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# Muscarinic Antagonists Induce Different Receptor Conformations in Rat Adenohypophysis

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SUMMARY

We have employed a method based on ligand competition experiments, which is capable of detecting interactions among ligand-occupied binding sites, to study the interactions between rat adenohypophysis muscarinic receptors occupied by several muscarinic antagonists. In this method, one examines the binding of a labeled ligand (the primary ligand) in the absence and presence of a competing ligand. The inhibition of binding of the primary ligand by the competing ligand shows significant deviations from that expected assuming a population of noninteracting, heterogeneous binding sites. The deviations seen in the case of competition between *N*-methyl-4-piperidyl benzilate (4NMPB) and (−)-*N*-methyl scopolamine (a benzilate and tropate) are more pronounced than in the case of 4NMPB and (−)-3-quinuclidinyl benzilate (two benzilate derivatives). The occurrence of such deviations suggests the existence of site-site interactions among rat adenohypophysis muscarinic receptors. On the other hand, no deviations were observed in competition experiments in homogenates of rat cortex and medulla-pons. This finding correlates with the linear Scatchard plots (with no indications for site-site interactions or heterogeneity) obtained for the binding of muscarinic antagonists in these brain regions. A mathematical analysis demonstrates that the deviations from the expectations of the site-heterogeneity model observed in the rat adenohypophysis system (which shows similar binding patterns for all ligands employed) can occur only if the primary and competing ligands induce different conformational transitions upon binding to the receptor. It is concluded that different muscarinic antagonists can lead to different isomerization states of the receptor in the system.

## INTRODUCTION

The binding of muscarinic antagonists to various regions of rat and mouse brain yields binding isotherms that fit a single dissociation constant (1–4). On the other hand, competition experiments of muscarinic agonists with radiolabeled antagonists demonstrate that the binding of the agonists to these preparations does not obey a simple mass-action law, and yields Hill coefficients smaller than unity (5–7). This phenomenon was interpreted as indicating the existence of two or three distinct populations of muscarinic binding sites, which are recognized differently by agonists, but not by antagonists (5–7). An alternative explanation, which could not be ruled out, is that agonist binding induces negatively cooperative interactions between the binding sites. This possibility can be tested directly by a recently developed method, which employs equilibrium competition experiments to demonstrate the existence of cooperative interactions among ligand-occupied binding sites. A complete theoretical discussion of this approach has been presented (8), and the method was applied successfully to demonstrate negatively cooperative interactions among the subunits of rabbit-muscle glyceraldehyde-3-phosphate dehydrogenase (9, 10). In this approach, the binding of a labeled ligand (the primary ligand), which reveals binding isotherms indicative of negative cooperativity or site heterogeneity, is compared in the absence and presence of a competing ligand. The mechanisms of site heterogeneity (without site-site interactions) and negative cooperativity predict different effects of such competition on the binding of the primary ligand (8–10).

This method requires an accurate determination of the binding curves of the primary ligand, both alone and in the presence of the competing ligand. Although radiolabeled muscarinic agonists are available (6, 11), their affinities are low relative to antagonists. Thus, high agonist concentrations are required in binding experiments, yielding high nonspecific binding which renders agonist-binding isotherms inaccurate (6). Binding studies employing labeled antagonists do not suffer such limitations, as saturation occurs at low antagonist concentrations. However, the fit of the binding curves of muscarinic antagonists in various brain regions to a simple mass-

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action law eliminates their use as primary ligands in the proposed scheme of competition experiments (8). This problem does not exist in rat adenohypophysis, where the binding of several muscarinic antagonists was shown to yield curvilinear Scatchard plots (12, 13). Therefore, the rat adenohypophysis provides a convenient system wherein the existence of interactions among muscarinic binding sites can be explored by competition experiments.

In a recent study (14), we have shown that the pattern of inhibition of [$^3$H]4NMPB[1] binding by (−)3QNB (another benzilate derivative) suggests the existence of negatively cooperative interactions between antagonist-occupied muscarinic receptors. In the present communication, we study the effects of competition between a pair of two benzilates [4NMPB and (−)3QNB] and between a benzilate and a tropate [4NMPB and (−)NMS], exchanging the roles of the primary and competing ligand within each pair. The results show deviations from the primary and competing ligand within each pair. The results show deviations from the effects of competition expected on the basis of heterogeneous, noninteracting binding sites; these deviations are more pronounced in the benzilate-tropate pair than between two benzilate derivatives. A mathematical analysis of the deviations according to a model which allows site-site interactions indicates that the observed deviations can occur only if the primary and the competing ligand induce different conformations upon binding to the muscarinic receptors—namely, only if different antagonists lead to different isomerization states.

## EXPERIMENTAL PROCEDURES

*Materials.* [$^3$H]4NMPB (20 Ci/mmole), unlabeled 4NMPB, and (−)3QNB were prepared as described previously (2, 13, 15). [$^3$H](−)3QNB (33 Ci/mmole) and [$^3$H](−)NMS (53.3 Ci/mmole) were obtained from New England Nuclear Corporation (Boston, Mass.). Unlabeled (−)NMS was obtained from Sigma Chemical Company (St. Louis, Mo.). All other compounds were of the highest purity available.

*Animals.* Adult male rats (CD strain) were supplied by Levinstein's Farm (Yokneam, Israel). The rats were kept at 24 ± 2°, with 14 hr under fluorescent illumination and 10 hr of darkness daily. Food (Assia Maabarot, Tel-Aviv) and water were supplied ad libitum.

*Binding assays.* Homogenates of rat adenohypophysis were prepared in cold 0.32 M sucrose from glands of 20 male rats, using a motor-driven Teflon pestle (950 rpm) in a glass homogenizer, as described earlier (13). Direct binding of tritiated antagonists to the muscarinic receptors in these homogenates was measured by the centrifugation method, following a procedure described previously (13). The binding was performed in modified Krebs-Henseleit solution (13) (pH 7.3 at 25°). The ligands were incubated with the homogenate for 30 min; this period is sufficient to achieve equilibrium, as the concentration of bound labeled ligand remained unchanged upon further incubation of up to 2 hr (longer incubations are impractical because of increased loss of receptors). Nonspecific binding was measured in the presence of 10 μM unlabeled atropine, and specific binding was determined by subtracting the nonspecific binding from the binding in the absence of atropine.

*Competition experiments.* Equilibrium competition experiments were performed according to an approach that can distinguish between site heterogeneity without interactions and a mechanism that involves negatively cooperative interactions among ligand-occupied binding sites (8). In this method, one examines the effect of a constant concentration of a competing ligand on the binding pattern of the primary, labeled ligand (8–10, 14). The presence of an unlabeled competing ligand affects the dissociation (or association) constants measured for the primary ligand, as well as its Hill coefficient at half-saturation, which is determined by the ratios between the latter dissociation constants (8). For site heterogeneity without interactions, the extent of the effect on these parameters can be calculated quantitatively from the dissociation constants extracted from direct binding curves for each ligand separately. This occurs since the competing ligand ($Z$) inhibits the binding of the primary ligand ($X$) to each class of sites independently in a competitive manner, transferring the saturation function $\bar{Y}(X)$ for the binding of the primary ligand to the form:

$$\bar{Y}(X) = \alpha_H \frac{[X]}{K_H^X\left(1+\frac{[Z]}{K_H^Z}\right)+[X]} + \alpha_L \frac{[X]}{K_L^X\left(1+\frac{[Z]}{K_L^Z}\right)+[X]} \quad (1)$$

where $\alpha_H$ and $\alpha_L$ are the respective fractions of high- and low-affinity binding sites, displaying the dissociation constants $K_H^X$, $K_L^X$, $K_H^Z$, $K_L^Z$ for the binding of $X$ and $Z$, respectively.

In contrast, the effects of competition on the Hill coefficient and on the dissociation constants of the primary ligand in systems with negatively cooperative interactions among the binding sites cannot be calculated on the basis of the separate binding curves (8). Thus, deviations from the expected values calculated on the basis of site heterogeneity without interactions are indicative of the participation of negatively cooperative interactions in the binding mechanism (8–10, 14).

The competition experiments were performed following the procedure described for the binding assays, except that unlabeled competing ligands were introduced together with the radiolabeled primary ligand. The concentrations of the competing ligands were well over that of the binding sites (0.1 nM) and over their dissociation constants, thus ensuring that the concentration of free competing ligand remains essentially constant upon titration with the primary ligand, and that a large portion of the binding sites is initially occupied by the competing ligand (8–10).

*Data analysis.* Binding data in the presence and absence of competing ligands were computer-analyzed, employing the nonlinear regression curve-fitting program BMDPAR (November 1978 revision) (7, 16, 17) developed at the Health Science Computing Facility (University of California, Los Angeles, CA).

## THEORY

A model of heterogeneous, noninteracting sites makes specific predictions on the effect of competition on the binding constants of the primary ligand (8–10, 14). Under certain conditions, it can be shown that the deviations from the expectations of the site heterogeneity model do not only suggest site-site interactions, but also indicate that the primary ligand and the competitor induce different conformations upon binding.

For simplicity, let us consider a dimer (the simplest system that allows site-site interactions). The treatment will be performed in terms of association constants, which lead to simpler expressions.

The saturation function $\bar{Y}(X)$ for $X$ binding in the presence of a competing ligand $Z$ to a dimer with noninteracting sites is given by:

$$\bar{Y}(X) = \frac{K_{Ha}^{X,exp}[X]}{1+K_{Ha}^{X,exp}[X]} + \frac{K_{La}^{X,exp}[X]}{1+K_{La}^{X,exp}[X]} \quad (2)$$

where $K_{Ha}^{X,exp}$ and $K_{La}^{X,exp}$ are the association constants expected for the binding of $X$ in the presence of $Z$ according to the site heterogeneity model:

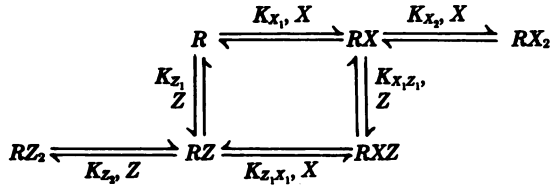
$$K_{Ha}^{X,exp} = \frac{K_{Ha}^X}{1+K_{Ha}^Z[Z]} \quad \text{and} \quad K_{La}^{X,exp} = \frac{K_{La}^X}{1+K_{La}^Z[Z]} \quad (3)$$

[1] The abbreviations used are: 4NMPB, *N*-methyl-4-piperidyl benzilate; [$^3$H]4NMPB, *N*-[$^3$H]methyl-4-piperidyl benzilate; (−)3QNB, (−)-3-quinuclidinyl benzilate; [$^3$H](−)3QNB, (−)-[$^3$H]quinuclidinyl benzilate; (−)NMS, (−)-*N*-methylscopolamine; [$^3$H](−)NMS, (−)-*N*[$^3$H]methylscopolamine.

$K^X_{Ha}$ and $K^Z_{Ha}$ are the intrinsic association constants for the binding of $X$ and $Z$ to the high-affinity sites. $K^X_{La}$ and $K^Z_{La}$ are defined similarly for the low-affinity sites.

The binding data can be fitted to Eq. 2. Deviations from the expected values of $K^{X,exp}_{Ha}$ and $K^{X,exp}_{La}$ (Eq. 3) are indicative of cooperative interactions (14). The observed constants in case of deviations are designated $K^{X,obs}_{Ha}$ and $K^{X,obs}_{La}$. Since the binding can be analyzed either in terms of site heterogeneity or in terms of a model which allows cooperativity, both the expected and observed constants can be expressed by the constants of the model allowing cooperativity. The ratio of $K^{X,obs}_{Ha}/K^{X,exp}_{Ha}$ and $K^{X,obs}_{La}/K^{X,exp}_{La}$ provides a direct measure of the deviation.

The most general model for the binding of competing ligands ($X$, $Z$) to a dimer is:




where $R$ is the dimer, and $RX_iZ_j$ ($1 \leq 1 + j \leq 2$) represent all receptor species with $i$-bound $X$ molecules and $j$ molecules of $Z$ (e.g., in site heterogeneity, $RX$ represents $R_HX + R_LX$; if ligand binding induces a conformational change to $R^*$, $RX$ represents $RX + R^*X$). The constants in the scheme are the thermodynamic association constants (8, 10): $K_{X_i} = [RX_i]/[RX_{i-1}][X]$; $K_{Z_j} = [RZ_j]/[RZ_{j-1}][Z]$; $K_{X_1Z_1} = [RXZ]/[RX][Z]$; and $K_{Z_1X_1} = [RXZ]/[RZ][X]$. The values of these constants are model-independent, but their interpretation depends on the model chosen.

When cooperative interactions exist, it is more convenient to use statistically corrected association constants ($K'$), which give a direct measure of the average affinity of the ligand per binding site (8, 10, 18):

$$K'_{X_i} = \frac{i}{n-i+1}K_{X_i};\ K'_{Z_j} = \frac{j}{n-j+1}K_{Z_j};\ K'_{X_iZ_j} = \frac{j}{n-i-j+1}K_{X_iZ_j};$$

and $K'_{Z_jX_i} = \frac{i}{n-i-j+1}K_{Z_jX_i}$. $n$ is the number of binding sites per receptor (2 for a dimer).

The saturation of a dimer by a single ligand $X$ can be described in the following general form (8):

$$\bar{Y}(X) = \frac{1}{2}\frac{\psi_1(X)[X] + 2\psi_2(X)[X]^2}{1 + \psi_1(X)[X] + \psi_2(X)[X]^2} \tag{4}$$

where $\psi_1(X) = K_{X_1} = 2K'_{X_1}$ and $\psi_2(X) = K_{X_1}K_{X_2} = K'_{X_1}K'_{X_2}$. For a dimer with two noninteracting sites, one obtains (8):

$$\psi_1(X) = K^X_{Ha} + K^X_{La} \text{ and } \psi_2(X) = K^X_{Ha} \cdot K^X_{La}$$

From these equalities it follows that:

$$K^X_{Ha} = \frac{\psi_1(X) + \sqrt{\psi_1^2(X) - 4\psi_2(X)}}{2} \tag{5}$$

$$K^X_{La} = \frac{\psi_1(X) - \sqrt{\psi_1^2(X) - 4\psi_2(X)}}{2} \tag{6}$$

Introducing $\psi_i(X)$ in terms of $K'_{X_i}$ in Eqs. 5 and 6 yields:

$$K^X_{Ha} = K'_{X_1}\left(1 + \sqrt{1 - \frac{K'_{X_2}}{K'_{X_1}}}\right) \tag{7}$$

$$K^X_{La} = K'_{X_1}\left(1 - \sqrt{1 - \frac{K'_{X_2}}{K'_{X_1}}}\right) \tag{8}$$

Similar expressions are obtained for $K^Z_{Ha}$ and $K^Z_{La}$ in terms of $K'_{Z_1}$ and $K'_{Z_2}$.

The treatment will be continued for $K^X_{Ha}$ only ($K^X_{La}$ can be treated in an equivalent manner).

Introducing $K^Z_{Ha}$, $K^Z_{La}$ (expressed according to Eqs. 7 and 8) in Eq. 3, one obtains:

$$K^{X,exp}_{Ha} = \frac{K'_{X_1}\left(1 + \sqrt{1 - \frac{K'_{X_2}}{K'_{X_1}}}\right)}{1 + K'_{Z_1}\left(1 + \sqrt{1 - \frac{K'_{Z_2}}{K'_{Z_1}}}\right)[Z]} \tag{9}$$

According to the general scheme which allows cooperativity, the observed saturation function for $X$ binding in the presence of $Z$ is (8):

$$\bar{Y}(X) = \frac{1}{2}\frac{\psi_1(X, Z)[X] + 2\psi_2(X, Z)[X]^2}{1 + \psi_1(X, Z)[X] + \psi_2(X, Z)[X]^2} \tag{10}$$

where $\psi_i(X, Z)$ are the observed $\psi_i$ values for $X$ binding in the presence of $Z$ (8):

$$\psi_1(X, Z) = \frac{2K'_{X_1}(1 + K'_{X_1Z_1}[Z])}{1 + 2K'_{Z_1}[Z] + K'_{Z_1}K'_{Z_2}[Z]^2}$$

and

$$\psi_2(X, Z) = \frac{K'_{X_1}K'_{X_2}}{1 + 2K'_{Z_1}[Z] + K'_{Z_1}K'_{Z_2}[Z]^2}$$

Thus, $K^{X,obs}_{Ha}$ and $K^{X,obs}_{La}$ are given by expressions similar to Eq. 5 and 6, with $\psi_i(X, Z)$ replacing $\psi_i(X)$:

$$K^{X,obs}_{Ha} = \frac{\psi_1(X, Z) + \sqrt{\psi_1^2(X, Z) - 4\psi_2(X, Z)}}{2} \tag{11}$$

Inserting the expressions for $\psi_i(X, Z)$, one obtains:

$$K^{X,obs}_{Ha} = \frac{K'_{X_1}(1 + K'_{X_1Z_1}[Z])}{1 + 2K'_{Z_1} + K'_{Z_1}K'_{Z_2}[Z]^2}\left(1 + \sqrt{1 - \frac{(1 + 2K_{Z_1}[Z] + K'_{Z_1}K'_{Z_2}[Z]^2)K'_{X_2}}{(1 + K'_{X_1Z_1}[Z])^2K'_{X_1}}}\right) \tag{12}$$

The deviation of the observed constant from that expected according to the site heterogeneity model is given by dividing Eq. 12 by Eq. 9:

$$\frac{K^{X,obs}_{Ha}}{K^{X,exp}_{Ha}} = \frac{(1 + K'_{X_1Z_1}[Z])\left[1 + K'_{Z_1}\left(1 + \sqrt{1 - \frac{K'_{Z_2}}{K'_{Z_1}}}\right)[Z]\right]}{(1 + 2K'_{Z_1}[Z] + K'_{Z_1}K'_{Z_2}[Z]^2)\left(1 + \sqrt{1 - \frac{K'_{X_2}}{K'_{X_1}}}\right)} \times \left(1 + \sqrt{1 - \frac{(1 + 2K'_{Z_1}[Z] + K'_{Z_1}K'_{Z_2}[Z]^2)K'_{X_2}}{(1 + K'_{X_1Z_1}[Z])^2K'_{X_1}}}\right) \tag{13}$$

This ratio can obtain any value, depending on the relationships between $K'_{X_1Z_1}$ (the affinity for $Z$ after the binding of one $X$ molecule) and $K'_{Z_1}$, $K'_{Z_2}$, $K'_{X_1}$, $K'_{X_2}$. A similar result is obtained for the effect of $X$ competition on $Z$ binding, except that $K'_{Z_1X_1}$ replaces $K'_{X_1Z_1}$ and the roles of $K'_{Z_i}$ and $K'_{X_i}$ are exchanged. The effects of $X$ and $Z$ do not have to be reciprocal, since $K'_{X_1Z_1}/K'_{Z_1X_1}$ can obtain any value.

In order to examine whether $X$ and $Z$ induce identical conformational changes, let us introduce the equivalent mathematical conditions, namely $K'_{X_1Z_1} = K'_{Z_2}$ (or $K'_{Z_1X_1} = K'_{X_2}$ while testing the effect of $X$ competition on $Z$ binding). This brings Eq. 13 to:

$$\frac{K^{X,obs}_{Ha}}{K^{X,exp}_{Ha}} = \frac{(1 + K'_{Z_2}[Z])\left[1 + K'_{Z_1}\left(1 + \sqrt{1 - \frac{K'_{Z_2}}{K'_{Z_1}}}\right)[Z]\right]}{(1 + 2K'_{Z_1}[Z] + K'_{Z_1}K'_{Z_2}[Z]^2)\left(1 + \sqrt{1 - \frac{K'_{X_2}}{K'_{X_1}}}\right)} \times \left(1 + \sqrt{1 - \frac{(1 + 2K'_{Z_1}[Z] + K'_{Z_1}K'_{Z_2}[Z]^2)K'_{X_2}}{(1 + K'_{Z_2}[Z])^2K'_{X_1}}}\right) \tag{14}$$

If the cooperativity in the binding of $X$ and $Z$ alone is similar ($K'_{X_2}/K'_{X_1} = K'_{Z_2}/K'_{Z_1}$), as is the case for the binding of the muscarinic antagonists

to rat adenohypophysis (see Table 1), Eq. 14 reduces to:

$$\frac{K_{Ha}^{X,obs}}{K_{Ha}^{X,exp}} = \frac{(1 + K'_{z_2}[Z])\left[1 + K'_{z_1}\left(1 + \sqrt{1 - \frac{K'_{z_2}}{K'_{z_1}}}\right)[Z]\right]}{(1 + 2K'_{z_1}[Z] + K'_{z_1}K'_{z_2}[Z]^2)\left(1 + \sqrt{1 - \frac{K'_{z_2}}{K'_{z_1}}}\right)} \cdot A \quad (15)$$

where

$$A = 1 + \sqrt{1 - \frac{(1 + 2K'_{z_1}[Z] + K'_{z_1}K'_{z_2}[Z]^2)K'_{z_2}}{(1 + K'_{z_2}[Z])^2 K'_{z_1}}}$$

$$= \frac{1 + K'_{z_2}[Z] + \sqrt{1 - \frac{K'_{z_2}}{K'_{z_1}}}}{1 + K'_{z_2}[Z]}$$

The denominator in $A$ $(1 + K'_{z_2}[Z])$ cancels out with the similar expression in the numerator of Eq. 15, which can be rearranged to:

$$\frac{K_{Ha}^{X,obs}}{K_{Ha}^{X,exp}} = \frac{(1 + 2K'_{z_1}[Z] + K'_{z_1}K'_{z_2}[Z]^2) + \sqrt{1 - \frac{K'_{z_2}}{K'_{z_1}}}(1 + 2K'_{z_1}[Z] + K'_{z_1}K'_{z_2}[Z]^2)}{(1 + 2K'_{z_1}[Z] + K'_{z_1}K'_{z_2}[Z]^2)\left(1 + \sqrt{1 - \frac{K'_{z_2}}{K'_{z_1}}}\right)} = 1 \quad (16)$$

Similarly, it can be shown that these conditions lead to $K_{La}^{X,obs}/K_{La}^{X,exp} = 1$. A similar result is obtained under these conditions when the roles of $X$ and $Z$ are exchanged—namely, for the effect of $X$ on $Z$ binding.

Therefore, when the binding pattern of the two competing ligands is similar (they exhibit similar cooperativity), a deviation from the association (or dissociation) constants expected according to the site heterogeneity model suggests that the two ligands induce *different* conformational changes in the receptor.

## RESULTS

*Competition experiments in rat adenohypophysis.* As discussed under Theory, the similarity (or nonsimilarity) in the binding patterns of the competing ligands are important for the analysis of the competition experiments. Scatchard plots showing the binding of the muscarinic antagonists employed in this study to rat adenohypophysis in the absence of competitors are shown as *insets* in Figs. 1 and 2. The dissociation constants and Hill coefficients for these ligands are given in Table 1. The binding parameters of all three antagonists are similar within the experimental error, reflecting the similarity in their binding patterns. The binding parameters are in accord with those reported earlier (13).

The results of the competition experiments with the various muscarinic antagonists are shown in Figs. 1 and 2 and in Table 2. Even when both the primary and the competing ligand are benzilate derivatives, a clear deviation of the observed dissociation constants from those expected according to site heterogeneity is observed (Fig. 1; Table 2). In view of the similar binding patterns of [$^3$H]4NMPB and [$^3$H](−)3QNB, the deviations suggest the existence of interactions among the muscarinic binding sites and indicate that the binding of 4NMPB and (−)3QNB induce different conformational changes in the receptor. Significant deviations (well above the standard error) are observed for the effect of (−)3QNB competition on both the high- and low-affinity sites of [$^3$H]4NMPB. Interestingly, the competition effects of this pair of ligands are not reciprocal, and the effect of

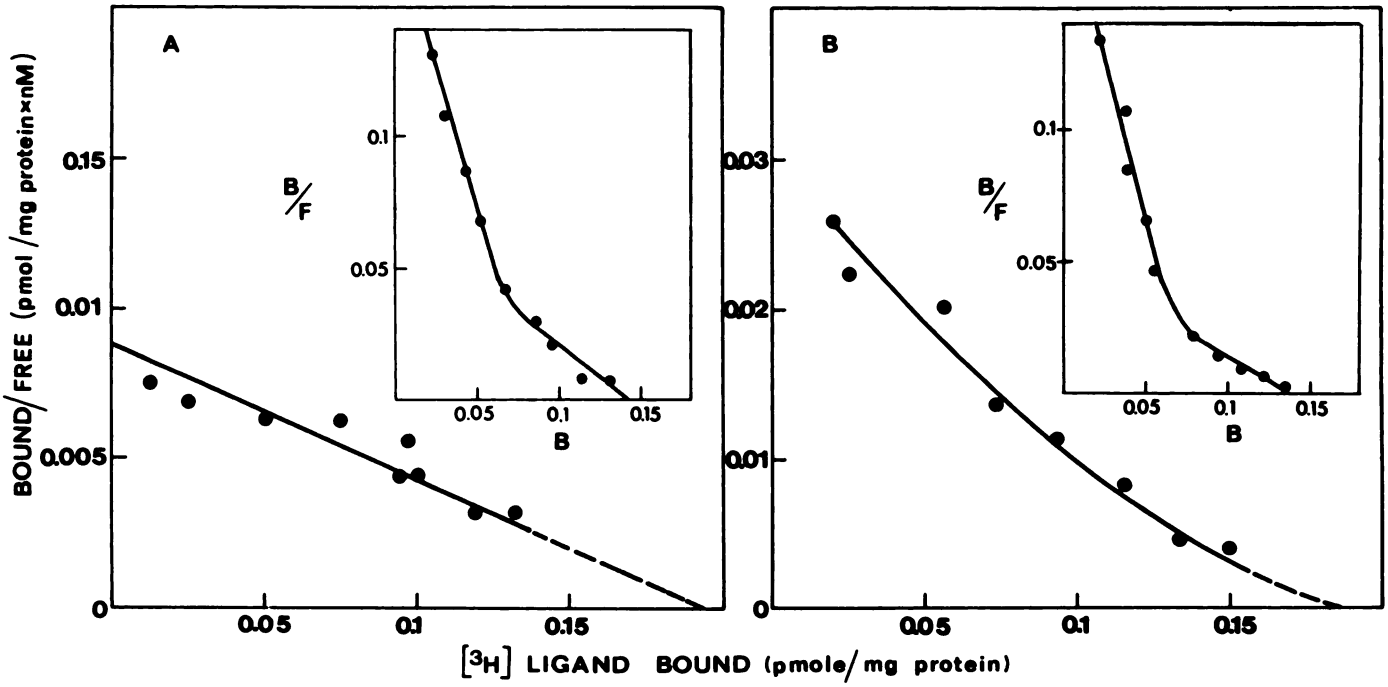



FIG. 1. *Representative Scatchard plots for [$^3$H]4NMPB and [$^3$H](−)3QNB binding to muscarinic receptors in rat adenohypophysis: effect of competition*

Each point represents the mean of three determinations, with standard error below 10%. Nonspecific binding was subtracted. A. [$^3$H]4NMPB binding in the presence of 6 nM (−)3QNB. *Inset*, [$^3$H]4NMPB binding in the absence of competitor. B. [$^3$H](−)3QNB binding in the presence of 6 nM 4NMPB. *Inset*, [$^3$H](−)3QNB binding without competition.

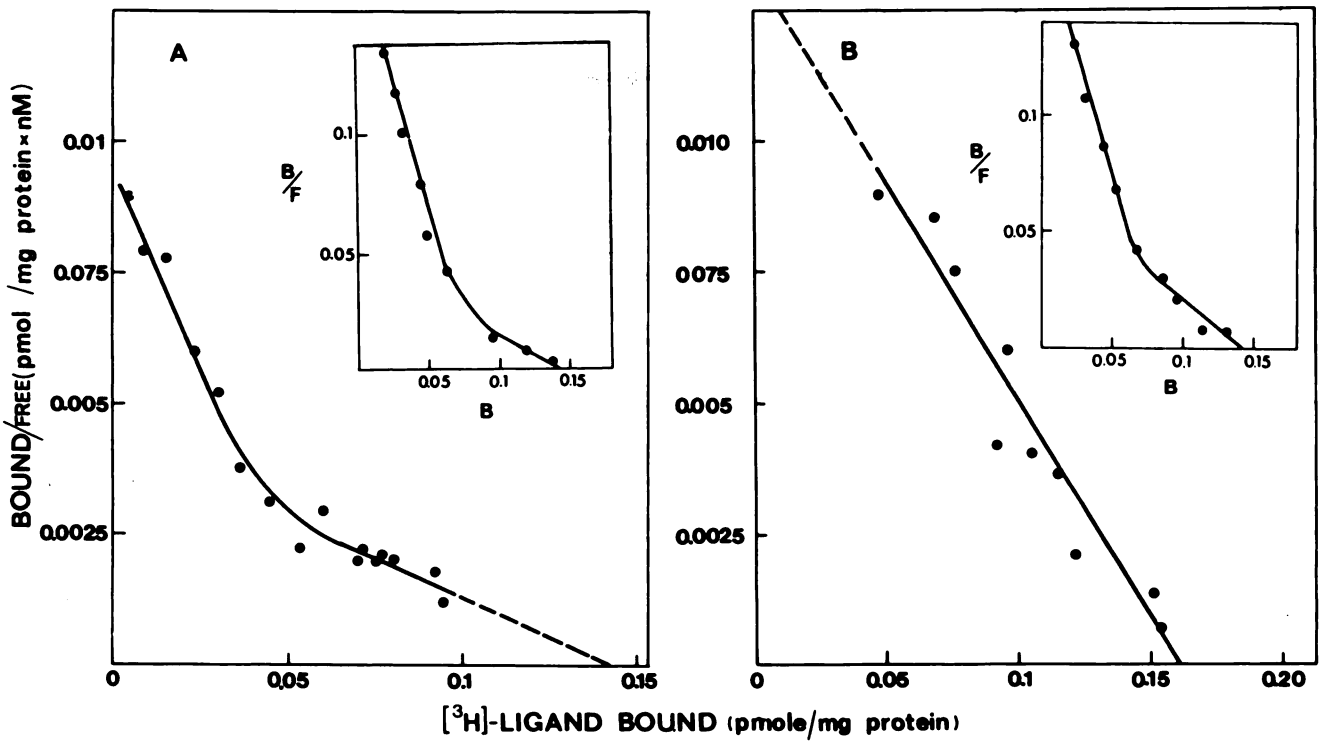



FIG. 2. *Representative Scatchard plots for [³H]4NMPB and [³H](−)NMS binding to muscarinic receptors in rat adenohypophysis: effect of competition*

Each point represents the mean of three determinations, with standard error below 10%. Nonspecific binding was subtracted. A. [³H](−)NMS binding in the presence of 6 nM 4NMPB. *Inset*, [³H](−)NMS binding without competition. B. [³H]4NMPB binding in the presence of 1 nM (−)NMS. *Inset*, [³H]4NMPB binding without competition.

4NMPB competition on [³H](−)3QNB binding shows a considerably smaller deviation (about 2-fold for the low-affinity site, and no deviation within the experimental error for the high-affinity site) from the expectations of the site heterogeneity model (Table 2). Such nonreciprocal effects are possible, since $K'_{X_1Z_1}/K'_{Z_1X_1}$ can obtain different values (the effect of a bound $X$ molecule on the binding of $Z$ can differ from that of a bound $Z$ molecule on $X$ binding).

The structural differences between tropates and benzilates are greater than those between two benzilate derivatives. This suggests that the difference between the conformational transitions induced by such ligands may be larger, thus leading to more pronounced deviations from the expectations of the site heterogeneity model. In order to explore this question, we performed competition experiments between the benzilate 4NMPB and the tropate (−)NMS. The results are depicted in Fig. 2 and Table 2. Indeed, the deviations observed in the effect of (−)NMS competition on [³H]4NMPB binding are the most significant and amount to a 9-fold deviation for the binding of [³H]4NMPB to the high-affinity site. A significant deviation (4- to 5-fold) is also observed for the effect of (−)NMS on [³H]4NMPB binding to the low-affinity site. The inhibition of [³H]4NMPB binding by (−)NMS is so much stronger than expected, assuming simple site heterogeneity, that it was necessary to use lower concentrations of (−)NMS as a competitor (1 or 2

TABLE 1

*Dissociation constants and Hill coefficients for the binding of muscarinic antagonists to rat adenohypophysis*

The results are averages of five separate experiments ± standard error. The dissociation constants $K_H$ and $K_L$ were obtained by fitting the data to Eq. 1 (with $[Z] = 0$), and represent the dissociation constants to the high- and low-affinity sites, assuming site heterogeneity. $\alpha_H$ and $\alpha_L$ are the fractions of the high- and low-affinity sites. The Hill coefficients ($n_H$) were derived from the slope of the respective Hill plots at midsaturation. The similarity of the Hill coefficients, $K_H$, $K_L$, $\alpha_H$, and $\alpha_L$ values indicates that the binding patterns of the three antagonists are identical, and thus their analysis according to a cooperative model should yield similar cooperativity. Indeed, analysis of the data according to Eq. 4, which allows cooperativity, yields $K'_{X_2}/K'_{X_1}$ values between 0.51 ± 0.12 and 0.58 ± 0.13 for all the antagonists employed, suggesting the same cooperativity within the experimental error.

| Ligand | $K_H$ | $K_L$ | $\alpha_H$ | $\alpha_L$ | $n_H$ |
|---|---|---|---|---|---|
| | nM | nM | | | |
| [³H]4NMPB | 0.41 ± 0.05 | 2.0 ± 0.3 | 0.40 ± 0.04 | 0.60 ± 0.06 | 0.65 ± 0.03 |
| [³H](−)3QNB | 0.35 ± 0.04 | 1.9 ± 0.3 | 0.41 ± 0.04 | 0.59 ± 0.05 | 0.60 ± 0.04 |
| [³H](−)NMS | 0.40 ± 0.04 | 2.2 ± 0.3 | 0.40 ± 0.03 | 0.60 ± 0.05 | 0.62 ± 0.04 |

TABLE 2

*Effects of competition on the dissociation constants of muscarinic antagonists in rat adenohypophysis*

$K_H^{X,obs}$ and $K_L^{X,obs}$ are the observed dissociation constants for the binding of the primary ligand in the presence of a competing ligand, obtained by fitting the data to Eq. 1 (with $K_H^{X,obs}$ in place of $K_H^X(1 + [Z]/K_H^Z)$, $K_L^{X,obs}$ in place of $K_L^X(1 + [Z]/K_L^Z)$, and with $\alpha_H$ and $\alpha_L$ as depicted in Table 1). $K_H^{X,exp}$ and $K_L^{X,exp}$, the expected dissociation constants according to the site heterogeneity model, were computed from the values of Table 1, using $K_H^{X,exp} = K_H^X(1 + [Z]/K_H^Z)$ and $K_L^{X,exp} = K_L^X(1 + [Z]/K_L^Z)$. The concentrations of the competing ligands are given in parentheses. The values of the constants are averages of five experiments ± standard error.

| Primary Ligand | Competing ligand | $K_H^{X,exp}$ | $K_H^{X,obs}$ | $K_H^{X,obs}/K_H^{X,exp}$ | $K_L^{X,exp}$ | $K_L^{X,obs}$ | $K_L^{X,obs}/K_L^{X,exp}$ |
|---|---|---|---|---|---|---|---|
| | | nM | nM | | nM | nM | |
| [$^3$H]4NMPB | (−)3QNB (6 nM) | 7.4 | 24.1 ± 2.0 | 3.3 | 8.3 | 24.1 ± 2.0 | 2.9 |
| [$^3$H]4NMPB | (−)3QNB (3 nM) | 3.9 | 12.4 ± 1.1 | 3.2 | 5.2 | 13.0 ± 1.0 | 2.5 |
| [$^3$H]4NMPB | (−)3QNB (1 nM) | 1.6 | 4.5 ± 0.5 | 2.8 | 3.1 | 7.1 ± 0.6 | 2.3 |
| [$^3$H](−)3QNB | 4NMPB (6 nM) | 5.4 | 4.8 ± 0.5 | 0.9 | 7.6 | 13.4 ± 1.1 | 1.8 |
| [$^3$H](−)3QNB | 4NMPB (3 nM) | 2.9 | 3.2 ± 0.4 | 1.1 | 4.8 | 7.6 ± 0.6 | 1.6 |
| [$^3$H](−)3QNB | 4NMPB (1 nM) | 1.2 | 1.1 ± 0.2 | 0.9 | 2.9 | 4.1 ± 0.5 | 1.4 |
| [$^3$H]4NMPB | (−)NMS (2 nM) | 2.5 | 22.8 ± 2.0 | 9.1 | 3.8 | 16.3 ± 1.4 | 4.3 |
| [$^3$H]4NMPB | (−)NMS (1 nM) | 1.4 | 12.0 ± 1.0 | 8.6 | 2.9 | 12.0 ± 1.2 | 4.2 |
| [$^3$H]4NMPB | (−)NMS (0.5 nM) | 0.9 | 7.1 ± 0.6 | 7.9 | 2.5 | 9.0 ± 0.8 | 3.6 |
| [$^3$H](−)NMS | 4NMPB (6 nM) | 6.2 | 5.8 ± 0.5 | 0.9 | 8.8 | 35.2 ± 3.0 | 4.0 |
| [$^3$H](−)NMS | 4NMPB (3 nM) | 3.3 | 3.3 ± 0.4 | 1.0 | 5.5 | 19.8 ± 2.1 | 3.6 |
| [$^3$H](−)NMS | 4NMPB (1 nM) | 1.4 | 1.1 ± 0.1 | 0.8 | 3.3 | 10.9 ± 1.1 | 3.3 |

nM instead of 6 nM) in order of obtain a measurable specific binding. Under these conditions, the lower-affinity sites would be only partially occupied by (−)NMS [48% with 2 nM (−)NMS according to the $K_L$ value in Table 1], and it is therefore possible that one observes only part of the possible effects of (−)NMS on the low-affinity site. Interestingly, the nonreciprocal nature of the competition effects, which was encountered earlier for the pair 4NMPB/(−)3QNB, holds also for the tropate-benzilate pair (Table 2). Thus, the competition effects of 4NMPB on [$^3$H](−)NMS binding do not significantly deviate from those predicted by site heterogeneity for [$^3$H](−)NMS binding to the high-affinity sites, and deviate by a factor of 4 from the expected effects on the binding to the low-affinity sites. These results, and especially the large deviations observed with (−)NMS as a competing ligand, provide further support for the existence of site-site interactions among the muscarinic binding sites in rat adenohypophysis, and strongly suggest that the binding of different antagonists leads to different conformational transitions in the receptors.

*Concentration dependence of competition experiments in rat adenohypophysis.* The deviations from the expectations of a model assuming no interactions among the binding sites should increase with the concentration of the competitor, since the occupation of binding sites by the competing ligand (accompanied by the propagation of conformational changes to neighboring sites) is the source of the deviations (8, 14). Therefore, one should aim to perform the experiment using the highest concentration of competitor which still allows an accurate determination of specific binding. Such conditions are favorable for the detection of such deviations (8). However, the dependence of the deviations on the competitor's concentration is not linear, since the occupation of one site (e.g., in a dimer) by the competitor could have a strong negatively cooperative effect on the binding of the primary ligand to the neighboring site. In such a case, a further increase in the concentration of the competing ligand would add only little to the deviation from the expectations of the site heterogeneity model. As shown in Table 2, this is indeed the situation with the rat adenohypophysis muscarinic receptors. Thus, for example, competition by 2 nM (−)NMS produces deviations that are only somewhat higher than those produced by 0.5 nM (−)NMS, although one can calculate from Table 1 that a different fraction of the receptors is initially occupied by the competitor [62% versus 34% of the total receptor population for 2 nM and 0.5 nM (−)NMS, respectively]. A similar pattern is observed with the other muscarinic ligands employed (Table 2). It is important to note that *any* deviations can be observed in systems with cooperative interactions among the binding sites, depending on the subunit structure of the oligomer and the specific interactions among ligand-occupied subunits (8). It is therefore not possible to predict the extent and the concentration dependence of the deviations; however, the demonstration of deviations from the competition pattern expected assuming noninteracting sites is sufficient to indicate the existence of site-site interactions.

*Competition experiments in rat brain homogenates.* Unlike the situation encountered in the adenohypophysis, the binding of muscarinic antagonists to homogenates of various brain regions yields linear Scatchard plots, with no indication of site heterogeneity or negative cooperativity (1–4). This suggests that, if there are interactions among the muscarinic receptors in these brain regions, they do not affect the binding of antagonists. In order to test whether this situation is valid also for muscarinic binding sites occupied by different antagonists, we conducted competition experiments employing 4NMPB, (−)3QNB, and (−)NMS in homogenates of rat medulla-pons and rat cortex. Representative Scatchard plots are shown in Fig. 3. The observed and the expected dissociation constants are depicted in Table 3. In all cases, the results fit a purely competitive inhibition, and

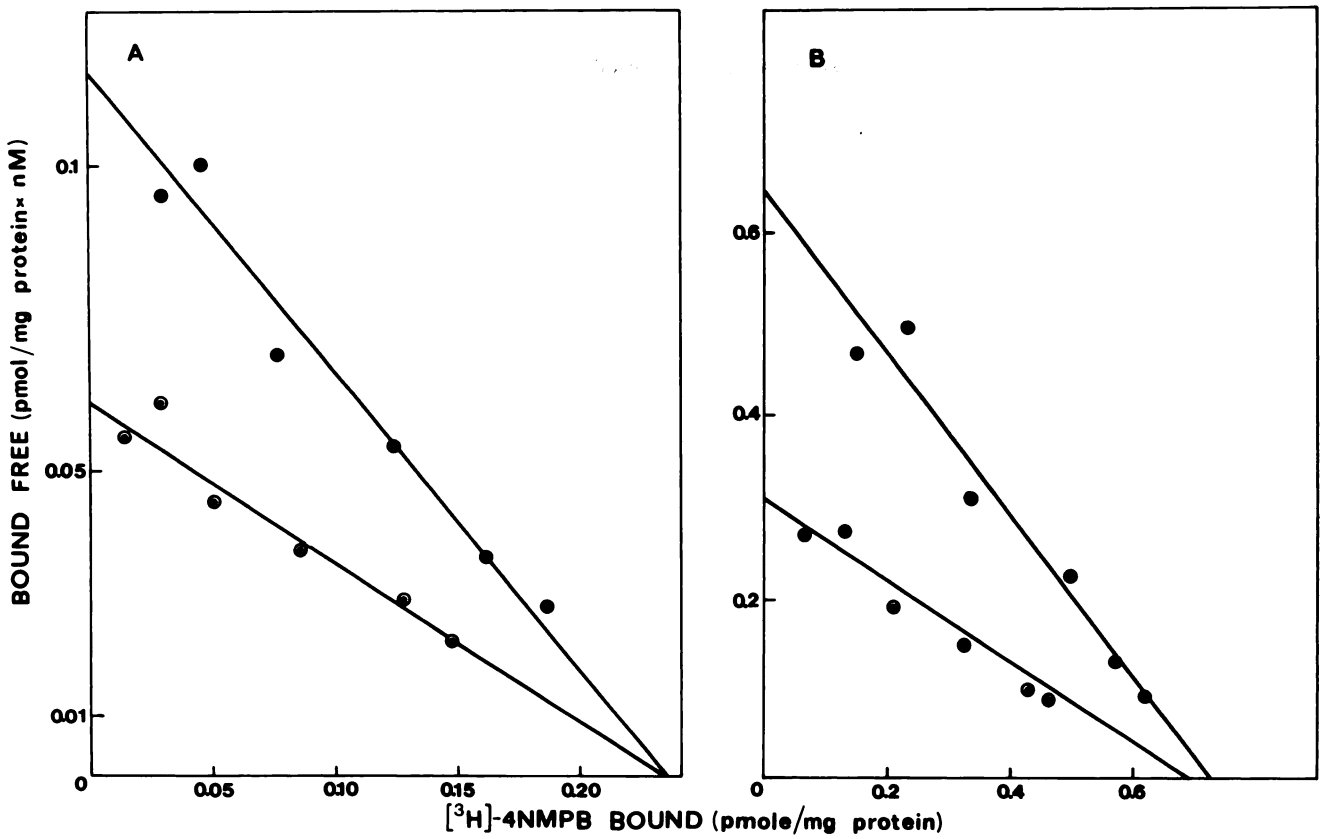



FIG. 3. *Representative Scatchard plots for the effect of (−)NMS competition on the binding of [³H]4NMPB in rat medulla-pons (A) and cortex (B) homogenates*

The binding shown is that of [³H]4NMPB alone (●) and in the presence of 1 nM (−)NMS (⊙). Each point represents the mean of three determinations, with standard error below 10%. Nonspecific binding was subtracted.

within the experimental error no deviations are observed from the dissociation constants expected assuming pure competition without interactions among the binding sites (Table 3).

## DISCUSSION

*Interactions between muscarinic binding sites.* On the basis of the inhibition of [³H]4NMPB binding by (−)3QNB, we previously suggested the existence of negatively cooperative interactions among antagonist-occupied muscarinic binding sites in rat adenohypophysis (14). The current findings on the effects of competition between several muscarinic antagonists on their dissociation constants strengthen this notion. In all cases (Figs. 1 and 2; Table 2), the inhibition of the binding of the primary ligand by the competing ligand is well above the inhibition expected assuming heterogeneous, noninteracting sites. Thus, interactions between the muscarinic binding sites are not limited to the inhibition of [³H]4NMPB binding by (−)3QNB but are reflected in

TABLE 3

*Effect of competition on the dissociation constants of muscarinic antagonists in rat medulla-pons and cortex*

The observed dissociation constants, $K_d^{X,obs}$, were obtained from the slope of the linear Scatchard plots obtained in these brain regions (e.g., in Fig. 3). The expected dissociation contants were calculated from $K_d^{X,exp} = K_d^X (1 + [Z]/K_d^Z)$, employing the values of the dissociation constants measured for the various antagonists in the absence of competition (in medulla-pons, 1.3, 0.25, and 0.70 nM for [³H]4NMPB, [³H](−)3QNB, and [³H](−)NMS; in cortex, 0.7, 0.5, and 0.4 nM for the same ligands, respectively). The concentration of the competing ligands was 1 nM in all cases. Each experiment was repeated three times.

| Brain region | Primary ligand | Competing ligand | $K_d^{X,exp}$ | $K_d^{X,obs}$ | $K_d^{X,obs}/K_d^{X,exp}$ |
|---|---|---|---|---|---|
| Medulla-pons | [³H]4NMPB | (−)3QNB | 6.5 | 8.2 ± 1.0 | 1.3 |
| | [³H](−)3QNB | 4NMPB | 0.5 | 0.6 ± 0.1 | 1.2 |
| | [³H]4NMPB | (−)NMS | 3.2 | 3.9 ± 0.5 | 1.2 |
| | [³H](−)NMS | 4NMPB | 1.2 | 1.1 ± 0.2 | 0.9 |
| Cortex | [³H]4NMPB | (−)3QNB | 2.1 | 2.4 ± 0.5 | 1.1 |
| | [³H](−)3QNB | 4NMPB | 1.2 | 1.1 ± 0.2 | 0.9 |
| | [³H]4NMPB | (−)NMS | 2.4 | 2.5 ± 0.5 | 1.0 |
| | [³H](−)NMS | 4NMPB | 1.0 | 1.1 ± 0.2 | 1.1 |

the competition effects of a variety of muscarinic antagonists. For all ligands employed, the deviation from the expectations of the site heterogeneity model was above the experimental error, and reached an order of magnitude for the effect of (−)NMS on [$^3$H]4NMPB binding (Table 2). This large discrepancy ensures that the deviations observed are real and do not stem from experimental inaccuracy.

It should be noted, however, that the competition experiments do not exclude site heterogeneity, which could exist along with site-site interactions (8). Evidence for muscarinic site heterogeneity in rat adenohypophysis is supplied by the effect of GTP on antagonist binding (19).

*Different isomerization induced by different antagonists.* In the theoretical section, it was shown that, when the separate binding of the primary and the competing ligands shows a similar extent of cooperativity ($K'_{X_2}/K'_{X_1} = K'_{Z_2}/K'_{Z_1}$), deviations from the expectations of the site heterogeneity model in competition experiments are possible only if the primary and the competing ligands induce different conformational alterations in the receptor upon binding. Since all of the antagonists employed in the present study exhibit similar binding patterns (Table 1), the deviations observed in the competition experiments (Table 2) indicate that different muscarinic antagonists induce different conformational transitions in the receptor. In this context, kinetic evidence for antagonist-induced conformational changes in the muscarinic receptors has been interpreted in terms of antagonist-induced isomerization of the receptors (7). The propagation of such isomerization to neighboring sites would produce cooperative effects, as seen in the competition experiments. Isomerization of noninteracting binding sites could *not* produce deviations from the competition effects expected according to the site heterogeneity model. We have demonstrated mathematically (14) that such systems behave in a manner analogous to that of heterogeneous, noninteracting sites without isomerization.

Interestingly, the deviations seen with the tropate-benzilate pair are more pronounced than with the benzilate-benzilate pair. This observation may indicate that a larger difference exists between the transitions induced by 4NMPB and (−)NMS than between those induced by the more closely related 4NMPB and (−)3QNB. This finding is in accord with the different degree of heterogeneity observed for the finding of tropates and benzilates to homogenates of various brain regions (7). Recent evidence obtained in cultured chick embryo heart cells (20) also supports the notion that tropates and benzilates induce different conformational transitions. The latter study reports that the ratio of the reverse to forward rate constants at the stage of conversion from a low-affinity to a high-affinity complex (namely, isomerization) is different for [$^3$H]NMS and [$^3$H](−)3QNB.

*Implications for the organization of muscarinic receptors in the membrane.* The detection of interactions among the muscarinic receptors suggests that they are organized in the membrane as oligomers containing two or more binding sites. This suggestion is supported by recent studies employing photoaffinity labeling using the radiolabeled reagent [*azido*-$^3$H]$^4$NMPB followed by gel electrophoresis (21–23). These studies indicated that muscarinic receptors in rat adenohypophysis exist as tetramers and dimers (23). The oligomeric structure of rat brain muscarinic receptors is also in accord with recent studies which were interpreted to suggest that two ligands may bind simultaneously to the muscarinic receptor (24).

The muscarinic sites may also interact with other binding sites, which could be present either on the same subunits which bind muscarinic ligands or on other polypeptides associated with them. Such interactions were proposed to be involved in the cooperative effects exerted on muscarinic receptors by the antiestrogen drug clomiphene (25), by bispyridinium oximes exhibiting antimuscarinic activity (26, 27), and by the neuromuscular blocking agent gallamine (28). A broad discussion of this subject has been presented recently (27).

The different binding patterns (linear versus curvilinear Scatchard plots) of muscarinic antagonists in rat brain (1–4) (Fig. 3) and in rat adenohypophysis (12, 13) (Figs. 1 and 2) could be explained by the participation of such additional membrane component(s), present in certain cell types and lacking in others, in the mediation of conformational changes within the muscarinic oligomers. Such a situation is in accord with the failure to detect interactions among the muscarinic binding sites in homogenates of rat medulla-pons and cortex (Table 3), although such interactions were found in the adenohypophysis (Table 2). However, the latter result cannot exclude the possibility that such interactions still occur among the muscarinic receptors in the various brain regions, and that they were not detected by the experimental procedure.

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