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Kinetic studies of the δ-opioid antagonist [^{3}H]DPN induced receptor binding on suspensions of mouse spleenocytes

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Abstract

Kinetic studies of binding of the δ-opioid antagonist [^{3}H]DPN with receptors of mouse spleenocytes are performed. Kinetic analysis of experimental data has shown that receptors of these cells possess activity toward the δ-opioid ligands. Presence of compounds that inhibit the conjugation of receptors with G-proteins, reduces receptor binding. Experimental data are computer simulated, and numerical values for various equilibrium as well as kinetic parameters of receptor binding and the G-protein cycle are obtained.

Keywords: Native mouse spleenocytes; Opioid receptor binding; Cycle of G protein; Computer simulation

1. Introduction

Hormone–receptor interaction plays an important role in the process of the transformation of an extracellular signal into a cellular response [1]. A great variety of cell-surface receptors mediate their action by a transducing pathway, involving the activation of guanine nucleotide-binding proteins (G proteins) [2,3]. The signal is recognized by the receptor and transmitted through a G protein to signal amplifiers which synthesize second messengers. In this transformation a receptor couples directly to a G protein, therefore the cycle of G protein and hormone–receptor interaction cannot be considered separately [4,5]. As a result of this coupling, the kinetics of the hormone–receptor interaction are strongly influenced by the dynamics of the concentrational change of guanine nucleotides GTP and GDP, and that it is essential to incorporate the GTP regenerating system into the extracellular signal transducing pathway [5]. Furthermore, various equilibrium as well as kinetic parameters of the G protein cycle enter a kinetic equation for the time evolution of the concentration of hormone–receptor complex, and the agonist- and antagonist-

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induced receptor binding is no longer described by the same mathematical expression.

In the present work we apply the developed kinetic theory for the hormone–receptor interaction [5] to the kinetics of antagonist-induced receptor binding. The concentration of the hormone–receptor complex is an experimentally observable parameter in the kinetic experiments and is a measure of the hormone–receptor interaction. Simulation of experimental data for receptor binding allows one to obtain numerical values for equilibrium and kinetic parameters of various steps involved in the function of the extracellular signal transduction system.

It is known that nervous and immune systems function in concert. For example, emotional stress makes the organism more sensitive to different diseases, while a positive emotional state supports the recovery from disease. This close connection between the nervous and immune systems is due to the fact that cells of both systems share the same receptors. It has been known for years that receptors of cells of the nervous system of various mammalian organisms possess certain activity for opioid hormones (μ-, κ- and δ-types) [1]. It is therefore interesting to study the kinetics of the opioid hormone-induced receptor binding on a suspension of cells of the immune system. For this purpose we have chosen mouse spleenocytes.

2. Materials and methods

2.1. Preparation of cell suspension

Tritium-labeled DPN (diprenorphin), as well as unlabeled morphine and Naltrexon have been obtained from Amersham (England) and Sigma (USA), respectively. GTP γ (NH) (guanil-5′-il-imidotriphosphate) has also been obtained from Sigma (USA).

Native spleenocytes have been obtained from the spleen of a mouse [6]. Cells have been homogenized in the medium of 0.83% ammonium chloride (pH 7.4) at 37°C, and incubated in PBS medium for 30 min at 0°C.

2.2. Detection of receptor binding

The δ-opioid receptor binding data have been obtained using the method of radioactive ligands [7]. Experimental measurements have been performed at equal time intervals of 10 and 15 s. To determine the amount of binding, the cell suspension has been placed in a vessel containing labeled ligand and incubated with intermittent stirring at 37°C. The standard measurement procedure is as follows: equal aliquots have been taken from the reaction mixture and placed onto the filter of the filtration unit; reaction has been stopped by diluting a reaction mixture with one volume (6 ml) of Hanks medium (pH 7.2) at 0°C, and the formed complex of the ligand with the cell-surface receptor has been washed with 12 ml of Hanks medium (pH 7.2) at 0°C.

For an estimation of the amount of non-specific binding, the reaction mixture has been incubated for 30 min in the presence of unlabeled δ-opioid agonist morphine and δ-opioid antagonist Naltrexon in the concentration of 1 and 10 μM, respectively. To probe the kinetic dependence of receptor binding on different experimental conditions, a suspension of spleenocytes was preliminarily incubated for 2.5 h at 37°C in the presence of 5 μM solution of GTP γ(NH) (nonhydrolizable analogue of GTP).

2.3. Statistical analysis

Analysis of experimental data was performed using the program STATGRAPHICS. The procedure includes the establishment of the exponential trend, the analysis of residues, as well as the determination of the correlation coefficient (R^2), the maximal value of the model function ($B_{\max}$), and the standard deviation (σ). Computer simulation was done using Mathematica 3.0 (Stephen Wolfram).

3. Results

Studies of kinetics of the δ-opioid antagonist [^{3}H]DPN binding in suspensions of spleenocytes have shown that receptors of these cells possess activity for δ-opioid ligands. Experimental curves have exponential form: an exponential growth in the beginning with the saturation plateau at later

times (see Figs. 1 and 2). The level of binding does not decrease even after 2–3 h of an experiment have lapsed (experimental points are not shown).

In a separate experiment it was shown that the relative standard deviations of the level of receptor binding does not exceed 5%, and that the receptor binding is not controlled by the process of diffusion of the ligand to or on the surface of the cytoplasmic membrane.

The addition of an excess of unlabeled ligand that has comparable value of the kinetic constant for binding to the receptor with that of the labeled ligand, to a complex of the receptor with the labeled ligand, reduces the amount of receptor binding. It was found that addition of the 10^3- and 10^4-excess of the unlabeled δ-opioid agonist morphine and antagonist Naltrexon, respectively to the reaction mixture (the complex of [^{3}H]DPN with cell-surface receptors of spleenocytes), does not reverse receptor binding (data are not shown). This leads us to think that binding of [^{3}H]DPN to receptors of spleenocytes is an irreversible step: $H + R \overset{k_1}{\rightarrow} H \cdot R$ (H, R, and $H \cdot R$ stand for [^{3}H]DPN, unoccupied receptor and the complex of receptor with [^{3}H]DPN, respectively).

The suspension of spleenocytes were incubated in the presence of GTPγ(NH) (nonhydrolizable analogue of GTP). This compound penetrates through a cytoplasmic membrane, inhibits the coupling of receptors to a G protein and suppresses its function due to an increase in the concentration of G protein in its GTP-bound form. This brings about formation of an excess of the low affinity ($R \cdot G \cdot GTP$) over the high affinity ($R \cdot G \cdot GDP$) form of receptors. The analysis of experimental data (see Fig. 3) shows that in the

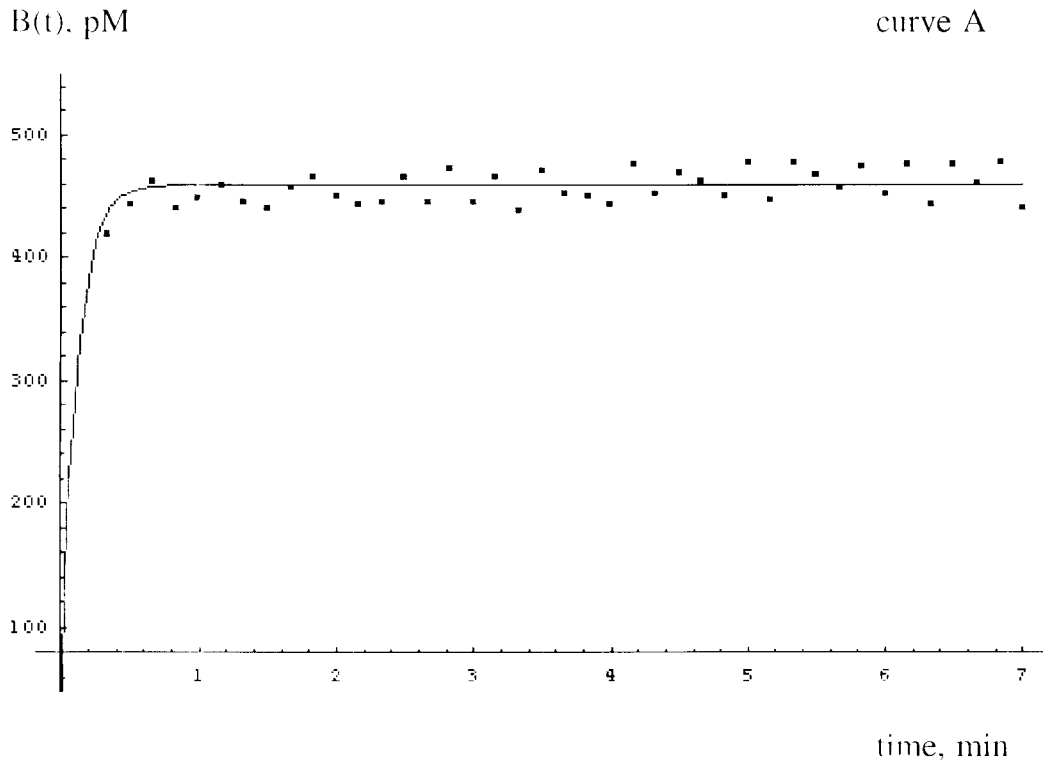

Fig. 1. Typical experimental kinetic curves for binding (superposition of specific receptor and non-specific binding) of the δ-opioid antagonist [^{3}H]DPN in suspension of mouse spleenocytes. Concentrations of cells and ligand are ~4 billions/ml, and 2.0 nM, respectively (curve A); ~4 billions/ml, and 1.7 nM, respectively (curve B). Dots correspond to experimental data points; solid curves are obtained by computer simulation of experimental data with Eq. (1).

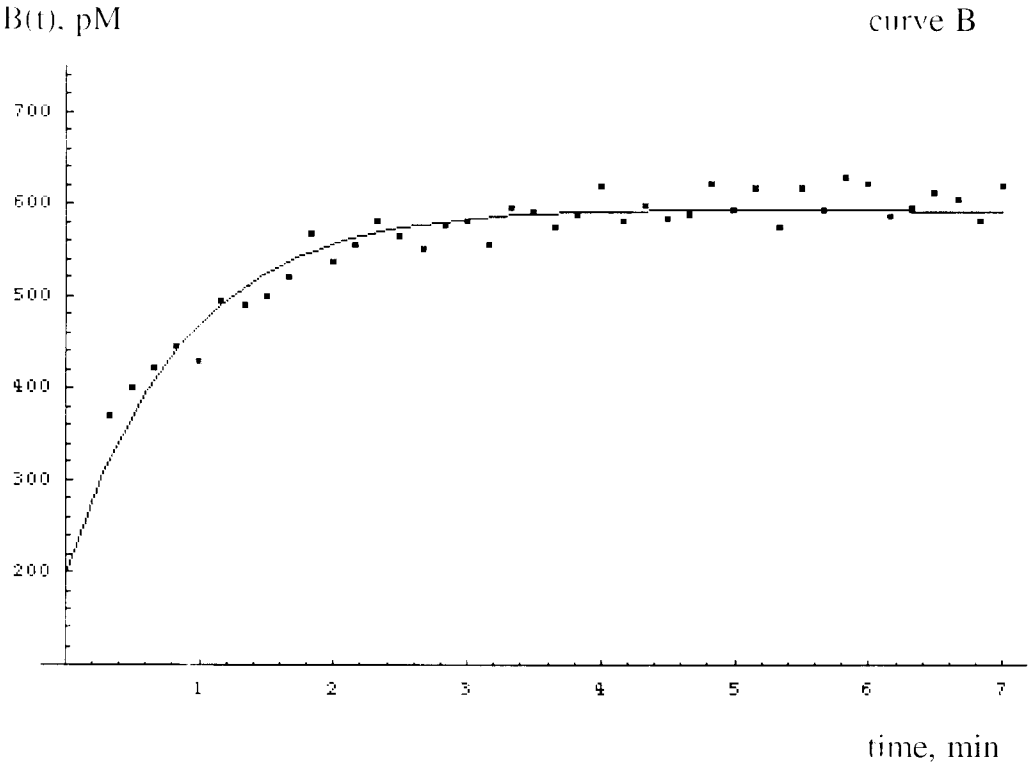

Fig. 1. (*Continued*)

presence of 5 μM solution of GTPγ(NH) the level of binding (top curve) is reduced by a factor of 1.5 (bottom curve). This means that the intracellular concentration of G protein is ~ 1 μM, and that the system of opioid receptors in suspensions of mouse spleenocytes mediate their action via the activation of G proteins.

4. Computer simulation

First, we briefly consider the structure (Fig. 3) of the external signal transduction system. The receptor (R) exists in high affinity (R_h) and low affinity (R_l) forms. Formation of the high affinity state of the receptor takes place when the former couples to the GDP-bound form of G protein ($G\cdot GDP$) and forms the first ternary receptor-G protein–GDP complex ($RG\cdot GDP$). The coupling of receptor to GTP-bound G protein, and the formation of the second ternary receptor-G protein–GTP complex ($RG\cdot GTP$) correspond to the formation of the low affinity state of the receptor. The activation of the receptor by extracellular ligand (H), i.e. the formation of the first quaternary hormone–receptor-G protein–GDP complex ($HRG\cdot GDP$), induces the release of GDP associated with the α-subunit of G protein (α), subsequent binding with GTP, and formation of the second quaternary hormone–receptor-G protein–GTP complex ($HRG\cdot GTP$). This leads to the dissociation of the second quaternary hormone–receptor-G protein–GTP complex to the hormone–receptor complex (HR) and GTP-bound G protein ($G\cdot GTP$). After the receptor has dissociated from the GTP-bound G protein, the dissociation of the bound hormone from the receptor in its low affinity state occurs. GTP-bound G protein dissociates into a dimer formed by β- and γ-subunits of heterotrimeric G protein ($\beta\gamma$) and a complex of GTP with α-subunit of G

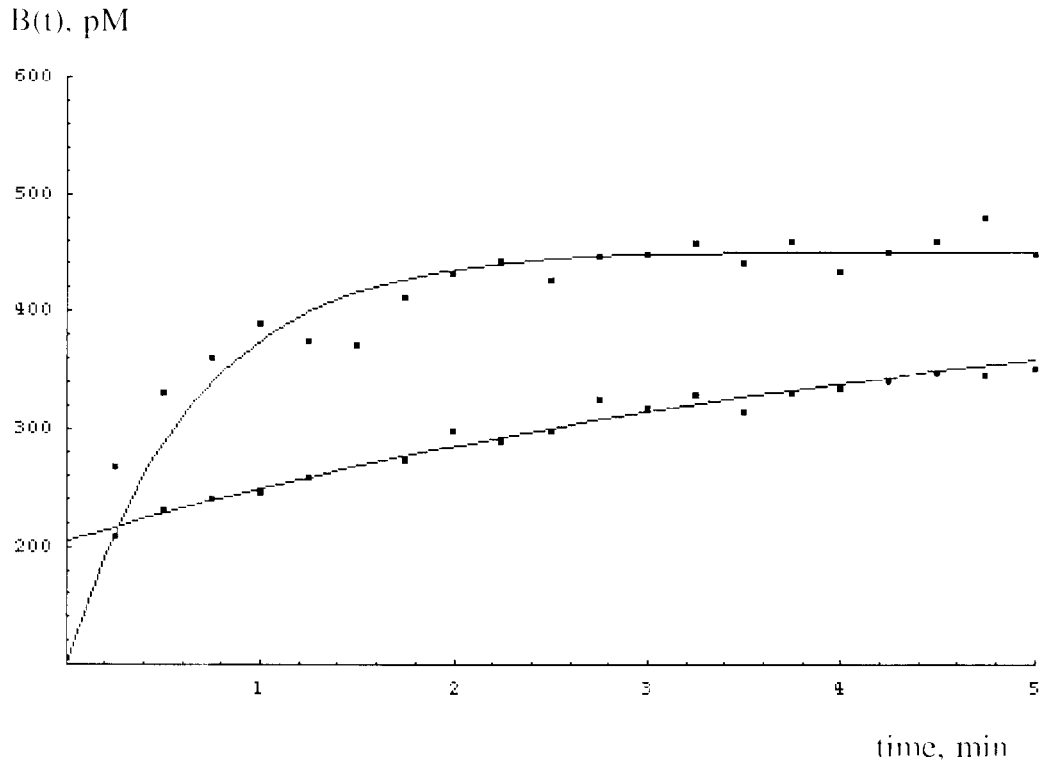

Fig. 2. Preliminary incubation of mouse spleenocytes for 2.5 h at 37°C in the presence of GTPγ(NH) (~5 μM) reduces the δ-opioid antagonist ([^{3}H]DPN) induced receptor binding. Dots correspond to experimental data points; both top and bottom solid curves are obtained by computer simulation of experimental data with Eq. (1). The top curve corresponds to receptor binding kinetics (superposition of specific receptor and non-specific binding) without the preincubation of cells in the presence of GTPγ(NH). The bottom curve corresponds to receptor binding kinetics (superposition of specific receptor and non-specific binding) with preliminary incubation of cells in the presence of GTPγ(NH). The level of binding is reduced by a factor of 1.5. Both curves correspond to the same concentrations of cells in suspension ~3.5 billions/ml, and the initial concentration of ligand ~2.0 nM.

protein ($\alpha \cdot GTP$). This complex activates an amplifier. After the hydrolysis of bound GTP into bound GDP has occurred ($\alpha \cdot GTP \rightarrow \alpha \cdot GDP$), the $\alpha \cdot GDP$ complex is released from an amplifier. The recombination of the $\alpha \cdot GDP$ complex with $\beta\gamma$-dimer terminates the cycle.

It turns out that to fully understand operation of the external signal transduction system, it is necessary to incorporate the GTP regenerating protein maintaining the cellular concentration of GTP into the G protein cycle [5]. This protein transfers the terminal phosphate from ATP to GDP coupled to G protein as

$$ATP + E \rightarrow ADP + E \cdot p_i$$

$$E \cdot p_i + G \cdot GDP \rightarrow E + G \cdot GDP.$$

Here E and $E \cdot p_i$ stand for the GTP regenerating protein in its free and inorganic phosphate (p_i) coupled form, respectively.

Detailed explanation for the proposed kinetic model of operation of the external signal transduction G protein mediated system, and the rigorous derivation of the equation governing the time evolution of the total concentration of hormone–receptor complex are given in reference [5] [see especially Fig. 3, Eqs (12–14), and Eq. (A.8) in reference [5]]. Here we present only the essential result (all denotations are same as in reference [5]). The expression for the time evolution of the total concentration of hormone–receptor

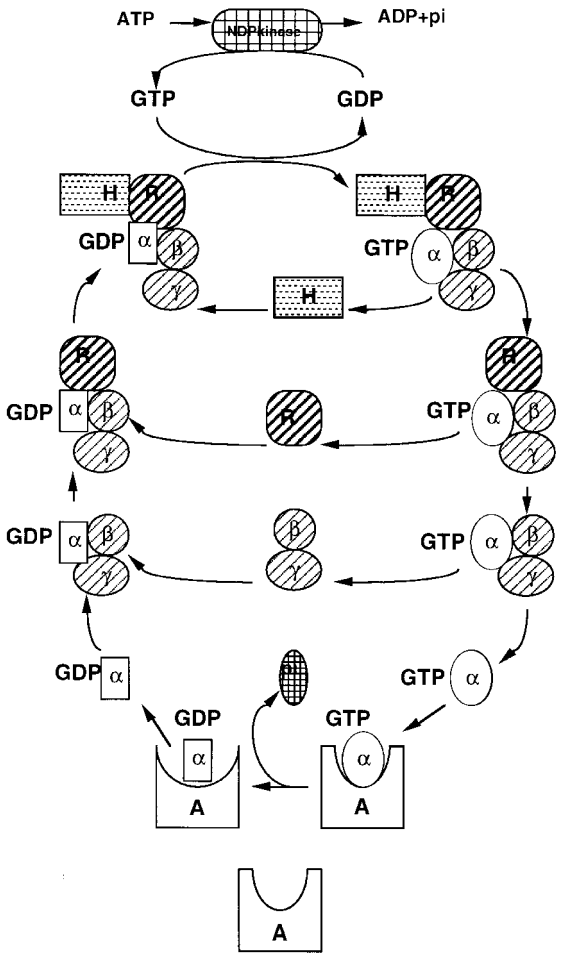

Fig. 3. The cycle of G protein. Abbreviations used: H, extracellular ligand (agonist, antagonist); R, free receptor; HR, the hormone–receptor complex; $RG\cdot GDP$, ternary receptor-G protein–GDP complex corresponding to the active form of the receptor (R_h); $G\cdot GDP$, the GDP-bound form of G protein; $HRG\cdot GDP$, the first quaternary hormone–receptor-G protein–GDP complex; $HRG\cdot GTP$, second quaternary hormone–receptor-G protein–GTP complex; $G\cdot GTP$, GTP-bound G protein; $\beta\gamma$-β-and γ-subunits of G protein dimer; $\alpha\cdot GTP$, the complex of GTP with α-subunit of G protein; $\alpha\cdot GDP$, the complex of GDP with α-subunit of G protein; *pi*-inorganic phosphate, E, the GTP regenerating protein. The cycle of G protein consists of three internal subcycles: the subcycle of the hormone H; the subcycle of the receptor R; and the subcycle of the $\beta\gamma$-dimer, and two external subcycles: the subcycle of the effector/amplifier A; and the subcycle of the GTP regenerating protein.

complex (Eq. 13 in reference [5]) reads:

$$(B(t)) = (B_{ns}) + \frac{(R_0)e^{-k_{in}t}(1-e^{-k_1(H_0)(t+K_2Z(t))})}{1+K_2z(t)} \times (1+K_2z(t)e^{-k_{-1}t}) \quad (1)$$

This expression can be used to study the kinetics of antagonist-receptor binding, provided the kinetic constant for dissociation of hormone (antagonist) from receptor (k_{-1}) is negligible in comparison with that for binding of hormone (antagonist) to receptor (k_1). (B) stands for receptor binding and is the concentration of the hormone–receptor complex; (B_{ns}) stands for non-specific binding; $z(t)$ takes care of a ratio of the current concentrations of the guanine nucleotides GTP and GDP (the GTP/GDP-ratio): $z(t) = (GTP)/(GDP)$ $Z(t)'$; is a time derivative of function $Z(t)$, such that $Z(t)' = z(t)$; (H_0) and (R_0) are the initial concentrations of hormone and receptor, respectively; k_{in} is the kinetic constant for the receptor inactivation (down regulation); K_2 is the equilibrium constant for the process of GDP/GTP-exchange: $HRG\cdot GDP + GTP = HRG\cdot GTP + GDP$, where $HRG\cdot GDP$ and $HRG\cdot GTP$ are the quaternary complex of hormone, receptor and G protein in its GDP- and GTP-coupled forms, respectively.

In the case of antagonist-induced receptor binding, concentrations of GTP and GDP do not change, and are equal to their initial concentrations, i.e. $(GTP) = (GTP)_0$, $(GDP) = (GDP)_0$ and the GTP/GDP-ratio is constant: $z = (GTP)_0/(GDP)_0$, and $Z = z\cdot t$.

Experimental data for binding of [^{3}H]DPN on suspension of mouse spleenocytes (Figs. 1 and 2) are computer simulated using the Eq. (1). Numerical values for various kinetic and equilibrium parameters are reported in Table 1.

5. Discussion

Suspensions of mouse spleenocytes possess receptor activity toward the δ-opioid ligands. There is a strong experimental evidence that the δ-opioid receptors are the receptors responsible for the binding of [^{3}H]DPN. That the level of binding does not decrease after it has reached saturation, and that the addition of an excess of unlabeled morphine and Naltrexon does not reverse the amount of binding, leads us to think that [^{3}H]DPN

Table 1
The estimated numerical values for various kinetic and equilibrium parameters of the cycle of G protein [the concentration of guanine nucleotides, the equilibrium constant for the GDP/GTP-exchange (K_2), the kinetic constant for inactivation (down regulation) of receptors (k_{in})], the hormone–receptor interaction (the kinetic constants for binding to (k_1) and dissociation from (k_{-1}) the receptor, the initial concentration of receptors (R_o) and centers of non-specific binding (B_{ns}) in suspension), as well as the correlation coefficient (R^2) and the ratio of the standard deviation (σ) to the maximal value of the model function (1) (B_{max}) in (%) for theoretical curves obtained by the computer simulation of the experimental kinetic curves for the δ-opioid antagonist ([^{3}H]DPN) induced receptor binding on native mouse spleenocytes. Correlation coefficient reflects the degree of correlation of the model function with experimental data; σ/B_{max} (%) measures the relative deviation of the model function from experimental data

Kinet./equil. and statistical parameters	Numerical values (Fig. 1, curve A)	Numerical values (Fig. 1, curve B)	Numerical values (Fig. 2, top curve)	Numerical values (Fig. 2, bottom curve)
GTP, M	2.5×10^{-5}	1.0×10^{-5}	2.0×10^{-5}	6.0×10^{-5}
GDP, M	3.0×10^{-5}	4.0×10^{-5}	6.0×10^{-5}	4.0×10^{-5}
k_1, 1/M min	4.0×10^{8}	2.0×10^{8}	1.8×10^{8}	5.8×10^{7}
k_{-1}, 1/min	0.001	0.005	0.004	0.004
k_{in}, 1/min	1.0×10^{-5}	1.0×10^{-5}	1.0×10^{-5}	1.0×10^{-5}
k_2	10.0	10.0	10.0	10.0
R_o, M	2.3×10^{-10}	2.4×10^{-10}	2.0×10^{-10}	1.4×10^{-10}
B_{ns}, M	0.3×10^{-10}	1.9×10^{-10}	1.0×10^{-10}	2.0×10^{-10}
R^2	0.92	0.92	0.90	0.91
σ/B_{max}(%)	5.5	5.6	5.7	5.6

is a typical antagonist on opioid receptors of mouse spleenocytes, and that [^{3}H]DPN binds to receptors irreversibly. This conclusion is supported by negligible numerical estimates obtained for the kinetic constants of hormone dissociation from receptor ($\sim 10^{-3}$ min^{-1}) and the receptor inactivation (down regulation) ($k_{in} = 1.0\times10^{-5}$ min^{-1}). Aside from non-specific centers of binding, there is only one type of receptor which is responsible for binding of [^{3}H]DPN. These receptors possess manifestly high affinity toward [^{3}H]DPN (the value for k_1 varies between 1.8 and 4.0×10^{8} M^{-1} min^{-1}).

Binding of [^{3}H]DPN to opioid receptors of mouse spleenocytes is connected to the function of the G protein. In the presence of an excess of GTPγ(NH), the level of binding is reduced from 2.0 to 1.4×10^{-10} M, the concentration of GTP is increased from 2.0×10^{-5} to 6.0×10^{-5} M, while the concentration of GDP is decreased from 6.0×10^{-5} to 4.0×10^{-5} M. Clearly, the obtained numerical estimate for the kinetic constant of receptor binding ($k_1 = 5.8\times10^{7}$ M^{-1} min^{-1}) in the presence of an excess of GTPγ(NH) corresponds to the low affinity form of receptor binding. In other words, the high affinity form of receptors of mouse spleenocytes exceeds the low affinity form in ability to bind a hormone approximately by a factor of 10. The numerical estimate for the equilibrium constant K_2 is greater than unity. This is because for G protein in mouse spleenocytes the complex of [^{3}H]DPN, receptor and G protein in its GTP-coupled form is structurally more preferable than the complex of [^{3}H]DPN, receptor and G protein in its GDP-coupled form. A share of non-specific binding varies between 0.3 and 2.0×10^{-10} M, and is not negligible in comparison with receptor binding. The initial concentration of opioid receptors varies between 1.4 and 2.4×10^{-10} M.

Upon the action of antagonist [^{3}H]DPN, concentrations of both GTP and GDP do not change. The current concentration of the high affinity receptors also does not change. This is why the

level of antagonist binding does not decrease, once it has reached saturation. This is of course due to the absence of any feedback between the antagonist-receptor interaction and function of the effector/amplifier system. Upon the action of the agonist, the amount of receptor binding decreases eventually to zero as time goes on. It is because the action of the agonist is exhaustive, i.e. it leads to the decrease of the current concentration of the high affinity form of receptors, and is due to a negative feedback between the agonist-receptor interaction and function of the effector/amplifier system.

The operation of the extracellular signal transduction G protein mediated system obeys the Le Chatelier principle: upon the action of an extracellular signal (external perturbation), the effector/amplifier which generates a cellular response and the G protein cycle which operates as the transmitter of the extracellular signal from the receptor (after dissociation of the hormone) to the effector/amplifier system and back to receptor, tends to decrease the effect of the external signal, that is the formation of GDP, the high affinity form of receptor $R \cdot G \cdot GDP$, and an increase in receptor binding by means of the GTP regenerating protein which facilitates the formation of GTP, the low affinity form of receptor $R \cdot G \cdot GTP$, and tends to decrease receptor binding. Experimentally measured concentration of the hormone–receptor complex reflects the balance between these opposite tendencies.

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