Evidence for a Tandem Two-site Model of Ligand Binding to Muscarinic Acetylcholine Receptors*

Received for publication, January 16, 2000, and in revised form, March 13, 2000
Published, JBC Papers in Press, April 3, 2000, DOI 10.1074/jbc.M000112200

Jan Jakubík‡, Esam E. El-Fakahany§, and Stanislav Tuček¶
From the ‡Institute of Physiology, Academy of Sciences of the Czech Republic, 14220 Prague, Czech Republic and the §University of Minnesota Medical School, Minneapolis, Minnesota 55455

After short preincubations with N-[3H]methylscopolamine ([3H]NMS) or R-([3H]quinuclidinyl benzilate ([3H]QNB), radioligand dissociation from muscarinic M1 receptors in Chinese hamster ovary cell membranes was fast, monoexponential, and independent of the concentration of unlabeled NMS or QNB added to reveal dissociation. After long preincubations, the dissociation was slow, not monoexponential, and inversely related to the concentration of the unlabeled ligand. Apparently, the unlabeled ligand becomes able to associate with the receptor simultaneously with the already bound radioligand if the preincubulation lasts for a long period, and to hinder radioligand dissociation. When the membranes were preincubated with [3H]NMS and then exposed to benzylcholine mustard (covalently binding specific site), [3H]NMS dissociation was blocked in wild-type receptors, but not in mutated (D99N) M1 receptors. Covalently binding [3H]propylbenzilylcholine mustard detected substantially more binding sites than [3H]NMS. The observations support a model in which the receptor binding domain has two tandemly arranged subsites for classical ligands, a peripheral one and a central one. Ligands bind to the peripheral subsite first (binding with lower affinity) and translocate to the central subsite (binding with higher affinity). The peripheral subsite of M1 receptors may include Asp-99. Experimental data on [3H]NMS and [3H]QNB association and dissociation perfectly agree with the predictions of the tandem two-site model.

The agonists and competitive antagonists of muscarinic acetylcholine receptors associate with a binding domain that is located within a cavity formed by the seven transmembrane segments of receptor molecules; the cavity opens into the extracellular space (1, 2). Most interactions between muscarinic receptors and their competitive antagonists can be explained in terms of simple bimolecular reactions, whereas the interactions between the receptors and the agonists are complicated by the associated receptor-G protein interactions (3–6). With regard to some antagonists, however, observations have been described that are difficult to explain in terms of simple single-step bimolecular reactions. Based on such observations, it has been proposed that the initial binding of the antagonist quinuclidinyl benzilate (QNB)1 is followed by a slow change in the conformation of the receptor molecule to a state that binds the ligand more avidly (receptor “isomerization”; Refs. 7–10) and that two “competitive” ligands may bind to the receptor simultaneously (10–12).

The present study of the complexity of the binding of muscarinic antagonists was started within the context of our investigations of allosteric modulations of muscarinic receptors (13–15). We wanted to know how the dissociation of muscarinic antagonists N-[3H]methylscopolamine ([3H]NMS) and [3H]QNB from the receptors is affected by changes in the concentration of the unlabeled ligand added to the system in order to reveal (“induce”) the dissociation, by the duration of the preceding radioligand association, and also by the treatment of receptors with a compound known to associate covalently with the classical binding site of muscarinic receptors (benzylcholine mustard (BCM); see “Experimental Procedures”) (16, 17). The data we obtained provide strong support for a model in which there are two tandemly arranged binding sites on each muscarinic receptor molecule, a “peripheral” one and a “central” one. In this model, classical ligands bind first to the former and then move to the latter site. The movement of the ligand from the peripheral to the central site may explain some of the phenomena which had been previously interpreted as consequences of receptor isomerization. The results and interpretations have been published as a symposium abstract (18).

EXPERIMENTAL PROCEDURES
Reagents—[3H]NMS and [propyl-3H]propylbenzilylcholine mustard ([3H]PBCM; N-propyl-N-chloroethyl-2-aminoethyl benzilate) were from NEN Life Science Products, and [3H]QNB was from Amersham Phamacia Biotech. Unlabeled QNB was from RBI (Nattick, MA), and NMS was from Sigma. BCM (N-methyl-N-chloroethyl-2-aminoethyl benzilate) was synthesized in the Institute of Experimental Medicine (St. Petersburg, Russia) and kindly provided by Dr. S. Shelkovnikov (Institute of Experimental Medicine, St. Petersburg, Russia).

Cells and Cell Membranes—Most experiments were performed on membranes of Chinese hamster ovary (CHO) cells stably transfected with the rat gene for muscarinic M1 receptors (rM1; membranes; Refs. 19 and 20) and on CHO cells stably transfected with the same gene in human embryonic kidney (HEK) cells (hM2; membranes; Refs. 19 and 20). Cells were grown in plastic dishes in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and 0.005% Genetin (14). They were harvested 7 days after subcul-

* This work was supported by grants from the Grant Agency of the Czech Republic (309/96/1287 and 309/99/014) and by NIH Fogarty International Collaboration Award (2-R03-TW00171). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
¶ To whom correspondence should be addressed: Inst. of Physiology AV CR, Vídeňská 1083, 14220 Prague, Czech Republic. Tel.: 420-2-4752620; Fax: 420-2-4752488; E-mail: tucek@biomed.cas.cz.

1 The abbreviations used are: QNB, R(-)-quinuclidinyl benzilate; BCM, benzylcholine mustard (N-methyl-N-chloroethyl-2-aminoethyl benzilate); CHO, Chinese hamster ovary; hM2 receptors, muscarinic receptors of the M2 subtype present in membranes of CHO cells stably expressing corresponding human gene; NMS, N-methylscopolamine; PBCM, propylbenzilylcholine mustard (N-propyl-N-chloroethyl-2-aminoethyl benzilate); rM1 receptors, muscarinic receptors of the M1 subtype present in membranes of CHO cells stably expressing corresponding rat gene.
turing, washed twice through centrifugation (3 min at 300 × g) and resuspension, and homogenized with an Ultra-Turrax homogenizer in a medium consisting of 136 mM NaCl, 5 mM KCl, 1 mM MgSO₄, 1 mM sodium phosphate buffer (pH 7.4), and 10 mM Na-HEPES buffer (pH 7.4). The homogenate was centrifuged twice for 10 min at 600 × g and combined supernatants were kept frozen at −20°C. On the day of experiment, membranes were sedimented by 15 min of centrifugation at 60,000 × g, and washed twice by resuspension and recentrifugation (see Ref. 15 for details).

Experiments with Covalently Associating Muscarinic Ligands BCM and [3H]PBCM—When BCM or PBCM are dissolved in water, their amine moieties give rise to aziridinium ions (the agents become “activated”), and this is followed by the formation of a covalent bond between the ligand and the receptor (17). BCM and [3H]PBCM were kept at −20°C in a stock solution in ethanol and, before being added to the incubation tubes, they were diluted with water and kept at room temperature for 60–90 min. Their interaction with receptors was stopped by adding 1 mM sodium thiosulfate. In experiments with radioligand association to BCM-pretreated membranes, the products of the reaction between BCM and sodium thiosulfate were removed by washing the membranes with centrifugation, but they remained in the incubation medium in experiments investigating the effect of BCM-pretreatment on radioligand dissociation. Details have been included in the description of individual types of experiments.

Radioligand Binding Experiments—Measurements of radioligand binding were performed essentially as described (15, 22, 23). Membranes corresponding to 600,000 cells were incubated at 25°C in a final incubation volume of 0.8 ml. The composition of the incubation medium corresponded to that of the homogenization medium (see above), with added ligands as indicated for individual experiments. Atropine (5 μM) was used to determine the nonspecific binding of [3H]NMS and [3H]QNB. The incubation was terminated by filtration through Whatman GF/C glass fiber filters in a Brandel cell harvester, and the radioactivity retained on the filters was measured by liquid scintillation spectrometry.

Several types of experiments were performed.

1) Experiments were designed to examine how [3H]NMS and [3H]QNB dissociation from their binding sites is affected by differences in the concentration of the unlabeled antagonist applied to induce dissociation. Membranes were preincubated with 100–1400 pM [3H]NMS or with 50 pM [3H]QNB for 60 min, after which unlabeled NMS or QNB were added at varying concentrations (1 μM to 1 mM) and the loss of bound radioactivity with time was followed.

2) Experiments were designed to examine how the dissociation of [3H]NMS and [3H]QNB from their binding sites is affected by shortening the preincubation with the radiolabeled antagonist. Membranes were preincubated with 100 pM [3H]NMS for just 2 min, or with 50 pM [3H]QNB for just 5 min, after which unlabeled NMS or QNB were added at three different concentrations and the time course of the loss of bound radioactivity was followed.

3) Experiments were designed to examine how the dissociation of [3H]NMS was affected by the irreversible ligand BCM (added after the membranes had been prelabelled with [3H]NMS). In these experiments, membranes were preincubated with a supersaturating (10 nM or 20 nM) concentration of [3H]NMS for 60 min, and then treated with a high concentration (100 nM or 10 μM) of BCM for 5 min. The action of BCM was stopped by adding 1 mM sodium thiosulfate, and the dissociation of [3H]NMS was started by adding 1 μM cold NMS (in experiments with the M₁ receptor subtype) or 5 μM cold atropine (in experiments with the M₂ receptor subtype).

4) Experiments were performed with [3H]NMS, [3H]QNB or [3H]PBCM association to their binding sites. Membranes were incubated with varying concentrations of [3H]NMS (50–400 pM), [3H]QNB (25–200 pM), or [3H]PBCM (20 mM), and the time course of radioligand binding was followed.

5) Experiments were designed to investigate how the association of [3H]PBCM to its binding sites is affected by mild pretreatment of membranes with BCM, and by the presence of unlabeled NMS, carbachol, alcuronium, or gallamine. In these experiments, membranes were preincubated with 5 nM BCM for 15 min, after which the action of BCM was stopped with 1 mM sodium thiosulfate and the membranes were washed in three cycles of centrifugation (15 min at 60,000 × g) and resuspending. The concentration of BCM applied was the lowest one with which it was still possible to achieve (during 15-min treatment) full blockade of the binding of 20 nM [3H]NMS (in experiments with the M₁ receptor subtype) or 5 nM [3H]NMS (in experiments with the M₂ receptor subtype) during a 2-h incubation.

Radioligand Binding Properties of rM₁ and rM₁-D99N Receptors—

![Figure 1: [3H]NMS dissociation from rM₁ receptors.](http://www.jbc.org/)

Mutant rM₁-D99N receptors differed from the wild-type rM₁ receptors in their lower affinity for classical muscarinic antagonists [3H]NMS and [3H]QNB, in accordance with earlier findings (19, 20). The Kᵣ values for the binding of [3H]NMS were 118 and 464 pM for the rM₁ and rM₁-D99N cells, respectively, and the corresponding Kᵣ values for the binding of [3H]QNB were 43.7 and 88.9 pM, respectively. Bₘ values expressed in fmol/10⁵ cells were 7.8 and 8.1 for the binding of [3H]NMS and 9.4 and 10.7 for the binding of [3H]QNB to membranes from the rM₁ and rM₁-D99N cells, respectively.

Treatment of Data—Our observations led us to conclude that there are two binding sites for “classical” antagonists and agonists on each muscarinic receptor: a peripheral one to which the ligands bind first, and a central one to which they move subsequently. Corresponding “two-site” model is proposed under “Discussion,” together with a set of differential equations defining its kinetics. With the use of the KINSIM (24) and FITSIM (25) program for chemical kinetics, these equations have been collectively fitted to data from experiments shown in Figs. 1–7, and the best fit kinetic constants obtained have been summarized in Table II. Individual data points in Figs. 1–7 are means of three experiments with incubations performed in triplicate. The curves in these figures have been drawn by computer using the proposed model and the best fit kinetic constants from Table II.

**RESULTS**

Dissociation of [3H]NMS and [3H]QNB from M₁ Muscarinic Receptors in the Presence of Different Concentrations of Unlabeled NMS or QNB—rM₁ membranes were preincubated with 100 pM [3H]NMS (Fig. 1A) or 400 pM [3H]QNB (Fig. 1B) for 60 min, after with 50 pM [3H]NMS for 90 min (Fig. 3A), and dissociation of the radioligand was then induced by adding different concentrations of unlabeled NMS (Fig. 1) or QNB (Fig. 3). It can be seen from Fig. 1 (A and B) that the rate of the dissociation of [3H]NMS from rM₁ membranes was fastest after the addition of 1 μM NMS, and slowest after the addition of 1 mM NMS. As shown in Fig. 1A, 62% of originally bound [3H]NMS dissociated...
during 10 min after the addition of 1 μM NMS, but only 35% dissociated after the addition of 1 μM NMS (see Table I for numerical values at selected data points). A similar relationship between the rate of dissociation of [3H]QNB from rM1 membranes and the concentration of unlabeled QNB was observed (Fig. 3A). Sixty min after the start of dissociation, 38% and 16% of originally bound [3H]QNB dissociated in the presence of 1 μM and 1 mM QNB, respectively. It is evident that the rate of dissociation of labeled ligands is inversely related to the concentration of the unlabeled ligands applied to reveal dissociation.

**Stippled curves in the left parts of Figs. 1 and 3 have been computed as best fits for an assumed monoeponential time course of dissociation. Solid lines correspond to the two-site model described under “Discussion” and to the kinetic constants summarized in Table II. It is apparent that the fit between individual data points and the computed curves is much better for the two-site model. This is confirmed by the plots of residuals shown in the right halves of Figs. 1 and 3.**

**Dissociation of [3H]NMS and [3H]QNB from Membranes Containing Muscarinic M1 Receptors with D99N Mutation—** In experiments shown in Fig. 2A, 66% of originally bound [3H]NMS dissociated from the rM1-D99N membranes 10 min after the start of dissociation in the presence of 1 μM NMS and 49% in the presence of 1 mM NMS. This compares to 62% and 35%, respectively, in the case of membranes with wild-type rM1 receptors (Fig. 1A). In experiments with [3H]QNB, 83% of the originally bound radioligand dissociated from rM1-D99N membranes 60 min after the addition of 1 μM QNB and 75% after the addition of 1 mM QNB (Fig. 3B); these values contrast with those observed in the case of membranes with wild-type rM1 receptors (38% and 16%, respectively, Fig. 3A). Apparently, the deceleration of dissociation produced by high concentrations of unlabeled ligands (NMS or QNB) is smaller at mutated D99N receptors than at receptors of the wild type. In addition, the D99N mutation had no effect on the rate of dissociation of [3H]QNB (Fig. 5), although not as much as in experiments with long preincubations. The D99N mutation had no effect on the rate of dissociation of [3H]NMS (Fig. 4) but accelerated the dissociation of [3H]QNB (Fig. 5), although not as much as in experiments with long preincubations.

**Association of [3H]NMS and [3H]QNB with rM1 and rM1-D99N Receptors—** As shown in Fig. 6, there was little difference between the time courses of [3H]NMS association to rM1 and rM1-D99N receptors as predicted by the one-site model and the two-site model, and as actually observed. On the other hand, the time course of [3H]QNB association to the rM1 receptors (Fig. 7A) was clearly different for the one-site and the two-site models, and the actual observations corresponded to what had been predicted by the two-site model. This is best seen from the plots of residuals in the right half of Fig. 7A.

**Association of [3H]PBCM with rM1 and rM1-D99N Receptors—** [3H]PBCM is known to covalently associate with the classical binding sites of muscarinic receptors with high specificity and in an atropine-sensitive manner (19, 26, 27). Fig. 6A reveals a conspicuous difference between the time courses of [3H]PBCM association to the membranes of cells expressing the wild-type and the mutant receptors. While the association of [3H]PBCM to mutant rM1-D99N receptors reached a maximum after about 45 min, the association to wild-type rM1 receptors continued during subsequent 2 h of observation and reached a nearly 2-fold value in comparison with the mutant, although the density of the [3H]PBCM binding sites (as determined in experiments with [3H]NMS saturation binding) was virtually the same in the two types of membranes (see legend to Fig. 8).

Pretreatment of cells with BCM under conditions that were just sufficient to completely prevent the binding of [3H]NMS diminished but did not prevent subsequent binding of [3H]PBCM (Fig. 8B). The rate of [3H]PBCM association to receptors mildly pretreated with BCM was substantially diminished (but

| Receptors | Preincubation with | Dissociation induced by | Duration of dissociation | Dissociated (mean ± S.E.) |
|-----------|-------------------|------------------------|-------------------------|--------------------------|
| rM1       | [3H]NMS (100 pm, 60 min) | 1 μM NMS | 10 min | 61.8 ± 0.4 |
|           |                   | 0.1 mM NMS | 10 min | 54.5 ± 0.3* |
|           |                   | 1 μM NMS | 10 min | 33.9 ± 0.4* |
|           |                   | 1 mM NMS | 10 min | 64.4 ± 0.3 |
|           |                   | 0.1 mM NMS | 10 min | 57.6 ± 0.5* |
|           |                   | 1 mM NMS | 10 min | 37.5 ± 0.03* |
| rM1-D99N  | [3H]NMS (350 pm, 60 min) | 1 μM NMS | 10 min | 66.7 ± 0.4 |
|           |                   | 0.1 mM NMS | 10 min | 62.1 ± 0.9* |
|           |                   | 1 μM NMS | 10 min | 48.9 ± 0.3* |
|           |                   | 1 mM NMS | 10 min | 69.4 ± 0.9 |
|           |                   | 0.1 mM NMS | 10 min | 65.0 ± 1.4 |
|           |                   | 1 mM NMS | 10 min | 53.5 ± 1.1* |
|           |                   | 1 μM QNB | 60 min | 40.1 ± 2.1 |
|           |                   | 1 mM QNB | 60 min | 28.5 ± 1.2* |
| rM1       | [3H]QNB (50 pm, 90 min) | 1 μM QNB | 60 min | 17.6 ± 1.3* |
|           |                   | 1 mM QNB | 60 min | 82.5 ± 0.5 |
|           |                   | 0.1 mM QNB | 60 min | 79.6 ± 1.1 |
| rM1-D99N  | [3H]QNB (50 pm, 90 min) | 1 μM QNB | 60 min | 74.1 ± 0.7* |

Selected data points from experiments with the dissociation of [3H]NMS and [3H]QNB from rM1 and rM1-D99N receptors

**TABLE I**

Data have been taken from experiments shown in Figs. 1–3. *, significantly different (p < 0.01) from both other means in the same group (analysis of variance and Bonferroni’s test).
Table II

\[
\begin{array}{|c|c|c|}
\hline
\text{Model of Dissociation} & \text{Graphs} & \text{Dotted lines} \\
\hline
\text{Tandem Two-site Arrangement of Muscarinic Binding Domains} & \text{Dotted lines} & \text{by computer as best fits for radioligand dissociation from a single-site model, whereas \textit{solid lines} describe dissociation from the tandem two-site model.} \\
\text{Graphs in the middle column are plots of residuals for the single-site model, and those in the right-hand column are plots of residuals in the tandem two-site model. Data are means of three experiments with incubations performed in triplicate.} \\
\hline
\text{not blocked) by 1 mM NMS or 1 mM carbachol, and virtually stopped by 1 mM alcuronium (an allosteric modulator of muscarinic receptors).} \\
\hline
\text{Association of} [3H]PBCM \text{ with} hM2 \text{ Receptors—Membranes of cells expressing} hM2 \text{ receptors were incubated for 2 h with suprasaturating concentrations of} [3H]NMS (5 nM) or [3H]PBCM (20 nM), and the binding of radiolabel was measured (Fig. 9, \textit{left two columns}). It was more than twice as high after incubations with [3H]PBCM than with [3H]NMS, suggesting that one molecule of the receptor associates with more than one molecule of [3H]PBCM. In subsequent experiments (Fig. 9, \textit{right part}), membranes were preincubated with 5 mM BCM for 15 min, after which BCM was reacted with sodium thiosulfate and the membranes were washed and incubated with [3H]NMS, or with [3H]PBCM and several orthosteric or allosteric ligands. While the binding of [3H]NMS was completely blocked by BCM pretreatment, [3H]PBCM still bound to membranes, and its binding was lower by approximately half than that in control membranes. This “extra” binding of [3H]PBCM was diminished by 100 \mu M NMS, 3 mM carbachol, 100 \mu M alcuronium, or 100 \mu M gallamine.} \\
\hline
\text{Deceleration of [3H]NMS Dissociation from rM1 and hM2 Receptors by BCM—In experiments shown in Fig. 10, rM1 membranes (Fig. 10A), rM1-D99N membranes (Fig. 10B), and hM2 membranes (Fig. 10C) were preincubated with [3H]NMS for 60 min, and the dissociation of [3H]NMS was started by the addition of unlabeled NMS or atropine at time 0. Portions of the membranes were exposed to BCM for 5 min in the end of the preincubation with [3H]NMS, before the addition of the unlabeled ligand. The exposure to BCM brought about a decrease (by close to 40%) of the initial [3H]NMS labeling and at the same time prevented further dissociation of [3H]NMS in the...} \\
\end{array}
\]
Tandem Two-site Arrangement of Muscarinic Binding Domains

Main Findings and Their Interpretation—Five observations appear to be most important conceptually.

1) After 60 or 90 min of preincubation, the rate of [3H]NMS or [3H]QNB dissociation from receptors was slowed down by increases in the concentration of unlabeled NMS or QNB, applied to reveal dissociation. A similar phenomenon has already been noted in work with atropine and other competitive antagonists (28–30), without a definite explanation. It seems apparent that unlabeled NMS or QNB bind to receptors simultaneously with [3H]NMS or [3H]QNB (thus creating ternary NMS-receptor-[3H]NMS or QNB-receptor-[3H]QNB complexes and slowing down dissociation), and yet that they prevent [3H]NMS and [3H]QNB reassociation.

2) If the prelabeling of the receptors had been shortened from 60 or 90 min to just 2 or 5 min, the dissociation of [3H]NMS or [3H]QNB was much faster and was not slowed down by higher concentrations of unlabeled NMS or QNB. It seems apparent that, during the short preincubation, the labeled ligand binds to a low affinity site (hence the fast dissociation rate after a short preincubation), while it translocates to a high affinity site. During the procedure of filtration and washing, the radioligand is more easily lost from the peripheral site (unless a covalent bond is formed, as in the case of BCM and PBCM), it becomes strong after the antagonist association is weak as long as the antagonist is attached to the peripheral site (unless a covalent bond is formed, as in the case of BCM and PBCM), it becomes strong after the antagonist association is weak as long as the antagonist is attached to the peripheral site.

3) The time course of [3H]NMS and [3H]QNB dissociation was monoexponential after short, but not after long, preincubation.

4) Covalently associating [3H]PBCM detected a higher number of binding sites than the loosely associating [3H]NMS.

5) BCM blocked [3H]NMS dissociation from prelabeled receptors.

All of these observations are easily explained on the generalizing assumption that there are two tandemly arranged binding sites for classical antagonists on each receptor molecule, a peripheral and a central one, and that the antagonists first bind to the peripheral site, from which they subsequently translocate to the central site. While the receptor-antagonist association is weak as long as the antagonist is attached to the peripheral site (unless a covalent bond is formed, as in the case of BCM and PBCM), it becomes strong after the antagonist moves to the central site. During the procedure of filtration and washing, the radioligand is more easily lost from the peripheral sites (i.e. a proportion of the non-covalent binding to them remains undetected), while the loss from the central sites is smaller. Moreover, if the peripheral sites become occupied by high concentrations of the unlabeled antagonist (added to the medium to induce dissociation of the radioligand), the rate at which the radioligand is able to leave the central sites is further slowed down.

Tandem Two-site Model—A system with the features just outlined has been schematically depicted in Fig. 11. In this...
because they completely overlap with those shown here. Ordinate of \([3H]QNB\) during preincubation was 50 pM. \([3H]QNB\) dissociation receptors after preincubation lasting only 5 min. The concentration of \([3H]QNB\) during preincubation was 50 pM. \([3H]QNB\) dissociation was induced by the addition of 1 mM QNB. Data obtained after the addition of 0.1 or 1 mM QNB have not been included because they completely overlap with those shown here. Ordinate, percentage of binding before the start of dissociation. Data are means of three experiments with incubations performed in triplicate.

system, antagonist (A) binding may be described by the following set of differential equations:

\[
d[A] = -k_{1.1} \times [A][R] + k_{-1.1} \times [AR] - k_{-1.3} \times [A][RA] + k_{-1.3} \times [ARA] \quad (\text{Eq. 1})
\]

\[
d[R] = -k_{1.1} \times [A][R] + k_{1.1} \times [AR] \quad (\text{Eq. 2})
\]

\[
d[AR] = k_{1.2} \times [A][R] - k_{-1.2} \times [RA] - k_{-1.3} \times [A][RA] + k_{-1.3} \times [ARA] \quad (\text{Eq. 3})
\]

\[
d[RA] = k_{1.3} \times [A][RA] - k_{-1.3} \times [AR] - k_{-3} \times [ARA] \quad (\text{Eq. 4})
\]

\[
d[ARA] = k_{2} \times [AR][A] - k_{1} \times [RA][A] \quad (\text{Eq. 5})
\]

The total amount of bound antagonist (Y) corresponds to the sum of [AR] + [RA] + 2 \times [ARA]. Consequently,

\[
dY = k_{1.1} \times [A][R] - k_{-1.1} \times [AR] + k_{1.3} \times [A][RA] - k_{3} \times [ARA] \quad (\text{Eq. 6})
\]

We used the KINSIM and FITSIM program and fitted these differential equations to data obtained in experiments with \([3H]NMS\) and \([3H]QNB\) association to and dissociation from rM1 and rM1-D99N binding sites. In view of the complexity of the system, it was not possible to obtain separate values for \(k_{1.3}\) and \(k_{-3}\) (describing the association and dissociation of
Tandem Two-site Arrangement of Muscarinic Binding Domains

\[ [3H]NMS \text{ and } [3H]QNB \text{ dissociation from the } D99N \text{ mutant are also followed in the right part of Fig. 8.} \]

\[ \text{FIG. 8. } [3H]PBCM \text{ association to membranes containing } M_1 \text{ receptors.} \]

\[ \text{A. comparison of } [3H]PBCM \text{ to } rM_1 \text{ receptors under control conditions (C), or after mild pretreatment of receptors with BCM (abolishing the binding of NMS).} \]

\[ \text{B. association of } [3H]PBCM \text{ to } rM_1 \text{ receptors was 7.8 fmol/10^6 cells, and that for the binding of } [3H]NMS \text{ to } rM_1 \text{ receptors was 8.1 fmol/10^6 cells.} \]

\[ [3H]NMS \text{ or } [3H]QNB \text{ to and from the peripheral site of a receptor whose central site is already occupied by } [3H]NMS \text{ or } [3H]QNB, \text{ respectively). Instead, we could only obtain best fit values of the } k_{-3}/k_{-2} \text{ ratio, which we call } K_{II} \text{ in Table I and which describes the affinity for } [3H]NMS \text{ or } [3H]QNB \text{ of the peripheral site of a receptor whose central site had already been occupied by the same ligand.} \]

\[ \text{The presence of the ligand at the central site had no effect on the affinity of the peripheral site for the same ligand, } K_{II} \text{ should be equal to } K_c. \]

\[ \text{The } K_{II} \text{ values were consistently higher than the } K_c \text{ values, however, which indicates that the binding of } [3H]NMS \text{ or } [3H]QNB \text{ to the central site diminishes the binding of the same ligand to the peripheral site (a negative cooperative effect). The extent of negative cooperativity has been expressed as factor } \alpha (31), \text{ taking that } \alpha = K_{III}/K_c. \]

\[ \text{We speculate that it depends on direct (steric and coulombic) interaction between the two ligand molecules.} \]

\[ \text{The best-fit values of individual parameters have been listed in Table II. Values designed as } K_c \text{ correspond to the product of } K_1 \times K_{II} \text{ and would represent the affinity of the receptor for } [3H]NMS \text{ if no formation of ternary complexes (receptor plus two molecules of NMS bound simultaneously) occurred.} \]

\[ \text{Figs. 1–3 demonstrate excellent agreement between experimental data and curves for } [3H]NMS \text{ and } [3H]QNB \text{ dissociation computed according to these values. After short preincubations (Figs. 4 and 5), the dissociation of } [3H]NMS \text{ and } [3H]QNB \text{ (presumably occurring from the peripheral sites) was fast and obeyed the } k_{off} \text{ values computed for the respective sites of the tandem two-site model.} \]

\[ \text{While the difference between curves for } [3H]NMS \text{ association according to the single-site and the tandem two-site model was small (Fig. 6), it is apparent from Fig. 7A that the correspondence between actual data concerning the association of } [3H]QNB \text{ and theoretical prediction was much better for the tandem two-site model than for the single-site one. The tandem two-site model offers good explanation of why BCM post-treatment stops } [3H]NMS \text{ dissociation (Fig. 10). It also enables to explain the difference between the extent of receptor labeling with } [3H]NMS \text{ and } [3H]PBCM \text{ (Figs. 8 and 9).} \]

\[ \text{It is generally believed that the aspartate residue in position 105 (Asp-105; within the third transmembrane segment) of muscarinic } M_1 \text{ receptors (and in homologous positions of the other muscarinic receptor subtypes) plays a key role in the binding of positively charged orthosteric muscarinic ligands to receptors (32–36). In all muscarinic receptor subtypes, there is another Asp present at the extracellular end of the third transmembrane domain (position 99 in the } M_4 \text{ receptor subtype; see Ref. 37), with a less well defined functional role. The observation that BCM virtually lost its ability to prevent } [3H]NMS \text{ dissociation from } rM_1 \text{ receptors with D99N mutation suggests that Asp-99 may be a part of the peripheral domain of the supposed tandem binding site. The finding that the rates of } [3H]NMS \text{ and } [3H]QNB \text{ dissociation from the D99N mutant receptors are less dependent on the concentration of the unlabeled ("excess") ligand than those from the wild-type receptors supports such view. It has been noted that Asp-99 plays a role} \]
in the interactions between the allosteric and orthosteric binding sites (20), but the question of topographical relations between the binding site for allosteric modulators and the peripheral part of the tandem orthosteric site requires special investigation.

Biphasic time courses of [3H]PBCM binding in Fig. 8 (A and B) raise the question of what determines the rate at which the irreversible ligand associates with the supposed peripheral and central parts of the orthosteric tandem site. We speculate that the association is rapid if it occurs to an empty receptor, or if it occurs at the peripheral site of a receptor the central site of which had already (“beforehand”) been occupied. In Fig. 8A, [3H]PBCM binds rapidly but non-covalently to the peripheral site and is quickly translocated to the central site where it stays long enough to make covalent bond. The association of the second molecule of [3H]PBCM with the peripheral site is slowed down. In the D99N mutant receptor, there is little binding at the peripheral site and the fast association concerns the central site. In Fig. 8B, BCM entered empty receptors during the preincubation and associated covalently with their central sites. Subsequent exposure to [3H]PBCM brought about fast covalent association of the radioligand with the peripheral sites. It may be noted that 1 mM NMS was not very efficient in preventing the binding of [3H]PBCM in the experiments shown in Fig. 8B; this may reflect the fact that the affinity of the peripheral sites for NMS is low in receptors in which the central sites had already been occupied by another ligand.

The tandem two-site model assumes that the ligand moves with regard to the receptor after the first association occurred. In a different context, a similar view has already been proposed by Saunders and Freedman (38), who suggested that muscarinic agonists first contact Asp-105 of the M1 receptors and then translocate to Asp-71 (in the middle of the second transmembrane segment). Acetylcholine is believed to undergo post-binding translocation between the peripheral and the central binding sites of acetylcholinesterase (39). The tandem two-site model provides explanation for most of the phenomena that had been interpreted in terms of receptor “isomerization” (see Introduction); it is indeed likely that ligand translocation is accompanied by some change in receptor conformation.

In an earlier study, Kurtenbach et al. (32) tried to distinguish whether [3H]PBCM associates with Asp-99 or Asp-105 of the M1 receptors and concluded that the association is mainly with Asp-105, but they (see also Ref. 40 by Curtis et al.) could not exclude that some association with Asp-99 does occur. On the other hand, Fraser et al. (19) observed that the binding of [3H]PBCM to M1 receptors was strongly diminished after Asp-99 had been mutated to Asn, perhaps reflecting the loss of the peripheral binding site, which we assume to be responsible for the decrease of [3H]PBCM binding observed in the present work (Fig. 8).

The tandem two-site model agrees with earlier kinetic observations indicating that the initial complex formed during [3H]NMS association with the receptor has a low affinity, and that the affinity with which the ligand is bound increases with time (41). An interesting aspect of the tandem two-site arrangement is that it may explain some of the differences in the results of measurements of the numbers of muscarinic binding sites encountered in literature. In particular, high numbers of muscarinic binding sites have been discovered frequently when [3H]QNB rather than [3H]NMS was applied for receptor labeling (42–44). Differences in results seem to depend on differences in the speed with which the ligand-receptor association becomes stabilized by ligand translocation from the peripheral to the central binding domain, and in the ease with which double occupancy of receptors by different ligands occurs.
Some of our observations could perhaps be interpreted on an alternative assumption, namely that the receptors work as dimers or oligomers (44, 45) with asymmetric properties of their binding sites, and that dimerization is impaired in D99N receptors. It would be difficult to explain, however, why the dissociation of [3H]NMS and [3H]QNB is fast after short pre-incubations and slow after long preincubations, and why the dissociation of [3H]NMS is blocked by an after-treatment with BCM. In conclusion, the assumption that the binding of muscarinic ligands to the classical binding site of the M₁ and M₂ muscarinic receptor subtypes is a dynamic process involving ligand translocation between two tandemly arranged binding domains provides excellent explanation of several phenomena that are difficult to interpret otherwise, and deserves further investigation.

Acknowledgments—We thank Dr. Lucie Bačáková for help with cell cultures and Dana Ungerová for unfailing technical assistance.

REFERENCES
1. Hulme, E. C., Birdsall, N. J. M., and Buckley, N. J. (1990) Annu. Rev. Pharmacol. Toxicol. 30, 633–673
2. Fraser, C. M., Lee, N. H., Pellegrino, S. M., and Kerlavage, A. R. (1994) Prog. Nucleic Acids Res. 49, 113–156
3. Caulfield, M. P. (1993) Pharmacol. Ther. 58, 319–379
4. Kenakin, T. (1997) Trends Pharmacol. Sci. 18, 456–464
5. Kurtenbach, E., Curtis, C. A. M., Pedder, E. K., Aitken, A., Harris, A. C. M., and Hulme, E. C. (1995) in Molecular Mechanisms of Muscarinic Acetylcholine Receptor Function (Wess, J., ed) pp. 19–32, Springer-Verlag, Heidelberg
6. Lu, Z.-L., and Hulme, E. C. (1999) J. Biol. Chem. 274, 7309–7315
7. Bonner, T. I. (1989) Trends Neurosci. 12, 148–151
8. Saunders, J., and Freedman, S. B. (1989) Trends Pharmacol. Sci., Suppl., 70–75
9. Curtis, C. A. M., Jones, P. G., and Hulme, E. C. (1993) Progr. Neurobiol. 41, 31–91
10. Curtis, C. A. M., Wheatley, M., Bansal, S., Birdsall, N. J. M., Eveleigh, P., Pedder, E. K., Poyner, D., and Hulme, E. C. (1989) J. Biol. Chem. 264, 489–495
11. Jakubík, J., and Tucek, S. (1998) Mol. Pharmacol. 53, 525–533
12. Jakubík, J., Bacáková, L., Liša, V., El-Fakahany, E. E., and Tucek, S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 8705–8709
13. Jakubík, J., Bačáková, L., El-Fakahany, E. E., and Tucek, S. (1997) Mol. Pharmacol. 52, 172–179
14. Gill, E. W., and Rang, H. P. (1966) Mol. Pharmacol. 2, 284–297
15. J. Pharmacol. Exp. Ther. 233, 707–714
16. El-Fakahany, E. E., and Lai, W. S. (1986) J. Pharmacol. Exp. Ther. 238, 554–563
17. Barshop, B. A., Wrenn, F. F., and Frieden, C. (1983) Trends Pharmacol. Sci. 13, 134–145
18. Jakubík, J., Bacáková, L., and El-Fakahany, E. E. (1997) Life Sci. 60, 1174
19. Fraser, C. M., Lampert, M., Robinson, D. A., Gocayne, J. D., and Venier, J. C. (1989) Mol. Pharmacol. 36, 840–847
20. Lee, N. H., Hu, J., and El-Fakahany, E. E. (1992) J. Pharmacol. Exp. Ther. 262, 312–316
21. Buček, P., Bonner, T. I., Buckley, C. M., and Bran, M. R. (1989) Mol. Pharmacol. 35, 469–476
22. Jakubík, J., Bačáková, L., El-Fakahany, E. E., and Tucek, S. (1995) J. Pharmacol. Exp. Ther. 274, 1077–1083
23. Jakubík, J., and Tucek, S. (1984) Br. J. Pharmacol. 113, 1529–1537
24. Barshop, B. A., Wrenn, F. F., and Frieden, C. (1983) Anal. Biochem. 130, 134–145
25. Zimmerle, C. T., and Frieden, C. (1989) Biochem. J. 258, 381–387
26. Birdsall, N. J. M., Burgen, A. S. V., and Hulme, E. C. (1979) Br. J. Pharmacol. 66, 337–342
27. Haga, K., and Haga, T. (1985) J. Biol. Chem. 260, 7927–7935
28. Birdsall, N. J. M., Hulme, E. C., Kromer, W., and Stockton, J. M. (1987) Fed. Proc. 46, 2525–2535
29. Waibelroek, M., Camus, J., Tastenoy, M., and Christophe, J. (1992) Br. J. Pharmacol. 105, 97–102
30. Waibelroek, M. (1994) Mol. Pharmacol. 46, 685–692
31. Ehlert, F. J. (1988) Mol. Pharmacol. 33, 187–194
32. Kurtenbach, E., Curtis, C. A. M., Pedder, E. K., Aitken, A., Harris, A. C. M., and Hulme, E. C. (1990) J. Biol. Chem. 265, 13702–13708
33. Spalding, T. A., Birdsall, N. J. M., Curtis, C. A. M., and Hulme, E. C. (1994) J. Biol. Chem. 269, 4992–4997
34. Page, K. M., Curtis, C. A. M., Jones, P. G., and Hulme, E. C. (1995) Eur. J. Pharmacol. 289, 429–437
35. Nordvall, G., and Hacksell, U. (1995) in Molecular Mechanisms of Muscarinic Acetylcholine Receptor Function (Wess, J., ed) pp. 19–32, Springer-Verlag, Heidelberg
36. Lu, Z.-L., and Hulme, E. C. (1999) J. Biol. Chem. 274, 7309–7315
37. Curtis, C. A. M., Jones, P. G., and Hulme, E. C. (1993) Progr. Neurobiol. 41, 31–91
38. Curtis, C. A. M., Wheatley, M., Bansal, S., Birdsall, N. J. M., Eveleigh, P., Pedder, E. K., Poyner, D., and Hulme, E. C. (1989) J. Biol. Chem. 264, 489–495
39. Tucek, S., Jakubík, J., Bačáková, L., and El-Fakahany, E. E. (1997) Life Sci. 60, 1174
40. In conclusion, the assumption that the binding of muscarinic ligands to the classical binding site of the M₁ and M₂ muscarinic receptor subtypes is a dynamic process involving ligand translocation between two tandemly arranged binding domains provides excellent explanation of several phenomena that are difficult to interpret otherwise, and deserves further investigation.
Evidence for a Tandem Two-site Model of Ligand Binding to Muscarinic Acetylcholine Receptors
Jan Jakubi?k, Esam E. El-Fakahany and Stanislav Tuc?ek

J. Biol. Chem. 2000, 275:18836-18844.
doi: 10.1074/jbc.M000112200 originally published online April 3, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M000112200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 44 references, 20 of which can be accessed free at http://www.jbc.org/content/275/25/18836.full.html#ref-list-1
Additions and Corrections

Vol. 275 (2000) 18836–18844

Evidence for a tandem two-site model of ligand binding to muscarinic acetylcholine receptors.

Jan Jakubík, Esam E. El-Fakahany, and Stanislav Tuček

Page 18839, Table II: During reanalysis of the data, we found a mistake in the implementation of KIN SIM package. After correction, we obtained different estimates of $k_{-2}^{\text{H}1}$ and $k_{-1}^{\text{H}1}$ constants and all dependent values ($K_{II}$, $K_{III}$, $K_{IV}$, and $K_d$) for QNB. The major difference is that we are not able to obtain $k_{-2}^{\text{H}1}$ and $k_{-1}^{\text{H}1}$ and consequently calculate dependent values for NMS with reasonable accuracy.

Page 18843, Fig. 10C: The change in this figure is in the curve of BCM-treated samples (squares), which is similar to the published version but the wrong set.

These changes do not affect the interpretation of the data nor the conclusions of the paper.

We suggest that subscribers photocopy these corrections and insert the photocopies at the appropriate places where the article to be corrected originally appeared. Authors are urged to introduce these corrections into any reprints they distribute. Secondary (abstract) services are urged to carry notice of these corrections as prominently as they carried the original abstracts.
Polyoma enhancer activator 3, an Ets transcription factor, mediates the induction of cyclooxygenase-2 by nitric oxide in colorectal cancer cells.

Yongmin Liu, Gregory L. Borchert, and James M. Phang

Page 18697, Fig. 3B: The labeling of the bars was incomplete. The complete figure is shown below.

Vol. 279 (2004) 37231–37234

Binding of xanthine oxidase to glycosaminoglycans limits inhibition by oxypurinol.

Eric E. Kelley, Andrés Trostchansky, Homero Rubbo, Bruce A. Freeman, Rafael Radi, and Margaret M. Turpey

Page 37233, Fig. 2 legend: The equation in the last sentence should read as follows:

\[(k'_{\text{inact}} = k_{\text{inact}} - K_D(k_{\text{inact}}/K_{\text{oxypurinol}})).\]