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INFLUENCE OF TEMPERATURE ON COOPERATIVE BINDING OF N-METHYLPIPERIDINYLBENZILATE WITH MUSCARINIC RECEPTOR FROM RAT CEREBRAL CORTEX

(*Presented by O. Eisen*)

Introduction

Several benzilic esters are potent muscarinic antagonists and their reversible binding to the receptor can be described by a single binding isotherm [1]

$$B_{\text{bound}} = \frac{B_{\max}[L]}{K_d + [L]}. \quad (1)$$

This equation corresponds either to a simple equilibrium binding scheme

$$R + L \overset{K_d}{\rightleftharpoons} RL \quad (2)$$

or to a more complicated scheme which involves reversible "isomerization» step of the receptor-ligand complex [2]

$$R + L \overset{K_A}{\rightleftharpoons} RL \underset{k_{-i}}{\overset{k_i}{\rightleftharpoons}} (RL). \quad (3)$$

In the latter scheme the apparent equilibrium constant K_d can be calculated as follows: $K_d = K_A \cdot K_{\text{isom}} = K_A \frac{k_{-i}}{k_i}$ and the second equilibrium is assumed to be slow as compared with the first binding step.

Both these reaction schemes (2) and (3) are undistinguishable in the equilibrium binding studies and the latter has been proved by a kinetic analysis of antagonist binding to the muscarinic receptor. This kinetic analysis has been made for two benzilic esters 3-quinuclidinylbenzilate and N-methylpiperidinylbenzilate at 25 °C and pH 7.4 [2]. However, a further kinetic study made with the former antagonist within a wider concentration interval showed a more complex nature of the binding process [3, 4] that involves also the cooperative regulation of the apparent rate of quinuclidinylbenzilate association with muscarinic receptor. This phenomenon of cooperative regulation can be explained by the presence of at least two different types of binding sites for 3-quinuclidinylbenzilate on the receptor. The binding sites which appear at high ligand concentration are responsible for the acceleration of the rate of the "isomerization" step. At the same time there is no increase in the number of binding sites for 3-quinuclidinylbenzilate as revealed by the equilibrium binding studies. These data show that dissociation of 3-quinuclidinylbenzilate from the regulatory sites is so rapid that the appropriate complexes cannot be detected by the common filtration technique.

In the present study the properties of these different binding sites and the cooperativity of antagonist binding with muscarinic receptor has been

further investigated at different temperatures, making use of another benzilic ester, N-methylpiperidinylbenzilate.

Materials and methods

[^{3}H]-4-N-methylpiperidinylbenzilate ([^{3}H]-NMPB, 70 Ci/mmol) was a generous gift from Prof. M. Sokolovsky, Tel Aviv University, Israel. All other chemicals of analytical grade were obtained from Sigma (USA) and Merck (Germany) and used without additional purification.

The membrane preparation of muscarinic receptors from rat cerebral cortex was prepared in 10 mM HEPES buffer pH 7.4 as described previously [5]. The membranes were incubated at different temperatures and the binding reaction was initiated by adding [^{3}H]-NMPB into the reaction mixture. The excess of ligand over receptor was used to obtain the pseudo first-order conditions of the association reaction. At certain time intervals aliquots were taken from the incubation mixture and filtered through GF/B filters (Whatman, England). The filters were immediately washed 4 times with 5 ml ice-cold 0.15 M NaCl solution. The nonspecific binding of [^{3}H]-NMPB to the membranes was measured under the same conditions, except that the membranes were preincubated 30 min with 10 μM atropine sulphate (Merck).

The observed pseudo first-order rate constants k_{obs} of [^{3}H]-NMPB binding with muscarinic receptor were determined by fitting the experimental data to the equation

$$B_t = B_n + B_s(1 - e^{-k_{obs}t}), \tag{4}$$

where B_t — total bound radioactivity at time t, B_n — nonspecifically bound radioactivity, B_s — maximal concentration of specifically bound radioactivity at t. Data fitting was done by means of a nonlinear regression program. The receptor [^{3}H]-NMPB complex dissociation rate constants were determined by the displacement of the labelled ligand upon addition of excess of unlabelled atropine sulphate or NMPB, as described in [3]. The observed rate constants of the dissociation process k_{diss} were calculated by fitting the data to the equation of the first order reaction

$$B_t = B_n + B_s \cdot e^{-k_{diss}t}, \tag{5}$$

where B_t, B_n, B_s and t correspond to the above-mentioned parameters.

The protein concentration was determined with a modified method of Lowry using bovine serum albumin as a standard [6].

The k_{obs} vs [[^{3}H]-NMPB] calculations were made on a "Nord-100" computer (Norsk Data, Norway), making use of a nonlinear regression program.

Results

1. Kinetics of [^{3}H]-NMPB association with receptor. The time-dependent increase in the binding of [^{3}H]-NMPB to membrane fragments from rat brain, assayed by filtration technique, can be related to the change of specific binding of the ligand with the receptor sites. The non-specific binding of this radioligand with the membrane fragments reaches its maximal value before time point of incubation measured i.e. when the first sample was filtered after 10 seconds from the moment of mixing reagents (Fig. 1). This fact simplified sufficiently kinetic measurements because only few time points had to be taken to determine the level of the non-specific [^{3}H]-NMPB binding with membranes.

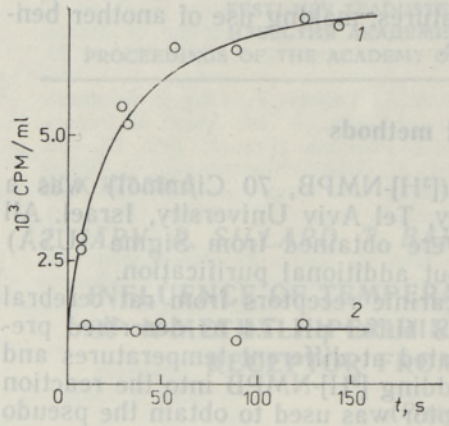
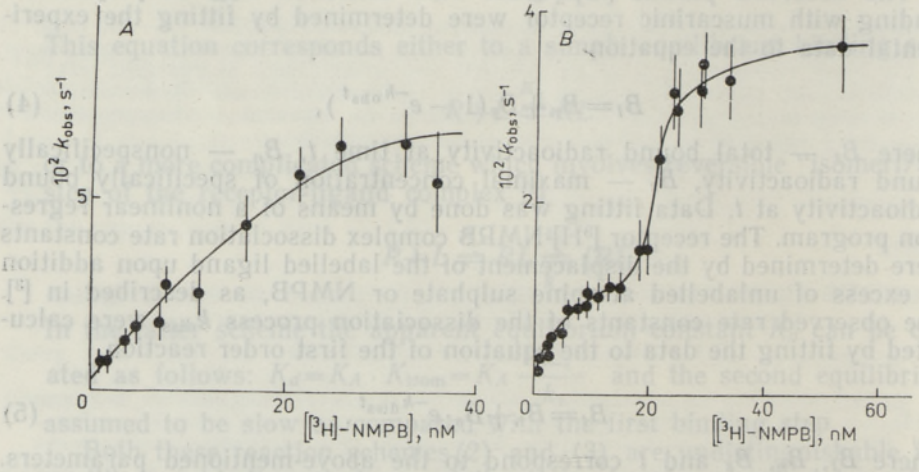


Fig. 1. Time course of [^{3}H]-NMPB (56.7 nM) binding to muscarinic receptor from rat brain (10 mM HEPES buffer, pH 7.4, 25 °C). *1* — total bound radioactivity; *2* — nonspecifically bound radioactivity in presence of 10 μM atropine.

All kinetic experiments were carried out under the pseudo first-order conditions where the radioligand concentration exceeds at least 10-fold the concentration of the totally bound [^{3}H]-NMPB. Under these conditions [^{3}H]-NMPB association with the membrane fragments followed the first-order reaction course with respect to the concentration of the receptor-ligand complex trapped on the Whatman GF/B-filters. From the kinetic curves obtained (Fig. 1) the rate constants k_{obs} were calculated at different ligand concentrations and at 0, 25 and 42 °C. At higher temperatures than 42 °C the experiments were difficult to conduct due to fast receptor inactivation.



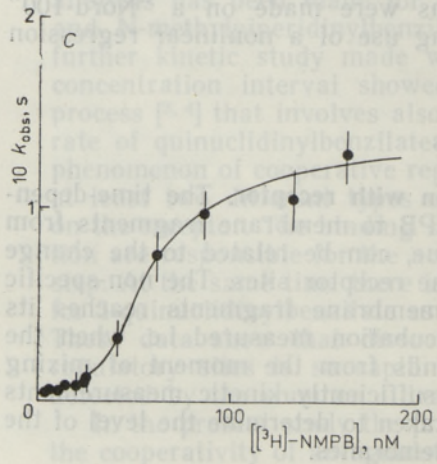


Fig. 2. The dependence of the observed association rate constant (k_{obs}) on the concentration of the antagonist [^{3}H]-NMPB, under pseudo first-order conditions. *A* — 42 °C; *B* — 25 °C; *C* — 0 °C.

The kinetic data obtained are shown in Fig. 2. It can be seen that the standard deviations of k_{obs} do not exceed 30%. The upper limit of ligand concentration used at 42 °C was determined by the reaction rate because fast reactions cannot be followed by the filtration method employed by us.

The kinetic data show that variation of temperature leads to remarkable changes in the shapes of k_{obs} vs [[^{3}H]-NMPB] plots (Fig. 2), which reveal clearly more than one inflection point at 0 and 25 °C. This can be explained with simultaneous binding of several ligand molecules to a single receptor complex and by cooperative regulation of the observed rate of the receptor-ligand complex formation reaction (3).

2. Kinetics of [^{3}H]-NMPB-receptor complex dissociation. Dissociation of [^{3}H]-NMPB from its complex with the membrane-bound muscarinic receptor was initiated by addition of excess of unlabelled NMPB or atropine. This proves the reversibility of the formation of the receptor-ligand complex. It has been found that the dissociation reaction follows the first-order reaction course up to the new equilibrium state. The mean values of k_{diss} obtained at temperatures between 0—42 °C are listed in Table 1.

Table 1

The values of NMPB-receptor complex dissociation rate constants k_{diss} at different temperatures (10 mM HEPES buffer, ph 7.4)

Temperature, °C	k_{diss}, s^{-1}
0	$(7.2\pm1.2)\ 10^{-5}$
12	$(1.4\pm0.4)\ 10^{-4}$
25	$(4.6\pm0.3)\ 10^{-4}$
42	$(2.3\pm0.3)\ 10^{-3}$

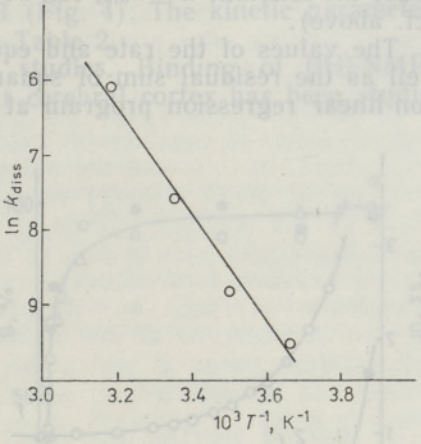


Fig. 3. The Arrhenius plot of [^{3}H]-NMPB-receptor complex dissociation constant k_{diss} (10 mM HEPES buffer, pH 7.4).

It should be noted that these rate constants agree well with the values of the intercepts found in k_{obs} vs [[^{3}H]-NMPB] plots and support the idea of the reversibility of the formation of the ligand receptor complex. From the dissociation kinetics data an Arrhenius plot can be constructed (Fig. 3) using the integrated form of the Arrhenius equation

$$\ln k_{diss} = -\frac{E_a}{RT} + \text{const.} \qquad (6)$$

As can be seen in Fig. 3 the dependence of ln k_{diss} is linear on $1/T$ within the used temperature interval 0°—42 °C and allows the calculation of the activation energy of the dissociation process $E_a = 14.3\pm5.4$ kcal·mol^{-1}.

3. Processing of the association kinetic data. The obtained data were analyzed according to the reaction scheme:

$$\begin{array}{ccc} R \overset{K_A}{\rightleftharpoons} RA & \overset{k_t}{\underset{k_{-t}}{\rightleftharpoons}} & (RA) \\ K_x \Big\downarrow\Big\uparrow & & \Big\downarrow\Big\uparrow \alpha K_x \\ RA_{n+1} & \overset{k_x}{\underset{k_{-x}}{\rightleftharpoons}} & A_n(RA). \end{array} \qquad (7)$$

If $\alpha = 1$, the following equation for the observed rate constant k_{obs} can be derived from the kinetic model above (7):

$$k_{obs}=\frac{k_i[A]}{K_A+[A]}\left(\frac{K_x^H}{K_x^H+[A]^H}\right)+\frac{k_x[A]^H}{K_x^H+[A]^H}\left(\frac{[A]}{K_A+[A]}\right)+k`. \quad (8)$$

This equation allows the estimation of the equilibrium constants K_A and K_x and rate constants k_i and k_x. The constant $k`$ characterizes the receptor-ligand complex dissociation rate and as the simplest approximation its value can be taken as equal to the observed dissociation rate constant k_{diss}, obtained from dissociation experiments at the appropriate temperature. This simplifies data processing according to Eq. (8) because the constant $k`=k_{diss}$ can be obtained from independent experiments (cf. above).

The values of the rate and equilibrium constants of this equation as well as the residual sum of squares Σz^2 were calculated by means of a non-linear regression program at the integer H values from 2 to 20. It has been found that a fast decrease of Σz^2 can be observed up to $H=7$, while a further increase in the Hill coefficient led to plateau in the Σz^2 vs H plot (Fig. 4). This means that a unique optimal parameter value for H cannot be determined proceeding from the kinetic data obtained at 25 °C. But it has been found that the kinetic parameters k_i, K_A, k_x and K_x, which were calculated at the H values above 7 remain within their error limits practically the same as those at H values equal to 7. Thus the values of these rate and equilibrium constants can be regarded as stable estimates not depending on the value of H in the $H \geqslant 7$ interval. These values for k_i, K_A, k_x and K_x are listed in Table 2.

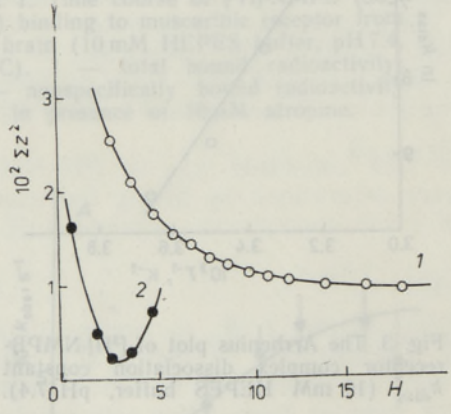


Fig. 4. Residual sum of squares Σz^2 for different integer values of the Hill coefficient (H) in processing of data obtained at 25 °C (*1*) and 0 °C (*2*).

Table 2

Kinetic parameters for [^{3}H]-NMPB interaction with muscarinic receptor at different temperatures (10 mM HEPES buffer, pH 7.4)

	0 °C	25 °C	42 °C
$10^2\ k_i$, s^{-1}	—	1.36±0.32	11.1±1.9
K_A, nM	—	5.53±3.16	27.6±7.3
$10^2\ k_x$, s^{-1}	1.25±0.04	3.57±0.35	—
K_x, nM	122.6±27.7	20.82±7.98	—
H	3	7	—
K_d, nM	0.3±0.1	0.5±0.1	0.4±0.1
K_{Isom}	—	0.03	0.02
Equation in the text	(10)	(8)	(9)

It can easily be seen that if $[A]<K_x$ Eq. (8) corresponds to a single binding isotherm

$$k_{obs}=\frac{k_i+[A]}{K_A+[A]}+k` \quad (9)$$

and describes well the k_{obs} vs [[^{3}H]-NMPB] plot obtained at 42 °C. The appropriate values of K_A and k_i are listed in Table 2. The initial part of k_{obs} vs [[^{3}H]-NMPB] plot up to 40 nM NMPB concentration obtained at 25 °C can be also described by Eq. (9), while at higher ligand concentration an additional sharp increase in the k_{obs} values takes place.

The kinetic data obtained at 0 °C resemble a common Hill plot and under the conditions $k_i=0$ and $K_A=0$ Eq. (8) simplifies to the following:

$$k_{obs}=\frac{k_x[A]^H}{K_x^H+[A]^H}+k`. \qquad (10)$$

In this case a clear minimum of Σz^2, the residual sum of squares, can be observed in the Σz^2 vs H plot at $H=3$ (Fig. 4). The kinetic parameters calculated at this H value are listed in Table 2.

4. [^{3}H]-NMPB equilibrium binding studies. Binding of [^{3}H]-NMPB with the membrane fragments from rat cerebral cortex has been studied under equilibrium conditions at 0, 25 and 42 °C. In all experiments the excess of free ligand over the concentration of the bound ligand was used to keep the reaction of NMPB association with receptor sites under the pseudo first-order conditions. The incubation time necessary to reach the equilibrium state at the lowest ligand concentration was calculated proceeding from the results of kinetic studies. The non-specific binding of [^{3}H]-NMPB has been determined in the presence of 10 μM unlabelled NMPB.

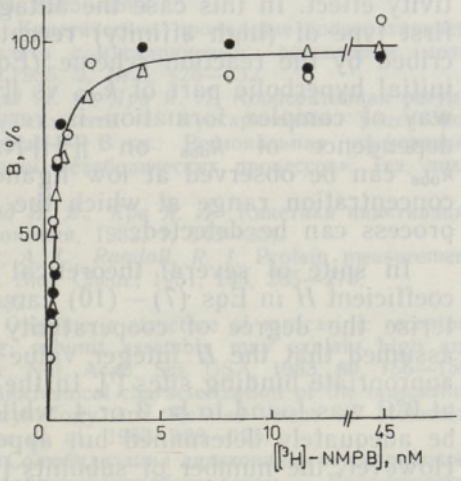


Fig. 5. Specific binding of [[^{3}H]-NMPB] to rat brain membranes at different temperatures: 0 °C — (●), 25 °C — (△), 42 °C — (○), under equilibrium conditions in 10 mM HEPES buffer, pH 7.4.

The binding curves shown in Fig. 5 can be satisfactorily described by a single binding isotherm and allow the calculation of the apparent value of the receptor-ligand complex dissociation constant K_d listed in Table 2.

The results obtained show that variation in temperature has almost no effect on the equilibrium binding constant of NMPB binding. Secondly, it is noteworthy that the capacity of brain membranes for the specific binding of [^{3}H]-NMPB remains constant at all temperatures studied up to 45 nM ligand concentration which exceeds more than 100-fold the K_d value.

Discussion

Muscarinic receptor interacts reversibly with [^{3}H]-NMPB and therefore only the complexes of slow dissociation rate can be determined by the filtration assay. For these "slowly dissociating" complexes of [^{3}H]-NMPB-receptor a single binding isotherm can be applied to describe the equilibrium binding data (Fig. 5) and there is no change in the stoichiometry of this complex within a relatively large interval of ligand concentration.

In kinetic experiments the same filtration assay was used and therefore the formation of the "slowly dissociating" receptor-ligand complex was also measured. However, the course of this reaction can be influenced

by the formation of other types of receptor-ligand complexes which are assumed to dissociate rapidly and therefore themselves cannot be directly assayed by the filtration method. In the kinetic analysis these "rapidly dissociating" [^{3}H]-NMPB-receptor complexes can be detected and in this study they were characterized by the apparent dissociation constants K_A and K_x. The former constant corresponds to the fast equilibrium binding step of the reaction scheme (3) and its value can be obtained from the hyperbolic part of k_{obs} vs [[^{3}H]-NMPB] plot. The second constant characterizes the interaction of one or more additional ligand molecules with the receptor. There should be at least two different types of binding sites for antagonist molecules on muscarinic receptor. The binding sites of the first type undergo isomerization step and thus are responsible for formation of the "slowly dissociating" complex detected on filters, while the second type of binding sites provides a possibility for the cooperative regulation of the former process.

It should be noted that the "slowly dissociating" receptor-antagonist complex forms at considerable rate without the assistance of the cooperativity effect. In this case the antagonist molecules interact only with the first type of (high affinity) receptor sites and the reaction can be described by the reaction scheme (Eq. 2). This conclusion is valid for the initial hyperbolic part of k_{obs} vs [[^{3}H]-NMPB] plots. At 0 °C the "direct" way of complex formation is very slow and instead of the hyperbolic dependence of k_{obs} on [[^{3}H]-NMPB] only a slight increase in k_{obs} can be observed at low ligand concentrations remaining below the concentration range at which the cooperative regulation of the binding process can be detected.

In spite of several theoretical limitations of the Hill equation, the coefficient H in Eqs (7)—(10) can be used to phenomenologically characterize the degree of cooperativity of the ligand binding system. It is assumed that the H integer value cannot be larger than the number of appropriate binding sites [7]. In the present case the best integer H value at 0°C was found to be 3 or 4, while at 25 °C this parameter value cannot be adequately determined but appears to be equal to or larger than 7. However, the number of subunits [8, 9, 10] and their nature in one muscarinic receptor complex as well as the actual number of binding sites in the whole complex cannot be established proceeding from the H values calculated above. It should be emphasized that these parameters give only a relatively crude approximation to the real stoichiometry of the complex. For this reason it is also rather difficult to discuss the meaning of the difference between the H values calculated for 0 and 25 °C (Table 2), although the number of cooperatively functioning subunits or binding sites on the same polypeptide chain may depend upon the state of protein or membrane structure and thus be governed by change in membrane fluidity caused by changes in temperature or by other physico-chemical factors [11]. Two models may accommodate our data.

First, a model which involves for each ligand-receptor complex only one site overcoming the conformational transition necessary for the formation of the "slowly dissociating" receptor-ligand complex. All other remaining binding sites serve as regulatory sites and do not form slowly dissociating complexes which may be determined by the filtration assay.

The second possible model involves equal number of binding and regulatory sites per one receptor complex. If the regulatory sites are not occupied, the binding sites function independently and give hyperbolic k_{obs} vs [A] plots as predicted by the reaction scheme (2). The interaction of ligand molecules with the regulatory sites gives increase in the rate of the "isomerization" step. This acceleration phenomenon is revealed, however, at some critical saturation level of the regulatory sites. This makes

the acceleration effect very sharp and determines the apparent Hill coefficient in the appropriate equations for k_{obs}.

In summary, the results of kinetic studies here and in our previous paper clearly show that muscarinic receptor may form different complexes with more than one antagonist molecule and that the binding of ligands may involve cooperative interactions between binding sites which control the conformational transitions of subunits. This complex nature of the antagonist binding process should be taken into account when the mechanism of binding of muscarinic agonists are studied by making use of radioactive reporter ligands which themselves are most often antagonists.

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TEMPERATUURI MÕJU N-METÜÜLPIPERIDINÜÜLBENSILAADI KOOPERATIIVSELE SIDUMISELE ROTI AJUKOORE MUSKARIINSE RETSEPTORIGA

On uuritud [^{3}H]-N-metüül-4-piperidinüülbensilaadi ([^{3}H]-NMPB) sidumise kineetikat roti ajukoore muskariinse retseptoriga erinevatel temperatuuridel (0°, 25° ja 42 °C) pseudo-esimest järku reaktsiooni tingimustes. Näiva kiiruskonstandi k_{obs} sõltuvus [^{3}H]-NMPB kontsentratsioonist 42 °C juures on hüperboolne, 25 °C juures on nimetatud sõltuvusel kaks käänupunkti: hüperboolse osa järel esineb k_{obs} väärtustes [^{3}H]-NMPB kontsentratsioonil *ca* 20 nM järsk tõus, mis saavutab piirväärtuse *ca* 30 nM kontsentratsioonil. 0 °C juures täheldati lihtsat sigmoidset k_{obs} sõltuvust [^{3}H]-NMPB kontsentratsioonist. Tasakaalulistes tingimustes tehtud katsed näitavad, et retseptoriga püsivalt seostunud ligandi-

molekulide arv samas kontsentratsioonivahemikus ei muutu. See viitab antagonisti kiirele dissotsiatsioonile regulatoorsetest tsentritest. Kõikidel uuritud temperatuuridel [^{3}H]-NMPB dissotsiatsoon ligand-retseptor-kompleksist allus esimest järku reaktsiooni kineetikale. Dissotsiatsiooni- ja assotsiatsiooniprotsesside aktivatsioonienergiate väärtusteks arvutati vastavalt 14,3 kcal/mol ja 23 kcal/mol, mis viitavad retseptorvalgu konformatsioonimuutustele ligandi sidumisel. Saadud andmete kirjeldamiseks on välja pakutud antagonistide muskariinse retseptoriga seostumise formaalkineetiline mudel, millele vastava võrrandiga on eksperimendiandmeid töödeldud. On arvutatud vastavad kiirus- ja tasakaalukonstandid. Esitatud andmete alusel saab väita, et muskariinse retseptoriga võib üheaegselt seostuda mitu ligandimolekuli, mis toimivad püsiva retseptor-antagonist-kompleksi moodustumisel kooperatiivselt.

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ВЛИЯНИЕ ТЕМПЕРАТУРЫ НА КООПЕРАТИВНОЕ СВЯЗЫВАНИЕ N-МЕТИЛПИПЕРИДИНИЛБЕНЗИЛАТА С МУСКАРИНОВЫМ РЕЦЕПТОРОМ КОРЫ МОЗГА КРЫС

Изучена кинетика ассоциации [^{3}H]-N-метил-4-пиперидинилбензилата с мускариновым рецептором коры мозга крыс при 0, 25 и 42 °С в условиях псевдопервого порядка. Предложена формально-кинетическая схема для описания связывания антагонистов с мускариновым рецептором. Установлено, что с мускариновым рецептором может одновременно связываться несколько молекул антагониста, которые, взаимодействуя кооперативно, образуют прочный комплекс — «рецептор—антагонист».