Effects of N-Ethylmaleimide on Conformational Equilibria in Purified Cardiac Muscarinic Receptors*

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Muscarinic receptors purified from porcine atria and devoid of G protein underwent a 9–27-fold decrease in their apparent affinity for the agonists quinuclidinyl benzilate, N-methylscopolamine, and scopolamine when treated with the thiol-selective reagent N-ethylmaleimide. Their apparent affinity for the agonists carbachol and oxotremorine-M was unchanged. Conversely, the rate of alkylation by N-ethylmaleimide, as monitored by the binding of [3H]quinuclidinyl benzilate, was decreased by antagonists while agonists were without effect. The receptor also underwent a time-dependent inactivation that was hastened by N-ethylmaleimide but slowed by quinuclidinyl benzilate and N-methylscopolamine. The destabilizing effect of N-ethylmaleimide was counteracted fully or nearly so at saturating concentrations of each antagonist and the agonist carbachol. Similar effects occurred with human M₃ receptors differentially tagged with the c-Myc and FLAG epitopes, coexpressed in SF9 cells, and extracted in digitonin/cholate. The degree of communoprecipitation was unchanged by N-ethylmaleimide, which therefore was without discernible effect on oligomeric size. The data are quantitatively consistent with a model in which the purified receptor from porcine atria interconverts spontaneously between two states (i.e. R = R*). Antagonists favor the R state; agonists and N-ethylmaleimide favor the comparatively unstable R* state, which predominates after purification. Occupancy by a ligand stabilizes both states, and antagonists impede alkylation by favoring R over R*. Similarities with constitutively active receptors suggest that R and R* are akin to the inactive and active states, respectively. Purified M₂ receptors therefore appear to exist predominantly in their active state.

Sulphydryl-specific reagents have been useful probes of the relationship between structure and function in muscarinic and other G protein-coupled receptors (e.g. Refs. 1–5). In recent studies, such reagents have been tagged with environmentally sensitive fluorescent probes or spin labels and used to detect a conformational change linked to activation (1–3). That change is thought to involve the rotation or tilting of the sixth transmembrane helix (1, 3–5). The hydrophilic nature of some reagents has been exploited to suggest that the conformational change leads to increased accessibility of the reactive residue (e.g. Refs. 4 and 5).

N-Ethylmaleimide is among the most widely used sulphydryl-specific reagents. In the case of muscarinic receptors, as with many others, it has been shown to affect receptor-G protein coupling and to have a differential effect on the binding properties of agonists and antagonists. With receptors in native membranes, the Hill coefficients for the binding of muscarinic agonists are increased from characteristically low values to values near or indistinguishable from 1; moreover, the shift in potency brought about by guanyl nucleotides is reduced or abolished (e.g. Refs. 6–9). The affinity of muscarinic antagonists generally is not affected by N-ethylmaleimide (7–12), whereas the affinity of agonists can be either increased (8, 10–14) or decreased (6, 9).

It has been suggested that N-ethylmaleimide reacts preferentially with one of two spontaneously interconverting states of the receptor, thereby driving the equilibrium toward the reactive conformation (10–12). Further support for the existence of multiple interconverting states, only one or comparatively few of which are functionally active, derives from the occurrence of constitutive activity as seen, for example, in mutants of the β₂ adrenergic receptor (15) or the M₅ muscarinic receptor (16). In the latter case, it was demonstrated that the ligand-regulated activity of 13 constitutively active mutants, differing only at position 465 in helix number 6, can be accounted for by shifts in a single equilibrium between an inactive and an active state (16). Also, the notion of spontaneously interconverting states is consistent with the constitutive activity and related properties of M₁–M₄ muscarinic receptors overexpressed in Chinese hamster ovary cells (17), and of M₅ muscarinic receptors coexpressed with comparatively large amounts of Gₛ (18).

In the present study, N-ethylmaleimide has been used to probe for multiple states of cardiac muscarinic receptors purified from porcine atria and devoid of G protein. The effects of the reagent on the binding properties of the receptor, and the countervailing effects of muscarinic ligands on the action of N-ethylmaleimide, have been studied with the system under both thermodynamic and kinetic control. The data are quantitatively consistent with a model in which the receptor interconverts spontaneously between two states that are intrinsic to the receptor alone. N-Ethylmaleimide appears to favor the state that is of weaker affinity for antagonists and also undergoes a comparatively rapid inactivation, a pattern that is shared by constitutively active receptors (e.g. Refs. 4, 19, and 20).

MATERIALS AND METHODS

Ligands, Detergents, Antisera, and Other Materials—N-[3H]Methylscopolamine was obtained as the chloride salt from PerkinElmer Life Sciences (lot 3406009, 83.5 Ci/mmol) and as the bromide salt from Amersham Biosciences (batch 27, 78.3 Ci/mmol). (−)-[3H]Quinuclidinyl benzilate was obtained from PerkinElmer Life Sciences (lot 3329907, 78.3 Ci/mmol). 

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N-Ethylmaleimide and Purified Muscarinic Receptors

G. Kaur and J. W. Wells, unpublished observations.

The muscarinic receptors purified from Sigma (ultra grade). Digitonin was obtained from Wako Bioproducts at a purity near 100% and at high purity from Roche Molecular Diagnostics and Calbiochem. The product from Roche Molecular Diagnostics was used to solubilize the vesicles from other sources was used to prepare and wash the columns used in various procedures. Sodium cholate was purchased from Sigma at a purity of 99% or better. Murine antibodies to the c-Myc epitope (9E10) conjugated to agarose were purchased from Santa Cruz Biotechnology, Inc. Murine antibodies to the FLAG epitope conjugated to horseradish peroxidase were from Sigma. Other materials were obtained from the sources indicated previously (21).

Muscarinic Receptors—Purified M2 receptor solubilized in digitonin/cholate (0.1% digitonin, 0.02% sodium cholate) was prepared from porcine atria essentially as described previously (22). Receptor was extracted from the sarcosomal fraction of the sarcosome gradient according to the procedure of Peters and Schmerlik (23), except that the first step of the extraction was carried out with 0.36% digitonin and 0.07% sodium cholate. Subsequent passage through DEAE-Sepharose (Amersham Biosciences), 3-(2-aminobenzyldihydroxy)tropane-Sepharose, and hydroxypatinate (CHT-II, Bio-Rad) was carried out as described previously (21, 22). The buffer used to elute the purified receptor from hydroxypatinate was exchanged for the same buffer D (20 mM KH2PO4, 20 mM NaCl, 1 mM Na2EDTA, 0.1 mM PMSF, 0.1% digitonin, and 0.02% sodium cholate, adjusted to pH 7.40 with KOH) (21) to obtain a stock solution that was divided into aliquots sufficient for one experiment and stored at −75 °C.

The specific activity of the purified preparation was 54 μmol of protein, as estimated from the maximal binding of [3H]quinuclidinyl benzilate (QNB). The corresponding purity was 28%, based on the maximal binding of [3H]quinuclidinyl benzilate (QNB). 1 The corresponding purity was 28%, based on the maximal binding of [3H]quinuclidinyl benzilate to controls from which the concentration of receptor was 6.5 pmol l in buffer A (20 mM HEPES, 20 mM NaCl, 5 mM MgSO4,1 mM Na2EDTA, 0.1 mM PMSF, 0.1% digitonin, and 0.02% sodium cholate, adjusted to pH 7.40 with KOH) for assays with solubilized or reconstituted receptors, respectively. An aliquot (5 pmol) of receptor, respectively. An aliquot (5 pmol) of receptor, respectively. An aliquot (5 pmol) of receptor, respectively. An aliquot (5 pmol) of receptor, respectively. An aliquot (5 pmol) of receptor, respectively. An aliquot (5 pmol) of receptor, respectively. An aliquot (5 pmol) of receptor, respectively. An aliquot (5 pmol) of receptor, respectively.

Human M2 muscarinic receptors tagged with the c-Myc or FLAG epitope were co-expressed in SF9 cells and extracted in digitonin/cholate (26). The final composition of the buffer was 15.7 mM KH2PO4, 15.7 mM NaCl, 0.78 mM Na2EDTA, 0.078 mM PMSF, 0.86% digitonin, and 0.17% sodium cholate, adjusted to pH 7.60 with KOH. The extract was stored at −75 °C and used without further purification in subsequent assays. Complementary DNA coding for the wild-type and c-Myc-tagged human M2 muscarinic receptor and cloned into a baculoviral vector was obtained from Biosignal, Inc. (Montreal). The construction of the baculovirus coding for FLAG-tagged human M2 receptor has been described elsewhere (26).

Binding with N-Ethylmaleimide—To prepare samples for use in the binding assays, an aliquot of purified receptor from porcine atria or the solubilized extract from SF9 cells (28–65 μl) was thawed and diluted to about 450 μl in buffer A (20 mM HEPES, 20 mM NaCl, 5 mM MgSO4, 1 mM Na2EDTA, 0.1 mM PMSF, 0.1% digitonin, and 0.02% sodium cholate, adjusted to pH 7.40 with NaOH) supplemented with N-ethylmaleimide. A fresh solution of N-ethylmaleimide was prepared for each reaction and was prepared in the same manner except that N-ethylmaleimide was omitted. The final concentration of N-ethylmaleimide in the reaction mixture was 10 mM except where stated otherwise; the concentration of receptor was 6.5–10 nm, as estimated from the binding of [3H]quinuclidinyl benzilate to controls from which N-ethylmaleimide was omitted. The reaction mixture was kept in an ice bath for 24 h, unless stated otherwise, and aliquots then were introduced directly into the binding assays. N-Ethylmaleimide was without effect on the binding of [3H]quinuclidinyl benzilate and therefore was not removed in most experiments. All volumes were recorded, and the final concentration of receptor was corrected as required in subsequent calculations.

When the reaction mixture was to contain N-ethylmaleimide together with either carbobalch or N-methylcopolamolipin, the ligand was preincubated with the undiluted thawed receptor in an ice bath for 30 min. N-Ethylmaleimide then was added, dissolved in deionized water at a concentration of 10 mM. The total volume of the reaction mixture was 20–200 μl. Controls were prepared in the same manner except for the omission of N-ethylmaleimide or the muscarinic ligand, as required. After the desired period of incubation in the ice bath, the volume of the reaction mixture was adjusted to 200 μl with buffer A, and the sample was desalted on a column of Sephadex G-50 (fine) (0.8 × 5.0 cm) previously equilibrated with buffer A. The eluate from the Sephadex column was used directly in the binding assays. To test for any effect of N-ethylmaleimide on the binding of [3H]quinuclidinyl benzilate, samples prepared with the reagent were kept in an ice bath for 24 h, diluted with buffer A, and then desalted on Sephadex G-50 as described above.

Reconstitution—Native and alkylated receptors were reconstituted with isolated membranes according to the procedure adapted from that described by Haga et al. (27). The G proteins were obtained as a mixture of isoforms purified from bovine brain (Calbiochem). Following incubation of the purified receptor for 24 h at 0 °C with or without N-ethylmaleimide, as described above, the native or alkylated product (3.6–7 pmol in 38–80 μl) was incubated with carbobalch (10 μl) for about 15 min, also at 0 °C. The receptor then was mixed (1:1) with a suspension of lipids (0.6 mg/ml L-α-phosphatidylcholine, 0.6 mg/ml α-phosphatidyl- \( L \)-serine, and 60 μg/ml cholesterol; Sigma) prepared in buffer B (20 mM HEPES, 160 mM NaCl, 6 mM MgCl2, 0.8 mM Na2EDTA, and 1 mM dithiothreitol, adjusted to pH 8.0 with KOH) supplemented with 0.18% sodium deoxycholate and 0.04% sodium cholate. The G proteins were added to the mixture (32–40 pmol of Gαi, 8–16 pmol Gβγ, 8–18 pmol of Gβ, and 64 pmol of Gs), which then was applied to a column of Sephadex G-50 (0.8 × 5.0 cm) pre-equilibrated with buffer B. The column was eluted with buffer B, and the reconstituted receptors emerged in the void volume (450 μl) at a concentration of 1.6–4.9 nm. The vesicles were uniform in size with a mean diameter of 30 ± 11 nm (n = 6), as determined previously by electron microscopy.2 Binding assays were performed on aliquots taken directly from the eluate of the Sephadex column.

Immunoprecipitation, Polyacrylamide Gel Electrophoresis, Western Blotting, and Immunodetection—The co-immunoprecipitation of c-Myc- and FLAG-tagged M2 receptors extracted from SF9 membranes was carried out and monitored as described previously (26). Polyacrylamide gels that were to be silver-stained were fixed for 30 min in a solution of 50% methanol and 10% acetic acid. The solution was rinsed off, and the gel was stained for 24 h at 4 °C in a solution of 5% tris(2-carboxyethyl)phosphine and 0.02% sodium citrate. Development was terminated by incubating the gel with sodium citrate (0.25 M) for 10 min. The gel was incubated further with dithiothreitol (5 μg/ml) for 5 min, rinsed, and stained for 30 min with a solution of silver nitrate (0.1%) (British Drug Houses). Bands were developed with 1.8% formaldehyde prepared in 3% sodium carbonate solution. As soon as the bands appeared, the development was terminated by incubating the gel with sodium citrate (2.3%) for 10 min. Densitometry scanning was performed at a resolution of 300 dots per inch, and the intensity was determined using 1D Image Analysis software (Kodak Digital Science). Further details have been described elsewhere (26).

Binding Assays—Binding was measured essentially as described previously (21). Solutions of the radioligand and any unlabeled ligands were prepared in buffer A or buffer C (25 mM KH2PO4, 4 mM HEPES, 250 mM NaCl, 10 mM MgCl2, 0.8 mM Na2EDTA, and 0.1 mM PMSF, adjusted to pH 7.60 with KOH) for assays with solubilized or reconstituted receptor, respectively. An aliquot (5 μl) then was added to the receptor (48 μl) in polypropylene microcentrifuge tubes pretreated with trimethylchlorosilane. The reaction mixture was incubated at 30 °C for the required period of time, and the bound radioligand was separated from the free ligand by centrifugation (15000 × g) for 1.5 min. The gel was washed with dithiothreitol (5 μg/ml) for 5 min, rinsed, and stained for 30 min with a solution of silver nitrate (0.1%) (British Drug Houses). Bands were developed with 1.8% formaldehyde prepared in 3% sodium carbonate solution. As soon as the bands appeared, the development was terminated by incubating the gel with sodium citrate (2.3%) for 10 min. Densitometry scanning was performed at a resolution of 300 dots per inch, and the intensity was determined using 1D Image Analysis software (Kodak Digital Science). Further details have been described elsewhere (26).

1 The abbreviations used are: QNB, quinuclidinyl benzilate; GMP-PyNHP, 5'-guanylyl-5'-yl imidodiphosphate; NEM, N-ethylmaleimide; NMS, N-methylscopolamine; PMSF, phenylmethylsulfonyl fluoride.

2 A. Ma, A. B. Pawagi, and J. W. Wells, unpublished observations.
of time under the conditions of the experiments. In assays with $N$-$[^3]$H]methylscopolamine, the time of incubation was as stated in the tables or figures. To follow the time dependence of binding, an aliquot of the radioligand (552–673 μl) in buffer A was added to the receptor (58–78 μl) in polypropylene microcentrifuge tubes pretreated with tri-methylchlorosilane. The reaction mixture was incubated at 30 °C, and aliquots (50 μl) were removed at the desired times and applied to Sephadex G-50 as described above. Assays were performed in duplicate or triplicate, and the receptor was kept in an ice bath until mixed with the ligand and incubated at 30 °C.

Non-specific binding was taken throughout as total binding in the presence of 1 mM unlabeled N-methylscopolamine. The value increased linearly with the concentration of unbound radioligand. Expressed as the fraction $N_S$, as defined in Equations 1 and 4 below, the mean estimate of non-specific binding from several representative experiments was 0.000080 ± 0.000009 for $[^3]$H]quinuclidinyl benzilate (n = 19) and 0.00018 ± 0.000002 for $N$-$[^3]$H]methylscopolamine (n = 16).

In assays with $N$-$[^3]$H]methylscopolamine, ethanol that accompanied the radioligand accelerated the inactivation of receptors pretreated with N-ethylmaleimide. Most or all of the ethanol therefore was evaporated under argon before the radioligand was taken up in buffer. In such cases, the concentration of ethanol in the reaction mixture was less than 1% (v/v) at the highest concentration of $N$-$[^3]$H]methylscopolamine and decreased proportionately at lower concentrations. Removal of ethanol had no effect on the binding of $N$-$[^3]$H]methylscopolamine to untreated receptors or on the binding of $[^3]$H]quinuclidinyl benzilate to either preparation. The radioligand therefore was used as supplied by the manufacturer in these assays.

Analysis of Data—All data were analyzed with total binding taken as the dependent variable ($B_{obs}$) and with the total concentrations of all ligands taken as the independent variables. Subsequent manipulations did not alter the relationship between the data and the fitted curve.

Except where stated otherwise, estimates of binding ($B_{%}$, $B_{\%}$) and total receptor ($R_{T}$) denote the concentration in the binding assay (pat).

The concentrations of ligands denote the total molar concentration in the binding assay.

Empirical analyses of the data were based on the Hill equation (Equations 1 or 2) and on Equation 3. The variables $[P]$, $[A]$, and $[AR]$ represent the total concentrations of the radiolabeled probe and an unlabeled ligand, respectively. In Equation 1, $B_{%}$ represents specific binding at the corresponding value of $[P]$, and $B_{\%}$ represents maximal specific binding; the parameter $EC_{50}$ denotes the concentration of unbound radioligand that yields half-maximal occupancy, and $n_H$ is the Hill coefficient. The parameter $NS$ represents the fraction of unbound radioligand that appears as non-specific binding (cf. Equation 14 in Ref. 28). Equation 1 was solved numerically (28). In Equations 2 and 3, the parameters $B_{\%}$ and $B_{\%}$ represent the asymptotic levels of binding with respect to the concentration of unlabeled ligand; $EC_{50}$ is the concentration of unlabeled ligand that reduces specific binding by one-half. In Equation 3, $F_{1}$ represents that fraction of specific binding defined by the inhibitory potency $IC_{50/1}$.

$$B_{\%} = B_{\%} + (B_{\%} - B_{\%}) \times \frac{IC_{50}}{EC_{50} + [A]^{n_H}} \quad \text{(Eq. 3)}$$

Semi-empirical and mechanistic descriptions of the data were based on Schemes I–III, in which there are one or more classes of mutually independent sites that bind either the radioligand (P) or an unlabeled ligand (A) in a mutually exclusive manner. It is assumed in Schemes I and II that the system is at thermodynamic equilibrium, whereas the formulation of Scheme III includes time as an explicit variable. In each case, the model was fitted to the data by means of Equation 4, in which the variables and parameters are as described above. An appreciable fraction of the radioligand bound to the receptor under at least some conditions in most experiments. Depletion therefore was accommodated in the calculation of $B_{\%}$.

$$B_{\%} = (B_{\%} + NS\times[P] - B_{\%}) \quad \text{(Eq. 4)}$$

Scheme I comprises a mixture of distinct sites ($R_j, j = 1, 2, \ldots, n$) wherein those of type $j$ bind $P$ and $A$ with the equilibrium dissociation constants $K_{Pj}$ and $K_{Aj}$, respectively, and constitute the fraction $F_j$ of all sites (i.e. $F_j = [R_j]/[R]$, where $[R_j] = [AR_j] + [AP_j] + [PR_j]$, and $[R] = \sum_j [R_j]$). Total specific binding of the probe was calculated according to Equation 5, and the required values of $[PR]_j$ were obtained as described below. Values of $EC_{50}$ were calculated from the fitted estimates of $K_{ij}$ (i.e. $P$ or $A$) and $F_j$ (n > 1), as described previously (21).

In Scheme II, each receptor of type $j$ can exist in two spontaneously interconverting states designated $R_j$ and $R_{ij}$. The ligands $P$ and $A$ bind to $R_j$ with equilibrium dissociation constants $K_{Pj}$ and $K_{Aj}$ (e.g. $P[R_j]/[PR_j] = K_{Pj}$), and the relative affinity of the ligand for the two states of the receptor is designated $\alpha_{Pj}$ and $\alpha_{Aj}$, respectively (i.e. $\alpha_{Pj} = K_{Pj}/K_{Pj}$, $\alpha_{Aj} = K_{Aj}/K_{Aj}$, $\alpha_{Aj}^* = [A]/[AR]/[AR]^*$). The parameter $K_{ij}$ represents the relative concentrations of $R_j$ and $R_{ij}$* at equilibrium in the absence of ligand (i.e. $[R_j]/[R_{ij}^*] = K_{ij}$). Sites of type $j$ constitute the fraction $F_j$ of all sites, as inferred in Scheme I (i.e. $F_j = [R_j]/[R]$), where $[R_j] = [AR_j] + [AP_j] + [AR_j^*] + [PR_j] + [PR_j]^*$. The specific binding of the probe was calculated according to Equation 6, and the required values of $[PR_j]$ and $[PR_j]^*$ were obtained as described below.

$$B_{sp} = \sum_{j=1}^{n} [PR_j] + [PR_j]^* \quad \text{(Eq. 5)}$$

In Scheme III, the receptor can exist in two spontaneously interconverting states ($R_j$ and $R_{ij}$) that convert irreversibly to a third and fourth state ($R_{ij}$ and $R_{ij}$, respectively). The equilibrium between $R_j$ and $R_{ij}$ is analogous to that between $R_{ij}$ and $R_{ij}$ for receptors of type $j$ in Scheme II (i.e. $[R_j]/[R_{ij}] = K_{ij}$, $[PR_j]/[PR_{ij}] = K_{ij}$). Analyses in terms of Schemes II and III required two classes of noninterconverting sites to accommodate both native and alkylated receptors. That heterogeneity is not shown explicitly in Scheme III, which depicts multiple states within a single class of sites, but it was accommodated in the model used in the calculations. The parameters $h_{ij}$ and $h_{ij}$ in Scheme III denote the first- and second-order rate constants for the binding of a radioligand (P) to receptors in state $j$, and $K_{ij}$ is the corresponding equilibrium dissociation constant (i.e. $h_{ij} = [AR_j]/[AR_j] = K_{ij}$). Similarly, the rate of interconversion between $R_j$ and $R_{ij}$ is determined by $h_{ij}$ and $h_{ij}$ (i.e. $h_{ij} = [AR_j]/[AR_j] = K_{ij}$), and that between $PR_j$ and $PR_{ij}$ is determined by $h_{ij}$ and $h_{ij}$ (i.e. $h_{ij} = [PR_j]/[PR_j] = K_{ij}$). The rate of conversion of $R_j$ and $PR_j$ (j = 1 or 2) to $R_{ij}$ and $PR_{ij}$, respectively, is determined by $h_{ij}$ and $h_{ij}$.

The specific binding of the probe at any time $t$ was calculated according to Equation 7, and the required values of $[PR_j]$ were obtained as described below.

$$B_{sp} = [PR_j] + [PR_j] + [PR_j] + [PR_j] \quad \text{(Eq. 7)}$$

The value of $B_{sp}$ in Equations 5 and 6 was calculated from the expansions in terms of the total concentration of receptor and the free concentrations of both ligands (i.e. $[P]$ and $[A]$). The required values of $[P]$ and $[A]$ were obtained by solving the pair of implicit equations.
The affinity of the alkylated receptor for $[^3H]$quinuclidinyl benzilate to native and alkylated receptors from porcine atria is illustrated in Fig. 2. Binding to the untreated receptor was faster at higher concentrations of the radioligand, becoming virtually instantaneous at saturating concentrations. Binding to the alkylated receptor was instantaneous at all concentrations of the radioligand used in the assays. Under all conditions, binding remained stable for up to 4 h after the maximal level was attained.

Alkylation reduced the apparent affinity of purified receptors for $N$-$[^3H]$methylscopolamine (Table II, Fig. 3), consistent with the effect on $[^3H]$quinuclidinyl benzilate, but the magnitude of the change increased with the time of incubation with the radioligand. Incubation for 15 min caused a 10-fold increase in $EC_{50}$ from 2.7 nm with the native receptor to 28 nM after alkylation; incubation for 3 h caused a 30-fold increase, from 2.7 to 85 nM. The relative capacity for $N$-$[^3H]$methylscopolamine and $[^3H]$quinuclidinyl benzilate was about 95% with the native receptor and decreased appreciably, to about 77%, after treatment with $N$-ethylmaleimide (Table II, Fig. 3). The decrease was not sensitive to the time of incubation with $N$-$[^3H]$methylscopolamine. Also, it was due entirely to a decrease in the absolute capacity for $N$-$[^3H]$methylscopolamine, because the binding of $[^3H]$quinuclidinyl benzilate was stable for up to 4 h (Fig. 2). If ethanol was not removed from $N$-$[^3H]$methylscopolamine, the relative capacity of alkylated receptors for $N$-$[^3H]$methylscopolamine and $[^3H]$quinuclidinyl benzilate was 0.51 ± 0.02 after incubation for 45 min ($n = 5$), 0.37 ± 0.02 after 2 h ($n = 3$), and 0.31 after 3 h ($n = 1$).

In assays with $N$-$[^3H]$methylscopolamine, the time-dependent nature of the effect on $EC_{50}$ suggests that the alkylated receptor was unstable under those conditions. To confirm that the increase in $EC_{50}$ was not wholly a consequence of instability, but derived at least in part from a decrease in affinity per se, the binding of $N$-$[^3H]$methylscopolamine was measured after equilibration for 24 h at 0°C. In three such experiments, with native and treated receptor taken in parallel, the mean value of log $EC_{50}$ increased upon alkylation from $-9.02 ± 0.07$ to $-8.14 ± 0.42$. The corresponding estimates of capacity are 551 ± 97 and 481 ± 100 pm for the native and alkylated

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The data shown in Figs. 3 and 7 were analyzed in terms of Scheme I, and the parametric values are listed in Tables II and III, respectively. The data in Fig. 3B also were combined with those in Fig. 4 and analyzed in terms of Scheme III, as described under “Discussion” and in the legend to Fig. 3. Similarly, the data represented in Fig. 7 also were combined with those in Fig. 9A and analyzed in terms of Scheme II, as described under “Discussion” and in the legends to Figs. 7 and 9. The use of Scheme I is essentially empirical, because the model offers no explanation for the interplay between $N$-ethylmaleimide on the one hand and muscarinic ligands on the other. In contrast, Schemes II and III describe the system in mechanistically explicit terms.
Effect of N-ethylmaleimide on the specific binding of [3H]quinuclidinyl benzilate to M₂ muscarinic receptor purified from porcine atria or extracted from Sf9 cells

Binding was measured at graded concentrations of [3H]QNB in experiments on two batches of receptor purified from porcine atria and one batch of extract from Sf9 cells. The number of experiments is shown in parentheses. Data from individual experiments were analyzed according to Equations 1, 4, and 5 (n = 2) to obtain estimates of \( n_k \), log EC\(_{50} \), and \( B_\text{max} \). Individual estimates of log EC\(_{50} \) and \( n_k \) were averaged to obtain the mean (± S.E.) listed in the table. Individual estimates of \( B_\text{max} \) were adjusted for dilutions prior to the binding assay to obtain an imputed value for the stock solution. The mean is shown relative to that for unreacted receptor from the same batch taken as 100. The absolute values of \( B_\text{max} \) for the two batches of purified receptor are as follows: 0.77 ± 0.84 (1); 2, 1.11 ± 0.46 (13); 6.57 ± 0.14 (9); 3, 8.15 ± 1.82 (2); 5.51 (1); 4, 9.54 (1), 5.85 (1); 5, 7.45 (1). The values for receptor from Sf9 cells are as follows: 1, 5.07 ± 0.01 (3); 2, 1.71 ± 0.23 (3).

| Condition | Treatment with NEM | Log EC\(_{50} \) | \( n_k \) | \( B_\text{max} \) |
|-----------|---------------------|-----------------|----------|-----------------|
| Purified M₂ receptor from porcine atria | | | | |
| 1 | None (10) | -9.36 ± 0.05 | 0.90 ± 0.08 | 100 |
| 2 | 10 mM NEM, 24 h (22) | -8.09 ± 0.04 | 0.84 ± 0.02 | 88, 76 |
| 3 | 10 mM NEM, 24 h; then removed (3) | -8.15 ± 0.04 | 0.77 ± 0.03 | 64, 64 |
| 4 | 10 mM NEM, 4 days (2) | -7.92 ± 0.07 | 0.74 ± 0.02 | 75, 68 |
| 5 | 1 mM NEM, 24 h (1) | -8.24 | 0.71 | 86 |
| Solubilized human M₂ receptor from Sf9 cells | | | | |
| 1 | None (3) | -9.44 ± 0.04 | 1.06 ± 0.14 | 100 |
| 2 | 10 mM NEM, 24 h (3) | -8.60 ± 0.03 | 0.82 ± 0.03 | 34 |

Fig. 1. Effect of N-ethylmaleimide on the binding of [3H]quinuclidinyl benzilate to M₂ receptor purified from porcine atria. Alkylated receptor (△, *), and a control from which NEM was omitted (□, ○, □) were prepared as described under “Materials and Methods.” Binding to the two preparations was measured in parallel at graded concentrations of the radioligand, either alone (upper curves) or together with 1 mM NMS (baseline). Different symbols denote data from three different experiments. "C", "Q", and "□" were carried out on samples from two batches of purified receptor. The lines represent the best fit of Equations 4 and 5 (Scheme 1) to all of the data taken together. One class of sites was sufficient throughout. With the native receptor, there was no appreciable increase in the sum of squares with 1 rather than 3 values of \( K_\text{v} \) (\( p = 0.25 \)) (log \( K_\text{v} \) = -9.43 ± 0.03). With the alkylated receptor, separate values of \( K_\text{v} \) were required for data from different experiments (\( p < 0.05 \)) (log \( K_\text{v} \) = -8.67 ± 0.04 (△), -8.28 ± 0.04 (*), and -8.38 ± 0.05 (□)). The mean value of \( K_\text{v} \) for the native receptor is 519 ± 48 pm, and the ratios of \( K_\text{v} \), after and before alkylation are as follows: 0.77 (△), 0.72 (*), and 0.91 (□). Values plotted on the y axis were normalized as described under “Materials and Methods.”

The instability caused by N-ethylmaleimide was prevented by the agonist carbachol, which had a protective effect similar to that of N-methylscopolamine or quinuclidinyl benzilate. When the alkylated receptor was preincubated at 30 °C for 15 min, the capacity for [3H]quinuclidinyl benzilate was reduced to 19% of that in a control preincubated on ice (Fig. 6, cf. Fig. 5). In contrast, the capacity was virtually identical to that of the control (\( p = 0.25 \)) when 10 mM carbachol was included during the preincubation at 30 °C and then removed prior to the binding assays.
The binding of \(^{\text{[H]}}\)NMS and \(^{\text{[H]}}\)QNB was measured in parallel in each experiment. The number of experiments is shown in parentheses, and the data are illustrated in Fig. 3. The values listed in the table pertain to \(^{\text{[H]}}\)NMS, which was incubated with the receptor for different times as shown. The time of incubation with \(^{\text{[H]}}\)QNB was 2 h throughout. The values of \(n_{H}\) are the mean ± S.E. of the individual values estimated separately for each set of data (Equation 1). The estimates of log \(EC_{50}\) and relative capacity are from the best fit of Equations 4 and 5 (Scheme I) to the data shown in the same panel taken together (21). \(^{\text{[H]}}\)NMS required two classes for the native receptor and one class after alkylation. \(^{\text{[H]}}\)QNB required two classes of sites throughout. The values of log \(EC_{50}\) for \(^{\text{[H]}}\)QNB were \(-9.41 (n = 4)\) and \(-8.03 (n = 11)\) for native and alkylated receptor, respectively. The value of \(IR\), for \(^{\text{[H]}}\)NMS was estimated relative to that for \(^{\text{[H]}}\)QNB in the same experiment (i.e. \(f_{R,\text{NMS}} = [\text{R}]_{\text{NMS}}/[\text{R}]_{\text{QNB}}\)), and the individual values of \(f_{R,\text{NMS}}\) were averaged to obtain the mean (± S.E.).

### Table II

| Receptor | Time of incubation (h) | Log \(EC_{50}\) | \(n_{H}\) | Relative capacity |
|----------|------------------------|-----------------|--------|------------------|
| Native   | 0.75 (4)               | \(-8.57\)       | 0.95 ± 0.04 | 0.95 ± 0.01      |
| Alkylated| 0.25 (3)               | \(-7.56\)       | 0.81 ± 0.04 | 0.77 ± 0.03      |
|          | 0.75 (2)               | \(-7.37\)       | 0.83 ± 0.04 | 0.73 ± 0.07      |
|          | 3.0 (1)                | \(-7.07\)       | 1.04     | 0.81             |

**Binding of Unlabeled Ligands**—Native and alkylated receptors were examined for the inhibitory effect of five unlabeled ligands, three antagonists and two agonists, on the binding of \(^{\text{[H]}}\)quinuclidinyl benzilate (Fig. 7). To define all parameters in subsequent analyses and to test for internal consistency, binding also was measured at graded concentrations of \(^{\text{[H]}}\)quinuclidinyl benzilate and \(^{\text{[H]}}\)methylscopolamine (Fig. 7, A and D). In assays with the alkylated receptor and \(^{\text{[H]}}\)methylscopolamine, the time of incubation was 15 min to avoid or at least to minimize inactivation.

The data represented in all panels of Fig. 7 were pooled and analyzed in terms of Scheme I (\(n = 2\)), and the parametric values are listed in Table III. Although the fit was better with two classes of sites rather than one, the difference is not readily discernible in the fitted curves. Like \(^{\text{[H]}}\)quinuclidinyl benzilate and \(^{\text{[H]}}\)methylscopolamine, both scopolamine and unlabeled \(N\)-methylscopolamine bound more weakly to alkylated receptors than to the native preparation (i.e. \(\Delta \log EC_{50} \leq -1.3\), Table III). A substantial reduction in affinity therefore appears to be a common effect of \(N\)-ethylmaleimide on the binding of antagonists. In contrast, there was little if any change in the affinities of the agonists carbachol and oxotremorine-M (Fig. 7, C and F, Table III).

The decreased affinity of unlabeled antagonists indicates that the corresponding decrease in the affinity of \(^{\text{[H]}}\)quinuclidinyl benzilate and \(^{\text{[H]}}\)methylscopolamine is not a kinetic artifact of destabilization. Because of the protection afforded by muscarinic ligands, one of which is always present in assays at near saturating concentrations of \(^{\text{[H]}}\)quinuclidinyl benzilate, the inhibitory potency is expected to be unaffected by the instability of the vacant receptor after alkylation. Furthermore, the affinity of quinuclidinyl benzilate was the same when estimated from binding at graded concentrations of \(^{\text{[H]}}\)quinuclidinyl benzilate (Fig. 7D) and when inferred from the inhibitory effect of the unlabeled analogue (Fig. 7E) (Table III). Although there was some discrepancy between labeled and unlabeled \(N\)-methylscopolamine, \(N\)-ethylmaleimide reduced the affinities of both analogues by a similar amount. These observations support the conclusions that emerge from Scheme I and the parametric values listed in Table III; namely, that the effect of \(N\)-ethylmaleimide on the binding of antagonists derives from a change in affinity per se, whereas the affinity of agonists is essentially unchanged.

The binding of oxotremorine-M was sensitive to GMP-P(NH)P when purified \(M_2\) receptor was reconstituted with Go/i in phospholipid vesicles (Fig. 8). Reconstitution alone led to a more pronounced heterogeneity, and the Hill coefficient for the native receptor decreased from 0.86 ± 0.05 in solution (\(n = 3\), Fig. 7C) to 0.58 ± 0.04 in vesicles (\(n = 2\), Fig. 8). With the alkylated receptor, \(n_H\) decreased from 0.88 ± 0.02 (\(n = 3\)) in solution to 0.59 ± 0.02 (\(n = 2\)) in vesicles. There was no effect of GMP-P(NH)P on the binding of oxotremorine-M when receptors and G proteins were mixed in solution. GMP-P(NH)P increased the Hill coefficient to 0.77 ± 0.15 and 0.68 ± 0.08 with native and alkylated receptors, respectively. The data required two classes of sites when described empirically in terms of Equation 3, and the fitted curves are illustrated in Fig. 8. The overall effect of GMP-P(NH)P was estimated as the area between the curves.
obtained in the absence and presence of the nucleotide, and the value after alkylation was 68% of that obtained with the native receptor.

Control of Alkylation by Muscarinic Ligands—The effect of N-ethylmaleimide on binding suggests that muscarinic ligands might act to regulate alkylation. Purified M2 receptors therefore were reacted with N-ethylmaleimide in the presence of either N-methylscopolamine or carbachol, and the progress of the reaction was monitored by measuring the affinity of the receptor for [3H]quinuclidinyl benzilate. Binding assays were carried out prior to the addition of the alkylating agent and after incubation of the reaction mixture for different times up to 24 h. Samples with and without the muscarinic ligand were processed in parallel.
achieved at 5.0 and 3.9 nM [3H]QNB, samples were incubated at 30 °C, alkylated receptor). Upon the addition of buffer A with or without [3H]QNB, the replicates were transferred from 0 to 30 °C and maintained at that temperature for the length of time shown on the abscissa (Fig. 10). The capacities of samples preincubated at 30 °C without carbachol, log EC50 = -8.01, f2 = 0.19 ± 0.02; preincubated at 30 °C with carbachol, log EC50 = -8.11, f2 = 1.03 ± 0.02. Values plotted on the y axis were normalized to the mean value of [R].

FIG. 5. Stability of M2 receptor purified from porcine atria. Native (○, □) and alkylated (○, △) receptor was obtained by pretreatment of the purified product with or without NEM. Aliquots were mixed with buffer A (○, □) or with buffer A containing a small amount of [3H]QNB at final concentrations of 5.0 (○, native receptor) or 3.9 nM (△, alkylated receptor). Each aliquot contained sufficient material for one measurement, and each measurement was performed in triplicate. Upon the addition of buffer A with or without [3H]QNB, the replicates were transferred from 0 to 30 °C and maintained at that temperature for the length of time shown on the abscissa (Fig. 10). The capacities of samples preincubated at 30 °C without carbachol, log EC50 = -8.01, f2 = 0.19 ± 0.02; preincubated at 30 °C with carbachol, log EC50 = -8.11, f2 = 1.03 ± 0.02. Values plotted on the y axis were normalized to the mean value of [R].

FIG. 6. Stabilization of the alkylated receptor by carbachol. Purified receptor from porcine atria was reacted with NEM, and aliquots of the alkylated product were preincubated for 15 min as follows: on ice (○, □), at 30 °C (○, △), at 30 °C, diamond with ×, ○, and at 30 °C in the presence of 10 mM carbachol, □, diamond with +, △. All samples then were desalted on Sephadex G-50 (0.8 × 5 cm) and assayed for the binding of [3H]QNB. Each experiment included samples processed in parallel under each set of conditions, and the data from three separate experiments are shown (○, □, ○, ×, ○, diamond with ×, △). The lines represent the best fit of Equations 4 and 5 (Scheme 1, n = 20) to all of the data taken together. Single values of Km and P1 were common to all data acquired under the same conditions. The capacities of samples preincubated on ice were estimated in absolute units (i.e. [RI]). The capacities of samples preincubated at 30 °C were estimated relative to [RI], with a single value of the ratio f2 for the data from all three experiments. The fitted parametric values are as follows: preincubated on ice, log EC50 = -8.10, [R1] = 510 ± 19 pm (n = 3); preincubated at 30 °C without carbachol, log EC50 = -8.01, f2 = 0.19 ± 0.02; preincubated at 30 °C with carbachol, log EC50 = -8.11, f2 = 1.03 ± 0.02. Values plotted on the y axis were normalized to the mean value of [R].

[3H]quinuclidinyl benzilate in either case (no carbachol, F2 = 0.67 ± 0.01; with carbachol, F2 = 0.71 ± 0.01).

Oligomeric Status of M2 Receptor Extracted from S9 Cells—G protein-coupled receptors are known to occur as oligomers (22, 26, 33). The relationship between oligomeric status and binding therefore was examined in extracts from S9 cells coexpressing the c-Myc- and FLAG-tagged M2 receptors. Because the FLAG epitope communoprecipitated with the c-Myc epitope, as identified on Western blots (Fig. 10, A and B), at least some of the receptors were present as dimers or larger oligomers in vivo. No signal is obtained if membranes from cells infected separately with the two baculoviruses are mixed prior to solubilization (26). The coprecipitated receptor migrated primarily as two immunoreactive bands with molecular masses indicative of monomers (59.5 ± 1.6 kDa, n = 6) and dimers (93.4 ± 2.6 kDa, n = 6), based on the calculated value of 51,673 Da for a monomer (24, 25). Some blots also contained a minor band exhibiting a molecular mass of 165 ± 19 kDa (n = 3), perhaps indicating a trimer.

Tagged receptors extracted from S9 cells were stable at 30 °C; because preincubation for 20 min affected neither the capacity for [3H]quinuclidinyl benzilate nor the degree of communoprecipitation (Fig. 10). N-Ethylmaleimide was without effect on the degree of communoprecipitation, which was unchanged after preincubation at 30 °C in the absence of ligand or in the presence of either quinuclidinyl benzilate or N-methylscopolamine (Fig. 10). The alkylated receptor was functionally

As illustrated in Fig. 9A, N-methylscopolamine slowed the rightward shift induced by N-ethylmaleimide in the binding curve for [3H]quinuclidinyl benzilate. When the data were analyzed collectively in terms of Scheme I (Equation 5, n = 2), the shift can be described as a time-dependent interconversion of sites from a state of higher affinity (log Km = -9.31 ± 0.02) to a state of lower affinity (log Km = -8.29 ± 0.03). Native receptors were wholly in the state of higher affinity. After treatment for 30 min in the absence of antagonist, the low affinity sites represented about 60% of the total binding (F2 = 0.62 ± 0.02); after 30 min in the presence of N-methylscopolamine, they represented less than 40% of the total binding (F2 = 0.36 ± 0.03). After more prolonged treatment in the absence and presence of N-methylscopolamine, the low affinity sites represented more than 80% (4 h, F2 = 0.84 ± 0.02; 24 h, F2 = 0.83 ± 0.02) and about 60% (4 h, F2 = 0.61 ± 0.02; 24 h, F2 = 0.59 ± 0.02) of the total binding, respectively.

The rate of the reaction with N-ethylmaleimide was unaffected by carbachol. As illustrated in Fig. 9B, the binding profiles obtained after alkylation for 30 min with and without the agonist are virtually superimposable. In terms of Scheme I, about 70% of the sites were in the state of lower affinity for
Fig. 7. Binding of antagonists and agonists to M₂ receptor purified from porcine atria. Native receptor (A–C) and receptor treated with NEM (D–F) were prepared as described under “Materials and Methods.” Panels A and D, binding was measured at graded concentrations of [³H]QNB (○), [¹H]NMS (△) and [³H]METH (•, +). Different symbols denote data from different experiments (n = 3). The time of incubation with [³H]NMS was 45 (native receptor) or 15 min (alkylated receptor). Panels B and E, the binding of [³H]QNB was measured at two concentrations of the radioligand (B, 1.0–1.2 and 11–13 nM; E, 8.7–12 and 49–94 nM) and graded concentrations of unlabeled NMS (○, △), scopolamine (○, △), or QNB (+, +). Panels C and F, the binding of [³H]QNB (C, 1.1–1.4 nM; F, 11–14 nM) was measured at graded concentrations of carbachol (○) or oxotremorine-M (■). The data represented in all panels were combined and analyzed in terms of Scheme I (Equations 4 and 5, n = 2) to obtain the parametric values listed in Table III. The same data also were combined with the data represented in Fig. 1A and analyzed in terms of Scheme II (Equations 4 and 6). The lines in the figure represent the best fit of Scheme II, and the parametric values are listed in Tables IV and V. Values plotted on the y axis were normalized to the mean value of [R], obtained for [³H]QNB (native receptor, 610 ± 18 pm, n = 15; alkylated receptor, 572 ± 26 pm, n = 23). The capacity for [¹H]NMS in panels A and D was scaled relative to that for [³H]QNB according to the ratio listed in Table II (native receptor, 0.95; alkylated receptor, 0.77). There were fewer constraints with Scheme I (n = 2) than with Scheme II, and the sum of squares is 15% less for the data represented in Fig. 7, but the fitted curves from Scheme I are almost superimposable with those shown in the figure.

unstable, however, in that about 60% of the original sites were lost before or during the binding assay (Table I, Fig. 10C). The overall loss increased to 86% after preincubation for 20 min and to 97% after preincubation for 1 h (Fig. 10C).

Alkylation by N-ethylmaleimide was similar in its effect on the binding properties of M₂ receptors from Sf9 cells and porcine atria. Both preparations underwent a reduction in their affinity for [³H]quinuclidinyl benzilate (Table I) and an increase in the rate of inactivation at 30 °C (cf. Figs. 5 and 10C), although the latter effect was much greater with the recombinant receptor. Because N-ethylmaleimide was without effect on the degree of communoprecipitation from Sf9 extracts, the loss of function apparently was not accompanied by any change in the oligomeric status of the receptor.

DISCUSSION

Effects of N-Ethylmaleimide on the M₂ Muscarinic Receptor—Muscarinic ligands and the sulfhydryl-specific reagent N-ethylmaleimide exhibit a pattern of complementary effects at M₂ receptors purified from porcine atria. Alkylation caused a 9–27-fold reduction in the affinities of three muscarinic antagonists, as estimated in terms of Scheme I, whereas the affinities of two agonists were unchanged. Conversely, the reaction with N-ethylmaleimide was slowed by the antagonist N-methylscopolamine but not by the agonist carbachol. The receptor also underwent a time-dependent, irreversible loss of capacity that was faster after alkylation and slower in the presence of quinuclidinyl benzilate or N-methylscopolamine. The destabilizing effect of N-ethylmaleimide was counteracted fully or nearly so at saturating concentrations of either antagonist or the agonist carbachol. The protective effect of [³H]quinuclidinyl benzilate was greater than that of N-[³H]methylscopolamine, as indicated by the time dependence of binding. Because [³H]quinuclidinyl benzilate dissociates more slowly than N-[³H]methylscopolamine, it follows that the degree of stabilization appears to depend upon the residency time of the ligand on the receptor.

The change effected by N-ethylmaleimide in the affinity of antagonists was not a consequence of its destabilizing effect on the receptor, notwithstanding the protection afforded by N-[³H]methylscopolamine and [³H]quinuclidinyl benzilate. Although instability can account for the progressively weaker binding of N-[³H]methylscopolamine to alkylated receptors...
TABLE III  
Affinities of antagonists and agonists for native and alkylated receptor, estimated semi-empirically in terms of Scheme I

| Ligand                          | L  | NEM | Log $K_i$ | Log $K_d$ | Log $EC_{50}$ | $\Delta\log EC_{50}$ |
|--------------------------------|----|-----|-----------|-----------|----------------|---------------------|
| ($[^3]H$)Quinuclidinyl benilate | P  | −   | −9.78 ± 0.12 | −8.70 ± 0.09 | −9.13         |                     |
|                               | A, P | +  | −8.58 ± 0.08  | −7.70 ± 0.11  | −8.06         | −1.07               |
| N-Methylscopolamine           | P  | −   | −9.01 ± 0.08  | −7.23 ± 0.17  | −7.59         | −0.97               |
| Scopolamine                   | A  | +   | −9.05 ± 0.17  | −7.91 ± 0.14  | −8.36         |                     |
| Carbachol                     | A  | +   | −7.27 ± 0.15  | −6.68 ± 0.16  | −6.92         | −1.44               |
| Oxotremorine-M                | A  | −   | −7.95 ± 0.15  | −6.98 ± 0.12  | −7.37         |                     |
|                                | A  | +   | −6.39 ± 0.14  | −5.84 ± 0.16  | −6.07         | −1.30               |
|                                | A  | +   | −3.94 ± 0.18  | −2.99 ± 0.21  | −3.37         | +0.33               |

FIG. 8. Effect of N-ethylmaleimide and GMP-P(NH)P on the binding of oxotremorine-M to M₁ receptor purified from porcine atria and reconstituted with Gz/Gi. Native receptor (O, □) and receptor treated with NEM (●, ■) was prepared and reconstituted with G proteins as described under “Materials and Methods.” The binding of ($[^3]H$)QNB was measured at graded concentrations of oxotremorine-M alone (○, □) and together with 0.1 mM GMP-P(NH)P (∆, ■). The concentration of ($[^3]H$)QNB was selected to achieve ~50% occupancy in the absence of oxotremorine-M (native receptor, 1.0–1.1 nM; alkylated receptor, 12–15 nM). Each curve represents two experiments performed on different batches of purified receptor. The lines represent the best fit of Equation 3 ($n = 2$). Single values of $IC_{50}$, and $F_p$ were common to both sets of data acquired under the same conditions, and separate values of $B_{(A)}$ and $B_{(A)}$ were assigned to the data from each experiment. Values plotted on the x axis were normalized to the fitted asymptotes taken as 100 and 0. The parametric values are as follows: native receptor (no GMP-P(NH)P), $IC_{50}$ = $-5.48 ± 0.17$; native receptor (with GMP-P(NH)P), $IC_{50}$ = $-4.16 ± 0.19$; native receptor (GMP-P(NH)P), $IC_{50}$ = $-3.11 ± 0.53$; $F_p$ = $0.31 ± 0.22$; alkylated receptor (no GMP-P(NH)P), $IC_{50}$ = $-5.37 ± 0.24$; alkylated receptor (GMP-P(NH)P), $IC_{50}$ = $-3.32 ± 0.08$. $F_p$ = $0.75 ± 0.05$; alkylated receptor (with GMP-P(NH)P), $IC_{50}$ = $-4.48 ± 0.43$; $F_p$ = $-3.18 ± 0.17$. $F_p$ = $0.74 ± 0.14$.

(N-ethylmaleimide) and Purified Muscarinic Receptors

N-Ethylmaleimide and Purified Muscarinic Receptors

The data represented in Fig. 7 were analyzed simultaneously in terms of Scheme I (Equations 4 and 5, $n = 2$) to obtain the parametric values for all ligands (radiolabeled, $L = P$; unlabeled, $L = A$). All experiments were performed at least three times except for the inhibition of ($[^3]H$)QNB by unlabeled QNB in panel E, which was done once. Assays at two concentrations of ($[^3]H$)QNB generally performed in parallel (panels B and E). Single values of $K_i$ were assigned to all of the relevant data acquired with the same preparation of receptor (i.e., native or alkylated). In the case of QNB, the radioligand and the unlabeled analogue could be assigned single values of $K_i$ without affecting the sum of squares (i.e., $K_i = K_A$) (p = 0.37); in the case of NMS, the same constraint compromised the fit (p < 0.00001). The sum of squares was significantly lower with two classes of sites rather than one (p < 0.00001), and a single value of $F_p$ was common to all of the data ($F_p = 0.58 ± 0.06$). The mean values of $[R]_t$ obtained for ($[^3]H$)QNB are as follows: native receptor, 633 ± 19 pm (n = 15); alkylated receptor, 632 ± 28 pm (n = 23).

It has been reported that N-ethylmaleimide can react with G proteins, thereby precluding their interaction with the receptor (34) and inhibiting GTPase activity (35). Because the purified receptor was devoid of $\alpha_2$, $\alpha_3$, $\alpha_5$, and $\alpha_6$, the effects of alkylation found in the present investigation were independent of G proteins; rather, the observed changes in binding and stability apparently derived from an effect intrinsic to the receptor alone. When the purified receptor was reconstituted with Gz5 in phospholipid vesicles, the binding of agonists was affected by N-ethylmaleimide in the manner that is characteristic of muscarinic receptors in native membranes (e.g., Refs. 6, 8, and 9). In particular, the reagent decreased the magnitude of the effect of GMP-P(NH)P. The purified receptor therefore retained its native functional properties with respect to the interaction with Gz5 and the allosteric interaction between the agonist and GMP-P(NH)P; also, the related effects of N-ethylmaleimide are largely independent of whether alkylation occurs in the native membrane or after purification.

N-Ethylmaleimide did not affect the nature or quantity of oligomers formed by differently tagged human M₂ receptors, at least as monitored by the coimmunoprecipitation of c-Myc and FLAG epitopes from extracts of coinfected Sf9 cells. Cloned receptors and receptors from porcine atria underwent qualitatively similar changes in affinity and stability, with the two preparations differing only in the magnitude of the change. It therefore appears that neither the instability nor the decreased affinity for ($[^3]H$)quinuclidinyl benzilate arose from any effect of alkylation on the oligomeric status of the receptor.

Evaluation in Terms of Scheme II—Whereas the changes in affinity and stability appear to be mutually independent, the reciprocal nature of the various effects argues for a common cause. Many studies have shown that muscarinic and other G

and 4), yet the change in affinity was similar or the same for both radioligands (Table III). Also, there was a comparable change in the affinities of three unlabeled antagonists, as inferred from their inhibitory effect under conditions that are expected to preclude inactivation. In binding assays conducted overnight at 0 °C, treatment with N-ethylmaleimide was found to reduce the affinity but not the capacity of purified receptors for $N_\text{-}[^3]H$-methylscopolamine. The independent nature of the changes in affinity and capacity is illustrated further by a comparison of receptors from Sf9 cells and porcine atria. Whereas the destabilizing effect of alkylation was markedly greater with the Sf9 extract, the decrease in affinity for ($[^3]H$)quinuclidinyl benzilate was similar in the two preparations.

The mean values of $[R]_t$ obtained for ($[^3]H$)QNB are as follows: native receptor, 633 ± 19 pm (n = 15); alkylated receptor, 632 ± 28 pm (n = 23).

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N-Ethylmaleimide did not affect the nature or quantity of oligomers formed by differently tagged human M₂ receptors, at least as monitored by the coimmunoprecipitation of c-Myc and FLAG epitopes from extracts of coinfected Sf9 cells. Cloned receptors and receptors from porcine atria underwent qualitatively similar changes in affinity and stability, with the two preparations differing only in the magnitude of the change. It therefore appears that neither the instability nor the decreased affinity for ($[^3]H$)quinuclidinyl benzilate arose from any effect of alkylation on the oligomeric status of the receptor.

Evaluation in Terms of Scheme II—Whereas the changes in affinity and stability appear to be mutually independent, the reciprocal nature of the various effects argues for a common cause. Many studies have shown that muscarinic and other G
protein-coupled receptors can interconvert spontaneously between two or more states of different intrinsic activity. Agonists bind with higher affinity to the more active state and shift the equilibrium accordingly, whereas antagonists favor the less active state (e.g. Refs. 15, 16, 36, and 37). The molecular distinction between the two states remains unclear, because the effects of mutation and other perturbations typically are monitored by means of a receptor-elicited response. According to one view, however, the inactive and active states correspond to the free receptor and the receptor-G protein complex, respectively (38). An extension of that view posits that the two states of the receptor differ in their affinity for the ligand on the one hand and the G protein on the other (15, 39, 40). It also has been suggested that N-ethylmaleimide reacts with muscarinic receptors only in the agonist-specific state, thereby locking the receptor in that conformation and causing an observed increase in the affinity for agonists (10, 11).

A model that incorporates the notion of two interconverting states offers a plausible explanation for the effects of N-ethylmaleimide on purified receptors in the present investigation. The lines in Figs. 7 and 9A represent the best fit of Scheme II to the data, taken together, and the parametric values are listed in Tables IV and V. It was assumed that there were two noninterconverting forms of the receptor overall (n = 2). Native receptors were wholly of one form (e.g. Figs. 7, A–C, j = 1, F2 = 0), and receptors treated with N-ethylmaleimide for 24 h were wholly of the other (e.g. Fig. 7, D–F, j = 2, F2 = 1). This restriction presupposes that alkylation was complete after 24 h in the absence of N-ethylmaleimide, and it follows from the observation that the potency of [3H]quinuclidinyl benzilate was essentially the same after treatment with N-ethylmaleimide for 24 h and 4 days (Table I). The reaction otherwise was assumed to be incomplete: that is, after treatment for less than 24 h in the absence of N-ethylmaleimide or for any time in the presence of N-ethylmaleimide (Fig. 9A). Accordingly, those data were analyzed as a mixture comprising both forms of the receptor (i.e. 0 < F2 < 1).

It also was assumed that N-ethylmaleimide was without effect on the affinity of the ligand for either R or R*. All of the data therefore shared single values of K0.1 and a1.1 for each ligand (i.e. R1 = K1,2 and a1,1 = a1,2). With this constraint and the assumption of homogeneity after alkylation for 24 h, the effects of N-ethylmaleimide are attributed exclusively to a change from K1 to K2; that is, alkylation is assumed to cause a shift in the distribution of receptors between the two states.

Preliminary analyses with Scheme II indicated that the constraints described above were without effect on the sum of squares, but neither were they sufficient to yield a unique set of parametric values. The ambiguity arises in part from uncertainty over the absolute value of K0.1, particularly after alkylation. The relative value of K0.1 is better defined, however, and a lower bound of about 100 was determined by mapping the sum of squares with respect to the ratio of K0.1/K2. The value of K0.1 is about 0.05–0.1 at all acceptable values of K0.1/K2, and the results obtained with K0.1/K2 fixed at 100 are listed in Tables IV and V.

The good agreement between Scheme II and the data indicates that the model can account for all of the effects illustrated in Figs. 7 and 9A. Based on the fitted value of K0.1, purified M2 receptors were predominantly but not exclusively in the R* state in the absence of muscarinic ligands (log K0.1 = −1.28, Table V). In the presence of an antagonist, however, the net interconversion associated with comparatively high values of
calculated for antagonists in terms of Scheme II (Table V) agree closely with the corresponding values of EC_{50} obtained for alkylated receptors in terms of Scheme I (Table III). In contrast, the values of K_{i} and \( \alpha_{L}K_{i} \) from Scheme II (Table V) bracket the corresponding values of EC_{50} obtained for the native receptor from Scheme I (Table III) (i.e. \( K_{i} < EC_{50} < \alpha_{L}K_{i} \)). The intermediate values of EC_{50} are a consequence of the redistribution of sites from R* to R that occurs upon the binding of the antagonist to the native receptor (Table V).

The lower limit on \( K_{R}/K_{R*} \) is related to the marked preference of alkylated receptors for the R* state under all conditions. The extent of the redistribution effected by ligands is governed by \( \alpha_{L} \). Antagonists bind more tightly to R (\( \alpha_{L} > 1 \), Table IV) and therefore promote R over R* (\( K_{R} < \alpha_{L}K_{R*} \), Table V). Because R* predominates with native receptors in the absence of ligand and with alkylated receptors irrespective of ligand (Table V), the value of \( K_{R}/K_{R*} \) must approximate or exceed that of \( \alpha_{L}K_{R*} \). The ratio \( K_{R}/K_{R*} \) therefore is limited by \( \alpha_{L} \) (i.e. \( \alpha_{L} \leq K_{R}/K_{R*} \)), or by the single value of \( \alpha_{L} \) if N-ethylmaleimide is without effect on the relative affinity of the ligand for R and R* (i.e. \( \alpha_{L} = \alpha_{L} = \alpha_{L} \)). The fit therefore is compromised at values of \( K_{R}/K_{R*} \) that are substantially less than \( \alpha_{L} \); the value of \( \alpha_{L} \) is governed in turn by the difference in EC_{50} before and after alkylation.

Because N-ethylmaleimide was without effect on the binding of carbachol and oxotremorine-M, the relative affinity of agonists for R and R* is unclear. This is illustrated in Fig. 11, where the global sum of squares is shown to be independent of \( \alpha_{L} \) at lower values and to increase at higher values. The map indicates that the data are consistent with any value of \( \alpha_{L} \) smaller than about 10^{-0.2} for carbachol and 10^{0.3} for oxotremorine-M. This ambiguity arises from the preponderance of R* under all conditions with respect to the agonist (Table V). Because the system is predominantly in the state potentially of higher affinity for agonists, the distribution of sites between R and R* undergoes little or no change upon addition of the ligand. The state of lower affinity for agonists therefore is unobservable.

Scheme II also can account for the opposing effects of N-methylscopolamine and N-ethylmaleimide on the affinity of purified receptors for [3H]quinuclidinyl benzilate (Fig. 9A). Native receptor was assumed to be exclusively R_{1} or R_{1*}, as described above, and the progress of the reaction was modeled as the increase in the fraction of sites identified as R_{2} or R_{2*} (\( i.e. F_{2} = [R_{2}]/([R_{1}] + [R_{2}]) \)). Upon treatment with N-ethylmaleimide in the absence of N-methylscopolamine, the fraction \( F_{2} \) increased from zero initially to 0.72 ± 0.03 after 30 min and to 0.92 ± 0.02 after 4 h; the value after 24 h was set at 1. In the presence of N-[3H]methylscopolamine, the value of \( F_{2} \) increased to only 0.46 ± 0.03 after 30 min and to 0.70 ± 0.03 after 4 or 24 h. In contrast to N-methylscopolamine, carbachol was without effect on the rate of interconversion from R_{1} to R_{2} (Fig. 9B). This difference between N-methylscopolamine and carbachol parallels the different preference of antagonists and agonists for the two states of the receptor. It follows that the antagonist slowed alkylation by favoring a state, in this case the R state, that is comparatively unreactive to N-ethylmaleimide.

**Evaluation in Terms of Scheme III**—A kinetically determined variant of Scheme II can account for the opposing effects of antagonists and N-ethylmaleimide on the rate of inactivation (Figs. 4 and 5) and, in the case of the alkylated receptor, for the time-dependent, rightward shift in the binding of N-[3H]methylscopolamine (Fig. 3B). Scheme III was formulated with time as an independent variable and incorporates the possibility that the receptor can decay to a state or states that do not bind
Effect of N-ethy maleimide on the affinities of muscarinic ligands for purified M,
receptor, evaluated in terms of Scheme II

The data illustrated in Figs. 7 and 9A were analyzed in concert according to Equations 4
and 6. There were two classes of sites overall (n = 2), representing native receptor (j = 1)
and alkylated receptor (j = 2). The reaction with NEM was complete after 24 h, and the data in
the lower panels of Fig. 7 therefore were treated as a homogenous population of sites (j = 2).
The data in Fig. 9A were treated as a mixture of sites. Single values of Kj,
and aj were common to all of the data acquired with both native and alkylated receptor, as described in the text. QNB and NMS were present both as the radioligand (L = P) and as the unlabelled analogue (L = A). The two forms of QNB yielded consistent estimates of Kj,
and aj,
which therefore were optimized as a single value (i.e. aj = aj,
Kj = Kj), The same constraint with NMS led to a significant increase in the
sum of squares. Capacities were estimated as the total concentration of sites (Ri) and that fraction corresponding to alkylated receptor (i.e. Fj = [Rj]/([Rj] + [Rj])). The number of curves represented in Fig. 7 is shown in parentheses (native receptor, alkylated receptor). Further details are described in the legend to Table V.

| Ligand | Log Ke | Log aj | Log (aj,Kj) |
|--------|--------|--------|-------------|
| [3H]Quinuclidinyl benzilate (3, 3) | -10.41 ± 0.07a | 2.29 ± 0.08 | -8.12 |
| N-[3H]Methylscopolamine (3, 3) | -9.87 ± 0.08 | 2.18 ± 0.09 | -7.69 |
| N-Methylscopolamine (3, 6) | -9.68 ± 0.08 | 2.86 ± 0.08 | -6.82 |
| Scopolamine (6, 6) | -8.65 ± 0.08 | 2.64 ± 0.08 | -6.01 |
| Carbachol (3, 3f) | > -3.11 | < -0.2 | 3.31 |
| Oxotremorine-M (3, 3f) | > -4.85 | < 0.9 | -4.00 |

a Unlabeled QNB was used only in assays with the alkylated receptor.

b The value listed in the table is for the data in Fig. 7. A separate value was required for the data in Fig. 9A (log Ke = -10.68 ± 0.08), perhaps because a different batch of receptor was used in those experiments.

The parametric values listed for carbachol and oxotremorine-M correspond in each case to the upper bound on log aj and log aj obtained for other ligands. The latter were determined with the value of log aj for each agonist fixed at -2.

Effect of N-ethyl maleimide and muscarinic ligands on the distribution of purified M,
receptor between interconverting states, evaluated in terms of Scheme II

The data illustrated in Fig. 7 and 9A were analyzed in concert according to Equations 4 and 6. The distribution of sites between R and R* in the absence of ligand was evaluated as the single value of Kij, common to all data acquired with the alkylated receptor and the single value of the ratio Kij/Kij, common to all data acquired with the native receptor. The value of log (Kij/Kij,) was fixed at 2 to obtain the optimized value of log Kij (-3.28 ± 0.08) and the corresponding value of log Kij (-1.28) listed in the table. The values of log (aj,Kij) were calculated from the value of log Kij and the appropriate value of log aj from Table IV. The fraction of sites in the R state is the value of Kij, for the unliganded receptor and from aj,Kij for the receptor at saturating concentrations of the ligand. Further details are described in the legend to Table V.

| Receptor | Ligand (L) | Log Ke | Log (aj,Kij) | [R]/([R] + [R*]) | [LR]/([LR] + [LR*]) |
|----------|------------|--------|--------------|-------------------|-------------------|
| Native   | None       | -1.28  | 5.7          | 91                | 91                |
|          | [3H]Quinuclidinyl benzilate | 1.01 | 91          |
|          | N-[3H]Methylscopolamine | 0.90 | 89          |
|          | N-Methylscopolamine | 1.58 | 97          |
|          | Scopolamine | 1.36 | 96          |
|          | Carbachol | -1.48 | <3.2         |
|          | Oxotremorine-M | -0.38 | <29         |<29 |
| Alkylated | None       | -3.28  | 0.052        | 9.3               | 7.3               |
|          | [3H]Quinuclidinyl benzilate | -0.99 | 9.3          |
|          | N-[3H]Methylscopolamine | -1.11 | 7.3          |
|          | N-Methylscopolamine | -0.43 | 27          |
|          | Scopolamine | 0.64 | 19          |
|          | Carbachol | -3.48 | <0.033       |
|          | Oxotremorine-M | -2.38 | <0.412      |<0.412 |

The radioligand, at least at the concentrations used in the assays. The lines in Figs. 3B and 4 represent the best fit to the pooled data, and the rate constants are listed in the legend to Fig. 4.

Receptors in the state designated as R1 in Scheme III were stable under the conditions of the assays, with a half-life measured in days either with or without N-[3H]methylscopolamine (i.e. kR1 and kR1). The R2 state was almost as stable in the presence of antagonist (kR2). In the absence of ligand (kR2), however, the decay was 5-fold more rapid with the native receptor and 25-fold more rapid after treatment with N-ethylmaleimide. Alkylation therefore hastened inactivation by further destabilizing the labile R2 state and by promoting R2 over the stable R1 state. The protective effect of N-[3H]methylscopolamine derived in part from its preference for R1 over R2 and the attendant redistribution of sites away from the labile state (i.e. aj > 100, Table IV); also, either state of the receptor was more stable in the presence of a ligand. The latter effect predominated after alkylation, because the value of aj,Kij was such that most of the sites were in the R1 state even at saturating concentrations of antagonist (Table V). Similarly, occupancy per se also accounted for the stabilizing effect of carbachol (Fig. 6).

In the context of Scheme III, the rightward shift shown for N-[3H]methylscopolamine in Fig. 3B derived from the opposing effects of the antagonist and N-ethylmaleimide on the inactivation of alkylated receptors. Because of the decay illustrated in Fig. 4B, longer incubation times increased the concentration of N-[3H]methylscopolamine required to achieve a given level of binding. That led in turn to an increase in the value of EC50 over time. Scheme III also can account for a decrease in maximal binding, as described below, but the predicted effects differ from those illustrated in Fig. 3B. In terms of the model, the capacity for N-[3H]methylscopolamine was 73–89% of that for [3H]quinuclidinyl benzilate at each time of incubation. The shortfall does not derive from the nonbinding species R2 and R4, because the parameter [R], comprises all forms of the receptor. Also, the amounts of R3 and R4 were assumed to be zero at the outset, and the apparent loss of sites is expected to emerge over time.

The predicted effect of time on the binding of N-[3H]methylscopolamine to the alkylated receptor is illustrated in Fig. 12,
The present results recall an earlier suggestion—Purification and purified receptors suggests that solubilization or purification is accompanied by a decrease in affinity of antagonists for muscarinic receptors in membranes (e.g. Refs. 8–10). With agonists, however, alkylation typically leads to an increase in the Hill coefficient and a concomitant decrease in the magnitude of the shift effected by guanyl nucleotides (e.g. Refs. 7–10). The effect on overall affinity has been less consistent, probably because of the complexity of the changes; thus, agonists have been found to bind more tightly in some studies (e.g. Refs. 8, 10, 13, 14) and less so in others (e.g. Refs. 6 and 9). Factors that seem to account for such variablity include the temperature of the reaction (41), the concentration of the reagent (9, 10), and the stability of the receptor before and after alkylation. In some cases, the change in the binding of the agonist may arise indirectly from an effect on the G protein (e.g. Ref. 41). N-Ethylmaleimide was without effect on agonists in the present investigation, but the characteristic effects were recovered upon reconstitution of the purified receptor with Go/i. The loss of that sensitivity upon purification is accompanied by a decrease in the value of Kp.

Scheme II or variants thereof can account for the constitutive activity and related effects that have been described for mutants and, in some cases, the native forms of several G protein-linked receptors (15, 16, 36, 42). The emergence of a common pattern wherein a ligand-regulated equilibrium is affected differently by agonists and antagonists suggests that the states designated here as R and R* represent the inactive and active forms of the M2 receptor, respectively. That in turn implies that solubilization and purification lead to activation of the receptor. It also suggests that agonists favor R* over R and argues against the alternative possibility that they are indifferent.

**Fig. 11.** Effect of aL for carbachol and oxotremorine-M on the weighted sum of squares in analyses with Scheme II. The analysis described in the legends to Figs. 7 and 9A was repeated with log aL for either carbachol (solid line) or oxotremorine-M (dashed line) fixed at successive values over the range shown on the abscissa. The corresponding weighted sum of squares is plotted on the ordinate. When the value of log aL was mapped for oxotremorine-M, that for carbachol was fixed at −2 to stabilize the convergence. The dotted line indicates the value of log aL at which the corresponding sum of squares significantly exceeds the smallest value for that agonist (carbachol, log aL = −0.2; oxotremorine-M, log aL = 0.9) (p < 0.05). The shallow minimum in the map for oxotremorine-M (log aL = 0.3) derives from the small difference in the inhibitory potency before and after alkylation (Δlog EC50 = −0.11, Table III).

where the solid lines were simulated according to Scheme III with the parametric values listed in the legend to Fig. 4. The binding curve expected after incubation for 15 min is almost superimposable with the dashed line, which is the pattern that would be obtained at equilibrium if the receptor were stable. A comparatively short time of incubation therefore allows for equilibration of the radioligand while avoiding an appreciable loss of sites. Longer times result in a rightward shift and a progressive reduction in the apparent capacity. The failure to observe the latter effect with N-[3H]methylscopolamine may derive in part from variations in [R], that masked a decrease in maximal binding.

**Conformational Status of the Receptor and Activation upon Purification**—The present results recall an earlier suggestion that N-ethylmaleimide reacts preferentially with a conformation of the muscarinic receptor that exhibits higher affinity for agonists (10, 11). In those studies, however, alkylation was found to increase the affinity of membrane-bound receptors for agonists without affecting that for antagonists; conversely, agonists speeded up the reaction with N-ethylmaleimide, whereas antagonists were without effect. That pattern is opposite to the present observation that alkylation decreased the affinity of purified receptors for agonists but not agonists while antagonists but not agonists slowed the reaction. In terms of Scheme II, it appears that the membrane-bound receptors were almost exclusively in the R state, and that alkylation led to a decrease in Kp. The change caused only a minor redistribution of sites from R to R*, however, and it therefore had no appreciable effect on the binding of antagonists; nonetheless, it was sufficient to permit a major redistribution at saturating concentrations of agonist. The contrasting behavior of membrane-bound and purified receptors suggests that solubilization or purification causes an interconversion from R to R*.

N-Ethylmaleimide generally has been found to have little or no effect on the affinity of antagonists for muscarinic receptors in membranes (e.g. Refs. 8–10). With agonists, however, alkylation typically leads to an increase in the Hill coefficient and a concomitant decrease in the magnitude of the shift effected by guanyl nucleotides (e.g. Refs. 7–10). The effect on overall affinity has been less consistent, probably because of the complexity of the changes; thus, agonists have been found to bind more tightly in some studies (e.g. Refs. 8, 10, 13, 14) and less so in others (e.g. Refs. 6 and 9). Factors that seem to account for such variability include the temperature of the reaction (41), the concentration of the reagent (9, 10), and the stability of the receptor before and after alkylation. In some cases, the change in the binding of the agonist may arise indirectly from an effect on the G protein (e.g. Ref. 41). N-Ethylmaleimide was without effect on agonists in the present investigation, but the characteristic effects were recovered upon reconstitution of the purified receptor with Go/i. The loss of that sensitivity upon purification therefore was reversible, in accord with the notion that solubilization or purification is accompanied by a decrease in the value of Kp.
Wild-type muscarinic receptors in native membranes exhibit some constitutive activity (16, 17), but most are presumably in the R state (i.e. $K_R > 1$). With $M_2$ receptors transiently expressed in NIH 3T3 cells, for example, only 1.9% of the sites were found to inhibit the R* state in the absence of ligand (16). If antagonists bind preferentially to R ($\alpha > 1$), their apparent affinity for membrane-bound receptors, as estimated from Scheme I, is expected to approximate their intrinsic affinity for the R state (EC$_{50} \approx K_R$).$^6$ Purified receptors appear to exist predominantly in the R$^*$ state (i.e. $K_R < 1$); if agonists bind preferentially to R$^*$ ($\alpha < 1$), their apparent affinity is expected to approximate their intrinsic affinity for the R$^*$ state (EC$_{50} \approx \alpha K_L$). It follows that solubilization should be accompanied by a reduction in the apparent affinity of antagonists ($K_L < EC_{50} \leq \alpha K_L$) and an increase in that of agonists ($\alpha K_L < EC_{50} < K_L$), assuming that the effects of a decrease in $K_R$ are not obscured by concomitant changes in either $K_L$ or $\alpha$. 

Among muscarinic antagonists, solubilization of the receptor generally has been found to reduce affinity or to have little effect (e.g. Refs. 43–45). Antagonists therefore behave as expected if detergents were to decrease $K_R$ by varying degrees, depending upon the conditions. Detergents and N-ethylmaleimide also may have a cumulative effect, as suggested by the early observation that N-ethylmaleimide reduced the binding of [3H]quinuclidinyl benzilate only when pretreated membranes from porcine brain were solubilized in $\alpha$-lyso-phosphatidylcholine or when alkylated was carried out on the solubilized receptor (46). Solubilization has been reported to increase the affinity of the $M_3$-selective antagonist pirenzepine for $M_3$ receptors (45, 47), but such examples are rare and perhaps highly specific.

Muscarinic agonists generally bind with lower affinity after solubilization (48), in contrast to predictions based narrowly on Scheme II, but the decrease may be dominated by changes in the interaction between the receptor and the G protein. G proteins do not appear explicitly in Scheme II, and the nature of their involvement is unclear. Also, detergents have been shown to reduce the potency of both agonists and antagonists when purified $M_2$ receptors were compared in solution and after reconstitution in phospholipid vesicles (49). The solubilization of porcine brain in cholate was found to reduce the potency of eight muscarinic antagonists with little or no change in that of five agonists (44). Such observations suggest that solubilization may effect more than a decrease in $K_R$; for example, a concomitant increase in $K_L$ could potentiate the increase in EC$_{50}$ expected with antagonists but tend to offset the decrease expected with agonists.

In accord with the predictions of Scheme II, the affinity of isoproterenol for the $\beta_2$ adrenergic receptor and a constitutively active mutant was increased as expected if solubilization and purification were to cause a shift from the inactive to the active state (15). Also, the suggestion that N-ethylmaleimide destabilizes the $M_2$ receptor by favoring the active state is consistent with other evidence that activated G protein-coupled receptors are comparatively unstable. Constitutively active mutants of both the $\beta_2$ adrenergic receptor (15, 19) and the $\alpha_2$A adrenergic receptor (20, 50) have been shown to undergo spontaneous inactivation more rapidly than the corresponding wild-type receptors.

**Effects Inconsistent with Schemes I–III**—A final comment is in order concerning two anomalies that cannot be accommodated by either Scheme I or Schemes II and III. With native and alkylated receptors, the affinity of $N$-[3H]methylscopolamine in terms of Scheme I exceeded that of the unlabeled analogue. The difference was only about 1.6-fold with native receptors but increased to 3–7-fold after treatment with N-ethylmaleimide (Table III). Also, the relative capacity for $N$-[3H]methylscopolamine and [3H]quinuclidinyl benzilate was greater than 0.9 with native receptors but only about 0.77 after alklylation (Fig. 3B, Table II). Similar discrepancies emerge in terms of Schemes II and III. Labeled and unlabeled $N$-methylscopolamine revealed a difference of 1.5- and 7.4-fold in the value of $K_L$ and $\alpha K_L$, respectively (Table V), and the relative value of $R_i$ inferred for $N$-[3H]methylscopolamine and [3H]quinuclidinyl benzilate after alklylation was about 0.8 (Fig. 3B).

Because there was no discrepancy in the affinity of quinuclidinyl benzilate, the effect with $N$-methylscopolamine seems to be ligand-specific. It has been noted previously that $N$-[3H]methylscopolamine can be contaminated with scopolamine (21), which would yield artifactually low estimates of both the capacity and the dissociation constant, but scopolamine was absent from the product used in the present investigation. Triethylamine was identified in the mass spectrum, but its equilibrium dissociation constant at $M_2$ receptors exceeds 0.1 mm (51). Because the molar ratio of the impurity to $N$-[3H]methylscopolamine never exceeded 0.7, any effect on binding was negligible (21).

The appearance of both anomalies after treatment with N-ethylmaleimide raises the possibility that they derive from the reduced stability of alkylated receptors. That seems unlikely, however, because the shortfall in capacity for $N$-[3H]methylscopolamine cannot be accounted for in terms of Scheme III. Also, the apparent affinity of $N$-[3H]methylscopolamine exceeded that of the unlabeled analogue (Table III). In the event of instability, the affinity of unlabeled $N$-methylscopolamine inferred from its inhibitory effect at near saturating concentrations of [3H]quinuclidinyl benzilate should exceed the apparent but artifactually low affinity of the radioligand. The expected effect of instability on the binding of $N$-[3H]methylscopolamine can be seen in Fig. 3B, where the profile shifts rightward over time. Even after 3 h, however, the radioligand remained at least as potent as the unlabeled ligand (cf. Tables II and III).

The difference in the capacity of alkylated receptors for [3H]quinuclidinyl benzilate and $N$-[3H]methylscopolamine implies that about 20% of the sites are of anomalously low affinity for the latter. No corresponding shoulder is evident in the inhibitory profile of unlabeled $N$-methylscopolamine, which therefore appears to inhibit at sites to which $N$-[3H]methylscopolamine does not bind. Essentially the same pattern has been described previously for $M_2$ muscarinic and $D_2$ dopaminergic receptors, and the apparent paradox could be rationalized in terms of cooperative interactions between the radiolabeled and unlabeled ligands (22, 51, 52). Similar effects may account for the difference in the capacity of alkylated receptors for $N$-[3H]methylscopolamine and [3H]quinuclidinyl benzilate in the present investigation.

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$^6$ In terms of Scheme II, the concentration of a ligand L that achieves half-maximal occupancy of the receptor R is defined as follows: EC$_{50} = \alpha K_L(1 + K_L)/(1 + \alpha K_L)$. Affinity and potency are qualified as either high or low with reference to the value of 1/K$_L$, 1/(\alpha K$_L$), 1/EC$_{50}$, etc.
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