Isomerization of the Muscarinic Receptor • Antagonist Complex*

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The mechanism of binding of two antagonists, 3-quinuclidinyl benzilate and N-methyl-4-piperidinyl benzilate, to the muscarinic receptor was studied. The pseudo-first order rate constant of association showed a hyperbolic dependence on the concentration of the antagonist(s) indicating that the interaction involves two equilibria. The first binding equilibrium is reached rapidly and is characterized by dissociation constants 2.7 ± 0.4 nM and 6.7 ± 2.5 nM in phosphate buffer (0.05 M, pH = 7.4) for 3-quinuclidinyl benzilate and N-methyl-4-piperidinyl benzilate, respectively. The first binding equilibrium is followed by a slower isomerization step of the receptor–antagonist complex. The equilibrium constants for the isomerization step of the complex for both ligands were about 0.15. The overall constant of binding obtained as the product of the above constants shows good agreement with the results of equilibrium binding studies.

Muscarinic acetylcholine receptors have been characterized in the central nervous system and in the peripheral by binding of radiolabeled antagonists of very high affinity (cf. for review Refs. 1 and 2). The most extensively used ligands include 3-quinuclidinyl benzilate (3), N-methyl-4-piperidinyl benzilate (4), and propylbenzilylcholine mustard (5). The first two benzilic esters interact reversibly with the receptor binding sites and the single binding isotherms obtained for this process may be ascribed to a simple bimolecular association reaction (1, 3):

\[ A + R \overset{k_{\text{as}}}{\underset{k_{\text{dis}}}\rightleftharpoons} AR \]  

(1)

where \( R \) stands for receptor, \( A \) for antagonist, \( k_{\text{as}} \) and \( k_{\text{dis}} \) the association and dissociation rate constants, respectively.

The few kinetic studies on the reversible binding of antagonist to muscarinic receptors, however, have indicated that the value of the dissociation constant of receptor–antagonist complex calculated from the equilibrium binding measurements for the reaction in Equation 1:

\[ K_d = \frac{[A][R]}{[AR]} \]  

(2)

does not agree with the equilibrium constant calculated as the ratio of the rate constants \( k_{\text{as}}/k_{\text{dis}} \) (6). In addition to this, deviations from the first order kinetics of the dissociation of the receptor–antagonist complex were found in several studies (7–9). Other experiments showed that formation of the complex under the pseudo-first order conditions ([A] >> [R]) also deviated from first order kinetics (8–11). Among several possible reasons for these findings, it has been suggested that muscarinic receptor interaction with antagonist(s) involves more than one reaction step with different rates of equilibration (8–11). The simplest reaction scheme for this case proposes the existence of two different receptor–antagonist complexes, \( AR \) and \( AR^* \):

\[ R + A \overset{k_1}{\underset{k_{-1}}\rightleftharpoons} AR \overset{k_2}{\underset{k_{-2}}\rightleftharpoons} AR^* \]  

(3)

Kloog and Sokolovsky (7), in addition to the above scheme, have also proposed isomerization of the free receptor according to Equation 4

\[ R \rightleftharpoons R^* \]  

(4)

and ligand binding to both isomers leading to a cyclic reaction scheme.

Although deviations from linearity of semilogarithmic plots implicated a mechanism involving isomerization of the receptor–antagonist complex, they did not prove its existence. The presence of such isomerization steps in protein-ligand interactions has been demonstrated in several systems by showing hyperbolic dependence of the pseudo-first order rate constant of association on the ligand concentration (12). In view of this, we decided to carry out an extensive study of binding of benzilic esters to the muscarinic receptor, since these compounds are widely used for investigation of the interaction of the receptor with other antagonists and agonists and, therefore, the establishment of the formal kinetic mechanism for these ligands is crucially important for interpretation of later data.

The present study, using two different receptor preparations and two different antagonists, provides kinetic evidence for the existence of two consecutive equilibria in antagonist binding to the muscarinic receptor and provides data on the corresponding equilibrium constants.

MATERIALS AND METHODS

3-[\( ^{3} \)H]Quinuclidinyl benzilate (28.4 Ci/mmol) was purchased from New England Nuclear Co., Boston, Mass. N-[\( ^{3} \)H]methyl-4-piperidinyl benzilate (55.4 Ci/mmol) was prepared and generously donated by Drs. Yoel Kloog and Mordechai Sokolovsky, Tel-Aviv University. All other chemicals were of reagent grade.

Smooth muscle membranes from small intestine of starved male Sprague-Dawley rats were prepared by inverting the intestine and scraping off the mucosa. The transparent membranes obtained were chopped in a tissue slicer prior to homogenization in phosphate buffer (0.1 M, \( pH = 7.4 \)) containing 2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride. The homogenates were further dispersed by sonication 2 × 10 s. The homogenate obtained was centrifuged at 100,000 g × 10 min and the supernatant was subjected to further centrifugation at 100,000 g × 60 min. The resulting pellet was washed twice with 0.1 M, \( pH = 7.4 \) buffer containing 2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride.

The homogenates were further dispersed by sonication 2 × 10 s. The homogenate obtained was centrifuged at 100,000 g × 10 min and the supernatant was subjected to further centrifugation at 100,000 g × 60 min. The resulting pellet was washed twice with 0.1 M, \( pH = 7.4 \) buffer containing 2 mM EDTA and 0.1 mM phenylmethylsulfonyl fluoride.

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remained constant for 4 h at 25°C. Membranes from cerebral cortex were prepared as described above except for slicing and sonicating the material.

Equilibrium binding studies were carried out by the filtration method of Yamamura and Snyder (3) in phosphate buffer (0.05 M, pH = 7.4) using 0.1 to 0.4 mg of protein/sample. Specific binding was determined as difference in binding of the labeled ligand in the absence and presence of atropine (1.0 μM) or oxotremorine (10.0 μM). The incubation was carried out at 25°C for 2 h in the case of 3-QNB 1 and for 1 h in the case of 4 NMPB. Filtration and washing (with 20 ml of ice cold buffer) was completed within 30 s.

Table 1 shows that both antagonists bound with high affinity to receptors from smooth muscle under the equilibrium binding conditions and that the number of receptor sites labeled with both ligands was identical. The binding constants ($K_d$) were calculated by fitting the data on specific binding versus free antagonist concentration to a single binding isotherm (1).

Kinetic studies were carried out with the same filtration assay. The incubation was started by rapidly mixing the antagonist into a receptor suspension of 50 ml. The quantity $B_t$, total bound ligand at time $t$, was determined as difference in binding of the labeled ligand with and without the cold ligand. The value of $B_t$ was thus the sum of the specific and nonspecific binding at equilibrium (cf. Equation 5), and $B_{nonsp}$, nonspecific binding, was calculated by fitting the association rate curves to Equation 5 by a nonlinear regression program. Independent estimates of these quantities were obtained in the equilibrium binding experiments. Nonspecific binding varied with antagonist concentration and was saturated within 1 min in the case of both ligands. The dissociation rate of the receptor-antagonist complex was studied by incubating the receptors with labeled ligand (5 to 7 nm) until saturation was reached. At this time, atropine (10.0 μM) was added and aliquots were taken for filtration. Fitting of experimental data was carried out on IBM 360/75 computer by using the nonlinear regression program (BMDP 3R, University of California, Los Angeles).

RESULTS

Kinetics of 3-QNB and 4-NMPB binding to muscarinic receptors were studied under the pseudo-first order conditions involving a 7- to 1000-fold excess of the antagonist over the receptor concentration. It was found that the nonspecific binding reached saturation before the first samples were filtered (50 to 60 s) and therefore the total change in radioligand binding reflected only the specific binding. The kinetic curves were fitted to the equation:

$$B_t = B_{nonsp} + B_{eq}(1 - e^{-k_{obs}t})$$

where $B_t$ is the concentration of bound radioligand at time $t$, $k_{obs}$ is the pseudo-first order rate constant, and the values of $B_{nonsp}$ and $B_{eq}$ characterize the specific and nonspecific binding at equilibrium, respectively. These values were calculated by the nonlinear least square method for each kinetic curve. This way, possible errors due to the underestimation of $B_{eq}$ for slow reactions and those due to the influence of spontaneous denaturation of receptor protein could be avoided. The values of $B_{nonsp}$ derived from kinetic experiments coincide within the error limits with those obtained in equilibrium binding experiments in the presence of atropine (1 μM). The results obtained are illustrated in a plot of

$$\ln \frac{B_{eq} + B_{nonsp} - B_t}{B_{eq}}$$

versus $t$ (Fig. 1, A and B) that corresponds to the semilogarithmic linear transformation of Equation 5. It is evident from Fig. 1, A and B, that the kinetic data may be described by a single exponential during the first half-life periods of the binding reaction of 3-QNB or 4-NMPB to membranes of smooth muscle. Similar results (not shown) were obtained with the

1 The abbreviations used are: 3-QNB, 3-[3H]quinuclidinyl benzilate; 4-NMPB, N-[3H]methyl-4-piperidinyl benzilate.

Table 1

| Antagonist | $K_d$ (nM) | Concentration of binding sites (mol/g protein) |
|------------|------------|---------------------------------------------|
| 3-QNB      | 0.23 ± 0.03| $(6.4 ± 0.2) \times 10^{-10}$               |
| 4-NMPB     | 1.04 ± 0.09| $(6.3 ± 0.2) \times 10^{-10}$               |

Antagonist binding to the muscarinic receptor from smooth muscle under equilibrium conditions

Membranes (0.1 mg/ml) from small intestine were incubated in phosphate buffer (0.05 M, pH = 7.4) with several concentrations of the labeled antagonists in the presence and absence of atropine (1 μM) at 25°C for 2 h, and subsequently vacuum-filtered on Whatman GF/B filters. The incubation volume was 2 ml and the filters were washed with 20 ml of ice cold buffer.

Table 2

| Antagonist | $K_d$ (nM) | Concentration of binding sites (mol/g protein) |
|------------|------------|---------------------------------------------|
| 3-QNB      | 0.23 ± 0.03| $(6.4 ± 0.2) \times 10^{-10}$               |
| 4-NMPB     | 1.04 ± 0.09| $(6.3 ± 0.2) \times 10^{-10}$               |

[1] The abbreviations used are: 3-QNB, 3-[3H]quinuclidinyl benzilate; 4-NMPB, N-[3H]methyl-4-piperidinyl benzilate.
Differences between results obtained in these buffers of the data with Equation 5 for determination of the values of \( B_{eq} \) and \( B_{nonsp} \). The slopes of the lines in the figure correspond to the values of \( k_{diss} \).

**DISCUSSION**

The kinetic study of muscarinic receptor interaction with 3-QNB and 4-NMPB over a wide range of antagonist concentrations allowed discrimination between the reaction mechanisms given by Equations 1 and 3. The existence of a mechanism involving two consecutive equilibria (Equation 3) has previously been suggested on the basis of deviations from linearity of semilogarithmic plots of association and dissociation rates of the receptor-antagonist complexes with both 3-QNB (10, 11) and 4-NMPB (7-9). These deviations could have several reasons and, in fact, could not be detected under our experimental conditions (using different receptor preparations and phosphate buffer instead of Krebs-Ringer buffer (7-11)). Differences between results obtained in these buffers were also noted by Kloog and Sokolovsky. Nevertheless, the existence of consecutive equilibria in antagonist binding could be demonstrated even in phosphate buffer by showing the hyperbolic dependence of the apparent first order rate constant of the association on the antagonist concentration (Fig. 2). This type of evidence for isomerization of protein-ligand complex has been discussed previously (12).

Our data concerning binding of both 3-QNB and 4-NMPB to the receptor can be explained by assuming a fast binding step followed by a slower isomerization of the receptor-antagonist complex. The isomerization step is slow enough to be characterized by the filtration method. In contrast, the formation and dissociation of the preceding complex (AR) was too fast to be analyzed by the filtration assay under the conditions used in the present study. In such a case, the system can be described by a single exponential (12, 13). A more detailed description of the first equilibrium calls for the special methods of fast reaction kinetics, like those successfully applied in studies on the nicotinic acetylcholine receptor (13). It is interesting to note that the rapid protein-ligand complex formation was followed by a slower isomerization step also in the case of this acetylcholine receptor. Thus, the relatively slow isomerization seems to be general for acetylcholine receptors and may be connected with some of their biological functions in the cell membrane. The isomerization of the receptor-ligand complex involves probably a conformational change of protein molecule. This conclusion is supported by the data on the remarkable influence of temperature on the values of \( k_{diss} \) for 3-QNB binding to receptors of cardiac membranes (14).
It should be noted that the isomerization step increases the apparent affinity of benzilate binding to muscarinic receptor as the values of $K_{isom}$ are about 0.15 for both 3-QNB and 4-NMPB. The equilibration of the isomerization reaction is reached faster in the case of 4-NMPB, a property that may make this ligand a more convenient and valuable tool in binding studies than is 3-QNB.

The isomerization equilibrium involves monomolecular steps and, therefore, can not be shifted completely by addition of an excess of antagonist. As a result, a portion of receptor present in the fast dissociating complex escapes detection in filtration assay independently of the antagonist concentration used. This is important to consider when measuring the number of receptor binding sites by these ligands. In the case of both 3-QNB and 4-NMPB, the ratio of $[AR]/[AR^*]$ appears to be about 1:7, which explains the close agreement between results obtained measuring the number of receptors with these ligands. In general, however, the ratio of $[AR]/[AR^*]$ may be dependent on the ligand structure as well as on the reaction conditions.

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REFERENCES
1. Birdsall, N. J. M., and Hulme, E. C. (1976) J. Neurochem. 27, 7-16
2. Heilbronn, E., and Bartfai, T. (1978) Prog. Neurobiol. 11, 171-188
3. Yamamura, H. I., and Snyder, S. H. (1974) Proc. Natl. Acad. Sci. U. S. A. 71, 1725-1729
4. Kloog, Y., and Sokolovsky, M. (1977) Brain Res. 134, 167-172
5. Burgen, A. S. V., Hiley, C. R., and Young, J. M. (1974) Br. J. Pharmacol. 50, 145-151
6. Yamamura, H. I., and Snyder, S. H. (1974) Mol. Pharmacol. 10, 861-867
7. Kloog, Y., and Sokolovsky, M. (1978) Brain Res. 144, 34-48
8. Kloog, Y., Egozi, Y., and Sokolovsky, M. (1979) FEBS Lett. 97, 265-268
9. Kloog, Y., Egozi, Y., and Sokolovsky, M. (1979) Mol. Pharmacol., in press
10. Galper, J. B., Klein, W., and Catterall, W. A. (1977) J. Biol. Chem. 252, 8692-8699
11. Galper, J. B., and Smith, T. W. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 5831-5835
12. Strickland, S., Palmer, G., and Massey, V. (1975) J. Biol. Chem. 250, 4049-4052
13. Gruhnagen, H.-H., Iwatsubo, M., and Changeux, J.-P. (1977) Eur. J. Biochem. 80, 225-242
14. Cavey, D., Vincent, J. P., and Lázaro, M. (1977) FEBS Lett. 84, 110-114
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