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REVISED RADIORECEPTOR ASSAY FOR $ß_2$-ADRENOCEPTORS EXPRESSED ON PERIPHERAL MONONUCLEAR LEUKOCYTES

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ABSTRACT

Peripheral mononuclear leukocytes (pMNL) bear a population of $ß_2$-adrenoceptors. Radioreceptor assays with (-)-^{125}Iodocyanopindolol (^{125}I-CYP) are often used to determine the expression of these hormone receptors under physiological and pathological conditions. Doubts on the occurence of just one class of binding sites as well as the availability of new laboratory equipment prompted us to revise the procedure employed for investigation of these receptors. pMNL were harvested from venous human blood by density centrifugation with Lymphopaque®, Lymphoprep®, or Ficoll® yielding immunologically distinct pMNL fractions. Receptor binding assays were performed semi-automatically with ^{125}I-CYP in the range from 0.6-600 pmol/l. Analysis of the data (modified affinity spectra, Scatchard plot) revealed two classes of binding sites (high- and low affinity binding). The binding isotherms were sigmoidal in the concentration range from 0.6-3.0 pmol/l. Parameters estimated for the high affinity binding site may vary by a factor of 10, depending on the mathematical model employed.

INTRODUCTION

The expression of $ß_2$-adrenoceptors is often investigated in clinical research on peripheral mononuclear leukocytes (pMNL), with the aim to correlate changes in their characteristics to the pathophysiology and pharmacotherapy of hypertensive (1,2,3,4,5) and asthmatic (6,7,8,9,10) diseases. The estimated binding parameters depend largely on the assay method and also on the mathematical model employed for analysis of the binding isotherm. Until very recently, the evaluation of radioreceptor assays for $ß_2$-adrenoceptors was based on the assumption of one homogenous class of binding sites for the radioligand ^{125}Iodo-cyanopindolol (^{125}I-CYP) on pMNL (11,12). In such

a case, the so-called "Scatchard analysis" is frequently believed to be a suitable method for estimating binding parameters. Sandnes et al. (13) and Anhäupl et al. (14), however, reported strong evidence for the existence of two classes of binding sites for ^{125}I-CYP on pMNL, yielding curvilinear Scatchard plots. This requires nonlinear regression analysis of the binding data and, as far as the diagnostic use is concerned, standard conditions for the binding experiment. Therefore, we carried out a series of experiments in order to optimalize assay conditions for the determination of β_2-adrenoceptor expression on pMNL that would be suitable for clinical research.

MATERIALS AND METHODS

Isolation of human peripheral mononuclear leukocytes (pMNL)

Venous blood was withdrawn from the antecubital vein of healthy subjects into a syringe containing 10 mg/ml EDTA between 9:00 and 10:00 a.m. (15). Immediately after venipuncture, intact peripheral mononuclear leucocytes were harvested by density centrifugation for 30 minutes at 4°C and 1600 x g using Lymphopaque^R, Lymphoprep^R (both supplied by Nyegaard & Co. AS, Oslo/Norway) or Ficoll^R (Biochrom GmbH & Co, Berlin/FRG). pMNL were washed twice in HH buffer pH 7.4 (HEPES buffered Hank's salt solution: 1.26 mmol/l $CaCl_2$, 5.4 mmol/l KCl, 0.44 mmol/l KH_2PO_4, 0.8 mmol/l $MgSO_4$, 136.9 mmol/l NaCl, 0.42 mmol/l Na_2HPO_4, 4.3 mmol/l $NaHCO_3$, 25 mmol/l HEPES; the reagents were purchased from Merck Darmstadt/FRG) and finally resuspended in HH buffer at a concentration of 5 x 10^6cells/ml. Cells were counted both in a Coulter Counter (Coulter Electronics, Düsseldorf/FRG) and in a light microscope (Neubauer chamber). Using the trypan blue exclusion method, the proportion of non-viable cells (i.e. the cell fraction stained by the dye) was less than 5%.

Quantitative evaluation of lymphocyte subpopulations

Five portions (100 µl) of the pMNL suspension were incubated for 30 min at 4°C in the dark with 10 µl of commercially available FITC (fluorescein-isothiocyanate)-labelled monoclonal antibodies (Becton & Dickinson GmbH, Heidelberg/FRG). The antibodies were directed

against cell surface epitopes defined as CD3 (T-lymphocytes), CD4 ($T_{helper/inducer}$-cells), CD8 ($T_{suppressor/cytotox.}$-cells), CD14 (monocytes/macrophages), and CD19 (B-lymphocytes), respectively (CD, cluster definition). After washing out the excess antibody with buffered saline, labelled cells were fixed in 0.1% paraformaldehyde and stored at 4°C. Within one week of preparation, cells were run on a fluorescence-activated flow cytometer (FACScan^R, Becton & Dickinson GmbH, Heidelberg/FRG) to determine the fraction of fluorescence-labelled cells (% of gated cells). Forward and right angle light scatter gates were set to include mononuclear cells.

Radioreceptor assay

100 µl of the pMNL suspension were incubated in a total reaction volume of 1000 µl for 2h at 37°C with various concentrations of (-)-^{125}Iodocyanopindolol (^{125}I-CYP, 2000 µCi/mmol, Amersham Buchler, Braunschweig/FRG) in the range from 0.6-600 pmol/l. The reaction was started by manual addition of the lymphocyte suspension and stopped by centrifugation for 10 min at 10000 x g in an Eppendorf-Hermle centrifuge Model Z 380 (Eppendorf Hamburg/FRG). Radioactivity of the pellets (total binding T) was assessed by gamma-counting. Unspecific binding (U) of the radioligand was determined in parallel incubations with 10^{-5} mol/l (-)-timolol. Timolol was added in a volume of 20 µl. If not stated otherwise, a Tecan Robotic Sample Processor Model 5052 (dual arm system; Zinsser Analytic - Tecan Robotics, Frankfurt/Main FRG) was used for pipetting; software designed by B. Liebl for this type of experiments was employed.

Data analysis

Specific binding was calculated as a difference between total and unspecific binding (c_b = T - U). A binding equation for two independent classes of binding sites (see below) was fitted to the data by nonlinear regression analysis (16, or the commercially available programms Enzfitter^R or Sigmaplot^R) yielding estimates of the binding parameters for the high and low affinity binding sites. Prior to this computation, "affinity spectra" were assessed using a recently described computational procedure (17).

Table 1

Fractional distibution of lymphocyte subsets in venous blood obtained from three healthy men (28-36 years of age). Blood was withdrawn between 9:00 and 10:00 a.m.
(% of gated cells ± standard error of the mean)

	Lymphopaque^R	Lymphoprep^R	Ficoll^R
CD3	61.7 ± 3.8	66.2 ± 2.8	70.8 ± 2.0
CD4	33.0 ± 3.5	36.3 ± 4.2	40.1 ± 4.4
CD8	21.4 ± 3.0	21.7 ± 1.7	23.0 ± 1.6
CD14	15.4 ± 2.0	15.1 ± 3.0	14.3 ± 1.7
CD19	5.8 ± 1.2	8.1 ± 2.2	9.2 ± 2.0

CD3: pan T-lymphocytes; CD4: $T_{helper-inducer}$-cells;
CD8: $T_{suppressor/cytotox.}$-cells; CD14: monocytes/macrophages;
CD19: B-lymphocytes

RESULTS

Isolation of peripheral mononuclear leucocytes (pMNL)

Density centrifugation with Lymphopaque^R (density of 1.086 g/ml) yielded more cells than density centrifugation with Lymphoprep^R or Ficoll^R (density of 1.077g/ml). The fractional distribution of lymphocyte subsets was different for the three commercially available separation media (Tab. 1). In general, Lymphopaque^R yielded less T- and B- lymphocytes (CD3-, CD4-, CD8-, and CD19-positive cells, respectively) but slightly more monocytes (CD14-positive cells).
Cell counts in the Coulter Counter were somewhat larger than in the Neubauer chamber, presumably because some small detriments and erythrocytes were still detected in the sample. If the gate on the Coulter Counter was set to allow the passage of just mononuclear cells, cell counts were identical in both methods.

Radioreceptor assay

Affinity spectra investigated in a concentration range from 0.6 - 600 pmol/l ^{125}I-CYP revealed several classes of binding sites (Fig. 1), two of them being significant in the sense mentioned in our

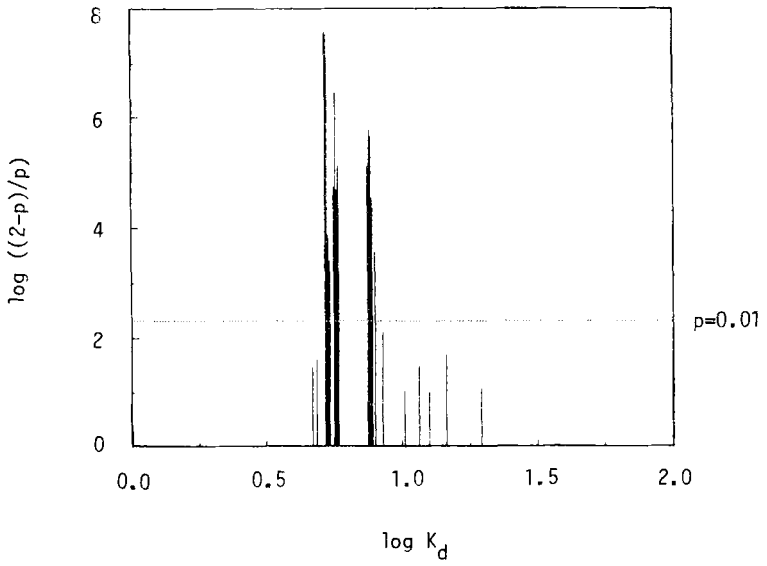


Fig. 1. Affinity spectra for the binding of ^{125}I-CYP to human peripheral mononuclear leukocytes in a concentration range of 0.6-600 pmol/l obtained by the STEP routine (17). Vertical lines denote estimates of Kd's (abscissa) from partial sequences of binding data. Ordinate: probability scale that expresses the significance of such an estimate (p is the significance of correlation coefficient for the "linearized Scatchard plot", cf ref. 17). Two clusters of binding sites (significance $p < 0.01$) can be seen in the graph.

earlier report (17). Binding isotherms within this concentration range were sigmoidal at low ligand concentrations, yielding a "paradoxical" course (an increase) in the corresponding Scatchard plot (Fig. 2). This confirms the existence of a multiple binding site population on pMNL, and suggests that the Hill coefficient of at least the high affinity binding site is larger than one. Thus, we have employed for nonlinear regression analysis a mathematical model of two classes of saturable binding sites assuming that both Hill coefficients may potentially differ from one:

$$c_b = B_{m1} * c_f^{n1} / (K_{d1} + c_f^{n1}) + B_{m2} * c_f^{n2} / (K_{d2} + c_f^{n2}) \qquad (1)$$

In this equation, B_m stands for maximal binding capacity of the cor-

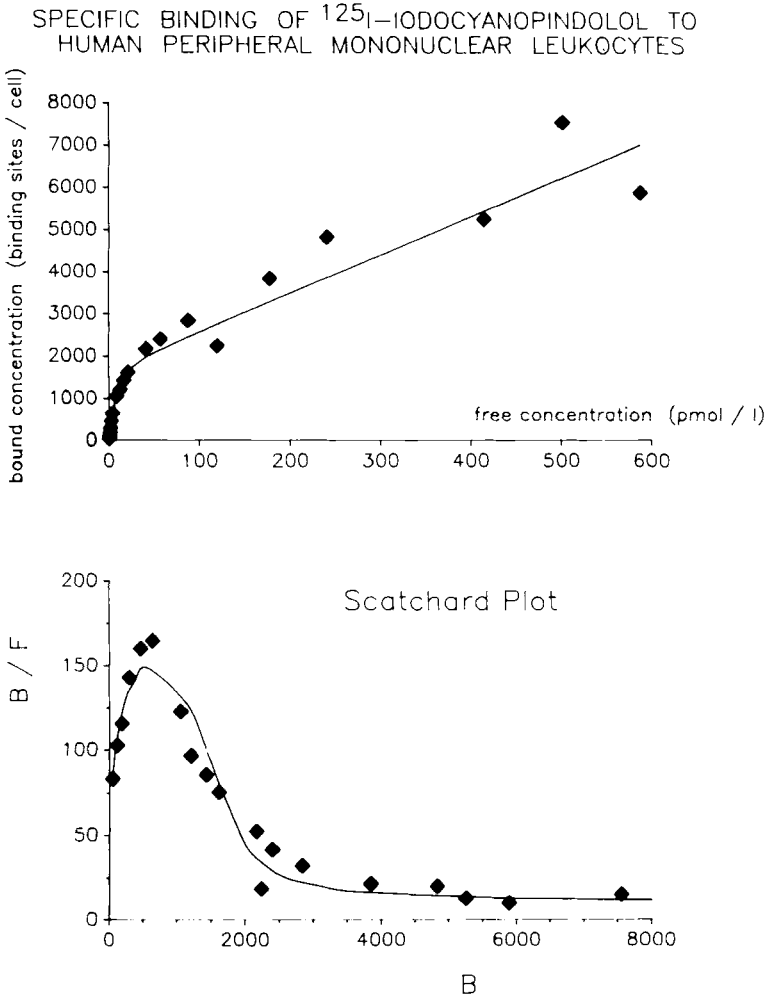


Fig. 2. Binding isotherm for the binding of ^{125}I-CYP to human peripheral mononuclear leukocytes in a concentration range of 0.6 - 600 pmol/l. Upper panel: Direct plot of the binding data (free/bound concentration). Lower panel: Scatchard plot. The mathematical model of one class of saturable binding sites (high affinity binding) and one class of non-saturated (or "unsaturable") binding sites (low affinity binding) including a Hill coefficient (equation 2) was iteratively fitted to the binding data.

Table 2

Binding parameters (parameter value ± standard error estimated by nonlinear regression analysis) of the high and low affinity ^{125}I-CYP binding sites expressed on pMNL obtained from a woman, 26 years of age. Radioreceptor assay with 19 concentrations of ^{125}I-CYP in the range of 0.6 - 600 pmol/l. Blood was withdrawn at 9:00 a.m.

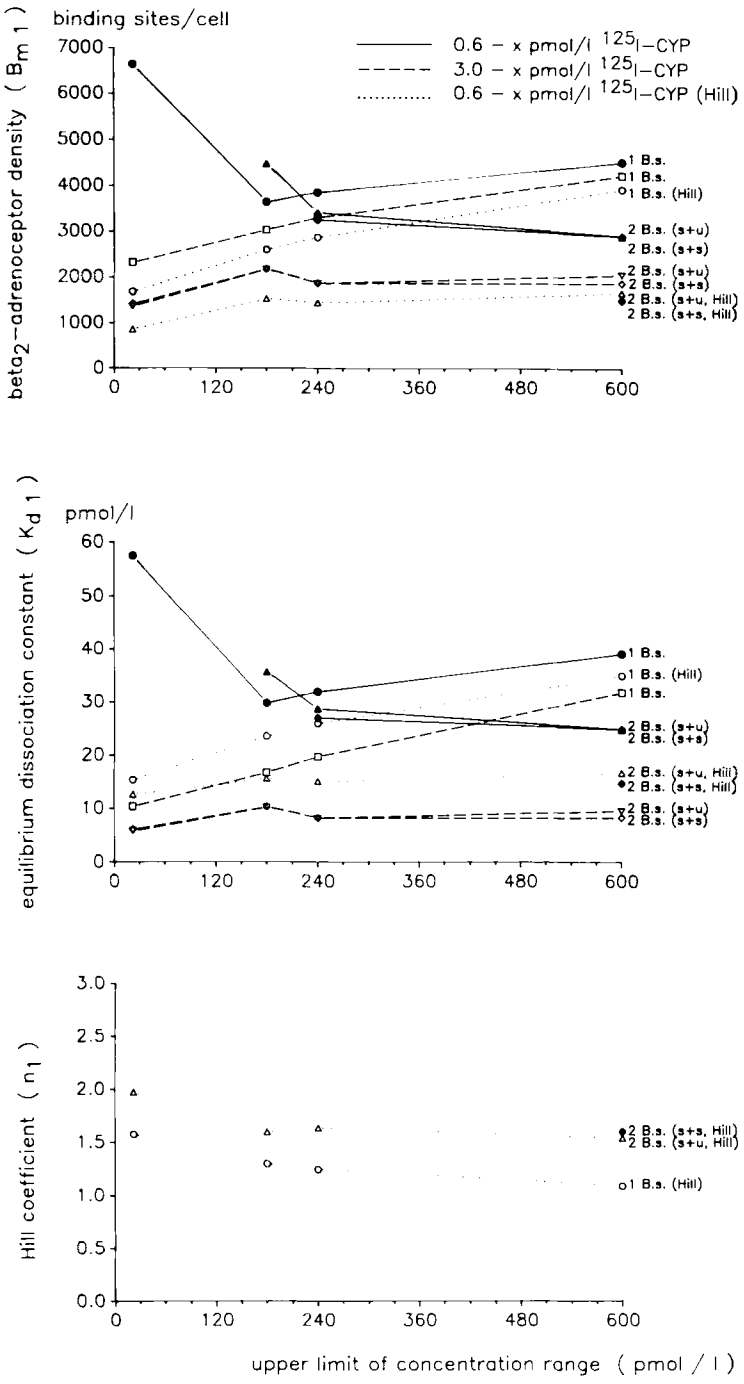
	2 saturable binding sites		1 saturable, 1 "unsaturable" binding site	
B_{m1}	1513 ± 692	sites/cell	1677 ± 185	sites/cell
K_{d1}	14.76 ± 2.47	pmol/l	16.75 ± 2.16	pmol/l
n_1	1.59 ± 0.27		1.53 ± 0.11	
B_{m2}	10062 ± 17984	sites/cell	------	
K_{d2}	1485 ± 5612	pmol/l	------	
n_2	1.14 ± 1.05		------	
C	------		9.08 ± 1.06	sites*l/cell/pmol

responding site, K_d for its dissociation constant and n for the Hill coefficient; indices 1 and 2 denote high and low affinity sites, respectively. Binding parameters estimated by nonlinear regression analysis (see above) are given in Tab. 2.

Table 2 indicates that the affinity of the low affinity binding site differs from the high affinity binding site by a factor of 100. K_d of the second binding site lies obviously above the upper limit of the ligand concentration range employed in the experiments. Parameter estimates are thus rather uncertain for the low affinity binding site, as seen from the large standard errors estimated by nonlinear regression analysis. We have therefore applied a simplified mathematical model, suitable for the combination of one saturable (high affinity) binding site with a non-saturated (or "unsaturable") low affinity binding site. In this model, the second term is replaced by a term expressing the binding linearly proportional to the ligand concentration (C is an empirical proportionality constant):

$$c_b = B_{m1} * c_f^{n1} / (K_{d,1} + c_f^{n1}) + C * c_f \qquad (2)$$

It should be mentioned that in case of non-saturated low affinity sites ($c_f << K_{d2}$) the new term should be precisely $(B_{m2} / K_{d2}) * c_f^{n2}$. However, since the estimated n_2 seems to be close to one (Tab. 2), linearity can be assumed. In Tab. 2, the C constant (9.08) is indeed close to the ratio B_{m2} / K_{d2} (6.8).



Without further statistical testing, it is obvious that estimates of binding parameters for the high affinity binding site, obtained by the two mathematical models, are very similar. The use of equation 2 in the ^{125}I-CYP concentration range of 0.6 - 600 pmol/l appears therefore more adequate.

DISCUSSION

Many clinical studies on the pathophysiology and pharmacotherapy of hypertension and bronchial asthma focus on the expression of ß-adrenoceptors. The receptor parameters are usually determined on blood cells (peripheral mononuclear leukocytes) that may be easily and repeatedly collected without causing undue discomfort to the patients. The estimated parameters, however, are highly variable and depend on methodology and concept of the performed assays, as well as on the reagents used.

For example, different $ß_2$-adrenoceptor densities have been described for various lymphocyte subpopulations (12,18,19). Results of radioreceptor assays may thus be influenced by variations in the fractional distribution of lymphocyte subsets in the pMNL fraction isolated from venous blood for the assays. This study demonstrates that, apart from physiological variations, this might occur by using different commercially available separation media for cell harvesting.

Until very recently, most of these studies have been carried out in a limited concentration range of the radioligand (10-150 pmol/l (11), or 5-80 pmol/l (9)). Estimates of binding parameters assumed the occurence of just one class of binding sites (high affinity binding). Such investigations led to the description of a positive correlation between the expression of $ß_2$-adrenoceptors (number of high affinity binding sites per cell) and the elevation of blood

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Fig. 3. Relationship between value of statistically estimated parameters for the high affinity ^{125}I-CYP binding site on pMNL, concentration range investigated (highest concentration indicated on x-axis), and mathematical model employed for the analysis of the binding isotherm.
1 B.s.: one saturable binding site; 2 B.s.: two independent, saturable binding sites; s + s: saturated high affinity and saturated low affinity binding sites; s + u: saturated high affinity and non-saturated low affinity binidng sites; "Hill": Hill coefficients were fitted).

pressure in hypertension (1,3). Reinhardt et al., however, noted in 1984 great variations in ^{125}I-CYP binding at higher concentrations (9). Extension of the concentration range to higher concentrations of the radioligand soon revealed a second, independent class of binding sites (13,14). Until now, the biological significance of these low affinity binding sites has not been clear. It has been suggested that they could represent a reduced receptor state resulting from the agonist-receptor interaction in vivo; the high affinity binding site is generally assumed to be the functional β_2-adrenoceptor (13,20).

The equilibrium dissociation constant of these low affinity binding sites for ^{125}I-CYP is in the order of 400 - 1500 pmol/l. An exact determination of the binding parameters of the low affinity binding site affords radioligand concentrations up to 2-8 nmol/l. The high radioligand concentrations present a considerable radiation exposure for the persons involved in the experiment. By employing the sample processor, radioactive exposure was minimized to the time of transfering the reagent blocks from the sample processor to the water - bath, then to the centrifuge, and back to the sample processor. After preparing the radioligand working solution, no further direct contact with radioactivity is necessary.

Nevertheless, under clinical conditions such assays are limited by the large quantity of cells needed for the analysis and by the high costs for the radioligand. Therefore, we suggest to carry out the radioreceptor assays in a concentration range from 0.6 - 150 pmol/l and to evaluate the binding data by employing the mathematical model of saturable high affinity binding and non-saturated low affinity binding expressed by equation 2. With this approach, we could not observe any difference in the number of β_2-adrenoceptors in patients suffering from bronchial asthma, as compared to healthy subjects; however, the affinity of the binding sites for the radioligand was significantly decreased in these patients (10).

Basically, any approach using a restricted range of ligand concentrations is not satisfactory. In the clinical research, however, certain compromises have to be met in order to make investigation at all possible. When the various evaluation procedures currently used in clinical reports were compared on the same set of data (Fig. 3), it became obvious that a mathematical model of one class of high affinity binding sites results invariably in an overestimation of the receptor parameters. Whether such an approach is at all acceptable, is disputable, for example in certain conditions, like in pediatrics, where only a very limited amount of blood may be drawn

from the patient for lymphocyte preparation. It has to be kept in mind, however, that no restricted concentration range is reliable: even a small variation in the binding isoterms will cause dramatic variations in the parameter estimates.

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