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MULTIPLE BINDING STATES OF MUSCARINIC ACETYLCHOLINE RECEPTORS IN MEMBRANES FROM NEUROBLASTOMA X GLIOMA HYBRID CELLS

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Membranes of neuron-like NG108-15 hybrid cells bind [^{3}H]quinuclidinyl benzilate (QNB) with high affinity and specificity. Greater than 90% of total [^{3}H]QNB binding is to sites having the pharmacological specificity of muscarinic acetylcholine receptors. Three significant features characterize the interaction of ligands with these sites: (1) Specific binding of [^{3}H]QNB at equilibrium follows a simple adsorption isotherm with an apparent K_D of 1 x 10^{-10} M; (2) Rates of [^{3}H]QNB association and dissociation are biphasic, and, as the binding reaction proceeds, the fraction of readily dissociable [^{3}H]QNB decreases; (3) Competition against [^{3}H]QNB for specific binding sites by antagonists gives a slope of 1 when analyzed on Hill plots, but competition for binding sites by agonists gives a slope of less than 1. A simple two-step model for activation is proposed to account for these features.

Muscarinic acetylcholine (ACh) receptors are found throughout the nervous system (1) and elsewhere (2,3), and mediate slow changes in membrane potentials (4), cyclic nucleotide concentrations (5), and phosphatidylinositol turnover (6). High affinity, reversible antagonists, [^{3}H]QNB (7) and [^{3}H]scopolamine (8), and an irreversible antagonist [^{3}H]propylbenzilylcholine mustard (9), have been introduced to quantify receptor binding sites.

Neuron-like neuroblastoma x glioma NG108-15 hybrid cells (10) bind both [^{3}H]QNB (11) and [^{3}H]scopolamine (8) and have proven useful for studying muscarinic ACh receptor properties. In NG108-15 cells muscarinic ACh receptors initially mediate a decrease in cellular cAMP (12). With sustained receptor stimulation, adenylate cyclase activity increases and becomes supersensitive to stimulation by prostaglandin E_1 (12). Additionally, continued activation of receptors accelerates their rate of degradation, leading to a 90% decrease in binding sites at steady state (11, 13). Similar receptor regulation occurs in other cultured cells (14,15).

Membranes from NG108-15 cells provide a homogeneous system for studying the mechanism of muscarinic ACh receptor activation. The present work focuses on the properties of muscarinic binding sites in these membranes in order to gain insight into the initial steps in activation. Although binding has a number of complex features, these features are compatible with a simple model for receptor-ligand interaction.

Abbreviations used: ACh, acetylcholine; QNB, quinuclidinyl benzilate

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MATERIALS AND METHODS

[^{3}H]QNB was obtained from Amersham-Searle. Oxotremorine was obtained from Aldrich and all other compounds were obtained from Signa.

NG108-15 cells of passage 15-19 were grown as previously described (12). Membranes were prepared by homogenizing cells in 0.05 M TrisCl pH 7.8 with 0.3 M sucrose, centrifuging at 30,000g x 30 minutes, homogenizing the pellet in 0.01 M TrisCl pH 7.8, and pelleting the membranes at 30,000g x 30 minutes. Final pellets were suspended in 0.05 M TrisCl pH 7.8, and aliquots were quick frozen and stored in a vapor phase N_2 freezer. Freezing and thawing had no effect on receptor measurements. The membrane fraction contained 23% of the original protein and all of the [^{3}H]QNB binding sites.

Binding of [^{3}H]QNB was measured using a filtration assay previously described (13). Except for rate experiments, duplicate measurements of binding were done for each condition, and the difference between duplicate samples routinely was less than 10% of their average. The concentration of the active stereoisomer of [^{3}H]QNB was 50% of the added isotope, since excess receptors from calf brain bound only one-half the racemic mixture. Measurement of specific binding by centrifugation gave the same values as measurements by filtration.

RESULTS

Equilibrium binding of [^{3}H]QNB At equilibrium, specific binding of [^{3}H]QNB to membranes from NG108-15 cells followed a typical adsorption isotherm (Fig. 1). Nonspecific binding to membranes was about 5% of specific binding, considerably lower than found in intact cells (8). Incubations were at 24° for 90 minutes, the minimum time needed for equilibration at all concentrations of [^{3}H]QNB (Fig. 2, below).

A Scatchard plot of specific [^{3}H]QNB binding (Fig. 1, inset) gave a single straight line, the intercept indicating a maximum number of binding sites in this experiment equal to 62 fmols per mg membrane protein. The number of binding sites varied between preparations, but triplicate cultures, grown and harvested together, gave receptor concentrations with a standard deviation of 5-15%. Linearity in a Scatchard plot often is interpreted as evidence for a single class of noninteracting binding sites, and the negative reciprocal of the slope is taken to equal the K_D. The apparent K_D calculated from Figure 1 was 1×10^{-10} M. In repeated experiments, the calculated K_D ranged from 6×10^{-11} M to 1×10^{-10} M, identical to values reported for muscarinic ACh receptors in other systems (7).

Rates of [^{3}H]QNB association and dissociation In order to verify the equilibrium determination of K_D, the kinetics of [^{3}H]QNB-receptor interaction were investigated. K_D should equal k_{off}/k_{on} for a simple binding reaction. Somewhat surprisingly, considering the linearity of the Scatchard plot, the rate of association had both rapid and slow phases (Fig. 2). With 3.8×10^{-10} M [^{3}H]QNB, about 40% of the binding occurred in the first 2 minutes, but equilibrium was not reached until about 90 minutes. Similar behavior was seen

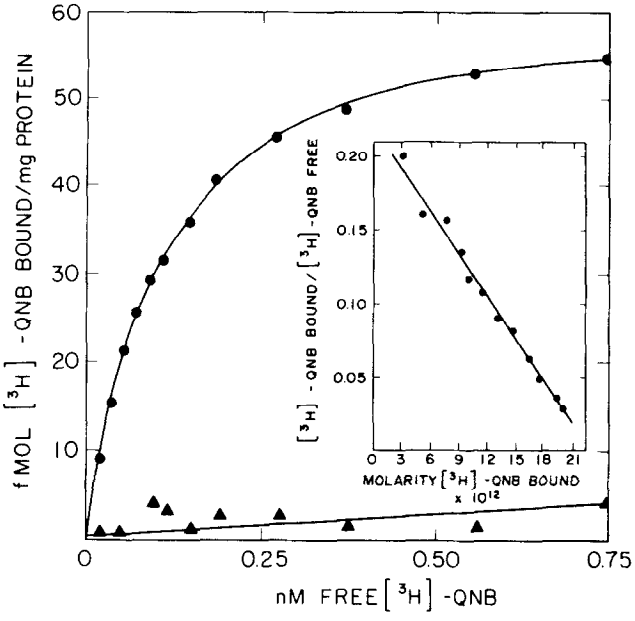

Figure 1 Concentration dependence of [^{3}H]QNB binding to NG108-15 membranes For total binding, membranes at 0.37 mg protein in 1 ml TrisCl pH 7.8 were incubated with increasing concentrations of [^{3}H]QNB for 90 minutes at 24°. Suspensions were diluted with 5 ml ice cold buffer and collected on fiberglass filters. Filters were washed 3x with 5 ml volumes of buffer and counted for tritium retained. Nonspecific binding was determined in the presence of 10^{-3} M oxotremorine. Specific binding was the difference between total and nonspecific binding. (●) Specific binding. (▲) Nonspecific binding.
Inset: Scatchard plot of specific binding.

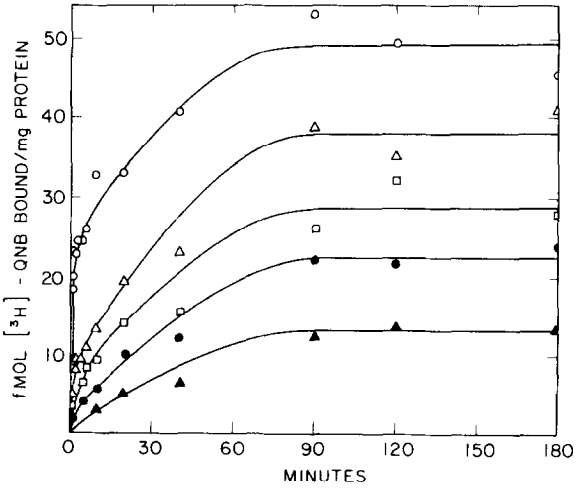

Figure 2 Rate of [^{3}H]QNB association Membranes at 0.26 mg protein per ml were incubated with incrasing [^{3}H](-)QNB and specific binding determined at the indicated times. (○) 380 pM, (△) 150 pM, (□) 110 pM, (●) 76 pM, (▲) 38 pM.

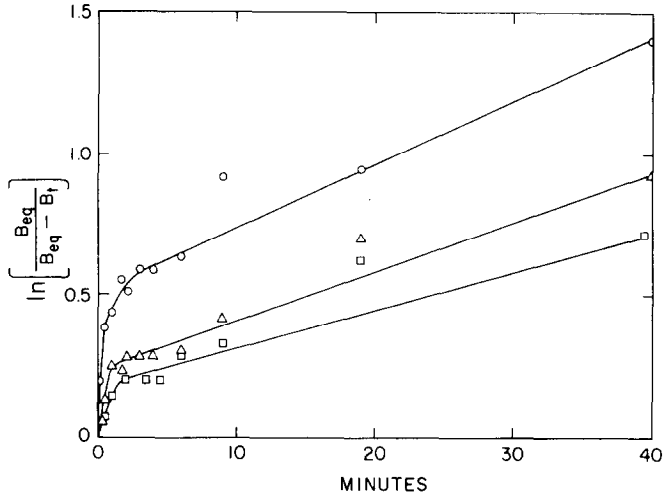

Figure 3 Logarithmic plot of [^{3}H]QNB association Data from Figure 2 were replotted with B_{eq} equal to binding at equilibrium and B_t equal to binding at time of sampling. (○) 380 pM, (△) 150 pM, (□) 110 pM.

with lower concentrations of [^{3}H]QNB. If [^{3}H]QNB were binding in a simple reversible manner to a single class of sites, a plot of ln $B_{eq}/(B_{eq} - B_t)$ versus time should be linear (16). Such a plot for the data of Figure 2 clearly was nonlinear (Fig. 3).

Dissociation of [^{3}H]QNB from the receptor also had rapid and slow phases (Fig. 4). Furthermore, with increasing duration of incubation, a decreasing percentage of bound [^{3}H]QNB dissociated rapidly (Fig. 4, inset). After 10 minutes incubation, the percentage of rapidly reversible binding was 50%, but after 90 minutes, the percentage was only 10%. At 90 minutes, the absolute amount of [^{3}H]QNB that dissociated rapidly was only half that measured at 10 minutes.

Competition for specific [^{3}H]QNB binding sites by cholinergic compounds

The affinity of binding sites for various cholinergic compounds was measured using equilibrium competition experiments. In these experiments, the concentration of added [^{3}H]QNB was constant at 5×10^{-10} M and competitors were added in increasing amounts. The data (Fig. 5) clearly show that [^{3}H]QNB binding sites had the pharmacological specificity of muscarinic ACh receptors (7). Potent muscarinic antagonists scopolamine and atropine blocked binding with half-maximal inhibition at 10^{-9} M and 2×10^{-9} M. Nicotinic antagonists, in contrast, blocked binding only at much higher concentrations. Acetylcholine, oxotremorine, arecoline, and carbachol, all activators of muscarinic ACh receptors, blocked binding half-maximally at 0.2, 0.3, 1, and 2×10^{-6} M, respectively.

Figure 5 also shows that agonist competition was qualitatively different than antagonist competition. Competition curves for agonists were broader than those for antagonists. The data of Figure 5 have been analyzed using Hill plots

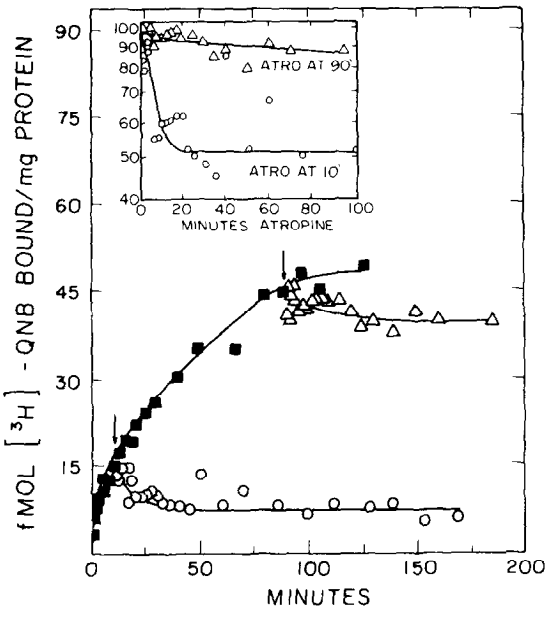

Figure 4 Rate of [^{3}H]QNB dissociation At 10 minutes (○), and at 90 minutes (△) after mixing membranes with [^{3}H]QNB, 10^{-6} M atropine was added to portions of the reaction mixture and [^{3}H]QNB remaining bound was measured at the indicated times. Rate of [^{3}H]QNB association was also determined (■). Inset: Percentage of [^{3}H]QNB specifically bound after adding atropine at 10 minutes (○) or at 90 minutes (△), logarithmic scale.

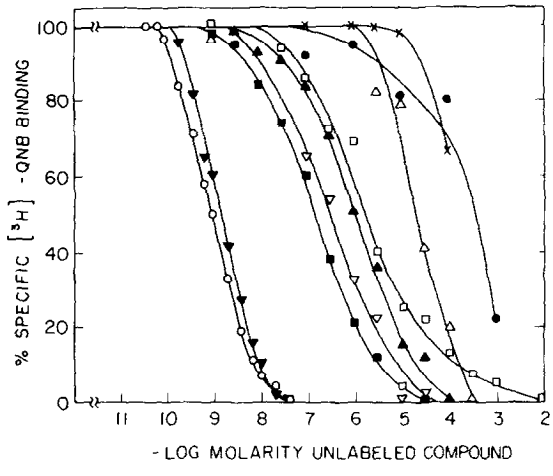

Figure 5 Competition with [^{3}H]QNB for binding sites by cholinergic drugs Specific binding of 2.5 x 10^{-10} M added [^{3}H](-)QNB was determined in the presence of 2.5 uM eserine sulfate and the indicated concentration of cholinergic drugs. 100% binding ranged from 31 fmols per mg protein to 49 fmols per mg protein in various preparations. Parameters of inhibition were independent of membrane preparations used. (○) scopolamine, (▼) atropine, (■) acetylcholine, (▽) oxotremorine, (▲) arecoline, (□) carbachol, (△) d-tubocurarine, (X) eserine, (●) hexamethonium.

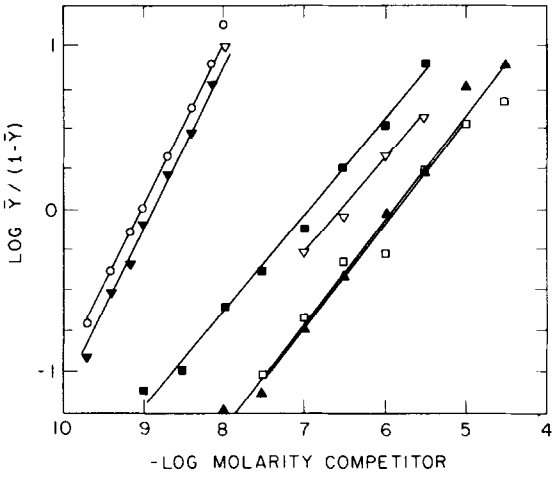

Figure 6 Hill plots of competition against [^{3}H]QNB The data from Figure 5 were replotted using the same symbols for the ligands. $\bar{Y}$ is the fractional inhibition of specific [^{3}H]QNB binding at the indicated concentration of competitor.

(Fig. 6), the slopes of which provide indices of apparent cooperativity or binding site heterogeneity (17). Slopes were clearly different for antagonists and agonists. Antagonist competition gave plots with slopes of 1, but agonist competition gave plots with slopes near 0.6.

DISCUSSION

Results presented show that the potent muscarinic antagonist [^{3}H]QNB binds to sites on membranes from NG108-15 cells with high affinity (Fig. 1), and that these sites have the pharmacological specificity of muscarinic ACh receptors (Fig. 5). Three important features, relevant to muscarinic receptor activation, characterize ligand-receptor interaction. First, at equilibrium, saturation of specific [^{3}H]QNB binding sites follows a simple adsorption isotherm. This usually is taken as evidence for a single class of binding sites. However, second, rates of [^{3}H]QNB association and dissociation are biphasic (Figs. 2-4), and the fraction of rapidly dissociating [^{3}H]QNB decreases with the time of reaction. These results indicate the presence of more than one binding state for antagonist. Third, agonists and antagonists compete against [^{3}H]QNB at equilibrium in a nonequivalent manner (Fig. 6). Agonist competition is characterized by Hill plots with slopes of less than one, whereas antagonist competition is characterized by slopes equal to one. This difference likely reflects the ability of agonists to activate receptors compared to the inability of antagonists to do so. Muscarinic receptors of heart muscle, which mediate different initial responses to ACh than the receptors of NG108-15 cells, have similar binding properties, as reported earlier (3). Some homologous features of binding also have been observed with muscarinic receptors of brain homogenates (18).

A simple scheme can account for these binding data as well as for the different physiological effects of antagonists and agonists. A two-step sequence is proposed for antagonist (I) and agonist (II) binding to receptors:

$$\text{(I-1)}\quad R + Ant \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R\text{-}Ant \qquad \text{(II-1)}\quad R + Ago \underset{k_2}{\overset{k_1}{\rightleftharpoons}} R\text{-}Ago$$

$$\text{(I-2)}\quad R\text{-}Ant \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R'\text{-}Ant \qquad \text{(II-2)}\quad R\text{-}Ago + E \underset{k_4}{\overset{k_3}{\rightleftharpoons}} R\text{-}Ago\text{-}E.$$

The sequences differ in their second steps. For antagonists, the second step is a simple, first-order rearrangement. The intermediate complex changes to a new conformation. For agonists, the second step is a bimolecular interaction. The intermediate complex interacts with an effector (E), activating E and promoting the initial response.

Scheme I for antagonists readily accounts for the equilibrium binding properties of [^{3}H]QNB. The equilibrium condition for the 2-step sequence leads to a linear equation for a Scatchard plot of (Bound/Free) versus (Bound):

$$B/F = \frac{k_1\,(k_3 + k_4)}{k_2 k_4}\,(B_{max} - B).$$

The model thus gives a straight-line Scatchard plot having an apparent dissociation constant of:

$$K_D^{app} = \frac{k_2 k_4}{k_1 (k_3 + k_4)}.$$

Although the differential equations for the kinetic properties of Scheme I were not solved, computer simulation showed that the model can account for the qualitative features of biphasic association and dissociation and for changing proportions of rapidly dissociating antagonist (19). This can be seen intuitively as well. Association would be biphasic if a rapidly reversible first step, quickly reaching steady-state, were followed by a slow rearrangement of the intermediate complex to give a high affinity terminal complex. Similarly, before equilibrium, dissociation would be biphasic because a measurable fraction of the bound antagonist would still be in the readily-reversible, intermediate complex. When the system reached equilibrium, however, most of the bound antagonist would be in the terminal complex, and dissociation would be limited by the slow reversal of the first-order step.

In Scheme II for agonist binding, an intermediate complex (R-Ago) is postulated to interact with an effector component (E) in a bimolecular second step in order to bring about a receptor-mediated response. Introducing a bimolecular interaction into a simple, 2-step binding sequence results in Hill plots for equilibrium binding that have slopes of less than one (17). Other models of binding,

such as negative cooperativity and heterogeneous binding sites (17), also could account for the equilibrium competition data for agonists, but Scheme II is appealing in its simplicity, its symmetry with respect to the model for antagonist binding, and its physiological relevance. Scheme II also leads to the prediction that when an effector is uncoupled from interaction with the intermediate complex, the binding of agonist should give a Hill plot with a slope of one. This prediction has been confirmed in binding experiments with solubilized muscarinic ACh receptors from calf brain (20). In addition, other evidence is consistent with Scheme II but not with the other two models. First, lack of changes in Hill coefficients for agonist binding following irreversible blockade of 95% of the receptors has been reported as evidence against negative cooperativity (21). Second, a recent report showing that only one labeled protein is observed following electrophoresis of alkylated receptors argues against heterogeneous binding sites (22).

Although a more complex model may perhaps be required to account for all the features of neuroreceptor activation, the relatively parsimonious schemes suggested here appear satisfactory for explaining the current binding data. Progress in receptor purification (23) hopefully will soon provide additional insight into the mechanism of muscarinic ACh receptor activation.

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