homogenates may be due to the lack of recycling of receptor from an intracellular compartment in intact cells during the washing step and subsequent binding assay with [³H]NMS. The component of binding resistant to AChM was less in homogenates (approximately 11%), which is consistent with the recycling hypothesis. McN-A-343 caused a concentration-dependent slowing in the rate of receptor alkylation by AChM when used at concentrations of 0.1 and 1.0

mM (Figure 6*b* and *c*, respectively). Global nonlinear regression analysis of the data according to equation 7 showed that the data were consistent with a competitive mechanism with the estimate of the dissociation constant of McN-A-343 being 4.77 ± 0.038 . There was no significant improvement in residual error when the data were fitted to the allosteric model (equation 5). We used the allosteric model, nonetheless, to estimate the minimum amount of negative cooperativity that would cause a significant increase in residual error ($P = 0.05$) assuming an allosteric mechanism. This minimum estimate of the log cooperativity factor ($\text{Log } \alpha$) was -4.02 . Consequently, although the data are consistent with a competitive mechanism, it is impossible to rule out an allosteric mechanism with high negative cooperativity (absolute value of $\text{Log } \alpha$ greater than 4.02).

We also analyzed the effects of ligands on the rate of receptor alkylation using the empirical approach described under “Methods,” which considers the effect of the ligand on the observed rate constant for receptor alkylation by AChM (Ehlert and Jenden, 1985). This effect is denoted by the ratio R , which represents the time constant for alkylation by AChM in the presence of a test ligand divided by that measured in its absence. Figure 7 shows a plot of $\text{Log } R$ against the log concentration of the inhibitor. If the mechanism for inhibition of alkylation is competitive, then the slope of the plot should increase with the inhibitor concentration and approach an asymptote having a slope of one. The concentration of ligand yielding an R value of 2 is equivalent to the observed dissociation constant of the inhibitor (K_{obs}) with $K_{obs} = (1 + XK_I)/K_i$, in which K_I and K_i denote the affinity constants of the aziridinium ion and the inhibitor, and X denotes the concentration of the aziridinium ion. When the concentration of aziridinium ion is low relative to the reciprocal of its affinity constant, then K_{obs} is approximately equivalent to the reciprocal of K_i . Figure 7*a* shows the results obtained with intact CHO hM₂ cells. The data with NMS and McN-A343 exhibit competitive-like behavior and were consistent with equation 12. The data for gallamine are consistent with an allosteric mechanism exhibiting a large amount of negative cooperativity (equation 13; $\text{Log } \alpha = -2.2$). That is, at low concentrations of AChM, the allosteric modulator exhibits a competitive-like inhibitory effect over a range of concentrations, but as the concentration of AChM increases, the effect of the modulator reaches a plateau at high concentrations. Figure 7*b* shows similar results obtained with McN-A-343 in homogenates of CHO hM₂ cells. The mechanism is consistent with competition.

A summary of the results of regression analysis of the data in Figure 7 is given in the figure legend. The data for McN-A-343 in homogenates and those for NMS and gallamine in intact cells were collected at two concentrations of AChM. To test whether the behavior of a ligand was consistent with competition (equation 12) or allosterism (equation 13), the data obtained at the two concentrations of AChM (i.e., six R values) were analyzed simultaneously, sharing the estimates of the parameters between the two sets of data. For McN-A-343 in intact cells, only one concentration of AChM was used, and the parameter estimates under this condition are based on the best fit to the three R values. The results this analysis are consistent with those described above in connection with Figure 3 – Figure 6.

Competitive binding experiments with acetylcholine mustard, gallamine and McN-A-343

The rate of alkylation of the M₂ muscarinic receptor by the aziridinium ion of AChM was negligible at 0°C (Figure 8*a*). This behavior provided a means to assess the interaction of AChM and its transformation products with the M₂ receptor using a standard competitive binding assay and to determine whether the binding constants so determined were in agreement with those estimated in the alkylation experiments. We measured the competitive inhibition of the binding of [³H]NMS by increasing concentrations of cyclized AChM (Figure 8*b*). To assess the potency of the aziridinium ion, aliquots of cyclized AChM were added directly into the competitive binding assay at 0°C. We also measured the competitive inhibition of the binding

of [^3H]NMS by increasing concentrations of the parent AChM and the alcoholic hydrolysis product of the aziridinium ion (Figure 8b). When the parent mustard and the alcoholic hydrolysis product were investigated, the incubation included 10 mM $\text{Na}_2\text{S}_2\text{O}_3$ to inactivate any aziridinium ion that might form during the assay, which is unlikely to be appreciable at 0°C . The result shows that the parent mustard at concentrations below 0.3 mM had no significant effect on [^3H]NMS binding, but inhibited about 15% of [^3H]NMS binding at a concentration of 1 mM. The alcoholic hydrolysis product of AChM had no significant effect on [^3H]NMS binding. In contrast, the aziridinium ion was a moderately potent inhibitor with a pIC_{50} value $\pm$ SEM of 3.87 ± 0.05 . This estimate was corrected for the competitive effect of [^3H]NMS to yield the true pK_i value (4.85). This estimate is similar to that estimated kinetically from the data shown in Figure 2 (4.62). The small discrepancy may be attributed to the difference in temperature between the two assays.

The influence of various concentrations of gallamine and McN-A-343 on the binding of [^3H]NMS at a fixed concentration is shown in Figure 8c. Gallamine caused a concentration-dependent inhibition of [^3H]NMS binding with IC_{50} for this effect being $6.5\text{ }\mu\text{M}$. Over the concentration range investigated, gallamine did not fully displace specific [^3H]NMS binding, but rather, the binding reached a plateau of 4.5% at high concentration of gallamine. Knowing this plateau value and the equilibrium dissociation constant of [^3H]NMS, it is possible to estimate a cooperativity value of 0.0043 for the interaction between [^3H]NMS and gallamine (Ehlert, 1988). It is also possible to estimate the dissociation constant of gallamine in the absence of [^3H]NMS to be $0.63\text{ }\mu\text{M}$ ($pK_2 = 6.2$). This value is similar to that estimated from the data in Figure 4 ($pK_2 = 6.35$), where allosteric inhibition of the kinetics of receptor alkylation was measured. In contrast, McN-A-343 caused a concentration-dependent inhibition of [^3H]NMS binding with complete displacement of specific binding occurring at a high concentration of McN-A-343. The IC_{50} value of McN-A-343 (0.33 mM) can be corrected to yield a dissociation constant of $30\text{ }\mu\text{M}$ ($pK_i = 4.52$). This value is similar to that estimated by competitive inhibition of the kinetics of receptor alkylation ($pK_i = 4.77$; Figure 6)

DISCUSSION

The aziridinium ion of AChM behaves as an agonist in isolated smooth muscle with a potency approximately one third that of acetylcholine, indicating that the compound quickly binds to the muscarinic receptor forming a reversible complex that elicits a response (Hirst and Jackson, 1972; Hudgins and Stubbins, 1972). Similar results have been observed in GTPase assays on membranes prepared from CHO cells transfected with the M_1 , M_2 and M_4 muscarinic receptors (Spalding et al., 1994). If cells or tissue expressing muscarinic receptors are first incubated with the aziridinium ion of AChM for periods of time up to an hour followed by washing, there is a subsequent loss of [^3H]radioligand binding, demonstrating that the compound binds irreversibly to muscarinic receptors (Robinson et al., 1975; Spalding et al., 1994). When CHO cells expressing M_1 and M_4 receptors (about 200 – 900 fmol/mg protein) were first treated with AChM, membranes prepared from these cells lack persistent agonist activity in GTPase assays, indicating that the covalent M_1 receptor-AChM complex is devoid of agonist activity (Spalding et al., 1994). Our results showing that treatment of CHO hM_2 cells with AChM causes an irreversible decrease in the binding capacity of [^3H]NMS with no change in affinity is consistent with these prior reports.

The observed rate constant for alkylation of the M_2 muscarinic receptor by the aziridinium ion of AChM increased as a mass-action-like function of the concentration of the aziridinium ion, suggesting that the rate of receptor alkylation is proportional to receptor occupancy. This model predicts that the concentration of aziridinium ion required for half-maximal receptor alkylation is equivalent to the dissociation constant of the reversible receptor complex. This appears to be the case as we found that the estimate of the affinity constant of the aziridinium ion was

approximately the same when measured from the kinetics of receptor alkylation or from competitive binding assays at temperatures (0°C) where little receptor alkylation occurs. Overall, these results are consistent with the hypothesis that the aziridinium rapidly equilibrates with the receptor to form a reversible complex having a log affinity constant of 4.62. The reversible complex then converts to a covalent complex at a relatively slower rate of 0.16 min⁻¹. This mechanism was first proposed for AChM based on the kinetics of its inactivation of muscarinic receptors in strips of the longitudinal muscle of the ileum (Robinson et al., 1975). The same mechanism also accounts for the interaction of an alkylating derivative of oxotremorine-M with muscarinic receptors in rat cerebral cortex (Ehlert and Jenden, 1985).

NMS caused a progressive, concentration-dependent slowing and ultimate cessation in the alkylation of the M₂ receptor by AChM when the aziridinium ion was used at concentrations ranging from 0.071 to 0.71 mM (assuming a 71% conversion of parent mustard into aziridinium ion; see Methods). The mechanism of inhibition was consistent with simple competition. Although gallamine caused a concentration dependent slowing in the rate of alkylation of the M₂ receptor by AChM (0.1 mM), this effect reached a plateau when the concentration of the mustard was raised to 1 mM. At this concentration, a near maximal slowing in the rate of alkylation was caused by gallamine at 0.1 mM, with little or no further slowing occurring at 1.0 mM. These results are consistent with a mechanism where gallamine binds to an allosteric site to reduce the affinity of the aziridinium ion of AChM for the orthosteric site such that its affinity is approximately only one percent that of its initial affinity. Thus, if the concentration of the aziridinium ion is sufficiently high to bind to the M₂ receptor with gallamine occupying the allosteric site, then alkylation should proceed without being slowed by a further increase in the concentration of gallamine.

In our simple allosteric model described in scheme 1, we assume that the rate constant (k_I) for alkylation of the aziridinium ion-receptor complex (XR) is the same as that of the aziridinium ion-receptor-gallamine complex (XRA). If the two rate constants differ, then the model shown in scheme 2 applies. In this model, k_I' and k_I'' denote the rate constants for alkylation of the XR and XRA complexes respectively. It can be shown that in our system, the observed estimate of the cooperativity between the binding of the allosteric modulator and the aziridinium ion of AChM (α_{obs}) is equivalent to ak_I''/k_I' . Since our estimate of the cooperativity between the aziridinium ion of AChM and gallamine is similar to that calculated for the interaction between gallamine and acetylcholine (Ehlert and Griffin, 2008), it seems unlikely that there is much difference between k_I' and k_I'' (i.e., $k_I''/k_I' = 1$ and $\alpha_{\text{obs}} = \alpha$). AChM is known to alkylate an aspartic acid residue at the orthosteric site (D105 in the M₁ sequence) (Spalding et al., 1994), and it appears unlikely that gallamine alters the conformation of the active site because it is without effect on the intrinsic efficacy of acetylcholine (Ehlert and Griffin, 2008). A more likely explanation is that gallamine alters access to the orthosteric site, perhaps through modification of a relay site (Ehlert and Griffin, 2008). Such a mechanism can easily account for a similarity in k_I' and k_I'' .

Our studies with McN-A-343 suggest that this compound competitively inhibits the alkylation of the M₂ muscarinic receptor by AChM, implying that at least part of the binding sites for these two molecules overlap. Alternatively, it is possible that McN-A-343 acts allosterically with AChM and exhibits a huge negative cooperativity ($-\text{Log } \alpha > 4$). This estimate represents the minimum required to explain our data. In studies on isolated atria, Christopoulos and Mitchelson (1997) suggested that McN-A-343 interacts competitively with carbachol, but calculated a minimum $-\text{Log } \alpha$ of 3.0 for McN-A-343 if the mechanism were actually allosteric. Thus, using our approach we have been able to push this minimum estimate further, suggesting that the simpler, alternative explanation is more likely - competitive inhibition. Site directed mutagenesis studies have revealed differences in the interaction of McN-A-343 and orthosteric agonists with the M₂ receptor, which has led to the conclusion that McN-A-343 interacts at

the allosteric site on the muscarinic receptor (May et al., 2007). Since the McN-A-343 molecule is considerably larger than acetylcholine, it is possible that part of McN-A-343 does interact with accessory regions of the orthosteric site that may include the allosteric site, whereas the quaternary ammonium part of the molecule competes with acetylcholine at the orthosteric site. Such a mode of interaction is consistent with competitive behavior (i.e., the sites occupied by acetylcholine and McN-A-343 overlap, and hence the binding of either ligand is mutually exclusive).

Our ability to discriminate an allosteric interaction from simple competition depends on the rapid equilibration of the test drug and the aziridinium ion of AChM with the receptor. This condition seems to occur in the case of gallamine because the results of functional studies with both high concentrations of gallamine (1 mM) and acetylcholine have shown that the response to acetylcholine is generated quickly and stably after pre-equilibration of the tissue with gallamine (Clark and Mitchelson, 1976). A similar result was obtained when the response to acetylcholine was antagonized by McN-A-343 (0.1 mM) (Christopoulos and Mitchelson, 1997). Also, in study of the binding of the allosteric ligand [³H]dimethyl W-84 to M₂ muscarinic receptors, it was shown that equilibrium was attained within seconds (Trankle et al., 2003). If gallamine did slow and eventually stop the association of AChM with the muscarinic receptor, then it would have behaved like a competitive antagonist in our assay. Our ability to detect gallamine as an allosteric modulator and estimate a cooperativity value consistent with that measured in functional studies with acetylcholine strongly suggests that AChM equilibrates rapidly with the muscarinic receptor in the presence of gallamine. We have found that gallamine exhibits a competitive like behavior when benzilylcholine mustard is used as the alkylating agent (unpublished observation). This behavior can be explained by the observation that gallamine, when bound to its allosteric site, prevents benzilylcholine mustard from accessing the orthosteric binding site. Apparently this situation does not occur with acetylcholine and its mustard derivative, presumably because of their smaller size. It appears that those instances in which a slowing in the kinetics of orthosteric ligand binding by gallamine and McN-A-343 has been reported, it is in connection with [³H]NMS and not a small orthosteric ligand.

Our method of characterizing the modulation of receptor alkylation kinetics is a powerful approach for discriminating high negative cooperativity from competitive inhibition, provided that small, site directed electrophiles with rapid kinetics are used.

Abbreviations

ACh, acetylcholine; AChM, acetylcholine mustard; CHO, Chinese hamster ovary; *EC*₅₀, concentration of agonist eliciting half-maximal response; KRB, Krebs Ringer bicarbonate; McN-A-343, 4-(trimethylamino)-2-butynyl-(3-chlorophenyl)carbamate; NMS, N-methylscopolamine; WIN 51,708, (17-β-hydroxy-17-α-ethynyl-5-α-androstano[3,2-b]pyrimido[1,2-a]benzimidazole).

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Figure 1. The effect of AChM on the binding of [³H]NMS to homogenates of CHO hM₂ cells
 Cellular homogenates were incubated with cyclized AChM for four min at 37°C, and the reaction was stopped by the addition of Na₂S₂O₃. The homogenates were washed by centrifugation and suspension in fresh buffer. Control homogenates were incubated similarly except for exposure to AChM. The specific binding of [³H]NMS was measured at various concentrations of radioligand. The symbols represent the mean values ± SEM from three control and two AChM-treated experiments, each done in triplicate. The data have been normalized relative to the binding capacity of [³H]NMS in control homogenates.

Figure 2. The kinetics of the alkylation of the M₂ muscarinic receptor by cyclized AChM

a CHO hM₂ cells were incubated at 37°C with various concentrations of cyclized AChM for the indicated times, washed and assayed for [³H]NMS binding. The concentrations of cyclized AChM shown in the figure denote the initial concentration of the parent mustard. The symbols represent the mean binding values $\pm$ SEM of two (1 mM AChM) and five (1, 10 and 100 μ M AChM) separate experiments, each done in triplicate. The SEM values vary from 3.0 to 0.2% making the span of the error bars smaller than the height of the symbols in many instances. The theoretical curve represents the least squares fit of equation 6 to the data. The concentration of [³H]NMS was 1 nM. *b*, The observed, first order rate constants for the loss of [³H]NMS binding at each concentration of AChM were calculated from the data in Figure 2*a* and are plotted against the log concentration of AChM. A simple one-site binding model was fitted to the data.

Figure 3. The effect of NMS on the rate of alkylation of the M₂ muscarinic receptor by cyclized AChM

CHO hM₂ cells were incubated at 37°C with AChM at concentrations of 0.1 mM (a) or 1.0 mM (b) for the indicated times in the absence and presence of various concentrations of NMS, washed and assayed for [³H]NMS binding. The concentrations of NMS are shown in the legend. The symbols represent the mean binding values ± SEM of two (a) and three (b) separate experiments, each done in triplicate. The SEM values in b vary from 1.6 to 0.4%, making the span of the error bars less than the height of the symbols. The concentration of [³H]NMS was 1 nM.

Figure 4. The effect of gallamine on the rate of alkylation of the M_2 muscarinic receptor by cyclized AChM

CHO hM₂ cells were incubated at 37°C with AChM at concentrations of 0.1 mM (*a*) or 1.0 mM (*b*) for the indicated times in the absence and presence of various concentrations of gallamine, washed and assayed for [³H]NMS binding. The concentrations of gallamine are shown in the figure. The symbols represent the mean binding values $\pm$ SEM of two (*a*) and three (*b*) separate experiments, each done in triplicate. The SEM values in *b* vary from 2.0 to 0.4%, making the span of the error bars less than the height of the symbols. The concentration of [³H]NMS was 1 nM.

Figure 5. The effect of WIN 51708 on the rate of alkylation of the M_2 muscarinic receptor by cyclized AChM

a, Homogenates of CHO h M_2 cells were incubated at 37°C with AChM (0.01 mM) for the indicated times in the absence and presence of various concentrations of WIN 51708, washed and assayed for [^3H]NMS binding. *b*, The experiment was the same as that shown in *a* except that intact CHO h M_2 cells were used and the concentration of AChM was 0.1 mM. The symbols represent the mean binding values $\pm$ SEM of two separate experiments, each done in triplicate. The concentration of [^3H]NMS was 1 nM.

Figure 6. The effect of McN-A-343 on the rate of alkylation of the M_2 muscarinic receptor by cyclized AChM

a, CHO hM_2 cells were incubated at 37°C with 1 mM AChM for the indicated times in the absence and presence of various concentrations of McN-A-343, washed and assayed for [3 H] NMS binding. Since treatment of intact cells with McN-A-343 (10 mM) alone decreased [3 H] NMS binding, the data obtained in the presence of McN-A-343 (10 mM) were expressed as a percent of [3 H]NMS binding in cells treated with McN-A-343 (10 mM). *b*, The experiment was the same as that shown in *a* except that homogenates of CHO hM_2 cells were used and the concentration of AChM was 0.1 mM. *c*, Same as *b* except that the concentration of AChM

was 1.0 mM. The symbols represent the mean binding values $\pm$ SEM of three (*a* and *b*) or two (*c*) separate experiments, each done in triplicate. The concentration of [³H]NMS was 1 nM.

Figure 7. The effects of NMS, gallamine and McN-A-343 on the observed time constant for alkylation ($1/k_{\text{obs}}$) of the M_2 muscarinic receptor by AChM

The Log ratio (R) of time constant for alkylation in the presence of the inhibitor divided by that measured in its absence is plotted against the log concentration of the inhibitor. The data in *a* were obtained in intact CHO hM₂ cells and those in *b* were obtained in homogenates of CHO hM₂ cells. The theoretical curves represent the best fit of equation 13 (gallamine) and 12 (NMS and McN-A-343) to the data. The data obtained for a given test ligand at the two concentrations of AChM (0.1 and 1.0 mM) were fitted simultaneously. Solid symbols and dashed lines represent data obtained with 0.1 mM AChM, whereas open symbols and solid lines denote data obtained with 1.0 mM AChM. The R values were calculated from the data shown in Figure 3, Figure 4 and Figure 6. The estimates of the parameters $\pm$ SEM are NMS, $pK_i = 10.06 \pm 0.12$; gallamine, $pK_2 = 6.53 \pm 0.11$, $\text{Log } \alpha = -2.20 \pm 0.10$; McN-A-343 in intact cells, $pK_i = 4.65 \pm 0.06$; McN-A-343 in homogenates, $pK_i = 4.65 \pm 0.08$.

Figure 8. Effects of AChM, its transformation products, gallamine and McN-A-343 on the binding of [^3H]NMS to intact CHO cells at 0°C

a, CHO hM₂ cells were incubated with various concentrations of the aziridinium ion of AChM for one hour at 0°C, washed and then assayed for [^3H]NMS binding at 0°C. *b*, The competitive inhibition of [^3H]NMS binding to the CHO hM₂ cells by the parent mustard, AChM, its aziridinium ion and alcoholic hydrolysis product was measured at 0°C. *c*, The specific binding of [^3H]NMS was measured at a fixed concentration (1 nM) in the presence of various concentrations of gallamine and McN-A-343. The symbols represent the mean binding values $\pm$ SEM of three experiments, each done in triplicate, with the exception of the parent mustard and alcoholic hydrolysis product, which were investigated in two experiments in triplicate.

Scheme 1. Simple model for the interaction of the aziridinium ion of AChM (X) with the M₂ receptor (R) in the presence of an allosteric modulator (A)

The aziridinium ion first forms a reversible complex with the receptor (XR), which converts to a covalent complex (X-R) at a relatively slower rate. K_1 and K_2 denote the affinity constants of the reversible complexes of XR and RA, α denotes the cooperativity factor for the allosteric interaction, and k_1 denotes the rate constant for alkylation of the receptor. In this simple model the rate constant for alkylation of the binary (XR) and ternary (XRA) complexes is the same.

Scheme 2. Complex model for the interaction of the aziridinium ion of AChM (X) with the M₂ receptor (R) in the presence of an allosteric modulator (A)

The aziridinium ion first forms a reversible complex with the receptor (XR), which converts to a covalent complex (X-R) at a relatively slower rate. K_1 and K_2 denote the affinity constants of the reversible complexes of XR and RA, and α denotes the cooperativity factor for the allosteric interaction. In this complex model the rate constant (k_1') for alkylation of the binary complex (XR) is different from that (k_1'') of the ternary complex (XRA).