INTERACTIONS BETWEEN ANTAGONIST-OCCUPIED MUSCARINIC BINDING SITES IN RAT ADENOHYPOPHYSIS

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1. Introduction

The binding of muscarinic antagonists to rat adenohypophysis yields curvilinear Scatchard plots [1,2], interpreted in terms of site heterogeneity [1]. However, negative cooperativity among the muscarinic sites could not be excluded [3]. To explore this possibility, we have employed a method based on ligand competition experiments, which tests directly the presence of site–site interactions [4]. By this method, one compares the binding of the primary ligand in the presence and absence of a competing ligand. For site heterogeneity without interactions, the presence of an unlabeled competing ligand will affect the Hill coefficient (measured at half-saturation) and the dissociation constants of the primary labeled ligand. The extend of the effect on these parameters can be calculated from the dissociation constants extracted from direct binding curves for each ligand separately [4]. In contrast, the effects of the competitor on the Hill coefficient and on the dissociation constants of the primary ligand in negatively cooperative systems, cannot be predicted from the separate binding curves [4]. Therefore, the failure of binding parameters obtained in competition experiments to meet the expected values calculated assuming site heterogeneity indicates the involvement of cooperative interactions in the binding mechanism.

Our results indicate that negatively cooperative interactions are involved in the binding of muscarinic antagonists to rat adenohypophysis.

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2. Experimental

2.1. *Materials*

N-[^{3}H]Methyl-4-piperidylbenzilate (N[^{3}H]M4PB) (29 Ci/mmol) and unlabeled muscarinic antagonists were prepared as in [1]. (–)-3-[^{3}H]quinuclidinylbenzilate ((–)3[^{3}H]QNB) (33 Ci/mmol) was from New England Nuclear (Boston MA). Adult male rats of the CD strain were supplied by Levinstein's farm (Yokneam) and maintained in an air-conditioned room at 24 ± 2°C for 14 h fluorescent illumination and 10 h darkness daily. Food (Assia Maabarot Ltd) and water were supplied ad libitum.

2.2. *Binding assays*

Direct binding of N[^{3}H]M4PB and (–)3[^{3}H]QNB to muscarinic receptors in homogenates of rat adenohypophysis (37°C, pH 7.3) was measured as in [1]. An incubation period of 30 min was performed, to ensure equilibrium. Specific binding was defined as total minus non-specific binding, i.e., binding in the presence of 10 μM unlabeled atropine.

2.3. *Competition experiments*

The method in [4] was employed to detect interactions among muscarinic binding sites. This method tests the effect of a constant concentration of a competing ligand on the binding pattern of the primary ligand [4–6]. Competition experiments were conducted as described for the binding assays, except for the presence of 6 nM unlabeled NM4PB or (–)3QNB. This concentration is well over that of the binding sites (0.1 nM) and over their dissociation constants, so that the concentration of free competing ligand is constant upon titration with the primary ligand, and a large portion of the binding sites is initially saturated with the competitor.

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2.4. *Data analysis*

Binding data were analyzed by computer with a non-linear regression curve-fitting program BMDPAR (November 1978 revision) [7–9], developed at the Health Science Computing Facility, University of California (Los Angeles CA).

3. Results and discussion

The dissociation constants derived from the binding of NM4PB and (–)3QNB alone are given in table 1. These constants agree well with [1].

Competition experiments were performed with N[^{3}H]M4PB as the primary ligand and unlabeled (–)3QNB or NM4PB as the competitor. The results are depicted in fig.1A and table 2. The inhibition by the competing ligand is 3–4-fold higher than expected assuming two classes of non-interacting sites (see table 2). This deviation is significant since it was reproduced in 5 separate expt, and is considerably above the standard deviations in the values of the dissociation constants (table 2). A similar deviation was observed also with (±)QNB as the competing ligand (not shown). The discrepancy between the observed and calculated dissociation constants indicates the existence of interactions among the muscarinic binding sites occupied by antagonists. The change in the Hill coefficient from 0.4 ± 0.02 for the binding of NM4PB alone to 1.03 ± 0.05 for NM4PB binding in the presence of (–)3QNB could fit either site heterogeneity or negative cooperativity [4].

To verify that the deviation from the behaviour expected for a system with site heterogeneity did not stem from an uncontrolled variable in the system, we investigated the binding of N[^{3}H]M4PB in the presence of a constant concentration (6 nM) of the same unlabeled ligand. Unlike the competition between 2 different ligands above, all the binding constants are known from direct binding studies, and the effects of competition can be calculated even for the case of negative cooperativity. The effect of competition by an unlabeled ligand on the Hill coefficient of the same labeled ligand in a dimeric system (the simplest cooperative system possible) is obtained upon replacing the competitor Z by the primary ligand X in eq. (11) from [4], yielding:

Table 1
Dissociation constants (nM) of muscarinic antagonists in rat adenohypophysis and medulla pons

Ligand	Adenohypophysis	Medulla-pons
N[^{3}H]M4PB	K_α = 0.4 ± 0.05 K_β = 2.0 ± 0.3	K_d = 0.85 ± 0.05
(–)3[^{3}H]QNB	K_α = 0.3 ± 0.04 K_β = 1.9 ± 0.3	K_d = 0.15 ± 0.02

The constants are av. 5 separate expt ± SE (standard error). K_α and K_β are the dissociation constants for the binding of antagonist to the high (α) and low (β) affinity sites in the adenohypophysis. In the medulla pons the binding curve is described by a single dissociation constant (K_d)

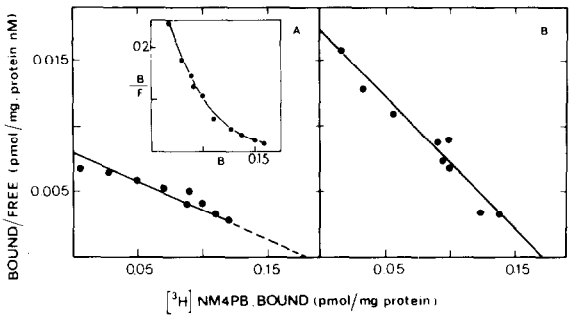

Fig.1. Scatchard plots of N[^{3}H]M4PB binding to muscarinic receptors in rat adenohypophysis: (A) binding in the presence of 6 nM unlabeled (–)3QNB; (B) binding in the presence of 6 nM unlabeled NM4PB; insert, binding of N[^{3}H]M4PB alone.

Table 2
Effect of competition on the dissociation constants (nM) of N[^{3}H]M4PB in adenohypophysis and medulla-pons

	Adenohypophysis		Medulla-pons	
Competing ligand	Exp.	Obs.	Exp.	Obs.
(–)3-QNB	8.3	24.5 ± 0.8	6.9	8.7 ± 0.9
NM4PB	6.0	9.3 ± 1.0	–	–

The expected and observed dissociation constants for N[^{3}H]-M4PB in the presence of unlabeled (–)3QNB or NM4PB are av. 5 expt ± SE (standard error). The concentrations of unlabeled competitors were 5 nM in the adenohypophysis and 1 nM in the medulla-pons. The distinction between high and low affinity sites (α,β) in the adenohypophysis (see table 1) does not hold any more, since the competitor transfers the Scatchard plot to a linear form (fig.1). The expected values of the dissociation constants were calculated from the dissociation constants for the binding of the 2 ligands separately (table 1), assuming pure competition. In the adenohypophysis, we assumed 2 classes of non-interacting sites, for which the antagonists compete with their respective K_α and K_β values

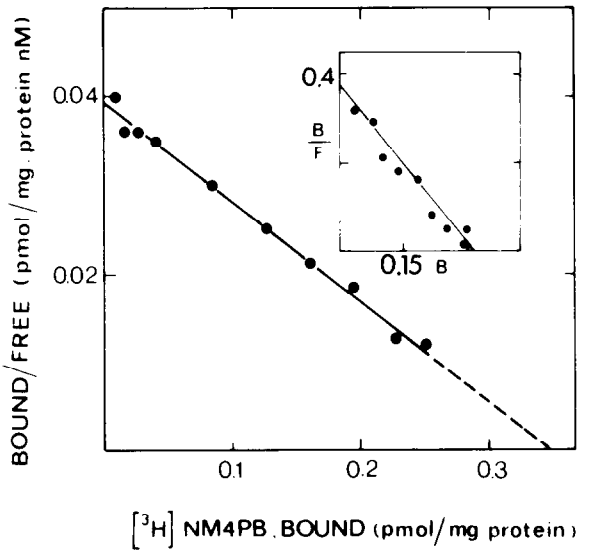

Fig.2. Scatchard plot of N[^{3}H]M4PB binding to muscarinic receptors in rat medulla-pons in the presence of 1 nM (−)3QNB: insert, binding of N[^{3}H]M4PB alone.

$$h(X^*) = 2/\left[1 + \left(\frac{K'_{x_1}}{K'_{x_2}}\right)^{\frac{1}{2}} \frac{(1 + K'_{x_2}[X])}{(1 + 2K'_{x_2}[X] + K'_{x_1}K'_{x_2}[X]^2)^{\frac{1}{2}}}\right] \quad (1)$$

where (h(X*) is the Hill coefficient observed for the binding of the labeled ligand X*, and K'_{x_i} are the intrinsic association constants (association constant/site) for the binding of the i-th molecule of X. The maximal effect is observed when the concentration of unlabeled X is well above $1/K'_{x_1}$ and $1/K'_{x_2}$, leading to a Hill coefficient of 1. The effect on the binding constants is obtained similarly by replacing Z with X in eq. (9,10) in [4], from which one obtains:

$$K'_{x_1}{}^* = K'_{x_1} \frac{1 + K'_{x_2}[X]}{1 + 2K'_{x_1}[X] + K'_{x_1}K'_{x_2}[X]^2} \quad (2)$$

$$K'_{x_2}{}^* = \frac{K'_{x_2}}{1 + K'_{x_2}[X]} \quad (3)$$

where $K'_{x_1}{}^*$ and $K'_{x_2}{}^*$ are the intrinsic association constants for the binding of labeled X observed in the presence of unlabeled X. Eq. (3) predicts a purely competitive inhibition for the binding of the second molecule of ligand, while eq. (2) also reduces to pure competitive inhibition when [X] is considerably higher than $1/K'_{x_1}$ and $1/K'_{x_2}$ (yielding $K'_{x_1}{}^* = 1/[X]$). Therefore, a linear Scatchard plot with a single dissociation constant equal to the concentration of unlabeled competing ligand is expected. These expectations were met for the binding of N[^{3}H]M4PB in the presence of unlabeled NM4PB (fig.1B, table 1), indicating that the deviations observed for competition between NM4PB and (−)3QNB (fig.1A) are real.

Since muscarinic antagonists bind to various brain regions (including medulla-pons [7]) with a single dissociation constant (with no indication of site-heterogeneity or negative cooperativity), it was interesting to test the effect of competition among antagonists in such a system. If there are no interactions between the binding sites, as suggested by the linear Scatchard plots for muscarinic antagonist binding, a purely competitive inhibition is expected. Such experiments were conducted in rat medulla-pons homogenates. In this system, the dissociation constant observed for the binding of N[^{3}H]M4PB, in the presence of 1 nM (−)3QNB (well above the concentration of binding sites and the dissociation constant for (−)3QNB) is the one expected assuming pure competition (fig.2, table 2).

Muscarinic receptors occupied by antagonists undergo isomerization [7]. To eliminate the possibility that the results obtained in the competition experiments between two different ligands in the adenohypophysis stem from isomerization and not from cooperative interactions, we have analyzed the effect of isomerization in systems with heterogenous, non-interacting sites. The analysis indicates that such systems behave in a manner analogous to that of systems without isomerization (see appendix).

Interactions among muscarinic binding sites in rat adenohypophysis may exist in addition to site heterogeneity. Evidence for the latter is supplied by the effect of GTP on muscarinic antagonist binding in rat adenohypophysis [10]; thus muscarinic receptors may exist as oligomers of $\geqslant$2 sites. Two ligands were found to bind simultaneously to muscarinic receptors in rat cortex [11].

Tropates and benzilates may induce different conformations upon binding to brain muscarinic receptors [7]. Thus, it will be interesting to test whether competition between tropates and benzilates yields different and more pronounced deviations.

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Appendix

Competition in the case of site heterogeneity plus isomerization

Rat brain muscarinic receptors were proposed to contain ⩾2 independent classes of binding sites: high affinity (R_H) and low affinity (R_L), comprizing the fractions α_H and α_L of the total sites [8]. A simple isomerization scheme suggested for receptors occupied by an antagonist X (shown here for the high-affinity sites) is [7]:

$$R_H^{**}Z \underset{}{\overset{K^Z_{H_2}}{\rightleftharpoons}} R_HZ \overset{Z,K^Z_{H_1}}{\rightleftharpoons} R_H \overset{X,K^X_{H_1}}{\rightleftharpoons} R_HX \overset{K^X_{H_2}}{\rightleftharpoons} R_H^*X$$

where $K^X_{H_1} = [R_HX]/[R_H]\,[X]$, $K^X_{H_2} = [R_H^*X]/[R_HX]$, and $K^Z_{H_1}$, $K^Z_{H_2}$ are defined similarly for the competitor Z

For generalization, Z is allowed to induce an isomerization (R_H^{**}) different than that of X (R_H^*). R_L also undergoes isomerization (*not shown*) to R_L^* and R_L^{**}, characterized by the equilibrium constants $K^X_{L_1}$, $K^X_{L_2}$, $K^Z_{L_1}$ and $K^Z_{L_2}$. The saturation function $\bar{Y}(x)$ (the fraction of sites occupied by X) for the binding of X alone is:

$$\bar{Y}(x) = \frac{[R_HX] + [R_LX] + [R_H^*X] + [R_L^*X]}{[R_H] + [R_HX] + [R_H^*X] + [R_L] + [R_LX] + [R_L^*X]}$$

$$= \alpha_H \frac{[R_HX] + [R_H^*X]}{[R_H]_{total}} + \alpha_L \frac{[R_LX] + [R_L^*X]}{[R_L]_{total}} \quad (1)$$

where $[R_H]_{total} = [R_H] + [R_HX] + [R_H^*X]$
and $[R_L]_{total} = [R_L] + [R_LX] + [R_L^*X]$

Introducing the expressions for $K^X_{H_1}$ and $K^X_{H_2}$ we obtain:

$$\bar{Y}(x) = \alpha_H K^X_{H_{obs}}[X]/(1 + K^X_{H_{obs}}[X]) + \alpha_L K^X_{L_{obs}}[X]/(1 + K^X_{L_{obs}}[X]) \quad (2)$$

where $K^X_{H_{obs}} = K^X_{H_1}(1 + K^X_{H_2})$, $K^X_{L_{obs}} = K^X_{L_1}(1 + K^X_{L_2})$

Thus, the only effect of allowing isomerization is that K^X_H and K^X_L (the association constants of X to the high and low affinity sites, respectively) are replaced by $K^X_{H_{obs}}$ and $K^X_{L_{obs}}$. In the presence of a constant concentration of a competing ligand Z, $[R_H]_{total}$ and $[R_L]_{total}$ in eq. (1) become ($[R_H] + [R_HX] + [R_H^*X] + [R_HZ] + [R_H^{**}Z]$) and ($[R_L] + [R_LX] + [R_L^*X] + [R_LZ] + [R_L^{**}Z]$), respectively. Using the expressions for $K^X_{H_1}, K^X_{H_2}, K^Z_{H_1}, K^Z_{H_2}, K^X_{L_1}, K^X_{L_2}, K^Z_{L_1}$ and $K^Z_{L_2}$, we obtain:

$$\bar{Y}(x) = \left[\alpha_{H_1} \frac{K^X_{H_{obs}}[X]}{1 + K^Z_{H_{obs}}[Z]} \Big/ \left(1 + \frac{K^X_{H_{obs}}[X]}{1 + K^Z_{H_{obs}}[Z]}\right)\right] + \left[\alpha_L \frac{K^X_{L_{obs}}[X]}{1 + K^Z_{L_{obs}}[Z]} \Big/ \left(1 + \frac{K^X_{L_{obs}}[X]}{1 + K^Z_{L_{obs}}[Z]}\right)\right] \quad (3)$$

where $K^X_{H_{obs}}$, $K^X_{L_{obs}}$ are as in eq. (2), while
$K^Z_{H_{obs}} = K^Z_{H_1}(1 + K^Z_{H_2})$ and $K^Z_{L_{obs}} = K^Z_{L_1}(1 + K^Z_{L_2})$

Thus, Z inhibits X binding to R_H and R_L in a purely competitive manner, according to $K^Z_{H_{obs}}$ and $K^Z_{L_{obs}}$ – the observed association constants for the binding of Z alone. Assuming more independent classes of sites, or more complex isomerization schemes (e.g., a cyclic scheme, where R*X and R**Z are also in direct equilibrium with R, or where free R* and R** also exist) yields similar results.