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An approach to analysis of radiolabeled ligand interactions with specific receptors

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Abstract

The aim of the study was to reveal general characteristics of the ligand–receptor interaction in the binding and displacement of radiolabeled ligands. The binding and displacement of DL-[^{3}H]propranolol hydrochloride ([^{3}H]propranolol) and L-[propyl-2,3,-^{3}H]dihydroalprenolol ([^{3}H]dihydroalprenolol), β-adrenoceptor antagonists, were studied with isolated rat red blood cells, their membranes and ghosts. The binding of [^{3}H]dihydroalprenolol and L-quinuclidinyl-[phenyl-4-^{3}H]-benzylate ([^{3}H]quinuclidinyl benzylate), a muscarinic acetylcholine receptor antagonist, was studied with cerebral cortex membranes. The ligand–receptor interaction corresponded to that for a model of two pools of receptors in the same effector system, with the binding of two ligand molecules to the receptors and was described by the following equation: $b = [(B_{m1}A^2)/(K_{d1}^2 + A^2)] + [(B_{m2}A^2)/(K_{d2}^2 + A^2)]$. The parameters of [^{3}H]propranolol binding to β-adrenoceptors were as follows: $K_{d1} = 0.74$ nM, $K_{d2} = 14.40$ nM, $B_{m1} = 24$ unit/cell, and $B_{m2} = 263$ unit/cell for native red blood cells from rats; $K_{d1} = 0.70$ nM, $K_{d2} = 19.59$ nM, $B_{m1} = 9$ fmol/mg protein, and $B_{m2} = 39$ fmol/mg protein for blood ghosts. The parameters of [^{3}H]quinuclidinyl benzylate binding to muscarinic acetylcholine receptors of cerebral cortex membrane were as follows: $K_{d1} = 0.43$ nM, $K_{d2} = 2.83$ nM, $B_{m1} = 712$ fmol/mg, $B_{m2} = 677$ fmol/mg. The analysis of the equilibrium binding and displacement of [^{3}H]propranolol and [^{3}H]dihydroalprenolol at β-adrenoceptors of membranes, ghosts and native red cells of rats, [^{3}H]dihydroalprenolol at β-adrenoceptors and [^{3}H]quinuclidinyl benzylate at muscarinic acetylcholine receptors of synaptosomal membranes from rat cerebral cortex demonstrated that the receptors bound two ligand molecules each and consisted of two discrete pools of high- and low-affinity receptors. Similar results were obtained for the displacement of [^{3}H]propranolol, [^{3}H]dihydroalprenolol and [^{3}H]quinuclidinyl benzylate by propranolol, dihydroalprenolol and quinuclidinyl benzylate.

Keywords: β-Adrenoceptor; Muscarinic acetylcholine receptor; Radioligand binding

1. Introduction

The first stage in the effect of a regulatory system on an effector cell is the binding of a neurotransmitter, hormone, peptide or other signalling molecule to the specific receptor. This process has been theoretically and experimentally analysed many times, using examples of physiological, enzymatic and radioligand reactions (Manukhin, 1968; Klotz and Hunston, 1971; Feldman, 1972; Molinoff et al., 1981; Dixon and Webb, 1982; Hieble et al., 1995; Gnagey and Ellis, 1996; Chidiac et al., 1997). Since all these reactions can be described by a hyperbolic curve, i.e., are governed by similar characteristics, the results obtained for each process can be used with certain limitations when studying the effect of biologically active substances on receptors at organic, cellular and subcellular levels. At the same time, standard treatment of experimental data does not always provide satisfactory results for the analysis. This fact suggests that not all aspects of ligand binding to a receptor have been taken to account. Therefore, based on the literature and our own data, the present work focuses on the general characteristics of the ligand–receptor interaction and possible ways to analyse them.

In the simplest instance, the interaction of a ligand (L) with its specific receptor (R) with the subsequent formation of the RL complex is necessary for the development, activation or inhibition of a physiological or biochemical reaction.

$$[R] + [L] \leftrightarrow [LR] \quad (1)$$

In this process, it is assumed that L and R interact according to the Law of Mass Action, i.e., the rates of [LR]

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complex formation (k_1) and dissociation (k_2) are proportional to concentrations of components in the system. If the process proceeds under conditions of ligand excess, that is, [L] ≫ [R], the concentration of free receptors is ([R] − [LR]) and when the equilibrium $K_d = (k_2/k_1)$ is achieved, Eq. (1) appears as follows:

$$([L][R - LR])/[LR] = K_d. \quad (2)$$

The process of ligand–receptor binding is characterised by the equilibrium dissociation constant (K_d), which is equal to the ligand concentration required for binding of 50% of active receptors. The association or affinity constant (K_a) quantitatively determines the affinity of a ligand for a given receptor. The term ''receptor'' or ''binding site'' implies a biochemical structure (specific binding protein or its part) capable of forming a reversible or irreversible complex with a specific ligand to induce, block or modify the intensity of a certain biological process. If the binding sites are homogeneous in their affinity for a ligand, there is no interaction between the binding sites, which could change their properties, and each site binds one ligand molecule. In this instance, the amount of bound receptors b at the ligand concentration [A] is determined by the following equation:

$$b = [(B_m A)/(K_d + A)] \quad (3)$$

where B_m is the concentration of binding sites in the effector system and K_d is the dissociation constant of the ligand–receptor complex. In Cartesian co-ordinates, ligand–receptor binding is described by a hyperbole, where the b value tends to B_m and reaches it at an infinitely high concentration of ligand [A]. In Scatchard co-ordinates (b,$b/$[A]), experimental points align themselves on a straight line which intersects the abscissa at a point equal to B_m and the ordinate at a point equal to $[B_m]/[K_d]$.

An effector system can contain not a single but two or more independent ligand binding sites (pools) that differ in their K_d (Feldman, 1972). The binding site independence implies that the process of ligand complexing with a receptor does not influence the kinetic constants of the ligand binding to other receptors. In such instances, the amount of bound ligand b is determined by a common equation:

$$b = [(B_{m1} A)/(K_{d1} + A)] + [(B_{m2} A)/(K_{d2} + A)] + \ldots + [(B_{mi} A)/(K_{di} + A)] \quad (4)$$

where B_{m1}, $B_{m2}, \ldots B_{mi}$ are the number of ligand binding sites in different receptor pools within the same effector system at dissociation constants K_{d1}, $K_{d2} \ldots K_{di}$, and [A] is the ligand concentration in the medium. In theory, several discrete pools of the same receptor type can occur in the effector system under consideration. However, the greater the number of such pools, the more cumbersome the analysis of the effector system. For two pools, Eq. (4) is simplified for graphical and mathematical analyses:

$$b = [(B_{m1} A)/(K_{d1} + A)] + [(B_{m2} A)/(K_{d2} + A)]. \quad (5)$$

In Cartesian co-ordinates, the plot of ligand binding to receptors in two discrete pools is also a hyperbole, where the b value tends to $B_{max} = B_{m1} + B_{m2}$. In Scatchard co-ordinates, the plot presents as a concave curve. Graphical and mathematical analyses allow one to isolate lines, asymptotes, corresponding to the ligand binding to receptors in each individual pool. The hyperbole asymptotes are the lines that intercept positions B_{m1}/K_{d1} and B_{m2}/K_{d2} on the y-axis and positions B_{m1} and B_{m2} on the x-axis, respectively. Their sum is equal to the positions intercepted by the curve extrapolated to x- and y-axes.

Finally, the specific binding protein receptor can possess several binding sites. In this instance, one receptor can bind two or more ligand molecules. For analysis of such systems, a number of models have been proposed (Klotz and Hunston, 1971; Feldman, 1972; Dixon and Webb, 1982). One of the earliest models was proposed by Hill (Dixon and Webb, 1982). If n ligand molecules bind to the receptor, then Eq. (1) appears as follows:

$$R + nL = L^nR. \quad (6)$$

If one considers that K_d is equal to the ligand concentration [L] which induces binding to one-half of the active receptors, then the K_d dimension at L^n is K_d^n, and Eq. (2) rearranges to

$$[L]^n([R] - [L^nR])/[L^nR] = K_d^n. \quad (7)$$

Consistently, Eq. (3) is as follows:

$$b = (B_m A^n)/(K_d^n + A^n). \quad (8)$$

After rearrangement and taking the logarithm, the Hill equation takes the following form:

$$\lg[b/(B_m - b)] = n \lg A - n \lg K_d. \quad (9)$$

In Hill co-ordinates, the slope is determined by the Hill coefficient (n) which cannot exceed the number of binding sites in the receptor molecule. The plot of ligand binding to two independent sites appears as an S-shaped curve with two branches corresponding to low and high ligand concentrations, i.e., the ligand binding to high- and low-affinity receptor pools. The curve in Hill co-ordinates can be approximated as a line with a slope (Hill coefficient) greater or smaller than 1. If the system has two discrete receptor sites, $n < 1$; if two ligand molecules bind to the same receptor, $1 < n < 2$. At $n = 2$, one may assume the occurrence of a single receptor site with two ligand molecules binding to each receptor.

In the general form, the reaction in which a variable number of ligand molecules (n) bind to a single receptor in

pools with different affinities is described by the following equation:

$$b = \left[\left(B_{m1}A^{n1}\right)/\left(K_{d1}^{n1} + A^{n1}\right)\right] + \left[\left(B_{m2}A^{n2}\right)/\left(K_{d2}^{n2} + A^{n2}\right)\right] + \ldots + \left[\left(B_{mi}A^{ni}\right)/\left(K_{di}^{ni} + A^{ni}\right)\right]. \quad (10)$$

The article presents analytical results obtained in experiments on the interaction of specific radiolabeled ligands with β-adreno- and muscarinic acetylcholine receptors on isolated cells and membranes. The instances under consideration of the ligand–receptor interaction correspond to two receptor sites in the same effector system and binding of two ligand molecules to a single receptor, i.e., is described by Eq. (11) at $n = 2$.

$$b = \left[\left(B_{m1}A^{n}\right)/\left(K_{d1}^{n} + A^{n}\right)\right] + \left[\left(B_{m2}A^{n}\right)/\left(K_{d2}^{n} + A^{n}\right)\right] \quad (11)$$

2. Material and methods

Red blood cells were isolated from Wistar male rats according to a commonly used method (Manukhin et al., 1993). The obtained suspension of intact red cells was used for radioligand analysis ex tempore. Red cell membranes and ghosts were obtained by the method described elsewhere (Tomoda et al., 1984; Smurova, 1994).

Synaptosomal membranes were isolated from the rat cerebral cortex according to the method of Henn and Henn (1982), with some modifications (Nesterova et al., 1995). Membrane preparations were stored at −50°C for 2 weeks.

Protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin (Reanal, Hungary) as a standard.

Radioligand assay was carried out according to the method of Manukhin et al., (1993). For studying the parameters of ligand binding to β-adrenoceptors the following specific antagonists were used: DL-[^{3}H]propranolol hydrochloride ([^{3}H]propranolol, 25 Ci/mmol, Amersham, UK) at concentrations from 0.80 to 22.22 nM and L-[propyl-2,3,-^{3}H]dihydroalprenolol ([^{3}H]dihydroalprenolol, 60 Ci/mmol, 38 Ci/mmol, Amersham) at concentrations from 0.42 to 6.67 nM. Specific binding was determined by the difference between the total binding and the binding in the presence of 10-μM DL-propranolol hydrochloride or L-alprenolol hydrochloride. In studies of competitive radioligand displacement by the antagonist, [^{3}H]propranolol was used at a concentration of 8.19 or 2 nM. Propranolol hydrochloride at concentrations from 0.01 to 10 μM was used as the displacing agent.

Muscarinic acetylcholine receptors were characterised using a specific antagonist L-quinuclidinyl-[phenyl-4-^{3}H]-benzylate ([^{3}H]quinuclidinyl benzylate, 41.5 Ci/mmol, Amersham) at concentrations from 0.3 to 9.0 nM in the presence of 10 μM atropine.

The major parameters of ligand–receptor interactions, K_{d1}, K_{d2}, IC_{501}, IC_{502}, B_{m1} and B_{m2}, were determined using Sigma Plot 5.0 software. For the initial treatment of experimental results, plots were constructed in Scatchard and Hill co-ordinates. The Hill coefficient (n) was calculated using a computer version of the least-squares method. The efficiency of ligand binding to diverse receptor pools was evaluated by using the equation $E = B/2K_d$. The efficiency (E) is an integral parameter that quantitatively characterises ligand binding at a ligand concentration equal to K_d (Manukhin, 1968). Theoretical curves were constructed from Eq. (8) for a single receptor pool and Eq. (11) for two receptor pools (Manukhin et al., 1990). The value of the equilibrium dissociation constant for the displacing agent (K_i, inhibitor constant) was calculated from the IC_{50} value by the method of Cheng and Prusoff (1973):

$$K_i = (IC_{50})/\left[1 + (A/K_d)\right], \quad (12)$$

where A is the radioligand concentration and K_d is the equilibrium dissociation constant for the radioligand.

Each experiment provided nine to eleven experimental points in triplicate. The significance of differences was evaluated by Student's t-test ($P < 0.05$). All values are presented as means ± S.E.M.

3. Results

The binding of the specific antagonists [^{3}H]propranolol and [^{3}H]dihydroalprenolol at β-adrenoceptors on membranes, blood ghosts and native rat red cells was studied at a broad range of concentrations using graphical and mathematical methods for calculation of ligand dissociation constants and densities of binding sites.

3.1. Equilibrium binding of ligands to β-adrenoceptors on native red cells of rats

As an example, results and analytical methods are presented for a series of experiments investigating [^{3}H]propranolol binding to β-adrenoceptors on rat red cells (Manukhin et al., 1995).

The value of [^{3}H]propranolol specific binding to β-adrenoceptors on rat red cells increased in the range of radioligand concentrations of 0.2–22.2 nM. The plot of ligand binding against ligand concentration was hyperbolic (Fig. 1). The non-linearity of the plot for [^{3}H]propranolol binding to erythrocyte β-adrenoceptors in standard Scatchard co-ordinates (Fig. 2) suggested a more complex ligand–receptor interaction than [L] + [R] = [LR], i.e., occurrence of several pools of ligand binding sites and/or binding of several ligand molecules to the receptor (Takeda et al., 1997).

For further analysis of the results, a plot was constructed in Hill co-ordinates (Fig. 3). Since the simultaneous binding of ligands to high- and low-affinity receptor pools is not seen at very high or very low ligand concen-

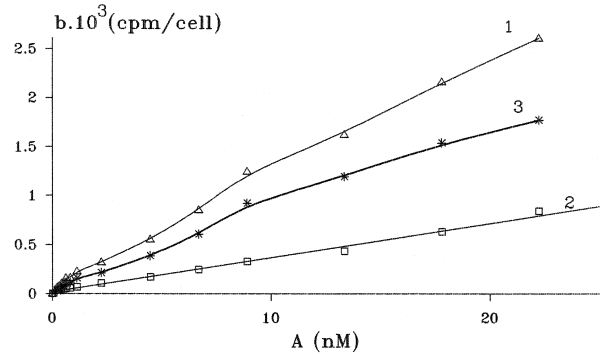

Fig. 1. Dependence of [^{3}H]propranolol binding to rat β-adrenoceptors on the ligand concentration. Abscissa: ligand concentration (nM); ordinate: density of binding sites (cpm/cell). (1) Total ligand binding; (2) non-specific binding against the background of 10 μM propranolol; (3) specific binding. Data are averaged from seven experiments.

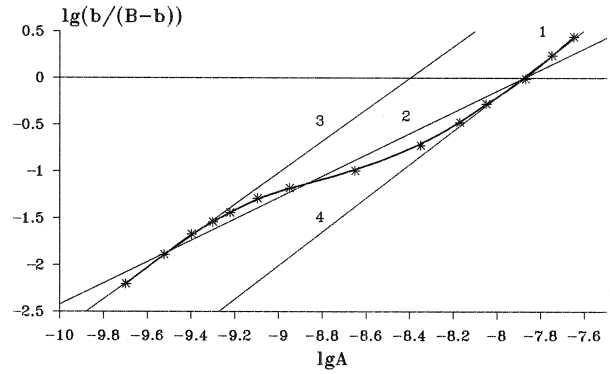

Fig. 3. Hill plots of [^{3}H]propranolol specific binding to β-adrenoceptors on rat red cells. (1) High-affinity receptor pool ($n = 2.07$); (2) low-affinity receptor pool ($n = 2.08$); (3) all receptors ($n = 1.14$). Calculated from Eq. (9).

trations, in these concentration ranges, the slope of Hill lines will approach to the number of binding sites on the receptor molecule (Chidiac et al., 1973; Dixon and Webb, 1982; Keleti, 1990; Gnagey and Ellis, 1996). In this instance (Fig. 3), the Hill coefficient for all experimental points was equal to 1.14, and for the points comprising 10% to 70% of ligand binding it was 1.45. For extreme points below 10% and above 70%, it was 2.07 and 2.06, respectively. This indicates the occurrence of two binding sites for the ligand on the receptor molecule. Therefore, the analysis of experimental results in terms of Hill co-ordinates suggests two discrete receptor pools and binding of two ligand molecules to a single receptor.

To confirm the results obtained by other methods of calculation, experimental data were mathematically analysed assuming the possible existence of one, two or more receptor pools and binding of one, two or more ligand molecules to a single receptor. The best results were obtained for the model of ligand–receptor interaction involving two heterogeneous receptor pools and assuming the addition of two ligand molecules to a single receptor. This means that the regularity of ligand–receptor binding corresponds to Eq. (1) for two pools of receptors with a heterogeneous affinity for the ligand (Table 1). Based on this calculation, experimental data are presented in $(b;b/\mathrm{A}^2)$ co-ordinates (Fig. 4). The plot of specific [^{3}H]propranolol binding constructed from mean values of seven experiments was concave, which indicates existence of two receptor pools (Molinoff et al., 1981; Gnagey and Ellis, 1996). The ligand interaction with each individual receptor pool is shown by straight asymptote lines on the plot.

To evaluate the consistency between estimated parameters and experimental data, a theoretical curve (Fig. 5) was constructed from Eq. (11) using parameters K_d, B_m and n. Experimental points (means of seven experiments) coincided well with the theoretical curve to confirm the validity of K_d and B_{max} calculations. From rearranged Eq. (11),

$$\left(b/\mathrm{A}^2\right) = \left[\left(B_{m1} + B_{m2}\right)/\left(K_{d1}^2 + K_{d2}^2\right)\right] - b/\left(K_{d1}^2 + K_{d2}^2\right)\right], \quad (13)$$

a theoretical curve was constructed using the $(b, b/\mathrm{A}^2)$ co-ordinates and the same parameters. Experimental points

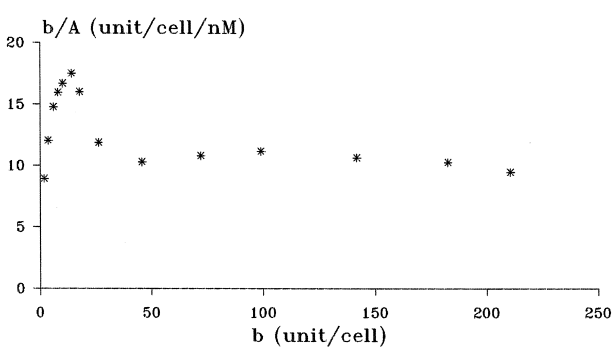

Fig. 2. Specific binding of [^{3}H]propranolol to β-adrenoceptors on rat red cells in Scatchard co-ordinates. Abscissa: density of ligand binding sites (unit/cell); ordinate: the ratio of ligand binding site density to ligand concentration (unit/cell/nM). Data are averaged from seven experiments.

Table 1
The use of different models of ligand–receptor interaction for calculating the parameters of [^{3}H]propranolol specific binding to β-adrenoceptors on native red cells from rats {7}
Notes: n = degree index for the ligand concentration in Eq. (11); K_{d1}, K_{d2} = dissociation constants for high- and low-affinity receptor pools; B_{m1}, B_{m2} = number of sites for ligand binding to high- and low-affinity receptors pools per cell; norm-square root of the sum of squared deviations of each experimental point from the estimated value. The number of experiments is indicated in braces { }.

Model	n	K_{d1} (nM)	K_{d2} (nM)	B_{m1} (unit/cell)	B_{m2} (unit/cell)	Norm
1 pool	1	82.5±15.6	–	1006±158	–	11.13
1 pool	2	10.7±0.90	–	250±15	–	32.93
2 pools	1	parameters are undefinable				
2 pools	2	0.74±0.07	14.4±0.41	24±2	263±5	5.21

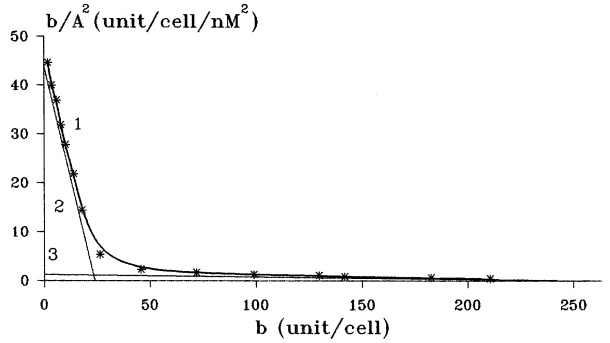

Fig. 4. Specific binding of [^{3}H]propranolol to β-adrenoceptors on rat red cells. Abscissa: density of ligand binding sites (unit/cell); ordinate: the ratio of ligand binding site density to squared ligand concentration (unit/cell/nM^2). (1) Specific binding to two receptor pools; (2) specific binding to the high-affinity pool; (3) specific binding to the low-affinity pool. Data are averaged from seven experiments.

were plotted on the theoretical curve (Fig. 4). The agreement of experimental points with those of the theoretical curve constructed in using more strict co-ordinates confirmed again the agreement between theoretical estimations and experimental data.

The results obtained allowed us to conclude that two β-adrenoceptor pools occur on native rat red cells, which differ in their dissociation constants and densities on the cell. The affinity of the high-affinity pool receptors for [^{3}H]propranolol was 20 times higher and their number was 10 times less. At the same time, the efficiency of ligand binding to the high-affinity receptor pool (E_1) was only twice as high because of different ratios between the major parameters of these two pools.

Since β-adrenoceptors are stereoselective and the [^{3}H]propranolol used by us contained both D- and L-forms, the functional characteristics of L-[^{3}H]dihydroalprenolol binding to β-adrenoceptors on native red cells were studied in the concentration range of 0.20–6.67 nM. The graphical and mathematical analysis of results was performed in the same way as in the experiments on equilibrium [^{3}H]propranolol binding. The β-adrenoceptors of native red cells were considered to exist in two discrete pools which significantly differed in their sensitivity to [^{3}H]dihydroalprenolol. The affinity of the high-affinity pool receptors was 17 times higher whereas their amounts differed only slightly (Table 2).

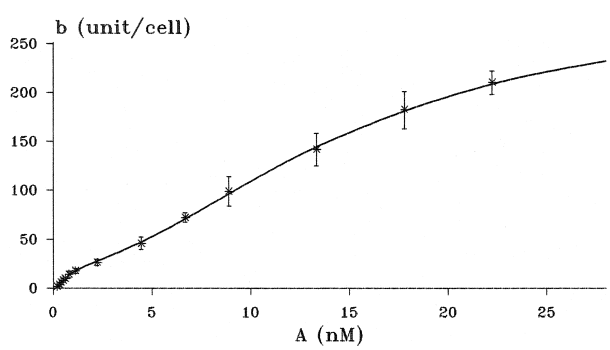

Fig. 5. Experimental points and the theoretical curve for [^{3}H]propranolol specific binding to two pools of β-adrenoceptors on rat red cells. Abscissa: ligand concentration (nM); ordinate: density of ligand binding sites (unit/cell). Theoretical curve is calculated at the following parameters: $K_{d1} = 0.74$ nM, $K_{d2} = 14.40$ nM, $B_{m1} = 24$ unit/cell, $B_{m2} = 283$ unit/cell. Data are averaged from seven experiments.

Table 2
The use of different models of ligand–receptor interaction for calculating the parameters of [^{3}H]dihydroalprenolol specific binding to β-adrenoceptors on native red cells from rats {6}
Notes: designations as in Table 1.

Model	n	K_{d1} (nM)	K_{d2} (nM)	B_{m1} (unit/cell)	B_{m2} (unit/cell)	Norm
1 pool	1	0.68 ± 0.09	–	121 ± 4	–	17.97
1 pool	2	0.46 ± 0.09	–	101 ± 5	–	39.81
2 pools	1	parameters are undefinable				
2 pools	2	0.22 ± 0.01	3.75 ± 0.44	71 ± 2	61 ± 4	5.46

Therefore, the graphical and mathematical analysis of equilibrium [^{3}H]propranolol and [^{3}H]dihydroalprenolol binding over a broad range of concentrations demonstrated that two molecules of specific antagonists bound to the β-adrenoceptor and that there were two discrete β-adrenoceptor pools on red cells, which significantly differed in their affinity to the ligand.

3.2. Equilibrium binding of ligands to β-adrenoceptors on membranes and blood ghosts from rat red cells

To determine the possible influence of intracellular factors on ligand binding to β-adrenoceptors, the ligand interaction with red cell membranes and ghosts was studied.

The plot of specific [^{3}H]dihydroalprenolol (0.5–20 nM) binding to β-adrenoceptors on isolated membranes from rat red cells was similar to that for native red cells. As in the instance with native red cells, the results obtained agreed to the greatest extent with the model of ligand–receptor interaction involving two receptor pools and assuming binding of two ligand molecules to a single receptor. The dissociation constant of high-affinity pool receptors was 9 times lower ($K_{d1} = 0.46 \pm 0.02$ nM, $K_{d2} = 3.97 \pm 0.14$ nM) whereas the number of receptors was similar in both pools (46 ± 1 and 47 ± 1 fmol/mg protein).

Another model for studying the functional characteristics of β-adrenoceptors is rat blood ghosts. Incubation of blood ghosts with increasing concentrations of the ligand (from 1 to 22 nM) led to increase in specific [^{3}H]propranolol binding, yielding a hyperbolic curve. The results obtained were consistent with there being two pools of ligand binding sites and binding of two ligand molecules to the receptor. The ligand affinity of high-affinity pool receptors was 28 times higher ($K_{d1} = 0.70 \pm 0.17$ nM and $K_{d2} = 19.59 \pm 2.59$ nM) while their number on the membrane was four times lower ($B_{m1} = 9 \pm 1$ fmol/mg pro-

tein, $B_{m2} = 39 \pm 4$ fmol/mg protein). The opposite ratios between the major parameters of different pools resulted in that the efficiency of ligand binding to the high-affinity receptor pool (E_1) being only six times higher.

Comparison of results on the equilibrium binding of [^{3}H]propranolol and [^{3}H]dihydroalprenolol to β-adrenoceptors on red cell membranes, blood ghosts and intact cells showed that the membrane isolation considerably reduced the number of ligand binding sites in both the high- and low-affinity receptor pools. It has been established that 1 mg of isolated membrane protein corresponds to 4×10^9 red blood cells. For high- and low-affinity pools of β-adrenoceptors on isolated membranes, the amount of [^{3}H]dihydroalprenolol binding sites comprised 7 units per individual red cell. The affinity of high-affinity pool receptors for [^{3}H]dihydroalprenolol was higher for intact preparations than for membrane preparations while for the low-affinity receptor pool, the affinity was similar (Table 2). In experiments with β-adrenoceptors of blood ghosts, the amount of detectable [^{3}H]propranolol binding sites per individual red cell comprised 2 unit/cell for the high-affinity pool and 6 unit/cell for the low-affinity pool. The decrease in adrenoceptor density may be due to the methodical procedures inactivating some of the receptors.

Graphical and mathematical analyses of equilibrium [^{3}H]propranolol and [^{3}H]dihydroalprenolol binding over a broad range of ligand concentrations revealed agreement of the major characteristics in the ligand–β-adrenoceptor interaction on membranes, blood ghosts and native red cells. It was found that a single receptor bound two molecules of the specific antagonist and that there were two discrete pools of β-adrenoceptors that differed in their affinity for the ligand. Isolating of the membranes and red cell ghosts decreased the number of binding sites for specific antagonists whereas the dissociation constants of the ligand–receptor complex changed to a lesser extent.

3.3. Competitive displacement by propranolol of [^{3}H]propranolol bound to β-adrenoceptors on native red cells from rats

Mathematical and graphical analyses of the competitive displacement of [^{3}H]propranolol (8 nM) by propranolol (0.01–10 μM) also revealed a two-component system consistent with the existense of two β-adrenoceptor pools and the binding of two ligand molecules to a single receptor (Table 3). A good agreement was observed between experimental values and the theoretical curve constructed from Eq. (10) in semilogarithmic co-ordinates (Fig. 6). This result confirmed once more the validity of the estimated parameters of the competitive displacement of [^{3}H]propranolol bound to red cell β-adrenoceptors. The presence of two receptor pools was observed in all 11 experiments.

The IC_{50} value determined in the competitive displacement experiments is not equal to the inhibitory constant of the displacing agent. Under certain conditions, the K_i is calculated from the IC_{50} by Eq. (12) using the method of Cheng and Prusoff (1973). At the radioligand concentration of 8 nM, the calculated inhibitory constant of displacing agent was 0.05 μM for the high-affinity receptor pool and 1.98 μM for the low-affinity receptor pool. These values considerably differed from the K_d values obtained for propranolol. The K_i value depends on the radioligand concentration, incubation time (Gnagey and Ellis, 1996) and the pattern of ligand interaction with the receptor molecule (Craig, 1993; Leff and Dougall, 1993). This is why we carried out experiments in which [^{3}H]propranolol (2 nM) was displaced by propranolol (0.01–0.4 μM) (Table 4). At the radioligand concentration of 2 nM calculated by the Cheng–Prusoff method, the propranolol K_i was 1.2 nM for the high-affinity receptor pool and 126.4 nM for the low-affinity receptor pool. Equilibrium dissociation constants of [^{3}H]propranolol were 0.74 ± 0.07 and 14.40 ± 0.41 nM for the high- and low-affinity receptor pools, respectively. The K_d and K_i values were close to each other only for the high-affinity receptor pool. The presented data, together with data from the literature (Gnagey and Ellis, 1996), provide evidence that it is possible to calculate the K_i of the displacing agent if the radioligand concentration is close to its K_d and the displacing agent is used in a narrow range of concentration affecting primarily a single receptor pool. Such conditions allow one to calculate the K_i value for the substance under investigation, which is close to the actual one. The K_i determined by the Cheng–Prusoff method depends to a considerable extent on experimental conditions and therefore characterises only the relative specific activity of the displacing agent.

The computer and graphical analysis demonstrate both qualitative and quantitative agreement of the data obtained in experiments on the competitive displacement and equilibrium binding of [^{3}H]propranolol to β-adrenoceptors on rat red cells. In both instances, the best result was obtained on the assumption that there are two heterogeneous pools of receptors where each receptor binds two ligand molecules. In experiments on the competitive displacement

Table 3
The use of different models of ligand–receptor interaction for calculating the parameters of the propranolol competitive displacement of [^{3}H]propranolol (8 nM) bound to β-adrenoceptors on native red cells from rats {11}

Notes: IC_{501}, IC_{502} = displacing agents concentrations which half-inhibit the specific binding of radioligand to high- and low-affinity receptor pools; other designations as in Table 1.

Model	n	IC_{501} (μM)	IC_{502} (μM)	B_{m1} (unit/cell)	B_{m2} (unit/cell)	Norm
1 pool	1	1.11 ± 0.04	–	88 ± 1	–	3.97
1 pool	2	0.81 ± 0.04	–	74 ± 1	–	10.80
2 pools	1	parameters are undefinable				
2 pools	2	0.56 ± 0.02	3.11 ± 0.19	53 ± 2	27 ± 1	1.33

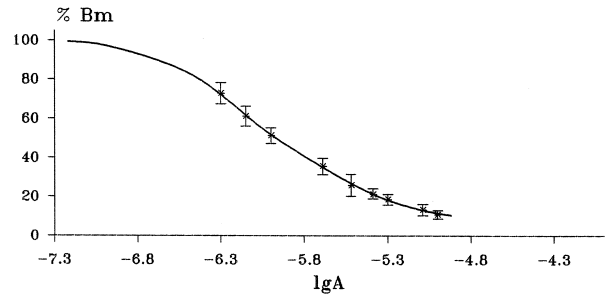

Fig. 6. Experimental points and the theoretical curve of competitive displacement of [^{3}H]propranolol by propranolol (8 nM). Abscissa: logarithm of displacing agent concentration (lg A); ordinate: specific binding of radioligand in percent of the control. Theoretical curve was calculated from the following parameters: $IC_{501} = 0.58$ μM, $IC_{502} = 3.11$ μM, $B_{m1} = 53$ unit/cell, $B_{m2} = 27$ unit/cell. Data are averaged from seven experiments.

of 8 nM [^{3}H]propranolol by propranolol, it was found that there were 81 adrenoceptors per red cell. In the equilibrium binding of [^{3}H]propranolol (8 nM), the adrenoceptor number was determined as 88 units. Theoretical calculations using Eq. (11) for the equilibrium binding of 2 nM [^{3}H]propranolol revealed 26 receptors on a cell, whereas propranolol displaced 2 nM [^{3}H]propranolol from 24 binding units on the cell at maximum. Therefore, the two methods for analysis of the ligand–receptor interaction (equilibrium binding and competitive displacement) provide, under definite conditions, consistent results for the estimated amount of receptors and can be used in experiments.

Further, similar investigations were performed on another common model, membranes from the rat cerebral cortex.

3.4. Equilibrium binding of [^{3}H]dihydroalprenolol to β-adrenoceptors on synaptosomal membranes from the rat cerebral cortex

Specific binding of [^{3}H]dihydroalprenolol (0.5–20 nM) to isolated membranes from the rat cerebral cortex is saturable. Mathematical and graphical analyses of experimental data showed that the obtained results were best matched to the model of ligand–receptor interaction involving two receptor pools, which assumes binding of two ligand molecules to a receptor (Table 5). The participation of two ligand molecules in the reaction was supported by the value of the Hill coefficient, which was 1.15 for all experimental points and 1.95 and 1.98 for the points below 10% and above 70%. In order to confirm the agreement of the parameters calculated from Eq. (11) with the data obtained, a theoretical curve was constructed, which matched the experimental points, as it did in previous experiments.

The two receptor pools significantly differed from each other in terms of all the parameters under analysis. The affinity of the high-affinity pool receptors for the ligand was 10 times higher while number of the receptors on the membrane was twice as less. The efficiency (E) of ligand binding to the high-affinity receptor pool was six times higher. Heterogeneity of [^{3}H]dihydroalprenolol binding sites was found in all experiments.

Therefore, the pattern of major characteristics of the ligand–receptor interaction was similar for β-adrenoceptors on the cerebral cortex membrane and red cells from rats.

Table 4
The use of different models of ligand–receptor interaction for calculating the parameters of the propranolol competitive displacement of [^{3}H]propranolol (2 nM) bound to β-adrenoceptors on native red cells from rats {6}
Notes: designations as in Table 3.

Model	n	IC_{501} (nM)	IC_{502} (nM)	B_{m1} (unit/cell)	B_{m2} (unit/cell)	Norm
1 pool	1	20.9 ± 3.8	–	23 ± 1	–	5.63
1 pool	2	12.3 ± 3.2	–	21 ± 1	–	10.40
2 pools	1	parameters are undefinable				
2 pools	2	4.4 ± 0.4	143.8 ± 8.5	14 ± 0.3	10 ± 0.3	0.73

Table 5
The use of different models of ligand–receptor interaction for calculating the parameters of the [^{3}H]dihydroalprenolol specific binding to β-adrenoceptors on membranes from the rat cerebral cortex {4}
Notes: designations as in Table 1.

Model	n	K_{d1} (nM)	K_{d2} (nM)	B_{m1} (fmol/mg)	B_{m2} (fmol/mg)	Norm
1 pool	1	5.29 ± 0.51	–	84 ± 3	–	6.28
1 pool	2	3.17 ± 0.43	–	63 ± 4	–	18.74
2 pools	1	0.63 ± 1.59	8.48 ± 5.14	11 ± 20	82 ± 13	4.97
2 pools	2	0.74 ± 0.09	7.63 ± 0.70	25 ± 2	48 ± 2	3.41

3.5. Equilibrium binding of [^{3}H]quinuclidinyl benzylate to muscarinic acetylcholine receptors on membranes from the rat cerebral cortex

Four subtypes of muscarinic acetylcholine receptors have been found in the cerebral cortex (Gnagey and Ellis, 1996). A non-selective antagonist [^{3}H]quinuclidinyl benzylate was used for studying the characteristics of muscarinic acetylcholine receptor binding to the ligand (Brann et al., 1993).

The specific binding of [^{3}H]quinuclidinyl benzylate to muscarinic acetylcholine receptors on membranes from the rat cerebral cortex was saturable (Fig. 7). The Hill coefficient calculated for all experimental points was 1.43. The value of the Hill coefficient $1 < n < 2$ indicated the occurrence of at least two discrete receptor pools and binding of more than one ligand molecule to each receptor. Therefore, experimental results were analysed on the assumption that either one or several receptor pools exist and that one

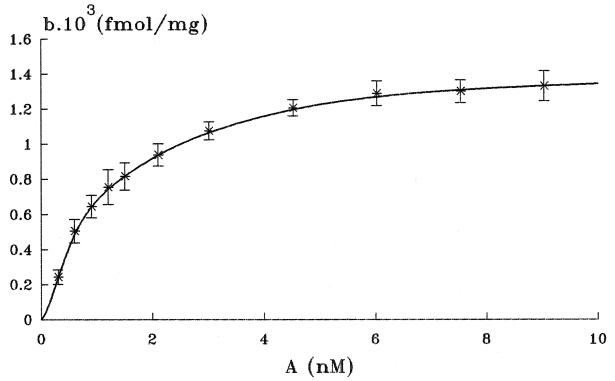

Fig. 7. Experimental points and the theoretical curve for [^{3}H]quinuclidinyl benzylate specific binding to muscarinic acetylcholine receptors on membranes from rat cerebral cortex. Abscissa: ligand concentration (nM); ordinate: amount of ligand binding sites (fmol/mg). Theoretical curve was calculated from the following parameters: $K_{d1} = 0.43$ nM, $K_{d2} = 2.83$ nM, $B_{m1} = 712$ fmol/mg, $B_{m2} = 677$ fmol/mg. Data are averaged from seven experiments.

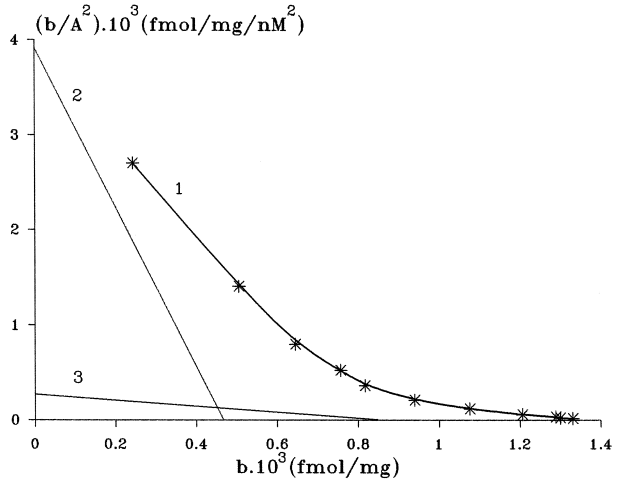

Fig. 8. Specific binding of [^{3}H]quinuclidinyl benzilate to muscarinic acetylcholine receptors on membranes from rat cerebral cortex. Abscissa: number of ligand binding sites (fmol/mg); ordinate: the ratio of ligand binding site number to squared ligand concentration (fmol/mg/nM^2). (1) Specific binding to two receptor pools; (2) specific binding to the high-affinity receptor pool; (3) specific binding to the low-affinity receptor pool. Data are averaged from seven experiments.

receptor binds either one of several ligand molecules. An optimum model of ligand–receptor interaction includes two receptor pools heterogeneous in their affinity for the ligand and assumes the binding of two ligand molecules to a single receptor (Table 6), i.e., corresponds to Eq. (11). Figs. 7 and 8 demonstrate theoretical curves constructed from Eqs. (11) and (13) and parameters from Table 6. Experimental points were plotted on the theoretical curves. The good agreement of experimental data with the theoretical data confirmed the validity of calculations. Asymptotes (Fig. 8) showed the regularity of ligand binding to high- and low-affinity receptor pools. The affinity of the receptors of the high-affinity pool was six times higher whereas the receptor density was only slightly higher than the receptor density of the low-affinity pool.

Therefore, the graphical and mathematical analysis of [^{3}H]quinuclidinyl benzylate binding over a broad concentration range demonstrated that a muscarinic acetylcholine receptor bound two molecules of the specific antagonist and the rat cerebral cortex contained two discrete pools of muscarinic acetylcholine receptors, which significantly differed in their affinity to the ligand.

Table 6
The use of different models of ligand–receptor interaction for calculating the parameters of the [^{3}H]quinuclidinyl benzilate specific binding to muscarinic acetylcholine receptors on membranes from the rat cerebral cortex {8}
Notes: B_{m1}, B_{m2} = number of sites for ligand binding to high- and low-affinity receptors pools (fmol/mg protein), other designations as in Table 1.

Model	n	K_{d1} (nM)	K_{d2} (nM)	B_{m1} (fmol/mg)	B_{m2} (fmol/mg)	Norm
1 pool	1	1.29 ± 0.05	–	1537 ± 18	–	64.6
1 pool	2	0.93 ± 0.08	–	1261 ± 41	–	278.8
2 pools	1	parameters are undefinable				
2 pools	2	0.43 ± 0.02	2.67 ± 0.13	712 ± 22	677 ± 19	22.6

4. Discussion

Using different methods to analyse the experimental results, we identified two receptor pools, low- and high-affinity ones, and demonstrated binding of two ligand molecules to a single receptor in several models. The two receptor pools are, in this instance, not different subtypes of β-adrenoceptors and muscarinic acetylcholine receptors because quinuclidinyl benzylate, propranolol and dihydroalprenolol are non-selective agonists and possess similar affinity for all subtypes of these receptors (Burgiser and Lefkowitz, 1984). Data in the literature also indicate heterogeneity of receptors of the same pharmacological subtype in their affinity for agonists and antagonists. For instance, high- and low-affinity [^{3}H]quinuclidinyl benzylate binding to muscarinic acetylcholine receptors has been shown on membranes from rat and golden hamster myocardium (Berrie et al., 1979; Chidiac et al., 1997), cerebral cortex and striatum (Aguilar et al., 1982; Roffel et al., 1991). The occurrence of two pools of binding sites for [^{125}I]iodocyanopindolol and [^{3}H]dihydroalprenolol has been demonstrated for β_2-adrenoceptors on human leukocytes (Haen et al., 1991), rat cerebral cortex (Molinoff et al., 1981) and β_1-adrenoceptors of turkey intact red cells (Andre et al., 1981), respectively.

At the same time, a number of studies have revealed only a single receptor pool in the same models. We believe that the non-selective antagonist [^{3}H]quinuclidinyl benzy-

late binds to a single pool of muscarinic acetylcholine receptors on cerebral membranes (Yamamura and Snyder, 1974; Gilbert et al., 1979). Generally, receptor heterogeneity manifests itself only with the use of antagonists over a broad range of concentrations (Sandnes et al., 1987; Anhaupl et al., 1988).

The functional heterogeneity of β-adrenoceptors depends apparently on the localisation of a certain portion of receptors in specialised membrane regions, where the receptor interaction with the regulatory protein is facilitated (Severne et al., 1986). It has been also suggested that muscarinic acetylcholine receptors with a high affinity for the ligand are physiologically relevant (Kaambre et al., 1984). Two saturable pools and one unsaturable pool of [^{125}I]iodocyanopindolol binding sites were found on β_2-adrenoceptors of human lymphocytes (Haen et al., 1991). In this study, the plot of specific ligand binding in Scatchard co-ordinates was similar to the curve obtained in our experiments with regard to equilibrium [^{3}H]propranolol binding to β_2-adrenoceptors on native red cells from rats (Fig. 2).

There are far fewer data in the literature on the binding of two molecules to a single receptor. This possibility is indirectly supported by the experimental data obtained on membranes from the myocardium (Wreggett and Wells, 1995) and shockfish *Torpedo califonica* (Dunn and Raftery, 1997), where Hill coefficients were more than 1.

It has been recently reported that the binding of [^{3}H]quinuclidinyl benzylate and other ligands to muscarinic acetylcholine receptors has a co-operative nature (Jarv, 1995; Lazareno and Birdsall, 1995; Wreggett and Wells, 1995; Chidiac et al., 1997). The concept of functioning of G-protein linked receptors assumes that ligands bind to two different binding sites which are simultaneously present on the receptor molecule, with formation of a triple complex including a receptor and two ligand molecules.

Two receptor pools are undetectable or can be detected unreliably if their K_d values are similar or at a ligand concentration which activates primarily the low- or high-affinity pool. Therefore, the use of graphical analytical methods alone often does not reveal the non-linearity of experimental point displacement in Hofsty, Scatchard, Laniwiver–Berk, Hill, etc. co-ordinates. Two receptor pools are well detected using the LIGAND computer program (Munson and Rodbard, 1980). However, the program does not provide for determination of more than one molecule binding to the binding site. The use of mathematical software that allows one to solve Eq. (11) provides a possibility for determining the number of discrete receptor pools and major parameters of the ligand–receptor binding reaction (K_d, B_{max}, n).

On the whole, the analysis of the equilibrium binding of [^{3}H]propranolol and [^{3}H]dihydroalprenolol to β-adrenoceptors on membranes, blood ghosts and native red cells from rats, [^{3}H]dihydroalprenolol to β-adrenoceptors and [^{3}H]quinuclidinyl benzylate to muscarinic acetylcholine receptors on synaptosomal membranes from the rat cerebral cortex showed that receptors bound two ligand molecules each and were represented by two discrete pools, one with high-affinity receptors and one with low-affinity receptors.

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