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Radioligand Method of Assessment of Human T-Lymphocytes' β-Adrenoceptors Activity

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Abstract—A new method of evaluation of beta-receptor's activity on the surface of human T-lymphocytes has been proposed based on the radioligand method. Optimal conditions for evaluation of specific binding to β_2-adrenoceptors of 0.5 fmol ligand per 1 million cells using [^{125}I] -cyanopindolol were found. The possibility of using β_2-adrenoceptor's activity assessment in clinical settings was demonstrated on human T-lymphocytes.

Keywords: β-adreneceptor, [^{125}I] -cyanopindolol, radioligand method

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INTRODUCTION

Studies of the changes of receptor interactions during various diseases with and without medical treatment are widely performed in clinical research. Among noninfectious diseases the cardiovascular and pulmonary pathologies are the most common and most important.

Cardiovascular diseases are the first among causes of death both in Russia and in other countries [1]. The actual pulmonary pathologies are broncho-obstructive diseases [2, 3]. Patients with such diseases are often treated with medicines affecting β-adrenoreceptors. The pulmonary diseases' treatment is based on the specific ligands of adrenoreceptors that are β-adrenomimetics (β-agonists), while β-adrenoblockators are applied during the treatment of cardiovascular diseases. Administration of the corresponding medicine (adrenimimetic or adrenoblockator) leads to the alterations of expression and affinity of adrenoreceptors. Long-term treatment of such medicines can decrease the efficiency of the chosen therapy and in some cases leads to the occurrence of side effects.

A few direct and indirect methods for the estimation of the status of the adrenoreceptor system have been developed recently. Direct methods estimate the affinity of the receptors to the specific ligands or the receptors' expression itself. Indirect methods assess the changes in status of secondary messengers, e.g., the level of cyclic AMP (cAMP) or the activity of proteinkinase A (PKA). One of the ways of estimation of lymphocyte adrenoreceptor complex is the evaluation of the severity adreno-dependent intercellular biochemical processes upon the influence of the agonist on the homogeneous cell population [4]. The study of secondary messengers (cAMP, PKA) allows the indirect estimation of β-adrenoreceptors expression [5-7]. However, this assay provides only the indirect information about lymphocytes' receptor activity and requires a considerable amount of blood for the analysis.

Real-time polymerase chain reaction (RT-PCR) and western-blot analysis with antibodies to β-adrenoreceptors are more sensitive. The probe size for these analyses is much smaller. Unfortunately, these methods estimate only the level of expression of β-receptor genes in the studied cells; RT-PCR demonstrates the transcription level, while western-blot, the level of translation [8]. The β-adrenorceptor activity itself, meaning the binding the specific ligands, could not be measured by these methods. Recently, the analysis of affinity and expression of β-adrenoreceptors are performed mainly on animal models in vivo and on the isolated cell lines in vitro [6, 7, 9–11].

The standard method of analysis of lymphocytes' β-adrenoreceptors activity includes the assessment of

Abbreviations: PBS – 5 mM potassium phosphate buffer with 0.14 M NaCl, pH 7.0; BSA—bovine serum albumin; PKA—protein kinase A.

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the specific binding by these receptors of the specific ligands labeled with radioactive ^{125}I or ^{3}H. Nonspecific binding is defined by the addition of the excess of unlabeled ligand. Specific binding is calculated by the difference between total and nonspecific binding; the receptors density and dissociation constant are defined in Scatchard coordinates using a linear regression coefficient. The dissociation constant for the receptor-ligand complex is calculated from the curves of the substitution of specific radiolabeled ligand by the nonspecific non-radioactive one using Cheng-Prusoff formula [13, 14]. However, this radioligand method of evaluation of the amount and affinity of β-adrenoreceptors is laborious, expensive and needs large blood samples, especially when applied to the new medicine's investigations. This type of analysis could hardly be applied in real clinical practice.

Here, we are presenting the data of modification of the radioligand method for assessment of human T-lymphocytes β-adrenoreceptor activity using [^{125}I]-cyanopindolol. This method could be applied for the analysis of β-adrenoreceptors' characteristics not only in model systems (such as cell lines or laboratory animals), but in the routine clinical practice.

RESULTS AND DISCUSSION

Adrenoreceptors by their structure are the guanine nucleotide binding proteins (G-proteins). β-adrenoreceptors could be divided on a few subtypes judging on their anatomical localization: β_1-adrenoreceptors are most common in the right atrium, β_2, in the lungs, β_3, in the adipose tissue [15, 16]. The analysis of the receptor activity includes the affinity and the expression (amount) of the receptors.

The ligand-receptor interaction depends on the ligand type (agonist or the reverse agonist) and is reflected in the change of cell activity. Agonists by binding the receptor activate the G-protein and increase the intercellular concentration of cyclic adenosine monophosphate (cAMP). These cellular alterations lead to the appearance of a clinical effect simultaneously with the changes in the receptor activity [17].

The agonists' administration as a medicine often leads to a decrease in the sensitivity to the ligand and to the desensitization [18–20] of the receptors. Application of antagonists, on the contrary, leads to an increase in the density of β-adrenoreceptors on the mammalian cell surface [22] and may affect their affinity [12]. It is worth noting that the changes in the receptor activity are observed not only upon the impact of pharmaceutical compounds, but also the cardiovascular and broncho-pulmonary diseases themselves are accompanied by the altered basic receptor activity. Patients with the ventricular extrasystole reveal a significant increase in the number of β_2-adrenoreceptors on the peripheral blood leukocytes when compared to the control group [23]. Patients with arterial hypertension also have an increased expression of the β_2-adrenoreceptors which correlate with the severity of the disease. Moreover, heart failure leads to a decrease in the maximal binding of the β_2-adrenoreceptors [24]. The examination of the patients with obstructive lung pathology reveals controversial data: some investigators demonstrate the initially low density of the β_2-adrenoreceptors on the blood cell surface, others do not notice any connection of the change in the receptor density on the blood cell surface with bronchial asthma development [25].

When the amount and affinity of β-adrenoreceptors are estimated by radioligand analysis [^{125}I]-cyanopindolol is usually used, while for selective analysis of β_1-adrenoreceptors [^{3}H]CGP12177 is applied (its chemical name is 4-[3[(1,1-dimethylethyl)amino]-2-hydroxypropoxy]-1,3-dihydro-2*H*-benzimidazole-2-on hydrochloride, specific ligand for β_1-adrenoreceptors) [26]. It is to be noted that specific activity of [^{125}I]-cyanopindolol is about 2000 Ci/mmol, while specific activity of [^{3}H]CGP12177 usually does not exceed 30–50 Ci/mmol.

This study aimed to modify the radioligand analysis for the quantitative estimation of β-adrenoreceptor activity on the human blood cells with potential application in clinical practice. Ligands labeled with tritium are 100 times less sensitive in the analysis than [^{125}I]-cyanopindolol. [^{125}I]-cyanopindolol allows the analysis of the sample with a sensitivity of 0.2–0.4 fmol ($0.2–0.4 \times 10^{-15}$ mol).

Specific cell binding we estimated as a difference between the binding of [^{125}I]-cyanopindolol in the presence of 1.7 μM non-radioactive ("cold") cyanopindolol and in its absence. This difference presents total specific activity of receptors binding cyanopindolol and usually is used as a characteristic of analysis specificity [27]. Total human lymphocytes were used as a model for the method development; moreover, the cells consisted of the combined lymphocyte fractions obtained from the blood samples of at least three donors. Specific binding of [^{125}I]-cyanopindolol was defined as 1 fmol per million cells (about 4000 imp./min/million cells). As manipulations of collection of blood samples and the separation of cells are time-consuming, we have compared the results of the specific binding of [^{125}I]-cyanopindolol with cells right after their separation and after long-term storage at 4°C in a PBS containing 100 μg/mL BSA. Twenty hours after the separation (overnight) specific binding hardly changed. Moreover, the detailed analysis of the binding and the Scatchard plotting by the method described previously [14] revealed that the constant of association of cyanopindolol with cells after 20 h corresponded to data of other investigators [14]. Thus, the separated lymphocytes could be stored overnight prior

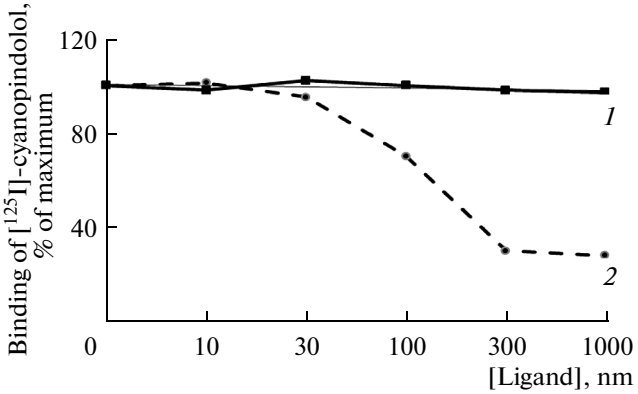

Fig. 1. Binding of [^{125}I]-cyanopindolol by lymphocytes in presence of beta-active ligands: β_1-ligand CGP-20712 (*1*) and β_2-ligand ICI 118551 (*2*). Each measurement of binding of [^{125}I]-cyanopindolol with T-lymphocytes was performed in triplicates. Standard deviation did not exceed 10%.

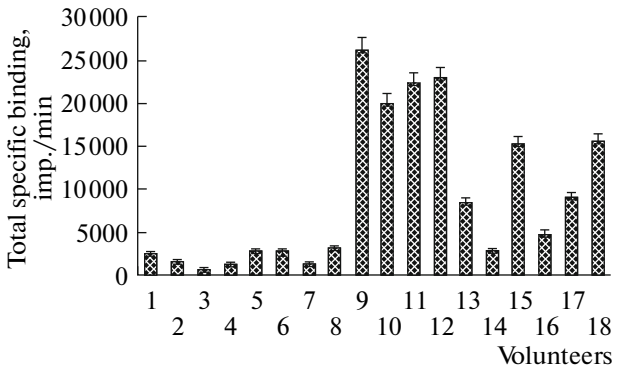

Fig. 2. Total specific binding of [^{125}I]-cyanopindolol on T-lymphocytes of 18 healthy volunteers. Binding is presented as imp./min per 1 million cells.

to the analysis. The continuation of the experiment had shown that prolonged storage impaired the specificity of the binding; and it decreased several times after tree days of storage. It was crucial not to freeze cells, as two cycles of freezing-thawing led to the almost complete loss of specific binding. A few series of our experiments with total lymphocytes had shown that the specific binding of [^{125}I]-cyanopindolol defined by the method from [27] was quite an objective indicator of its activity and finely correlated with cytological indicators of cell viability.

Unfortunately, the specific binding, as described above, is implemented by the several types of receptors, while only the data for individual types of receptors could be clinically important [28]. That is why we had chosen the concentrations of the "cold" non-radioactive ligands specific for β_1- or β_2-adrenoreceptors, which could competitively substitute [^{125}I]-cyanopindolol. CGP-20712 (1-[2-((3-Carbamoyl-4-hydroxy)phenoxy)ethylamino]3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol dihydrochloride) was used as the specific β_1-ligand, and ICI 118551 ((±)-erythro-(S*,S*)-1-[2,3-(Dihydro-7-methyl-1*H*-inden-4-yl)oxy]3-[(1-methylethyl)amino]-2-butanol hydrochloride) was used as the β_2-ligand. Both these compounds are widely applied in various studies of the β-adrenoreceptors specificity [27, 29].

We had analyzed the competitive binding by lymphocytes' receptors of [^{125}I]-cyanopindolol and CGP-20712 and ICI 118551 ligands in various concentrations (Fig. 1). Even very high concentrations (up to 1 μM) of CGP-20712 did not induce a substantial decrease in lymphocytes' binding of [^{125}I]-cyanopindolol (curve *1*, Fig. 1). It provides the indirect proof that healthy human lymphocytes bear a limited number of β_1-adrenoreceptors and in this case, it could not be defined with [^{125}I]-cyanopindolol. ICI 118551 competitively substitute [^{125}I]-cyanopindolol with the increase of the "displacer." Nevertheless, the threshold concentration had been discovered: there is no further substitution of the labeled ligand occurred with concentrations higher than the threshold (curve *2*, Fig. 1). About 30% of the [^{125}I]-cyanopindolol was left. Such an effect could be explained as [^{125}I]-cyanopindolol is not a strictly specific ligand for the β-adrenoreceptors and binds quite effectively with other receptors, e.g., with serotonin ones [30–32]. That is why we suggest that the decrease of the binding of [^{125}I]-cyanopindolol in the presence of specific ligand ICI 118551 characterizes exactly the β_2-adrenoreceptor activity of lymphocytes.

Using these data, we have analyzed the receptor activity of human T-lymphocytes and the possibility of the application of this assay for detection of alterations of cell receptor activity in response to the treatment with the drugs or other chemical compounds. The values for the receptor activity of T-lymphocytes for total specific activity and for β_2-adrenoreceptors are shown in Figs. 2 and 3, correspondingly. Total specific receptor activity means the difference between the amount of [^{125}I]-cyanopindolol bound by cells in the presence of 1.7 μM non-radioactive cyanopindolol, and in its absence. Specific activity of β_2-adrenoreceptors of T-lymphocytes was defined as the difference between the amount of [^{125}I]-cyanopindolol bound by cells in the presence of 0.16 μM ICI 118551, and in its absence. The concentration of the ligand ICI 118551 (0.16 μM) was based on the total lymphocyte data, where it provided about 50% of the maximally possible competitive substitution of [^{125}I]-cyanopindolol (Fig. 1). The data presented demonstrate that the activity of β_2-adrenoreceptors, estimated by the ability of specific binding of [^{125}I]-cyanopindolol, vary significantly, and now we could not

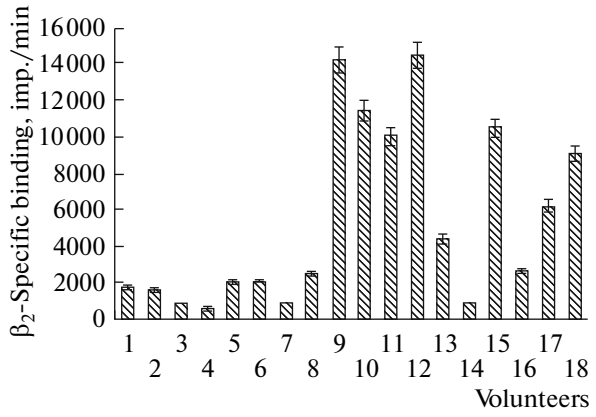

Fig. 3. β_2-Specific binding of [^{125}I]-cyanopindolol on T-lymphocytes of 18 healthy volunteers in presence of 0.16 μM ICI 118551. Binding is presented as imp./min per 1 million cells.

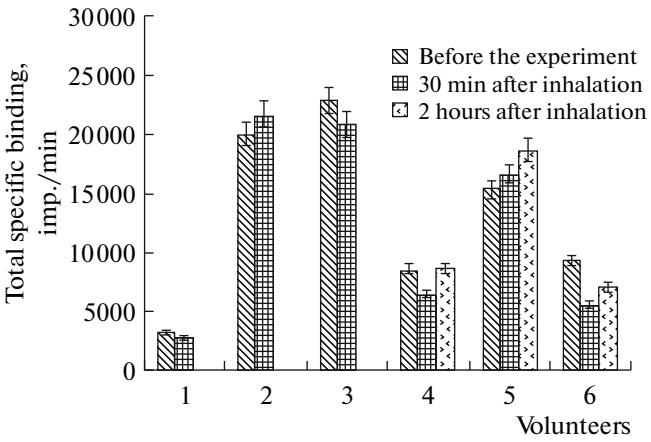

Fig. 4. Total specific binding of [^{125}I]-cyanopindolol on T-lymphocytes of 6 healthy volunteers before the experiment and 30 min (volunteers 1–6) and 3 healthy volunteers before the experiment and 2 hours (volunteers 4–6) after the inhalation of short-acting β_2-agonist (salbutamol). Binding is presented as imp./min per 1 million cells.

speak about any "normal" or even "average" activity. Moreover, the fluctuations of total receptor activity in different individuals (Fig. 2) did not correlate with the fluctuations of the specific β_2-receptor activity in the same individuals (Fig. 3).

We think that not the absolute values of the labelled-ligand-binding cells, but the dynamics of the alterations of these values after the impact of the external factors, e.g., during the functional test, are of the most importance. The short-acting β_2-agonist (active compound was salbutamol) was used as an active substance. T-lymphocytes were separated as described in the Experimental section. Blood samples were obtained before and after the salbutamol inhalation (approximately 400 μg). It should be pointed out that the changes in the total receptor activity, i.e., specific binding of cyanopindolol, shown in Fig. 4, did not correlated with the changes of β_2-adrenoreceptor activity shown in Fig. 5. All results are summarized in the table. Alterations detected 30 minutes after the inhalation of beta-agonist had shown a decrease in the β_2-receptor binding (only 1 out of 6 volunteers did not demonstrate any changes). As an error of measures in the triplicate probes did not exceed 10%, we assume significant changes in the receptor activity of more than 20%.

It should be noted that the alterations of the total binding after the effect of salbutamol was not always correlated with the changes of the specific β_2-receptor binding (Figs. 4 and 5). Three individuals were ana-

Total specific binding of [^{125}I]-cyanopindolol by T-lymphocytes and β_2-specific binding in the presence of 160 nM β_2-ligand ICI 118551 in healthy volunteers*

Volunteer	Total specific binding			β_2-specific binding		
	before the experiment	30 min after inhalation	2 hours after inhalation	before the experiment	30 min after inhalation	2 hours after inhalation
1	3300 ± 230	2824 ± 850		2502 ± 551	1928 ± 162	
2	20015 ± 2001	21697 ± 2171		11477 ± 1864	10680 ± 571	
3	22894 ± 1345	20870 ± 1107		14486 ± 407	12448 ± 350	
4	8558 ± 283	6518 ± 215	8554 ± 475	4366 ± 283	3614 ± 335	4294 ± 264
5	15285 ± 670	16610 ± 1162	18666 ± 1587	10485 ± 578	10640 ± 1273	10659 ± 1077
6	9264 ± 472	5534 ± 303	7043 ± 413	6195 ± 370	4113 ± 401	5044 ± 792

* Mean from the 3 independent measurements (imp./min per 1 million cells). Here the results obtained before the experiment are presented, 30 minutes and 2 hours after the inhalation of short-acting β_2-agonist (salbutamol).

lyzed additionally 2 hours after the inhalation and revealed the restoration of receptor activity almost up to the initial level (Figs. 4 and 5, donors 4–6). Moreover, despite the substantial difference in the absolute binding value of [^{125}I]-cyanopindolol by T-lymphocytes from different donors, similar dynamics was observed for the changes in the activity of β_2-adrenoreceptors (Figs. 4 and 5).

In sum, this assay surely does not provide the information about exact number of β2-adrenoreceptors on the T-lymphocyte surface, because the amount of the ligand (in our case, [^{125}I]-cyanopindolol) bound by cells, depends not only on the number of receptors located on the external cell side, but on the receptors' affinity and on their availability for the soluble ligands. That is why we believe, that in this case, it is more correct to speak of receptor activity and its dynamics upon the influence of β_2-agonist, that is probably more important for clinical practice than the estimation of expression and activity of the receptors.

Based on these results a few important preliminary conclusions could be drawn.

1. We could quantitatively estimate the receptory activity of exactly β_2-adrenoreceptors in the cells by competitive binding (substitution) of [^{125}I]-cyanopindolol by specific ligand ICI 118551 in final concentration 0.16 μM.

2. The difference between the amount of [^{125}I]-cyanopindolol bound in the presence of β-specific ligand and without it is a quantitative characteristic of β-adrenoreceptors exposed on the cell surface (in this case, T-lymphocytes).

3. Suggested modification of the radioligand analysis allows significant detection of the dynamics of receptor response of T-lymphocytes when exposed to the body as a whole.

We believe that further development of this assay for the evaluation of cells' receptor activity will assist the application of the data in clinical practice.

EXPERIMENTAL

Materials

Specific ligands CGP-20712 (β_1-adreno blocker) and ICI 118551 (β_2-adreno blocker), cyanopindolol, bovine serum albumin were purchased from Sigma (United States). Salbutamol (trade name Ventolin), short-time β_2-agonist, was purchased from Glaxo-SmithKline Pharmaceuticals (Poland). It is available as a dosed inhalation aerosol (100 μg/dose). Radioactive Na^{125}I dissolved in NaOH solution in concentration 1000MBq/mL was provided by V/O Isotop (Russia).

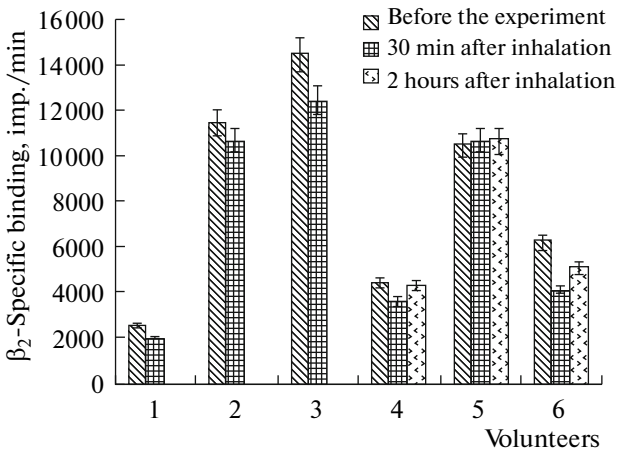

Fig. 5. β_2-Specific binding of [^{125}I]-cyanopindolol on T-lymphocytes of 6 healthy volunteers before the experiment and 30 min (volunteers 1–6) and 3 healthy volunteers before the experiment and 2 hours (volunteers 4–6) after the inhalation of short-acting β_2-agonist (salbutamol). Binding is presented as imp./min per 1 million cells.

Blood Samples and Cells Separation

The study included 20 healthy volunteers older than 18 years without cardiovascular pathology and bronchial obstruction; no-one received medicines affecting β-adrenoreceptors. All volunteers were subjected to medical examination before the blood samples had been taken; they also were examined by standard methods to evaluate the status of cardiovascular and broncho-pulmonary systems. For the experiments performed on T-lymphocytes blood samples had been taken thrice from each individual: first time, before the start of experiment (control), second time, 30 minutes, and third time 2 hours after the inhalation with 400 μg salbutamol.

Mononuclear lymphocytes were separated as described previously [33]. T-lymphocytes were isolated from the total lymphocyte fraction with Pan T Cell Isolation Kit II human 1 × 1 mL, 1 × 2 mL (Miltenyi Biotec, Germany) according to the manufacturer's instructions.

Preparation of [^{125}I]-Cyanopindolol

Radioactive isotope ^{125}I was included in the cyanopindolol molecule by the method described previously with some modifications [34]. The reaction mixture for the radioiodification contained 1 μg cyanopindolol and 1 mCi Na^{125}I in 50 μL of 0.2 M potassium phosphate buffer (pH 7.0). The reaction was initiated by the addition of 10 μL of chloramine to solution (10 mg/mL), incubated for 1 min at room temperature and then the reaction was stopped by the addition of 10 μL of sodium thiosulfate (20 mg/mL). After incubation for 5 min at room temperature, 1 μL

of "cold" sodium iodide (6 mg/mL) was added. The target product was isolated with HPLC on the Nucleosil 100 C-18 column (5 μM, 4 × 150 mm) in the ion-pairing mode: the elution was performed in a gradient of concentration of acetonitrile from 0 to 100% in 10% acetic acid for 20 min with the rate 0.5 mL/min, the eluate was detected by UV absorption at λ 280 nm. An HPLC chromatograph (Gilson, France) was used. Fractions containing [^{125}I]-cyanopindolol were combined, dried, dissolved in 70% ethanol and stored at –20°C.

Total Specific Binding of [^{125}I]-Cyanopindolol with Cells

All reactions and measurements were performed in triplicates. One hundred μL of water solution of [^{125}I]-cyanopindolol in PBS with concentration 1000 imp./min/μL, 10 μL of unlabelled cyanopindolol in concentrations varying from 10^{-8} to 10^{-6} M (or 10 μL water) and 100 μL of cell suspension with concentration 5–10 million cells/mL were incubated for 1 hour at 37°C with careful mixing on the shaker, rotating at 100 rpm in 1.5-mL centrifuge tubes (Eppendorf). The process was stopped by the addition of 400 μL of ice-cold water to each probe. Cells were centrifuged in the Microspin12 BioSan (Latvia) at 2000 *g* for 10 min, supernatant was discarded, and the pellet was carefully suspended in 200 μL of ice-cold PBS and again centrifuged in the same conditions. Supernatant was discarded; the pellet was suspended carefully in 200 μL of ice-cold PBS and centrifuged at 10000 *g* for 2 min. Supernatant was discarded, and the pellet in the tubes was analyzed on a γ-counter Wallac Wizard 1470 (PerkinElmer) measuring the amount of radioactive material in each probe. The efficiency of the count was about 60%.

Specific β_2-Binding of [^{125}I]-Cyanopindolol with Lymphocytes

All reactions and measurements were performed in triplicates. One hundred μL of water solution of [^{125}I]-cyanopindolol in PBS with a concentration of 1000 imp./min/μL, 10 μL of unlabelled ligand (CGP-20712, or ICI 118551, or cyanopindolol) in various concentrations (or 10 μL water) and 100 μL of cell suspension with concentration 10 million cells/mL were incubated for 1 hour at 37°C with careful mixing on the shaker, rotating at 100 rpm in the 1.5-mL centrifuge tubes (Eppendorf). Further manipulations were analogous to those in the experiments for total specific binding (see above).

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