Rate Constants of Agonist Binding to Muscarinic Receptors in Rat Brain Medulla

EVALUATION BY COMPETITION KINETICS*

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The method of competition kinetics, which measures the binding kinetics of an unlabeled ligand through its effect on the binding kinetics of a labeled ligand, was employed to investigate the kinetics of muscarinic agonist binding to rat brain medulla pons homogenates. The agonists studied were acetylcholine, carbamylcholine, and oxotremorine, with N-methyl-4-[3H]piperidyl benzilate employed as the radiolabeled ligand. Our results suggested that the binding of muscarinic agonists to the high affinity sites is characterized by dissociation rate constants higher by 2 orders of magnitude than those of antagonists, with rather similar association rate constants. In contrast, the major differences between the kinetic binding parameters of agonists and antagonists to the low affinity agonist binding sites are in the association rate constants, which were 2–5 orders of magnitude lower for agonists. This demonstrates that there are basic differences in the interactions of agonists with the low and high affinity sites. Our findings also suggest that isomerization of the muscarinic receptors following ligand binding is significant in the case of antagonists, but not of agonists. Moreover, it is demonstrated that in the medulla pons preparation, agonist-induced interconversion between high and low affinity bindings sites does not occur to an appreciable extent.

The binding kinetics of tritiated antagonists to muscarinic cholinergic receptors were extensively investigated during the past few years (1–6). Such studies have contributed to the understanding of the mechanism of antagonist-receptor interactions, and have led to the formulation of the isomerization model, where the receptor undergoes a conformational change following the binding of antagonists (1–4, 6). The kinetic studies also revealed differences among various classes of muscarinic antagonists, namely tropates and benzilates (3, 6, 7).

The kinetics of agonist binding to the muscarinic receptor are of special interest not only due to their possible physiological roles, but also in view of several interesting phenomena. Although most antagonists were found to bind with a similar affinity to all the muscarinic binding sites, direct and indirect equilibrium binding studies have suggested the existence of different states of the muscarinic receptors, which bind agonists with different affinities (8). These affinity states may undergo interconversion (a transfer of part of the population with high affinity towards agonists to the low affinity state and vice versa) by several agents and treatments (9–13). Such interconversion was also detected in vivo during development of mouse brain (14), rat brain (15, 16), chicken heart (17), and in various stages of the estrous cycle in female rat anterior pituitary (18) and preoptic hypothalamus (19).

Two major obstacles interfere with direct studies of the interactions of agonists with the muscarinic receptor: (a) the low affinity of agonists necessitates the use of high concentrations of labeled agonists, yielding high nonspecific binding (8); (b) rapid dissociation rates render direct kinetic studies with agonists even more difficult.

In equilibrium binding studies, the problem of low affinity of agonists has been overcome by the use of indirect binding experiments, namely, competition between highly affine, labeled antagonists and unlabeled agonists (3,8). An analogous approach to kinetic studies could provide important information on the nature of agonist-receptor interactions, which cannot be obtained by direct binding studies. In order to enable such indirect kinetic studies, we have developed a theoretical basis for a "competition kinetics" method (20). In this method, the binding kinetics of a labeled ligand are measured in the presence and absence of a competing unlabeled ligand, and the rate constants of the latter are evaluated by nonlinear regression analysis of the integrated rate equations developed according to the specific model chosen. In the accompanying manuscript (20) we have demonstrated the validity of the method and its applicability to measurements of ligand-receptor interactions employing antagonist binding to rat brain muscarinic receptors as the experimental system. In the present paper, we report the application of competition kinetics to determine the kinetic constants of unlabeled muscarinic agonists in rat brain medulla pons. This preparation was chosen due to two reasons: (a) it contains approximately equal proportions of high and low affinity sites for agonist binding (11), thus enabling kinetic analysis of the interaction of both sites with agonists. (b) The existence of two subclasses (M1 and M2) of muscarinic receptors, which bind non-classical muscarinic agonists (e.g. pirenzepine) with different affinities, was proposed recently (21, 22). An alternative explanation for this antagonist binding heterogeneity is that it stems mainly from ligand-induced conformation changes (14, 23, 24). In view of this controversy, we decided to employ medulla pons preparations, which were suggested to contain almost exclusively (around 90%) the M2 subtype (25). This would avoid complications which could arise due to the presence of mixtures of the two subtypes. Such complications were also eliminated by the use of [3H]4-NMPB,† an antagonist that

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† The abbreviation used is: 4-NMPB, N-methyl-4-piperidyl benzilate.

8795
bonds to all the muscarinic sites with the same affinity.

Our results indicate that muscarinic agonists bind differently to low and high affinity agonist binding sites: while the most significant differences between the binding of agonists and antagonists to the high affinity agonist binding sites in the dissociation rate constant, in the case of the low affinity agonist binding sites the major differences occur between the association rate constants. Moreover, our findings suggest that ligand-induced isomerization of the muscarinic binding sites occurs upon the binding of antagonists, but not agonists, to the receptor.

**Experimental Procedures**

**Materials**—[^3H]4-NMPB (69 Ci/mmole) was prepared as described previously (26). Unlabeled carbachol, acetylcholine, and physostigmine were from Sigma. Oxotremorine was a product of Aldrich.

**Medulla Pons Homogenates—**Adult male rats (CD strain) were from Levenshtein’s Farm (Yokneam, Israel). The rats were maintained at 24 ± 2 °C. Food (Assia Maabarat, Tel Aviv) and water were supplied ad libitum. Rats aged 3–4 months (190–250 g) were decapitated and their brains removed. The medulla pons was dissected out in a cold room. Homogenates were prepared in cold 0.32 M sucrose from the medulla pons of two rats for each experiment, using a motor driven Teflon pestle in a glass homogenizer.

**Equilibrium Binding—**Binding of [^3H]4-NMPB to muscarinic receptors in homogenates from medulla pons was determined by a filtration assay, following a formerly described procedure (26). The binding was performed in modified Krebs-Henseleit solution (25 mM Tris-HCl, 118 mM NaCl, 4.7 mM KCl, 1.9 mM CaCl2, 0.54 mM MgCl2, 1 mM NaH2PO4, 11.1 mM glucose, pH 7.4) at 22 °C. In the case of acetylcholine, the membrane suspension was pre-treated with 10 µM physostigmine, which inhibit over 90% of the acetylcholinesterase activity without affecting agonist or antagonist binding. The ligands were incubated with the homogenate for 45 min, a period sufficient to achieve equilibrium in this system (bound ligand concentration did not change upon incubation for 1 more h). Nonspecific binding was determined by subtracting the initial guesses of the constants by 2 orders of magnitude; this did not result in significant changes in the best-fit values in 95% of the cases (the remaining 5% were discarded).

Equilibrium competition curves were fit to the sum of Equations 1 and 2 ((RAn) + (R’An)) is the time-dependent concentration of bound antagonist. The kinetic data were computer-analyzed by fitting to this sum, using a nonlinear regression curve fitting library program (28), as described in detail in the accompanying manuscript (20). This procedure yielded the best fit kinetic constants k+1, k-1, k+, k-. The possibility of local minima in the curve-fitting procedure was eliminated by changing the initial guesses of the constants by 2 orders of magnitude; this did not result in significant changes in the best-fit values in 95% of the cases (the remaining 5% were discarded).

Analysis of the binding kinetics of unlabeled agonists (deduced from competition experiments with [^3H]4-NMPB) was performed by employing the same nonlinear regression procedure (a detailed description is given in Ref. 20). In the case of low agonist concentrations, which occupy almost exclusively the high affinity agonist binding sites, the data were fitted to the sum of Equations 1, 2, and 6 (see ‘Theory’ in Miniprint). At high agonist concentrations, where the low affinity sites are also significantly occupied, the data were fitted to the sum of Equations 6 and 7 developed in the theoretical section (see Miniprint). For the experiments of competition kinetics with unlabeled agonists, the test for true (non-local) minima by changing the initial guesses failed in about 20% of the cases; the latter curves were discarded.

**Results**

**Direct Binding Kinetics—**In the competition kinetics method employed in the present study, two ligands (an unlabeled agonist and a labeled antagonist) compete on the same receptor sites. The rate constants characterizing the binding of the unlabeled agonist to the receptor can be derived through its effect on the binding kinetics of the labeled antagonist, providing that the latter are known from independent experiments. Thus, we have measured directly the binding kinetics of[^3H]4-NMPB (the labeled antagonist employed throughout this study) to the muscarinic receptors in medulla pons homogenates. The kinetic constants derived from a series of such curves (Fig. 1–3) are depicted in Table I. These constants are in the same range as those obtained for[^3H]4-NMPB binding to the muscarinic receptors in rat brain cortex in the accompanying report (20) (Table I). It should be noted that the ratio between the isomerization constants k+k- is

\[^7]

Portions of this paper (including Equations 3–7, Schemes b and c, and Appendices I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-3277, cite the authors, and include a check or money order for $3.20 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
around unity. Thus, the isomerization step induced by $[^{3}H]4$-NMPB binding cannot be neglected, and has to be taken into consideration.

**Agonist Binding to High Affinity Receptor Sites**—Equilibrium competition studies of muscarinic agonists with radiolabeled antagonists demonstrated that unlike antagonists, agonist binding to muscarinic receptors does not obey a simple mass-action law, and yields Hill coefficients smaller than unity (8). This phenomenon was interpreted as indicating the existence of multiple-affinity populations of muscarinic binding sites, which are recognized differently by agonists, but not by antagonists (8). In order to reduce the number of parameters fitted in the experiment, one can therefore employ low agonist concentrations which result in negligible occupancy of the low affinity agonist binding sites. The results of experiments on antagonist binding kinetics, conducted in the presence of such low agonist concentrations, are shown in Figs. 1–3. These kinetic curves can be analyzed to yield the kinetic parameters of agonist binding to the high affinity sites. In order to establish whether the ligand-induced isomerization may indeed be neglected in the case of agonist binding (as was assumed in Scheme b, under "Theory," Miniprint Section), we have analyzed the results assuming that agonist-induced isomerization does exist. The equations employed to fit the data in this case are given in Appendix II (see Miniprint). The results of this analysis are depicted in Table II, along with the results obtained using an analysis which ignores the agonist-induced isomerization step. Both the association and dissociation rate constants of agonists to the high affinity receptor state are essentially identical in the two modes of analysis, suggesting that the contribution of agonist-induced isomerization is not significant. Moreover, the ratio

![Fig. 1. Representative curves of $[^{3}H]4$-NMPB binding kinetics in the presence of unlabeled oxotremorine. The concentration of $[^{3}H]4$-NMPB was 2 nM in all cases. The medulla pons homogenate contained 0.3 mg of protein/ml (Rg = 0.3 pmol/mg protein). In the absence of agonist (0 μM oxotremorine) the solid lines represent the computer fit to the sum of Equations 1 and 2. In the presence of a low oxotremorine concentration (20 pM), which occupies almost exclusively the high affinity sites, the computer fit was performed using the sum of Equations 1, 2, and 3 (with $[Rg]$ replaced by $[Rg]/(1 - u)$ in Equations 1 and 2). In the presence of a high oxotremorine concentration (25 μM), which occupies significantly both high and low affinity sites, the sum of Equations 6 and 7 was employed in the fitting procedure. Each point in the curves is the mean of three determinations, with standard error below 10%. Every second data point was plotted, in order to avoid overcrowding in the initial times.](image1)

![Fig. 2. Representative curves of $[^{3}H]4$-NMPB binding kinetics in the presence of carbamylcholine. The $[^{3}H]4$-NMPB concentration was 2 nM, and the medulla pons homogenate contained 0.3 mg of protein/ml. The computer fit in the absence (0 μM) and in the presence of low (1 μM) or high (50 μM) concentrations of carbamylcholine was performed as described in the legend to Fig. 1. Each point is the mean of three determinations, with standard error below 10%. Every second data point was plotted, in order to avoid overcrowding in the initial times.](image2)

![Fig. 3. Representative curves of $[^{3}H]4$-NMPB binding kinetics in the presence of acetylcholine. These experiments were conducted in the presence of 10 μM physostigmine, in order to prevent acetylcholinesterase activity. The $[^{3}H]4$-NMPB concentration was 2 nM, and the homogenate contained 0.3 mg of protein/ml. The computer fit in the absence (0 μM) and in the presence of low (0.5 μM) or high (25 μM) acetylcholine was performed as described in Fig. 1. Each point is the mean of three determinations, with standard error below 10%. Every second data point was plotted, in order to avoid overcrowding in the initial times.](image3)
Acetylcholine studies obtained for antagonists (Tables I and II, Ref. 20). On the other hand, no such differences are observed between the constants explain their lower affinities to the muscarinic sites. Representative competition kinetics curves obtained at high agonist concentrations are shown in Figs. 1–3 was analyzed separately to yield the rate constants. The constants in the table are the mean ± S.E. of these values. The asymptotic S.E. (in the fit of each separate curve) were much lower (below 1%).

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$k_1$</th>
<th>$k_1$</th>
<th>$k_2$</th>
<th>$k_3$</th>
<th>$k_2/k_1$</th>
<th>$K_a$</th>
<th>$K^*_a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>(0.3 ± 0.1) x 10^6</td>
<td>0.7 ± 0.2</td>
<td>0.28 ± 0.07</td>
<td>0.1 ± 0.02</td>
<td>0.36 ± 0.09</td>
<td>0.61 ± 0.03</td>
<td>0.42 ± 0.02</td>
</tr>
<tr>
<td>Medulla pons</td>
<td>(0.34 ± 0.001) x 10^6</td>
<td>0.42 ± 0.07</td>
<td>0.14 ± 0.03</td>
<td>0.16 ± 0.06</td>
<td>1.11 ± 0.34</td>
<td>0.65 ± 0.03</td>
<td>0.6 ± 0.03</td>
</tr>
</tbody>
</table>

The values of $k_3/k_2$ and $K^*_a$ were calculated separately for each curve; the S.E. values in the table were computed from the spread of these calculated values. Calculation of S.E. from the mean ± S.E. of the rate constants yields larger values, 40–60% for $k_3/k_2$ and 90–105% for $K^*_a$.

### TABLE II

Rate constants of muscarinic agonist binding to high affinity agonist binding sites derived from competition kinetics

Experiments were performed using medulla pons homogenates as described in the legends to Figs. 1–3. The [H]4-NMPB concentration was 2 nM in all cases. The various rate constants are defined in Scheme c ("Theory," Miniprint Section) in the absence of agonist-induced isomerization, and in Scheme d (Appendix II, Miniprint Section) for the case which assumes such an isomerization. $a$ is the fraction of high affinity agonist binding sites (as $a = [R]^f/[R]^f + [R]^s$), as determined in the competition kinetics experiments without isomerization. The $a$ values obtained in independent equilibrium experiments were 0.55, 0.60, and 0.60 for acetylcholine, carbamylcholine, and oxotremorine, respectively. $K_a$ is the agonist dissociation constant from the high affinity sites calculated from $k_3$ and $k_2$ without isomerization according to $K_a = k_3/k_2$. $K^*_a$ is this agonist dissociation constant determined at equilibrium. The fit in the absence of agonist-induced isomerization was performed using the sum of Equations 1, 2, and 6 (with $[R]^f(1 - a)$ in place of $[R]^f$ in Equations 1 and 2). The fit in the case assuming agonist-induced isomerization was performed as described in Appendix II (see Miniprint). The values are the mean ± S.E. of three experiments similar to those depicted in Figs. 1–3. Each experiment was performed three times, and the curves were analyzed separately. The asymptotic S.E. were lower (<1%).

<table>
<thead>
<tr>
<th>Competing Ligand</th>
<th>$a$</th>
<th>$K_a$</th>
<th>$K^*_a$</th>
<th>$nM$</th>
<th>$K_a$</th>
<th>$K^*_a$</th>
<th>$nM$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine</td>
<td>0.57 ± 0.04</td>
<td>108 ± 10^4</td>
<td>82 ± 10</td>
<td>0.27 ± 0.05</td>
<td>29.2 ± 5</td>
<td>202 ± 25</td>
<td></td>
</tr>
<tr>
<td>(0.5 μM)</td>
<td></td>
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</tr>
<tr>
<td>Carbamylcholine</td>
<td>0.57 ± 0.04</td>
<td>90 ± 9</td>
<td>150 ± 10</td>
<td>0.31 ± 0.07</td>
<td>25.0 ± 5</td>
<td>223 ± 20</td>
<td></td>
</tr>
<tr>
<td>(1 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxotremorine</td>
<td>0.61 ± 0.03</td>
<td>17 ± 2</td>
<td>14 ± 2</td>
<td>1.2 ± 0.4</td>
<td>20.3 ± 6</td>
<td>271 ± 23</td>
<td></td>
</tr>
<tr>
<td>(0.2 μM)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*The S.E. values in these columns were computed from the spread of the values calculated separately for each curve. The S.E. calculated from the mean ± S.E. of the rate constants are larger; they are in the ranges of 35–65% and 20–30% for $K_a$ and $K^*_a$, respectively.

The dissociation rate constants of agonists from the high affinity receptor sites are 2 orders of magnitude higher than those obtained for antagonists (Tables I and II, Ref. 20). On the other hand, no such differences are observed between agonists and antagonists regarding their association rate constants. The far higher values of the agonist dissociation rate constants explain their lower affinities to the muscarinic receptors as compared with antagonists.

**Agonist Binding to the Low Affinity Receptor Sites**—At high agonist concentrations, both the high and low affinity agonist binding sites are occupied by agonist. Thus, competition kinetics experiments conducted in the presence of relatively high agonist concentrations (high enough to occupy significantly both types of sites, but low enough to enable detection of the binding of the labeled agonist) can be employed to explore the rate constants of agonist binding to the low affinity sites. Representative competition kinetics curves obtained at high agonist concentrations are shown in Figs. 1–3. Since the rate constants characterizing the interactions of the agonists with the high affinity sites are already known at this stage (Table II), it was possible to insert $k_3$ and $k_3$ (Table II) along with $k_1$, $k_1$, $k_3$, $k_3$ (Table I) and thus reduce the parameters in the fit to $a$, $k_3$, and $k_4$. The parameters obtained in this manner for acetylcholine, carbamylcholine, and oxotremorine are depicted in Table III. We have also tested the possibility that agonist-induced isomerization of the low affinity sites contributes significantly to the measurements, using an analysis analogous to that described in the case of agonist binding to the high affinity sites. This analysis did not alter the values of $k_3$ and $k_4$ and yielded $k_3/k_2/\text{K}_{20}$ (defined analogously to $k_3/k_2/\text{K}_{20}$, Appendix II, Miniprint Section) values between 50 and 200, suggesting that agonist-induced isomerization is negligible also in the case of the low affinity sites.

It is interesting to note that the rate constants characterizing the interaction of oxotremorine with the low affinity agonist binding sites differ extensively from those of acetylcholine and carbamylcholine (Table III). Although the rate constants of oxotremorine binding to the high affinity sites are also different from those of the other two agonists ($k_3$ for oxotremorine is 4–5-fold higher), the differences are much more striking in the case of the low affinity sites. These differences may be the reason for the significantly higher...
affinity of oxotremorine to the low affinity sites as compared with the affinities of acetylcholine or carbamylcholine. These findings are in accord with earlier reports on differences in the binding characteristics of and the biochemical response to oxotremorine as compared with other muscarinic agonists (30, 31).

Proportion of High and Low Affinity Agonist Binding Sites—Another important parameter obtained from the competition kinetics experiments is \( \alpha \), the fraction of high affinity agonist binding sites. The advantage of the competition kinetics method is that it enables the extraction of the \( \alpha \) value using a single agonist concentration, unlike the situation in equilibrium binding studies, which require a whole set of agonist concentrations to determine \( \alpha \). Using the competition kinetics method one can therefore compare the \( \alpha \) values obtained at different agonist concentrations. The results (Tables II and III) demonstrate that similar \( \alpha \) values are obtained using high and low agonist concentrations. In addition, the \( \alpha \) values of all three agonists tested were essentially identical (Tables II and III). These \( \alpha \) values are in accord with those obtained in equilibrium binding measurements both in the present study (Table II, legend) and in previous reports (11, 12, 32, 40).

**Comparison between the rate constants characterizing the binding of agonists and antagonists to the muscarinic receptors in the medulla pons homogenates reveals some interesting features. Thus, the association rate constants of the agonists to the high affinity sites are rather similar to those of antagonists (Tables I and II; Ref. 20). On the other hand, the dissociation rate constants of the agonists from these sites are 2 orders of magnitude higher than those of antagonists. It therefore follows that the lower affinities demonstrated by agonists to their high affinity sites stem from their higher rate of dissociation from these sites. An analogous comparison between the rate constants of agonists and antagonists can also be made regarding the low affinity agonist binding sites (Tables I and III; Ref. 20). Such a comparison indicates that the dissociation rate constants of the agonists (except oxotremorine, which differs markedly also from the other two agonists) from the low affinity sites are similar to those of antagonists. In this case, the difference between agonists and antagonists resides in the association rate constants, which are 2–5 orders of magnitude lower for agonists. Thus, the higher equilibrium dissociation constants characterizing the binding of agonists to the low affinity sites stem from lower association rate constants, rather than from higher dissociation rate constants. This demonstrates that there are basic differences between the interactions of agonists with the low and high affinity sites. Since the high affinity state towards agonists is believed to represent a form of the muscarinic receptor which is coupled to a guanylnucleotide binding protein (for review see Ref. 33), these findings indicate that the coupled receptor may interact differently with agonists.

It should be noted that the agonist equilibrium dissociation constants calculated from the appropriate rate constants are in accord with the actual dissociation constants determined in equilibrium (Tables II and III). Moreover, in the case of acetylcholine, the results of direct binding of acetyl-[\( ^3 \)H]choline to the high affinity sites (\( k_d = 0.15 \times 10^9 \text{ M}^{-1} \text{ min}^{-1} \), \( K_{eq}^{2+} = 76 \text{ nM} \)) are in accord with the \( k_d \) and \( K_{eq}^{2+} \) obtained for acetylcholine by competition kinetics (Table II). This agreement demonstrates once more the validity of the results obtained by the competition kinetics method. The equilibrium dissociation constants found in the present study are in accord with former results (8, 14, 32, 40).

**Ligand-induced Isomerization of the Muscarinic Receptors**—The findings reported in this study demonstrate significant differences between the ability of agonists and antagonists to induce isomerization of the muscarinic binding sites. Thus,

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**TABLE III**

<table>
<thead>
<tr>
<th>Competing agonist</th>
<th>( K_a^{2+} )</th>
<th>( K_a^{3+} )</th>
<th>( k_a )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetylcholine (25 \mu M)</td>
<td>0.57 ± 0.05</td>
<td>22 ± 3^a</td>
<td>25 ± 3</td>
</tr>
<tr>
<td>Carbamylcholine (50 \mu M)</td>
<td>0.62 ± 0.04</td>
<td>43 ± 4^a</td>
<td>48 ± 5</td>
</tr>
<tr>
<td>Oxotremorine (5 \mu M)</td>
<td>0.65 ± 0.05</td>
<td>15 ± 0.2^a</td>
<td>14 ± 0.2</td>
</tr>
</tbody>
</table>

^a The \( K_a^{2+} \) values were computed separately for each curve, and the S.E. values were calculated from the spread of these calculated values. The S.E. calculated from the mean ± S.E. of the rate constants are larger, and range from 40 to 60%.

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for [3H]4-NMPB binding, the ratio between the isomerization constants (k_{on}/k_{off}) is 1.1 in the medulla pons (Table I); this antagonist yielded a ratio of 0.4 in rat brain cortex, where another antagonist (→N-methylscopolamine) yielded a ratio of 1.0 (20). It therefore follows that in all these cases the isomerization induced by antagonist binding is significant and cannot be neglected, as suggested earlier (1-4, 6, 33).

In contrast, analysis of agonist binding kinetics assuming the existence of agonist-induced isomerization yields very high ratios between k_{on}/k_{off} (50-200) for both the high and low affinity binding sites (Table II; see also “Results”). This high ratio suggests that the isomerization induced by agonist binding to the medulla pons muscarinic receptors is negligible. This suggestion gains further support from the finding (Tables II and III; see also “Results”) that both the association and dissociation constants of the agonists are similar whether or not agonist-induced isomerization is included in the binding scheme. It should be noted that ligand-induced isomerization was also observed in the case of the nicotinic acetylcholine receptor. However, unlike the situation encountered with the muscarinic receptors, isomerization of the nicotinic receptor was reported to be induced by agonists (34-37), while the existence of antagonist-induced isomerization is controversial (34, 36).

It is interesting to note that the binding of muscarinic agonists was proposed to involve two attachment points, while that of antagonists was proposed to involve an additional portion (hydrophobic) of the molecule (39, 39, and references therein). It is therefore possible that the binding of antagonists to an additional domain in the muscarinic binding site orients them differently within this site, and enables them to induce a conformational change in the receptor in a different manner from agonists. Finally, it should be noted that agonist-induced isomerization of the medulla pons muscarinic receptors which occurs on a time scale different from that of the kinetic experiments (10 s to 60 min) cannot be ruled out, especially since conformational changes of some type are associated with the muscarinic receptors, isomerization of the nicotinic receptor was reported to be induced by agonists (34-37), while the existence of antagonist-induced isomerization is controversial (34, 36).

Lack of Agonist-induced Interconversion—The proportion of high affinity agonist binding sites (α) is normally defined in equilibrium binding studies, through extrapolation of the binding data to high ligand concentrations. Since these data are obtained using a series of agonist concentrations, an obvious assumption is incorporated into this type of analysis is that α does not depend on agonist concentration. The competition kinetics experiments provide a direct test for this assumption, since α can be independently extracted from the fit of each separate kinetic binding curve. The α values derived in this manner for all three agonists at high agonist concentrations (Table II) are essentially identical to those obtained at low agonist concentrations (Table III). They are in excellent agreement with the α values obtained in equilibrium binding experiments (Table II, legend; Refs. 12, 32, and 40). These findings demonstrate that α does not change with the agonist concentration, and suggest that there is no significant agonist-induced interconversion between the high and low affinity agonist binding sites in the medulla pons preparation. This conclusion is in accord with the finding (Tables II and III; Refs. 12, 32, and 40) that the α values in the medulla pons preparation are similar for all the muscarinic agonists tested.

It should be noted that there are reports on differences between the α values of different agonists in various other brain regions (for review, see Ref. 33). Therefore, the current experiments do not rule out the possibility that agonists may induce interconversion of muscarinic binding sites in those brain regions. This subject is currently under study in our laboratory. Moreover, guanylnucleotides and transition metal ions are capable of inducing interconversion of muscarinic agonist binding sites both in the medulla pons and in other brain regions (for review, see Ref. 33). Since studies on interconversion with these affectors are conducted in the presence of agonists, it still has to be established whether agonist-induced interconversion can occur in the presence of these affectors.

Acknowledgments—Enlightening discussions with Drs. A. P. Minto and Y. Kloo are gratefully acknowledged. We thank Ronit Calron for providing excellent technical assistance, and David Sagin for constructing the computer programs employed in this study.

REFERENCES
Binding Kinetics of Muscarinic Agonists

13.17-24


28. James, F., and Roos, M. (1972) Minuit Program, Cern, Switzerland


1) Inasmuch to solve these equations using the Laplace transform (given in Appendix I), Following the inverse transform, one obtains the solutions for $[A^a]^*$ and $[A^a]^*_{\infty}$, which are the experimentally-determined quantities in the system

\[
\begin{align*}
\frac{d[A^a]^*}{dt} &= k_2 [A^a]^* - k_{-2} [A^a]^*_{\infty} + k_{-1} [A^a]^* + k_3 [A^a]^*_{\infty} - k_1 [A^a]^*_{\infty} \\
\frac{d[A^a]^*_{\infty}}{dt} &= k_{-1} [A^a]^* + k_1 [A^a]^*_{\infty} - k_2 [A^a]^*_{\infty} - k_{-2} [A^a]^*_{\infty} \\
\end{align*}
\]

where $k_1$, $k_2$, $k_3$, and $k_{-1}$, $k_{-2}$, $k_{-3}$ are the roots of the polynomial DEQN, which is the denominator of eqns. (2-4) in Appendix I.

The binding of the labeled antagonist to the low affinity agonist binding sites is not perturbed by the agonist and is therefore given by the sum of eqns. (1) and (2), with $[A^a]^*_{\infty}$ replaced by $[A^a]^*_{\infty}$ (the concentration of the low affinity sites). Addition of eqn. (3) to this sum yields $[A^a]^*_{\infty} = [A^a]^* + [A^a]^*_{\infty}$, which is the time-dependent concentration of bound labeled antagonist to both high and low affinity agonist binding sites.

The kinetic data were fitted to the expression of this sum employing a non-linear regression curve-fitting procedure (28), as described in detail in the accompanying manuscript (29). The values of the constants $k_1$, $k_2$, $k_3$, and $k_{-1}$, $k_{-2}$, $k_{-3}$, which are known from direct determination of the binding kinetics of the antagonist alone, are introduced into eqns. (1), (2) and (3) along with $[A^a]^*$, $[A^a]^*_{\infty}$, and the program computes the best-fit values of $k_1$, $k_2$, and $k_3$.

Kinetics of agonist binding to the low and high affinity receptors: As high agonist concentrations, both the high and the low affinity agonist binding sites are occupied by the agonist. Due care has to be taken to ensure that the solution for $[A^a]^*_{\infty}$ is already present in eqn. (4). The solution for $[A^a]^*_{\infty}$ follows the same steps as shown for agonist binding to the high affinity sites, except that in this case $k_1$ and $k_2$ replace $k_{-1}$ and $k_{-2}$, and 1-4 replaces a:

\[
\begin{align*}
\frac{d[A^a]^*_{\infty}}{dt} &= k_{-2} [A^a]^*_{\infty} - k_{-1} [A^a]^*_{\infty} + k_2 [A^a]^* + k_3 [A^a]^*_{\infty} - k_1 [A^a]^*_{\infty} \\
\end{align*}
\]

The mass-action equation for the total concentration of $[A^a]$, $[A^a]^*$ and $[A^a]^*_{\infty}$ is:

\[
\begin{align*}
\frac{d[A^a]}{dt} &= k_1 [A^a]^*_{\infty} + k_{-1} [A^a]^* + k_2 [A^a]^*_{\infty} - k_{-2} [A^a]^*_{\infty} + k_3 [A^a]^* - k_2 [A^a]^*_{\infty} - k_{-3} [A^a]^*_{\infty} \\
\end{align*}
\]

The total receptor concentration, $[A^a]$, is given by $[A^a] = [A^a]^* + [A^a]^*_{\infty}$. Defining $a$ as the proportion of the total concentration of high affinity binding sites for agonist will give:

\[
\begin{align*}
[A^a]^* = [A^a] - [A^a]^*_{\infty} \quad \text{and} \quad [A^a]^*_{\infty} = (1 - a) [A^a] \\
\end{align*}
\]

Inserting this equation in the former one and extracting $[A^a]^*$ the kinetics of antagonist binding are $k_1$ can be described by the following differential equations:

\[
\begin{align*}
\frac{d[A^a]^*}{dt} &= k_2 [A^a]^* - k_{-2} [A^a]^* + k_{-1} [A^a]^* + k_3 [A^a]^*_{\infty} - k_1 [A^a]^*_{\infty} \\
\frac{d[A^a]^*_{\infty}}{dt} &= k_{-1} [A^a]^* + k_1 [A^a]^*_{\infty} - k_2 [A^a]^*_{\infty} - k_{-2} [A^a]^*_{\infty} \\
\end{align*}
\]

The experimentally-determined quantities in the system are $[A^a]^*_{\infty}$ and $[A^a]^*_{\infty}$, which are the denomenator of DEQN, which is the denominator of eqns. (2-4) in Appendix I.

The binding of the labeled antagonist to the low affinity agonist binding sites is not perturbed by the agonist and is therefore given by the sum of eqns. (1) and (2), with $[A^a]^*_{\infty}$ replaced by $[A^a]^*_{\infty}$ (the concentration of the low affinity sites). Addition of eqn. (3) to this sum yields $[A^a]^*_{\infty} = [A^a]^* + [A^a]^*_{\infty}$, which is the time-dependent concentration of bound labeled antagonist to both high and low affinity agonist binding sites.

The kinetic data were fitted to the expression of this sum employing a non-linear regression curve-fitting procedure (28), as described in detail in the accompanying manuscript (29). The values of the constants $k_1$, $k_2$, $k_3$, and $k_{-1}$, $k_{-2}$, $k_{-3}$, which are known from direct determination of the binding kinetics of the antagonist alone, are introduced into eqns. (1), (2) and (3) along with $[A^a]^*$, $[A^a]^*_{\infty}$, and the program computes the best-fit values of $k_1$, $k_2$, and $k_3$.

The experimentally-determined quantities in the system are $[A^a]^*_{\infty}$ and $[A^a]^*_{\infty}$, which are the denomenator of DEQN, which is the denominator of eqns. (2-4) in Appendix I.

The binding of the labeled antagonist to the low affinity agonist binding sites is not perturbed by the agonist and is therefore given by the sum of eqns. (1) and (2), with $[A^a]^*_{\infty}$ replaced by $[A^a]^*_{\infty}$ (the concentration of the low affinity sites). Addition of eqn. (3) to this sum yields $[A^a]^*_{\infty} = [A^a]^* + [A^a]^*_{\infty}$, which is the time-dependent concentration of bound labeled antagonist to both high and low affinity agonist binding sites.

The kinetic data were fitted to the expression of this sum employing a non-linear regression curve-fitting procedure (28), as described in detail in the accompanying manuscript (29). The values of the constants $k_1$, $k_2$, $k_3$, and $k_{-1}$, $k_{-2}$, $k_{-3}$, which are known from direct determination of the binding kinetics of the antagonist alone, are introduced into eqns. (1), (2) and (3) along with $[A^a]^*$, $[A^a]^*_{\infty}$, and the program computes the best-fit values of $k_1$, $k_2$, and $k_3$.
Solving these equations for $[R_yA^*_n]$ and $[R_yA^*_n]$: 

\[(A-4)\]  
\[
[R_yA^*_n] = \frac{a_{k_1}([A^*_n][R_y] + \kappa_{k_2}([A^*_n][R_y])^2 + (h_{k_1} - h_{k_2})([A^*_n][R_y])}{DENOM}
\]

where,

\[
DENOM = \beta + \gamma + \delta + \epsilon ([A^*_n][R_y])^2 + 2(\delta_{k_1} + \epsilon_{k_2})[A^*_n][R_y] + \beta_{k_1} + \gamma_{k_2} - \alpha_{k_1} + \alpha_{k_2}
\]

Eqs. (A-4) can be written as:

\[(A-5)\]  
\[
[R_yA^*_n] = \frac{a_{k_1}([A^*_n][R_y] + \kappa_{k_2}([A^*_n][R_y])^2 + (h_{k_1} - h_{k_2})([A^*_n][R_y])}{DENOM}
\]

where $A$ and $B$ are as defined for eqn. (5) under the Theory section, and $\alpha + \beta + \gamma + \delta$ is equal to $DENOM$ in eqn. (A-4). $\alpha_{k_1}, \beta_{k_1}, \gamma_{k_2}$ and $\delta_{k_2}$ are therefore the roots of the polynomial $DENOM$. When these roots are all different from each other (as occurs in the system investigated in this study), the inverse Laplace transform for these equations (tabulated in ref. 41) yields eqns. (6) given in the Theory section.

Appendix II - Kinetic Analysis of Agent Binding to the High Affinity Agonist Binding Sites Excluding an Internalization Step of the Agent-Receptor Complex

Introducing an immobilization step for the complex of the agent with the high affinity binding site for agonists under the conditions underlying scheme c (low agonist concentrations, which leave the low affinity binding sites for agonists uncoupled by agonist) yields scheme e:

\[(6)\]  
\[
R_yA^*_n \stackrel{k_{-2}}{\rightarrow} R_yA^*_n \stackrel{k_{1-1}}{\rightarrow} R_yA^*_n \stackrel{\kappa_{1-1}}{\rightarrow} \frac{1}{[A^*_n]} \stackrel{k_{-3}}{\rightarrow} R_yA^*_n
\]

The reaction mechanisms given for the competition of the agonist and the antagonist on the high affinity binding sites for agonists is similar to that given for two competing antagonists (20). Thus, solution for $[R_yA^*_n]$ and $[R_yA^*_n]$, similar to eqns. (11) and (12) in the accompanying manuscript, can be derived.

\[(A-6)\]  
\[
[R_yA^*_n] = \frac{a_{k_1}([A^*_n][R_y] + \kappa_{k_2}([A^*_n][R_y])^2 + (h_{k_1} - h_{k_2})([A^*_n][R_y])}{DENOM}
\]

\[(A-7)\]  
\[
[R_yA^*_n] = \frac{a_{k_1}([A^*_n][R_y] + \kappa_{k_2}([A^*_n][R_y])^2 + (h_{k_1} - h_{k_2})([A^*_n][R_y])}{DENOM}
\]

where $A = k_{-2} + k_{1-1} + k_{2-1} + k_{-3} + k_{-1} + k_{1-1} + k_{2-1} + k_{-3}$, $C = k_{-2} + k_{1-1} + k_{2-1} + k_{-3} + k_{1-1} + k_{2-1} + k_{-3}$, $f = k_{1-1}$, $g = k_{2-1}$, $h = k_{-3}$, $i = k_{1-1}$, $j = k_{2-1}$, and $k = k_{-3}$.

The binding of the labeled antigen to the low affinity agonist binding sites is not perturbed by the agonist, and is therefore given by the sum of eqns. (1) and (2), with $[R_y]$ replaced by $([A^*_n][R_y]$ (the concentration of the low affinity sites). Addition of eqns. (A-6) and (A-7) in this sum yields $[R_yA^*_n] + [R_yA^*_n] = [R_yA^*_n] + [R_yA^*_n]$, which is the time-dependent concentration of bound labeled antigen to both high and low affinity binding sites.

The kinetic data were fitted to the expression of this sum, employing a non-linear regression curve-fitting procedure (20). The values of the constants $k_{-2}$, $k_{1-1}$, $k_{2-1}$ and $k_{-3}$ (which are known from direct determination of the binding kinetics of the antigen alone) as well as the value of $\alpha$ (which is known from the analysis of agonist binding to the high affinity receptor sites for agonist without an immobilization step of the complex agonist-receptor) were introduced into eqns. (1), (2), (A-6) and (A-7), and the program computes the best fit values $\beta_2$, $\beta_3$, $\beta_4$, $\beta_5$, $\beta_6$ and $\beta_7$. 

\[
\beta_2, \beta_3, \beta_4, \beta_5, \beta_6, \beta_7
\]