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Evidence for Paired M_2 Muscarinic Receptors

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SUMMARY

Binding assays involving various antagonists, including *N*-[^{3}H]methylscopolamine, [^{3}H]quinuclidinyl benzilate, AFDX-116, pirenzepine, and propylbenzilylcholine mustard, disclosed only a single population of M_2 muscarinic receptors in membranes from the rat "brainstem" (medulla, pons, and colliculi). However, competition curves between *N*-[^{3}H]methylscopolamine and various agonists, including oxotremorine, *cis*-dioxolane, and acetylethylcholine mustard, showed approximately equal numbers of guanine nucleotide-sensitive high affinity (H) sites and guanine nucleotide-insensitive low affinity (L) sites. This 50% H phenomenon persisted in different buffers, at different temperatures, after the number of receptors was halved (and, thus, the remaining receptor to guanine nucleotide-binding protein ratio was doubled), after membrane solubilization with digitonin, and when rabbit cardiac membranes were used instead of rat brainstem membranes. Preferential occupation of H sites with acetylethylcholine mustard, and of L sites with quinuclidinyl benzilate or either mustard, yielded residual free receptor populations showing predominantly L and H sites, respectively. Low concentrations of [^{3}H]-oxotremorine-M labeled only H sites, and the B_{max} for these sites was 49% of the B_{max} found with [^{3}H]quinuclidinyl benzilate plus guanine nucleotide. These and other results are most consistent with the idea that H and L receptor sites exist on separate but dimeric receptor molecules and with the hypothesis that only the H receptors cycle between high and low affinity, depending upon interactions between this receptor molecule and a guanine nucleotide-binding protein.

Muscarinic receptors belong to a large family of membrane proteins whose actions are mediated via the activation of heterotrimeric G proteins (1, 2). The binding of agonists but not antagonists to these proteins leads to the formation of a high affinity "ternary" complex between agonist, receptor, and G protein. The binding of GTP or its nonhydrolyzable analogs to the G protein in ternary complexes is believed to initiate the dissociation of one subunit of the G protein and to destabilize ternary complexes, leading to a lower affinity agonist-receptor complex. In their classical studies of the binding of agonists to β-adrenergic receptors, Lefkowitz and colleagues (3, 4) found that a simple two-state ternary complex model was sufficient to explain their data. An important feature of their results was that the percentage H varied with agonist efficacy from 52 to 92% and dropped near 0% in the presence of guanine nucleotides.

Despite the high degree of structural homology between muscarinic receptors and β receptors (5–7), the binding properties of muscarinic receptors are different from those found for β receptors. The first suggestion that muscarinic receptors had unusual binding properties came from studies of brain receptors by Birdsall *et al.* (8). They found that competition curves between agonists and ^{3}H-antagonist were best modeled assuming three rather than two affinity states for agonists. Subsequent investigators confirmed the utility of using a three-state model for the analysis of some experiments, although at least as often only two affinity states for agonists were found (see Ref. 9). It should be noted that many studies of muscarinic receptors in which three affinity states were reported, including the original work with cerebral cortex (8), were carried out with tissue preparations that are now known to contain receptors derived from several of the five known genes (m1–m5) for these receptors (10). Such studies clearly do not show that a single receptor can have three affinity states. However, Birdsall *et al.* (8) also studied the brainstem and many investigators have worked since with the mammalian heart (e.g., Refs. 9, 11, and 12) to document the phenomenology of three states, and these tissues are believed to express mRNA that is overwhelmingly for m2 receptors (13–15). The heart and the brainstem also appear to have uniform receptor populations with respect to the binding of antagonists that distinguish m1–m5 receptors

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ABBREVIATIONS: G protein, guanine nucleotide-binding protein; AECM, acetylethylcholine mustard; Gpp(NH)p, guanyl-5′-yl imidodiphosphate; H, guanine nucleotide-sensitive receptor site capable of showing high affinity for agonists; L, guanine nucleotide-insensitive receptor site showing only low affinity for agonists; NMS, *N*-methylscopolamine; PBCM, propylbenzilylcholine mustard; QNB, quinuclidinyl benzilate; PEI, polyethylenimine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CHAPSO, 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate.

(15, 16). There is, therefore, wide agreement that m2 receptors alone can show three or two affinity states for agonists.

In their appraisal of the binding of ligands to canine cardiac receptors, Mattera *et al.* (11) found another new feature of muscarinic receptors, namely Hill coefficients of 1.3–1.5 for the binding of [^{3}H]QNB in the presence but not the absence of Gpp(NH)p. They noted that the presence of positive cooperativity requires at least 2 mol of ligand binding sites/mol of receptors and that the dependence of cooperativity upon guanine nucleotide indicates complexes between antagonist, receptor, and G protein. They found that binding data for agonists could be rationalized equally well assuming either three affinity states or the presence of bivalent receptor sites that could sometimes show negative cooperativity for binding agonists. They preferred the cooperative model because of their findings with QNB in the presence of Gpp(NH)p. Either bivalent receptors (having two binding sites/monomeric receptor protein) or pairs of monomers (dimers or higher multiples) could explain their data. Sokolovsky and colleagues (17) have suggested that muscarinic receptors from the heart and brainstem can move during electrophoresis as dimers, although their results have not been confirmed.

A further unusual feature of agonist binding to muscarinic receptors, which is compatible with the idea of bivalent or dimeric receptors, is the frequent presence of nearly equal numbers of H and L sites. For example, in 23 experiments with carbachol and hamster cardiac receptors, Wells and colleagues (9) found a mean value of 50% H. Nonetheless, they found variable two- and three-state phenomena and percentage H values, for different agonists and different regions of the heart, and concluded that the traditional ternary complex model was inadequate to explain their binding data (18). Others have considered the possibility that multiple effector proteins may contribute to the multiple agonist affinity states seen with cardiac muscarinic receptors (19), an idea that is supported by evidence for at least three G proteins (G_i, G_o, and G_k) in the heart that couple to muscarinic receptors (1). However, Schimerlik and colleagues (20) found 53% H values for the binding of carbachol to pure soluble M_2 receptors from the porcine heart, thus demonstrating that G protein is not essential to stabilize H sites on muscarinic receptors or to explain 50% H phenomena. They also provided the critical fact that soluble M_2 receptors bind only 1 mol of QNB/mol of monomeric receptor protein. Further work from this laboratory has shown that pure soluble atrial muscarinic receptors have the hydrodynamic properties of monomers (21) and that reconstitution of these receptors with a small excess of pure G_i, in vesicles, yields 51–54% H values for the binding of agonists (22, 23).

The phenomenon of equal numbers of H and L sites is not limited to m2 receptors. Potter and co-workers (24, 25) found that rabbit hippocampal muscarinic receptors showed only two affinity states for a number of agonists, with values between 45 and 55% H. Because 1 nM [^{3}H]pirenzepine ($K_d \approx 3$ nM) was used for the assays, because the tissue has mRNA primarily for m1 and m3 receptors (13), and because the affinity of pirenzepine is about 11-fold higher for m1 than m3 receptors (26), the results may be taken as applicable to m1 receptors. We proposed that the best working model for the presence of 50% H was dimeric receptors (24), because Haga and Haga (27) had found only 1 mol of antagonist binding/mol of purified bovine cerebral muscarinic receptors. With respect to m3 receptors, we have found that the 50% H phenomenon is also characteristic of muscarinic receptors in the rat submandibular gland,[1] which expresses a nearly pure population of these receptors (10). Finally, Galper *et al.* (28) found 48% H for competition between *cis*-dioxolane and [^{3}H]QNB, using chick heart receptors, which are homologous to mammalian m4 receptors (29).

The results of genetic studies have shown that a number of amino acid residues on each monomeric receptor protein may contribute to affinity phenomena. Most attention has focused on four highly conserved aspartate residues in the second and third putative transmembrane helices of all muscarinic and β-adrenergic receptors. Any of these residues could, in theory, serve as a specific counter-ion for the onium head groups of muscarinic and adrenergic agonists. From the results of point mutations of these aspartates in m1 and β_2 receptors (6, 7, 30) and attempts to localize the sites of binding of PBCM to specific residues of m1 receptors (7, 31–33), it appears that one deeply placed aspartate in the third transmembrane helix is essential for the binding of agonists and antagonists and that another more superficial aspartate contributes significantly to binding phenomena. It is also known that there are two acidic residues, with pK values of about 5.4 and 6.8, on muscarinic receptors, whose protonation strongly affects the binding of ligands (33, 34). The binding of [^{3}H]NMS is affected by the protonation of a "site" with a pK of about 5.9, the binding of AFDX-116 appears to be due to a site with a pK of 6.8, and sites with pK values of 5.4 and 6.8 may both contribute to the binding of antagonist molecules like methoctramine (34). Other amino acid residues are also believed to be important for establishing the nature of the active site, although not as the counter-ions for onium headgroups (30). Venter *et al.* (5) have suggested that different residues in β receptors may subserve high and low affinity binding. Saunders *et al.* (35) have gone one step further, on the basis of the binding and structural characteristics of a series of new muscarinic agonists and antagonists, to suggest that relatively large lipophilic antagonists bind primarily to a superficial aspartate, whereas smaller hydrophilic agonists can bind both to this aspartate and, with higher affinity, to a more deeply placed residue. These data provide a potential structural basis for the idea of bivalent receptors.

In this study we have examined the 50% H phenomenon further, using freshly prepared M_2 receptors associated with their native G protein(s), [^{3}H]QNB plus a guanine nucleotide to assess the total numbers of receptors, and a variety of structurally different agonists. The degree to which H and L sites can bind ligands independently was examined further after selective blockade of each site. The results focus attention on the binding of agonists to two sites, as well as on the presence of two affinity states. Moreover, evidence for guanine nucleotide-sensitive H sites, but guanine nucleotide-insensitive L sites, leads to a new working hypothesis, namely that only half of the QNB receptors, the H sites, can ever show high affinity.

Materials and Methods

cis-Dioxolane, oxotremorine, and (−)-QNB were purchased from Research Biochemicals Inc. (Natick, MA). AECM was synthesized by A. Fisher (Israel Institute for Biological Research, Ness-Ziona, Israel) (36). [*N-methyl*-^{3}H]pirenzepine (87 Ci/mmol), [*N-methyl*-^{3}H]NMS (71.3 Ci/mmol), [L-*benzilic*-4,4′-^{3}H]QNB (32.9 Ci/mmol), [*methyl*-^{3}H]

[1] L. T. Potter, L. A. Ballesteros, and H. E. Hanchett. Binding of agonists to glandular M_3 receptors. Manuscript in preparation.

oxotremorine-M (87 Ci/mmol), and nonradioactive PBCM were purchased from Dupont/New England Nuclear Corp. (Boston, MA). Pirenzepine and AFDX-116 were provided through the courtesy of Boehringer Ingelheim Pharmaceuticals (Ridgefield, CT). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Adult male Sprague Dawley rats, weighing approximately 200 g, were anesthetized with diethyl ether and guillotined. Brains were removed to ice, the cerebellum was discarded, and the colliculi, underlying hindbrain, medulla, and pons were separated from the remainder of the brain by a cut that was just anterior to the superior colliculi and angled downward to a line between the substantia nigra and pons. The region taken, here called "brainstem," includes most of the major brain nuclei rich in "M_2" receptors (15), as revealed by the binding of [^{3}H]QNB in the presence of pirenzepine (37) and the binding of [^{3}H]AFDX-116 (38), but no regions containing significant amounts of "M_1" receptors (15).

Membranes were prepared as described previously (24, 25, 39). In brief, brainstem tissue (~0.3 g/rat) was homogenized in 20–25 ml of ice-cold 50 mM sodium phosphate buffer, pH 7.4, containing 10 mM Na_3EDTA and 0.1 mM freshly prepared phenylmethylsulfonyl fluoride, followed by sedimentation of membranes and their resuspension in 20 mM Tris·HCl buffer, pH 7.4, containing 1 mM $MnCl_2$ (Tris-Mn buffer). These steps remove and inactivate proteases and tend to dissociate agonist-receptor complexes (39); we believe these steps are important for the quantitative study of H sites. All assays with membranes were performed with freshly prepared membranes from 5 mg of tissue.

Competition curves between counterligands and ^{3}H-antagonists for M_2 receptors in membranes were carried out as follows. Membranes were incubated in sextuplicate, at 25° for 60 min, in 10 ml of Tris-Mn buffer containing 0.1 nM [^{3}H]NMS or 1 nM [^{3}H]QNB and one of 15 concentrations of counterligand. Membranes were collected by filtration on Whatman 934AH glass fiber filters. Filters were rinsed three times with 4 ml of ice-cold buffer, placed in counting vials, dried in an oven at 80°, and counted at an efficiency of about 46% in 4 ml of Cytoscint ES (ICN Schwartz/Mann, Cleveland, OH).

AECM and PBCM were activated to their alkylating aziridinium ions by preparation in Tris-Mn buffer at 25°, 30 min before each experiment. These ions react slowly with Tris buffer; hence, concentrations refer to the parent compounds. Eserine (3 μM) was included in all media for experiments with AECM, to minimize the hydrolysis of AECM by esterases. At temperatures near 0°, agonist mustards are known to interact reversibly with muscarinic receptors (e.g., Ref. 40). However, at warmer temperatures, the aziridinium ion of AECM first binds, presumably reversibly, and then is capable of alkylating receptors (41). Because equilibrium does not occur during warm incubations of AECM or PBCM with receptors, the experiments shown in Figs. 3 and 6 are considered "protection" experiments, and the term "apparent" is used before affinity constants.

Competition between oxotremorine and 0.1 nM [^{3}H]NMS for solubilized M_2 receptors was carried out as described above, with the following modifications. The phosphate-EDTA buffer used for homogenization contained 0.2 mM Gpp(NH)p. Sedimented membranes were resuspended in Tris-Mn buffer (membranes from 1 g of tissue formed a 10-ml suspension) and mixed with an equal volume of ice-cold 1.5% digitonin, 0.3% sodium cholate, in the same buffer. The suspension was kept on ice for 1 hr and recentrifuged. Membrane protein from 10 mg of tissue was assayed at a final digitonin concentration of 0.015%. Filters were soaked in 0.3% PEI for 1–2 hr before use. This method for recovering solubilized receptors (42) is believed to depend upon the adsorption of acidic receptor protein to polycationic PEI-coated glass.

Competition curves between counterligands and 5 nM [^{3}H]oxotremorine-M for M_2 receptors in membranes were obtained as described for ^{3}H-antagonists, except for an incubation volume of 1 ml and the use of filters soaked in 0.3% PEI.

In some experiments, 30 μM Gpp(NH)p was included during incubation to reduce the numbers of receptors showing high affinity.

In some experiments, the initial pellet of membranes was resuspended in 100 ml of ice-cold 10 mM potassium phosphate buffer containing 25 mM sodium pyrophosphate and 2 mM Na_3EDTA (pH 7.4). After 60–90 min on ice, the suspension was recentrifuged, and membranes were resuspended in Tris-Mn buffer for binding assays.

In some experiments, H and L sites were blocked partially (~50%) and nonselectively by preincubation of membranes, at 25° for 60 min, in 10 ml of Tris-Mn buffer containing 0.16 nM (−)-QNB. Membranes were then sedimented and resuspended in fresh buffer for further assays. In some experiments, H sites were reduced selectively by similar preincubation with 10 nM AECM. In other experiments, L sites were reduced selectively by similar preincubation with 100 nM oxotremorine (to protect H sites) and 1 nM (−)-QNB.

In one set of experiments, membranes from the atria of adult albino rabbits were prepared and assayed exactly as described for membranes from the rat brainstem, in order to compare results obtained with the receptors from these tissues. In another set of experiments, membranes from the rat brainstem were homogenized, resuspended, and assayed in three additional buffers at pH 7.4, to compare results in different media. All other conditions (volumes, temperature, time, etc.) remained as described above. The buffers used were original Krebs-Henseleit solution, as used by Birdsall *et al.* (8); 100 mM NaCl, 10 mM $MgCl_2$, 20 mM HEPES buffer, as used by Hammer and Giachetti (43); and 100 mM NaCl, 2 mM $MgCl_2$, 20 mM Tris·HCl buffer, as used by Burgen (12). Phenylmethylsulfonyl fluoride was added to each buffer, to a concentration of 0.1 mM, just before homogenization.

Competition curves were fitted to a one- or two-site binding model using the iterative, nonlinear, least squares regression analysis of the GraphPAD program (GraphPAD Software, San Diego, CA). For one-site analyses, data points were fitted both to a sigmoid curve with variable slope, to determine Hill coefficients, and to a mass action curve with a Hill coefficient of 1.0. The mass action curves are shown. Points in each figure in this paper are means of sextuplicate determinations from one of two to five typical experiments. Standard deviations for these determinations are not shown, because they averaged 1–2% of the means for ^{3}H-antagonist assays and 2–4% of the means for assays with [^{3}H]oxotremorine-M. Lines in the figures are computer-generated curve fits; they are given with the goodness-of-fit value (r^2). Fifty percent inhibitory concentrations (IC_{50} values) and the proportions of H and L sites were derived from the curves. Dissociation

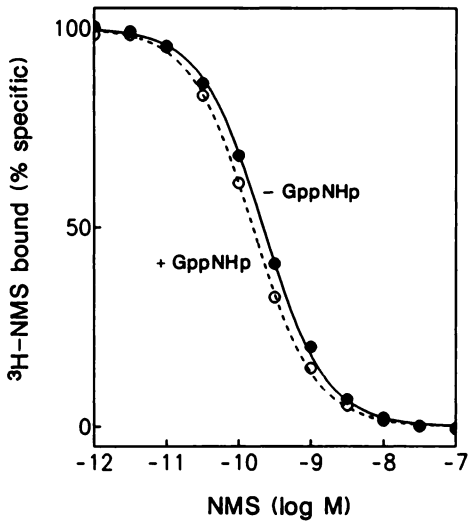

Fig. 1. Competition between NMS and 0.1 nM [^{3}H]NMS for M_2 receptors in brainstem membranes in the presence and absence of 30 μM Gpp(NH)p. Without Gpp(NH)p, the data were: 0% (nonspecific binding) = 157 cpm; 100% = 6287 cpm; goodness-of-fit value (r^2) = 1.000; K_d = 0.129 nM; Hill coefficient = 0.98. The average K_d for four such experiments was 0.125 nM. With Gpp(NH)p, the data were: 0% = 168 cpm; 100% = 8426 cpm; r^2 = 1.000; K_d = 0.064 nM; Hill coefficient = 0.99. The average K_d for three such experiments was 0.063 nM.

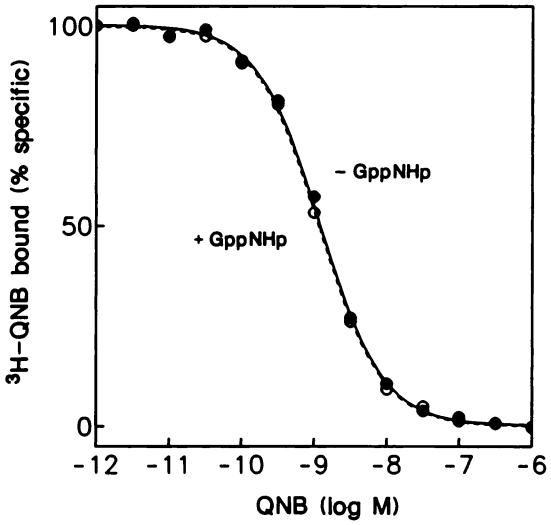

Fig. 2. Competition between (–)-QNB and [^{3}H]QNB for M_2 receptors in brainstem membranes in the presence and absence of 30 μM Gpp(NH)p. Without Gpp(NH)p, the data were: 0% = 672 cpm; 100% = 7356 cpm; r^2 = 0.999; K_d = 0.266 nM; Hill coefficient = 1.03. With Gpp(NH)p, the data were: 0% = 751 cpm; 100% = 8480 cpm; r^2 = 0.999; K_d = 0.173 nM; Hill coefficient = 1.06. The average K_d for three such experiments with Gpp(NH)p was 0.171 nM, and the average B_{max} was equivalent to 57.1 ± 4.2 pmol of receptors/g of tissue.

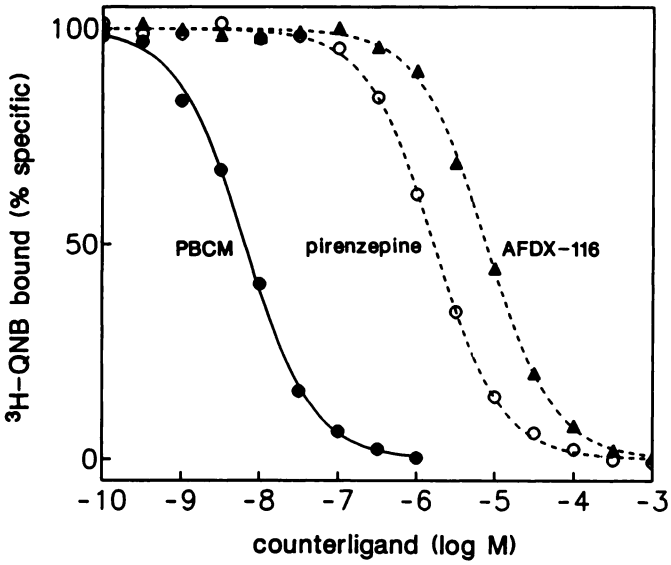

Fig. 3. Protective effect of various antagonists on the binding of 1 nM [^{3}H]QNB to M_2 receptors in brainstem membranes in the presence of 30 μM Gpp(NH)p. The data for PBCM were: 0% = 1082 cpm; 100% = 7822 cpm; r^2 = 0.999; apparent K_i = 0.94 nM; Hill coefficient = 0.94. For pirenzepine the data were: 0% = 605 cpm; 100% = 8947 cpm; r^2 = 1.000; K_i = 241 nM; Hill coefficient = 0.99. With AFDX-116 the results were: 0% = 605 cpm; 100% = 9247 cpm; r^2 = 0.999; K_i = 1626 nM; Hill coefficient = 0.98. Note that all the curves in Figs. 1–3 are mass action curves.

constants (K_i, K_H, or K_L; K_d in the case of self-competition) were calculated from IC_{50} values using the Cheng-Prusoff equation, $K = IC_{50}/(1 + L/K_d)$, where L is the free concentration of the radioligand after binding and K_d is its affinity. Because ligand depletion averaged 10% for assays with 0.1 nM [^{3}H]NMS, the final free L value was taken as the average value of 0.095 nM. Ligand depletion was negligible with 1 nM [^{3}H]QNB or 5 nM [^{3}H]oxotremorine-M and was ignored. Unless otherwise noted, the K_d values used for correcting of IC_{50} values were: [^{3}H]NMS without Gpp(NH)p, 0.125 nM; [^{3}H]NMS with Gpp(NH)p, 0.063 nM; and [^{3}H]QNB with Gpp(NH)p, 0.171 nM (see Results). To determine whether the data were better fit to a one- or two-site model, the residual sums of the squares of the respective fits were compared by a partial F test.

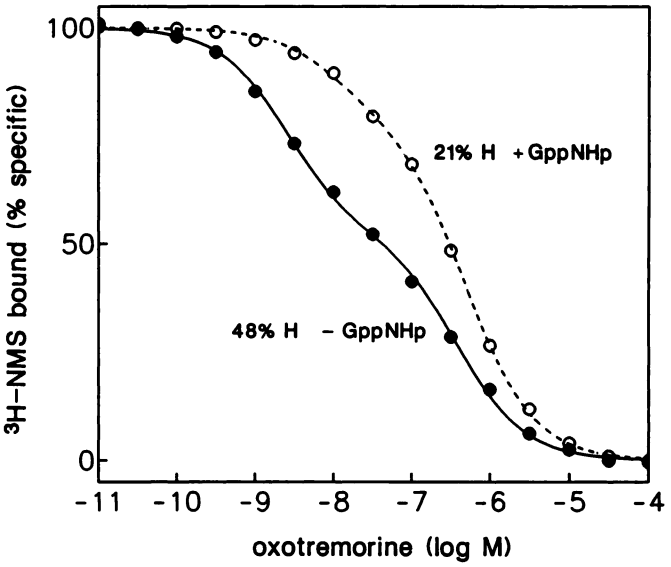

Fig. 4. Competition between oxotremorine and 0.1 nM [^{3}H]NMS for M_2 receptors in brainstem membranes with or without 30 μM Gpp(NH)p. Both curves have two components. Without Gpp(NH)p, the results were: 0% = 130 cpm; 100% = 4649 cpm; r^2 = 1.000; K_H = 1.4 nM; K_L = 221 nM. The data with Gpp(NH)p were: 0% = 152 cpm; 100% = 8757 cpm; r^2 = 1.000; K_H = 4.4 nM; K_L = 199 nM. Note that there is little change in K_L with Gpp(NH)p.

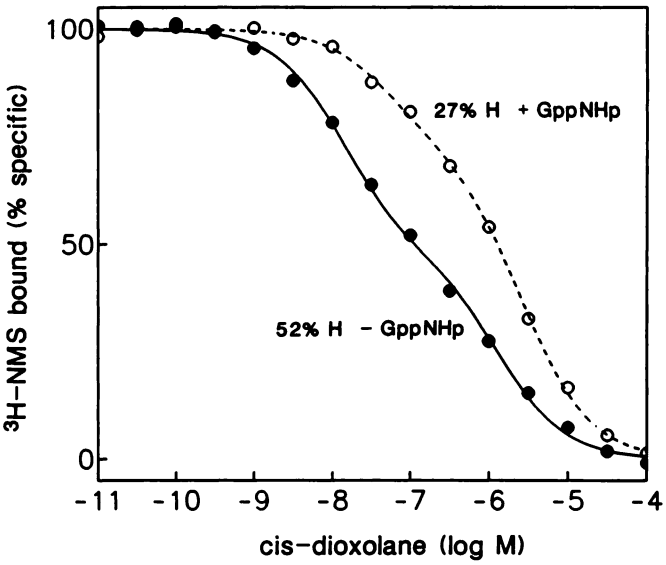

Fig. 5. Competition between *cis*-dioxolane and 0.1 nM [^{3}H]NMS for M_2 receptors in brainstem membranes with or without 30 μM Gpp(NH)p. Both curves have two components. Without Gpp(NH)p, the data were: 0% = 216 cpm; 100% = 5015 cpm; r^2 = 0.999; K_H = 7.8 nM; K_L = 735 nM. Results with Gpp(NH)p were: 0% = 222 cpm; 100% = 8442 cpm; r^2 = 0.999; K_H = 21.1 nM; K_L = 1042 nM.

Results

Binding of antagonists to M_2 receptors. Fig. 1 shows competition curves between NMS and [^{3}H]NMS in the absence and presence of 30 μM Gpp(NH)p. In each case, only a single

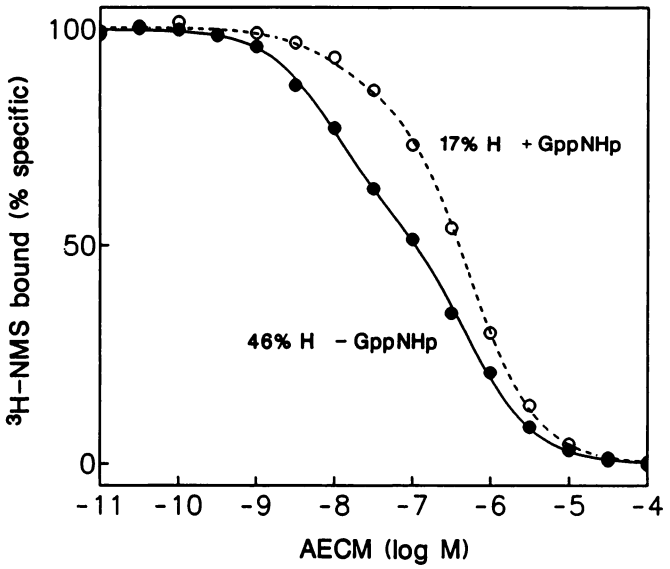

Fig. 6. Protective effect of the aziridinium ion of AECM on the binding of 0.1 nM [^{3}H]NMS to M_2 receptors in brainstem membranes. Each curve has two components. The data without Gpp(NH)p were: 0% = 106 cpm; 100% = 9480 cpm; r^2 = 1.000; apparent K_H = 9.9 nM; apparent K_L = 225 nM. With Gpp(NH)p, the data were: 0% = 109 cpm; 100% = 4420 cpm; r^2 = 1.000; apparent K_H = 5.4 nM; apparent K_L = 295 nM. Note little change in K_L with Gpp(NH)p, despite alkylation of H sites with AECM.

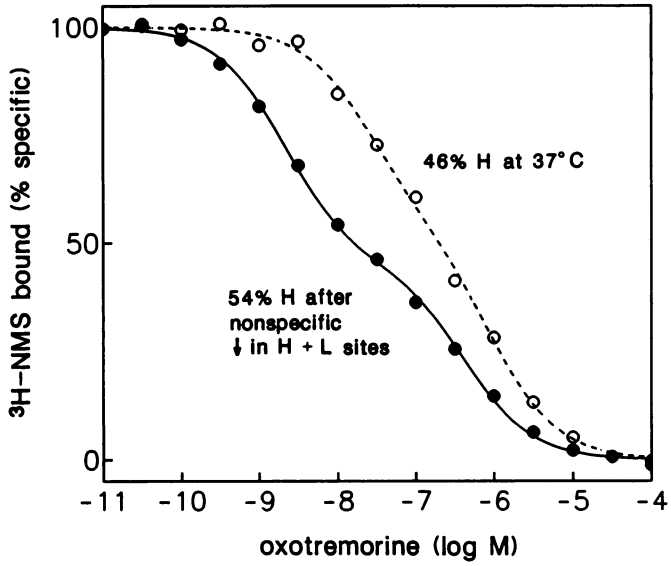

Fig. 7. Competition between oxotremorine and 0.1 nM [^{3}H]NMS for M_2 receptors in brainstem membranes at 37° and after nonspecific reduction in the number of receptors by preincubation with (−)-QNB. The *left curve* is very similar to the control curve shown at the left in Fig. 4. At 37°, 0% = 86 cpm; 100% = 6029 cpm; r^2 = 0.999; K_H = 13.0 nM; K_L = 491 nM (based on a K_d for [^{3}H]NMS of 0.105 nM at 37°). With fewer receptors, 0% = 172 cpm; 100% = 2540 cpm; r^2 = 0.999; K_H = 1.2 nM; K_L = 243 nM. Note that changes in temperature and receptor levels do not have a significant effect on the 50% H phenomenon.

population of binding sites was apparent, with a Hill coefficient close to 1.0. The affinity was slightly higher with Gpp(NH)p than without it. Very similar results were obtained from competition curves between QNB and [^{3}H]QNB (Fig. 2). Again, Hill coefficients were close to 1.0 and antagonist affinity was slightly higher in the presence of Gpp(NH)p. Several other antagonists also yielded binding curves that indicated a ho-

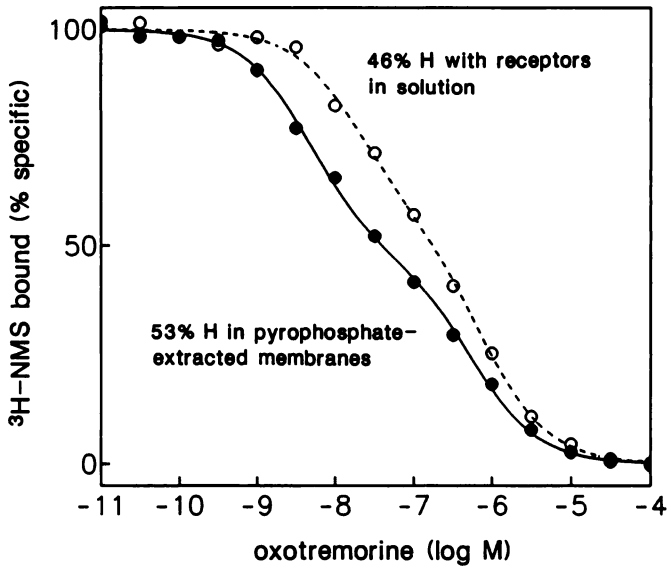

Fig. 8. Competition between oxotremorine and 0.1 nM [^{3}H]NMS for M_2 receptors in pyrophosphate-extracted brainstem membranes and receptors in digitonin solution. The *left curve* is virtually identical to the control curve shown at the left in Fig. 4. The extracted membranes showed: 0% = 114 cpm; 100% = 5388 cpm; r^2 = 0.999; K_H = 2.7 nM; K_L = 301 nM. In solution, the data (for receptors from 10 rather than 5 mg of tissue) were: 0% = 72 cpm; 100% = 4332 cpm; r^2 = 0.999; K_H = 11.0 nM; K_L = 406 nM (based on a K_d for [^{3}H]NMS of 0.121 nM in solution). Note that changes that usually cause receptor dispersion do not significantly alter the 50% H phenomenon.

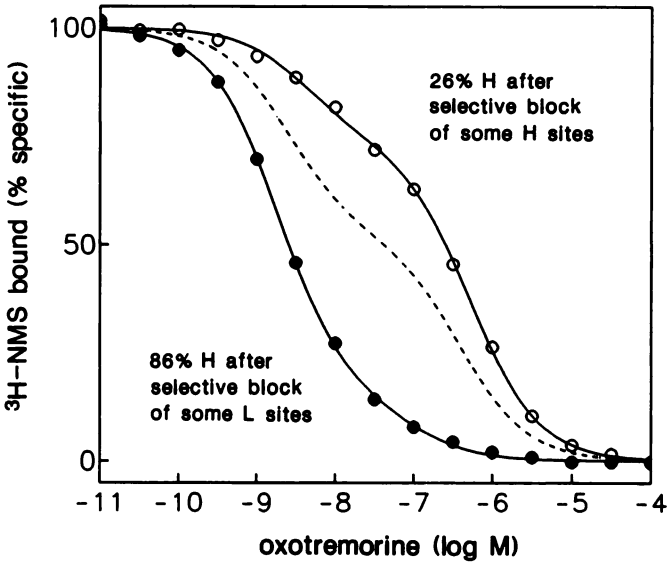

Fig. 9. Competition between oxotremorine and 0.1 nM [^{3}H]NMS for M_2 receptors in brainstem membranes that were preincubated under conditions that preferentially blocked H sites with AECM or L sites with (−)-QNB. Each curve shows two components. The data for the *left curve* were: 0% = 114 cpm; 100% = 2414 cpm; r^2 = 1.000; K_H = 1.1 nM; K_L = 52.6 nM. Note little change in K_H by comparison with the value of 1.4 nM in Fig. 4, despite blockade of most L sites. The *middle curve* is the control curve from Fig. 4. The results for the *right curve* were: 0% = 128 cpm; 100% = 2790 cpm; r^2 = 1.000; K_H = 2.5 nM; K_L = 295 nM. Note little change in K_L by comparison with the value of 221 nM in Fig. 4, despite blockade of many H sites.

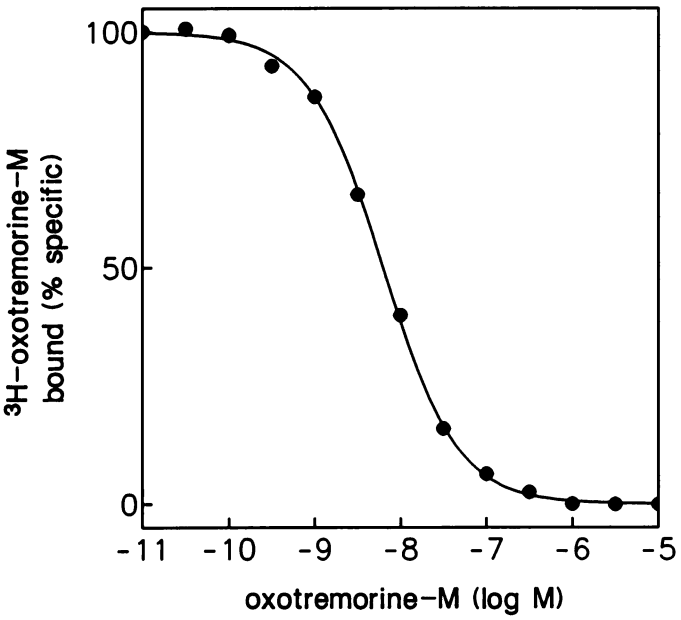

Fig. 10. Competition between oxotremorine-M and 5 nM [^{3}H]oxotremorine-M for M_2 receptors in brainstem membranes. The curve is a mass action curve. The data were: 0% = 1507 cpm; 100% = 10,955 cpm; r^2 = 1,000; K_d = 1.45 nM; Hill coefficient = 0.96; B_{max} = 14,093 cpm. Mean values for three such experiments plus three saturation curves are given in the text and indicate an average B_{max} of 28.1 pmol of H sites/g of tissue. This is half the number of receptors found with [^{3}H]QNB (fig. 2).

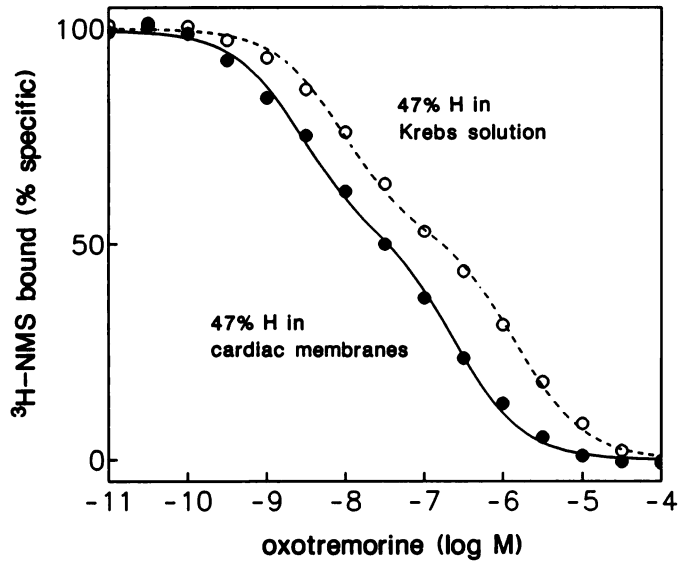

Fig. 11. Competition between oxotremorine and 0.1 nM [^{3}H]NMS for M_2 receptors in rabbit atrial membranes in Tris-Mn buffer and for M_2 receptors in rat brainstem membranes in Krebs solution. Both curves show two components. Data are given in Table 1.

mogeneous population of binding sites, including pirenzepine, which is selective for m1 receptors (10, 15), AFDX-116, which is selective for m2 receptors (10, 15), and PBCM, which is generally considered nonselective (Fig. 3). Positive cooperativity was not found with any of these five antagonists in the presence of Gpp(NH)p.

Binding of agonists to two affinity states of M_2 receptors. Fig. 4 shows competition curves between the agonist oxotremorine (a tertiary amine) and [^{3}H]NMS, in the absence and presence of Gpp(NH)p. Similar experiments using the

TABLE 1

Data for competition assays between 10^{-11} to 10^{-4} M oxotremorine and 0.1 nM [^{3}H]NMS, using rat brainstem membranes in different buffers and using rabbit atrial membranes in Tris-Mn buffer

Conditions	H sites	Binding		IC_{50}		r^2
		0% value	100% value	H sites	L sites	
	%	cpm	cpm	nM	nM	
Tris-Mn buffer (Fig. 4)	48	130	4649	2.5	389	1.000
Krebs solution (Fig. 11)	47	171	5726	9.1	1470	0.999
100 mM NaCl, 20 mM HEPES, 10 mM $MgCl_2$ (43)	50	196	5972	4.2	854	0.998
100 mM NaCl, 20 mM Tris·HCl, 2 mM $MgCl_2$ (12)	54	145	5609	7.3	1523	0.999
Atrial membranes in Tris-Mn buffer (Fig. 11)	47	115	3000	2.5	245	0.999

agonists *cis*-dioxolane (a quaternary amine) and AECM (an alkylating aziridinium derivative of acetylcholine) are shown in Figs. 5 and 6. The binding curves for each agonist in the absence of Gpp(NH)p were very well fitted using a two-site binding model, and in each case the numbers of H and L sites were approximately equal. In the presence of 30 μM Gpp(NH)p, most of the binding sites showed low affinity. After corrections for the affinity of [^{3}H]NMS with and without Gpp(NH)p, it was clear that the affinities of oxotremorine, *cis*-dioxolane, and AECM for L sites were very similar before and after the addition of Gpp(NH)p. It may, therefore, be concluded that half of the labeled sites, the L sites, were not regulated by Gpp(NH)p and that the other half, the H sites, could assume either high or low affinity, depending upon receptor interactions with a G protein.

All other potent agonists tested, including acetylcholine, arecoline, bethanechol, carbachol, methacholine, muscarine, oxotremorine-M, propionylcholine, and RS-86, also showed the 50% H phenomenon under the conditions described. The mean percentage H value in 20 experiments was 52.0%. These data will be presented elsewhere, in relation to the relative affinities of different agonists for H and L sites on m1–m4 receptors.

A number of variations in assay conditions failed to have a significant effect on the nearly equal proportions of H and L sites observed during competition between oxotremorine and [^{3}H]NMS in Tris-Mn buffer. Fig. 7 shows the (lack of) effect of raising the temperature from 25° to 37°, a change that would be expected to alter multicomponent equilibrium processes. Fig. 7 also shows the lack of effect of reducing the free receptor level before competition, by preincubation of membranes with (−)-QNB. Reduction of the receptor level would be expected to have a major effect on the ratio of H to L sites if the ratio were dependent upon the stoichiometry between freely interacting receptors and a lower concentration of receptor-specific G proteins (18). Fig. 8 shows the lack of effect of extracting membranes with 25 mM sodium pyrophosphate before competition assays. This treatment might be expected to alter 50% H phenomena if receptor-receptor interactions were in some way dependent upon receptor anchoring in patches by myosin-like, pyrophosphate-extractable proteins. Finally, Fig. 8 also shows the lack of effect on percentage H values of dissolving membrane proteins with digitonin before competition assays.

Selective blockade of H and L sites. If H and L affinity states are dependent upon the presence of distinct populations of receptor sites on one or more M_2 receptor molecules, then it should be possible to block each kind of site selectively and find that most of the remaining sites have the affinity of the other site. Fig. 9 shows the results of an experiment in which AECM was used to occupy some H sites selectively first, followed by a competition experiment between oxotremorine and [^{3}H]NMS to study the remaining sites. The result was clear; most of the remaining sites had low affinity. Fig. 9 also shows the results of an experiment in which L sites were blocked selectively. Membranes were first incubated in oxotremorine to protect H sites, with sufficient QNB to occupy most L sites. The membranes were then sedimented and resuspended to remove free ligands; L sites remained blocked because the half-time for the dissociation of QNB under the assay conditions was 840 min (data not shown). Subsequent assessment of the remaining receptors by competition between oxotremorine and [^{3}H]NMS showed that 86% of the remaining sites had high affinity. The results were qualitatively similar when either AECM or PBCM was used as the ligand to occupy L sites (data not shown). These experiments demonstrate that H and L sites on M_2 receptors have many of the characteristics of independent receptors. In comparison, Baker and Posner (41) have shown previously that partial occupation of cardiac muscarinic receptors with AECM reduced the subsequent binding of [^{3}H] oxotremorine-M more than that of [^{3}H]NMS. Baker *et al.* (44) have also shown that irreversible adrenergic agonists selectively occupy and then block the high affinity sites of pulmonary β receptors. Their results and the present results are clearly similar. For further comparison, Birdsall *et al.* (45) showed that preincubation of cortical membranes with carbachol plus PBCM altered subsequent competition curves between carbachol and a ^{3}H-antagonist, with a large loss in low affinity sites. They concluded that high and low affinity agonist-binding sites were not interconvertible under the conditions of their experiments. However, membranes from the rat cerebral cortex are now known to have m1–m4 muscarinic receptors, with differing affinities for agonists. Hence, the loss of low affinity sites in their experiments could have been due to more blockade of one receptor subtype than of another by PBCM. Thus, it is not possible to conclude from the results of Birdsall *et al.* (45) that the L sites of a single receptor subtype can be blocked selectively with nearly irreversible antagonists.

Selective binding of [^{3}H]oxotremorine-M to H sites. Fig. 10 shows a competition curve between oxotremorine-M and 5 nM [^{3}H]oxotremorine-M. The binding data showed a single class of binding sites. Rosenthal/Scatchard analyses of binding data with 0.5–50 nM [^{3}H]oxotremorine-M also revealed only a single class of binding sites (data not shown). Mean values (± standard deviations) from three self-competition experiments and three saturation analyses were K_d = 1.33 ± 0.96 nM, Hill coefficient = 0.96 ± 0.05, and B_{max} = 28.1 ± 2.6 pmol/g. This B_{max} for [^{3}H]oxotremorine-M was 49% of the average number of total brainstem receptors found with [^{3}H] QNB in the presence of 30 μM Gpp(NH)p. (Fig. 2). We conclude that the concentrations of [^{3}H]oxotremorine-M that we studied bound selectively to H sites. In comparison, Galper *et al.* (28) used 0–1000 nM *cis*-[^{3}H]dioxolane to study muscarinic receptors in embryonic chick hearts and found two classes of sites, with 36% having high affinity. In their experiments, *cis*-dioxolane also competed with [^{3}H]QNB for two sites, with 48% H. Further, Mattera *et al.* (11) found ~65% as many sites with 150 nM [^{3}H]oxotremorine-M as with saturating concentrations of [^{3}H]QNB in membranes from adult canine hearts.

Agonist binding in different buffers and with cardiac M_2 receptors. Fig. 11 shows a competition curve between oxotremorine and [^{3}H]NMS in which rabbit atrial membranes in Tris-Mn buffer were used instead of rat brainstem membranes. The results were almost identical to those in Fig. 4. The present result of 47% H is very similar to the result of 52% H obtained by Wells and co-workers (9), using oxotremorine, [^{3}H]NMS, and hamster atrial membranes suspended in a modified Krebs solution, containing HEPES buffer instead of bicarbonate buffer, at 30°.

Fig. 11 also shows a competition curve between oxotremorine and [^{3}H]NMS in which rat brainstem membranes were studied in Krebs-Henseleit solution. As in Tris-Mn buffer, the binding curve was very well fitted using a two-site binding model; the percentage H value was 47%. Data obtained using two other buffers also yielded excellent two-site analyses and approximately equal numbers of H and L sites (Table 1). It is evident that the 50% H phenomenon is not dependent upon the medium used to prepare and assay brainstem membranes.

Discussion

In this study, we found a uniform population of M_2 muscarinic receptors in the rat brainstem with the antagonists QNB, NMS, pirenzepine, AFDX-116, and PBCM. It is probable that these receptors are identical to genetically defined m2 receptors (10, 13–15, 33). Despite the appearance of only one population of receptors, competition curves between various agonists and [^{3}H]NMS showed the presence of equal populations of H and L sites. The change in the affinity of H sites but not L sites upon the addition of guanine nucleotide leads to the hypothesis that only H sites can show high and low affinity transitions that are dependent upon interactions between receptors and G protein with and without guanine nucleotide. Low concentrations of [^{3}H]oxotremorine-M clearly occupied H sites preferentially, and these sites could be blocked independently of L sites by using AECM. Similarly, L sites could be blocked independently of H sites with a variety of nearly irreversible ligands (QNB, AECM, and PBCM), after protection of H sites with an efficacious agonist. After preferential blockade of H or L sites, the remaining sites showed remarkably little change in their properties. The 50% H phenomenon was found with cardiac M_2 receptors. These data support the idea that M_2 receptors, like M_1 receptors (24), have paired ligand binding sites as well as two affinity states for agonists.

A variety of explanations could, in theory, account for the phenomenon of equal numbers of H and L sites. These include artifacts due to complex equilibrium states or damage to receptor-G protein complexes, equal numbers of two G proteins, limiting G protein to receptor ratios, bivalent receptors, equal numbers of two independent receptor proteins, and dimeric receptors. These possibilities are discussed below.

In our view, the possibility that artifacts produce the 50% H phenomenon is made very unlikely by observations of about 50% H in experiments involving a variety of muscarinic receptors (m1–m4) from different tissues and species, with many agonists, several antagonists, different assay conditions, and

even pure M_2 receptors in solution and after reconstitution (20, 22, 23).

The possibility that there are two separate and equally important G proteins that interact with m2 and other muscarinic receptors in membranes cannot be excluded. In fact, there is evidence that striatal D_2 dopamine receptors copurify during affinity chromatography with approximately equal amounts of two different-sized G proteins, each of which is sensitive to pertussis toxin (46). In membranes and in solution, D_2 receptors show two agonist affinity states, which are regulated by guanine nucleotides. However, there is no suggestion that these affinity states are due to separate D_2 receptor complexes. More important, Schimerlik and colleagues have shown that pure M_2 receptors can show about 50% H in solution (20) and after reconstitution with a single pure G protein (22, 23). These data do not support the idea that the 50% H phenomenon is dependent upon two G proteins, even though there may be circumstances in which one receptor subtype does interact with equal amounts of two G proteins.

The numbers of H and L sites on m1–m4 receptors could appear equal if the stoichiometry between freely interacting receptors and G proteins was approximately 2:1 (18). Four considerations make this very unlikely. First, the concentration of receptors in membranes is believed to be considerably less than that of G proteins. Second, direct reconstitution experiments with muscarinic receptors and a small excess of G proteins have yielded ~50% H values (22, 23, 47). Third, when we decreased the receptor level by half, there was no change in the 50% H phenomenon (Fig. 7). Finally, when we alkylated H sites with AECM, there was no redistribution of remaining receptors (most of which had L sites) to interact with unchanged G proteins and achieve high affinity (Fig. 9). It should be noted that these data do not exclude the possibility that there are stable complexes of dimeric receptors with one G protein in normal membranes (see below).

The idea that there may be bivalent muscarinic receptors finds some structural basis in the evidence for two aspartate residues that modify the binding of agonists and antagonists to m1 receptors (7, 31–33) and the presence of two acidic residues that modify the binding of the m2-selective antagonist methoctramine to cardiac M_2 receptors (33, 34). However, the 50% H phenomenon and the idea of bivalent receptors are not both compatible with current information about any muscarinic receptor. An essential requirement to reconcile 50% H and bivalent receptors is the presence of two antagonist binding sites on each monomeric receptor molecule. This idea is incompatible with evidence for only 1 mol of binding sites/mol of monomeric M_1 or M_2 receptor protein (20, 27). It is, further, incompatible with extensive evidence for homogeneous antagonist binding sites on m1–m5 receptors, because the binding of antagonists to two different sites on one receptor should produce two binding profiles. Finally, it is extremely unlikely that the occupation of one site (H or L) on bivalent receptors would have virtually no effect on the binding characteristics of the other site (see Fig. 9). We conclude that H and L sites are on separate receptor monomers.

Current evidence does not support the idea that H and L sites are present on two different subtypes of muscarinic receptors. On the contrary, the brainstem and heart contain nearly homogeneous mRNA for m2 receptors (13–15), the receptors in these tissues interact homogeneously with many subtype-selective antagonists, the receptors appear to be of uniform size (21), they show a single pK for binding [^{3}H]NMS (34), they show nearly homogeneous low affinity for agonists in the presence of G proteins and guanine nucleotides in membranes (e.g., Ref. 12 and this paper) and in cells (48), they show nearly homogeneous low affinity for agonists in the absence of G proteins in solution and after reconstitution (22, 23, 27, 49), and receptors with H and L sites copurify during affinity chromatography (21).

On the other hand, it is possible that H and L sites are on physically distinct m2 receptor proteins. In order to achieve two such populations of m2-like muscarinic receptors, it would be necessary to have either two mRNAs (from separate genes or alternative RNA splicing) or one receptor pool that divides in two because of posttranslational changes. Alternative splicing of the mRNA for D_2 dopamine receptors has been noted in the region that generates the third intracellular loop of this protein (50). The amino acid sequence in the third intracellular loop of m2 muscarinic receptors is known to control the ability of these receptors to interact with G proteins (51). Hence, alterations in this region of some m2-like receptors could produce L isoreceptors that are incapable of interacting with G protein. An m2 receptor that is incapable of interacting with G protein would have no obvious biological function by itself and is highly unlikely as an independent receptor monomer. However, distinct m2 monomers could easily be one basis for the dimerization of these receptors or could result from dimerization because of posttranslational changes.

The presence of equal numbers of H and L sites is most compatible with the idea that muscarinic receptors are paired at some point in their posttranslational lifetime. As a working hypothesis, we propose that muscarinic receptors normally exist in membranes as dimers. Because the reconstitution of M_2 receptors without G protein appears to be sufficient to yield a low level of high affinity binding sites for agonists [3–16% (22, 49)], it is suggested that receptor-receptor interactions alone may be sufficient to begin the process that permits H receptors to assume high affinity for agonists. Obviously, agonists may also induce and/or stabilize the dimerization of receptors. Because percentage H values can rise to near 50% after the reconstitution of receptors with G protein (22, 23, 47), it is suggested that dimers are further stabilized by the presence of G protein. The most parsimonious explanation for the presence of one guanine nucleotide-regulated H receptor and one guanine nucleotide-unregulated L receptor is the association of only one G protein molecule with each receptor dimer. In shorthand, $2R + G \rightarrow LHG$. The two receptors that take part in this dimerization could be identical or could be different isoreceptors.

One of the most powerful tests of any hypothesis is its ability to explain, reconcile, or at least fit with unusual phenomena. Unusual binding phenomena are practically the rule with muscarinic receptors and several categories of such data are discussed below. (a) The dimer-single G protein hypothesis clearly fits with extensive evidence for homogeneous receptor protein, two agonist affinity states, and effects of guanine nucleotides that are limited to half the receptor population. (b) The dimeric receptor hypothesis is consistent with the evidence of Galper *et al.* (28) that the binding of an agonist to the high and low affinity forms of chick heart muscarinic receptors involves two independent parallel reactions and that agonist binding does

not mediate affinity changes, suggesting that high affinity receptors do not require agonist binding for their formation. Our data concerning the selective binding of AECM to H sites (Fig. 9) indicate further that the prior or simultaneous occupation of L sites is not a prerequisite for the formation of H sites. (c) The *LHG* hypothesis is compatible with the evidence of Poyner *et al.* (52) that only half of the muscarinic receptors of the cortex, heart, and glands [M_1–M_3 (15)] behave in solution in the detergent CHAPSO as if they were ever associated with G protein. (d) If, indeed, muscarinic receptors are paired in membranes, whether or not they are isoreceptors, it is possible that their positions are different and/or that the proteins become different by reason of distinct glycosylation, phosphorylation, etc. Such differences could easily be affected by the presence of guanine nucleotides. Some differences in receptor structure, position, or conformation may help explain why the numbers of binding sites for [^{3}H]NMS can vary from about 50% (e.g., Refs. 53 and 54) to 100% (e.g., Refs. 48 and 55) of the total number of binding sites for [^{3}H]QNB, why Bartfai and his colleagues (56) found two separate affinity states for (−)-[^{3}H]QNB, why receptor desensitization often involves about half the total receptors (e.g., Ref. 55), and possibly why Mattera *et al.* (11) found different Hill coefficients for the binding of [^{3}H]QNB in the presence and absence of Gpp(NH)p. As noted by Mattera *et al.* (11), changes in interreceptor cooperativity can also account for their findings. Some differences in the position and microenvironment of the monomers in dimers may also help account for the different binding sites that appear to be presented to the new lipophilic versus hydrophilic ligands studied by Saunders *et al.* (35) and for the different pK values of the binding sites of [^{3}H]NMS and AFDX-116 (34). (e) Schimerlik and his colleagues found that M_2 receptors could show 53% H in solution without G protein (20) and also that these receptors could behave as monomers in solution (21). Any of four explanations could account for those apparently disparate findings. First, receptor purification may yield receptor dimers that are dissociated by the Triton X-405 detergent used for hydrodynamic studies. Second, agonists may induce or stabilize receptor dimerization in solution. Third, receptor purification may yield monomeric H and L receptors that have become different by reason of posttranslational changes during their existence as dimers in membranes. Fourth, H and L monomers may be isoreceptors with different agonist-binding properties in solution. (f) Whereas several reconstitution studies have yielded ~50% H for receptors and G proteins, Haga and colleagues (49) have also found that the reconstitution of M_2 receptors can yield up to 83% H values. At first glance, this result is incompatible with the *LHG* hypothesis. However, it is the predicted result if identical receptors are reconstituted with a large excess of G protein in a large volume of lipid, because the most likely result is then *RG* complexes that show a high percentage H, rather than receptor-receptor interactions. An alternative explanation is that the procedures used by Haga and co-workers for the solubilization and/or purification of M_2 receptors were selective for H over L isoreceptors. (g) Finally, an extremely puzzling situation is the highly reproducible observation of either two or three agonist affinity states under slightly different conditions. As discussed by Mattera *et al.* (11), the presence of cooperativity between receptor sites can explain three or more affinity states. Because we have seen three agonist affinity states in the presence of saturating levels of [^{3}H]QNB (39) but only two states in the presence of sub-K_d levels of [^{3}H]NMS (this paper) and [^{3}H] pirenzepine (24, 25), we suspect that one factor that helps to produce more than two states is double labeling of receptor dimers. Specifically, in the near absence of antagonist, one expects agonist-H/free L and agonist-H/agonist-L, producing two affinity states. In the presence of saturating concentrations of an antagonist, the first of these is not present and, instead, one expects agonist-H/antagonist-L, agonist-H/agonist-L, and antagonist-H/agonist-L. This could produce a third affinity state, provided that the affinity of agonist for L sites is usually modified by the occupation of H sites by an agonist rather than an antagonist.

Several very interesting questions are raised by the dimeric receptor hypothesis that are beyond the scope of this discussion. One is the question of the physiological and biochemical meaning of paired receptor sites. In this regard, it may be noted that the concentrations of acetylcholine achieved at synapses, say 1–100 μM, are probably sufficient to occupy L sites, because binding data for acetylcholine under physiological conditions suggest a K_d of about 20 μM.[2] A second question is whether the apparent isomerization of receptors, in membranes (57) and in solution (58), upon the binding of antagonists is a purely intrareceptor phenomenon or whether it may also relate to interreceptor interactions. A third question is whether the allosteric effects of nonclassical antagonists like gallamine (59) and tetrahydroaminoacridine (60) are exerted only within monomers or also between receptor monomers. Our data for the cooperative binding of tetrahydroaminoacridine plus [^{3}H]NMS to hippocampal muscarinic receptors in solution favors intrareceptor allosteric effects but does not exclude dimeric effects (60). Finally, if muscarinic receptors can exist as dimers, other receptors that interact with G proteins may also dimerize. Preliminary data concerning glucagon receptors have suggested dimers (61). Vauquelin and colleagues (62, 63) have explored a variety of ~50% H phenomena with β receptors, including agonist binding, inactivation of receptor complexes with *N*-ethylmaleimide, and desensitization. They concluded that artifacts, different receptor populations, and limited G protein to receptor ratios did not explain their binding data, and they came to the conclusion that a structural heterogeneity of receptors and/or different microenvironments for receptors were necessary. Their data fit the dimeric hypothesis. With respect to β receptors, it is also interesting that Lefkowitz and his colleagues (3, 4) found values in the range of 50–100% H for different agonists, whereas we found values in the range of 0–50% H for different agonists for hippocampal M_1 muscarinic receptors (25). These data imply that two G proteins can interact with receptor dimers under some conditions.

This study has provided only indirect evidence for receptor dimers. Further direct physical evidence for dimers of muscarinic receptors (17) is necessary to test the *LHG* hypothesis.

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