ISSN 1068-1620, Russian Journal of Bioorganic Chemistry, 2019, Vol. 45, No. 2, pp. 129–134. © Pleiades Publishing, Ltd., 2019.
Russian Text © E.V. Smolyakova, Yu.S. Skoblov, N.A. Skoblova, O.Yu. Agapova, L.G. Ambatiello, A.A. Klimova, T.V. Kuznetsova, V.P. Masenko, S.Yu. Nistor, A.V. Rvacheva, I.E. Chazova, K.A. Zykov, 2019, published in Bioorganicheskaya Khimiya, 2019, Vol. 45, No. 3, pp. 295–301.

Specificity and Selectivity of a Modified Radioligand Method for Estimating the Binding Activity of β1 Adrenergic Receptors in Human T Lymphocytes

E. V. Smolyakova[a, 1], Yu. S. Skoblov[b], N. A. Skoblova[b], O. Yu. Agapova[c], L. G. Ambatiello[a], A. A. Klimova[a], T. V. Kuznetsova[a], V. P. Masenko[a], S. Yu. Nistor[c], A. V. Rvacheva[c], I. E. Chazova[a], and K. A. Zykov[d, c]

[a] *National Medical Research Center of Cardiology, Ministry of Healthcare of the Russian Federation, Moscow, 121552 Russia*
[b] *Shemyakin–Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow, 117997 Russia*
[c] *Evdokimov Moscow State University of Medicine and Dentistry, Ministry of Healthcare of the Russian Federation, Moscow, 127473 Russia*
[d] *Research Institute of Pulmonology, Federal Medical and Biological Agency of Russia, Moscow, 115682 Russia*

Received September 3, 2018; revised October 7, 2018; accepted October 17, 2018

Abstract—A radioligand-based method was proposed for quantifying the binding activity of β1-adrenergic receptors (ARs) on the surface of human T lymphocytes. Using the transfected HEK293 cell lines expressing β1-AR (ADL-7A) and β2-AR (A2R9) as a model, the conditions for [^{125}I]cyanopindolol reliable detection of specific ligand binding to β1-adrenergic receptors at the level of 1–1.5 fmol per 1 million cells were selected. The real possibility of performing this analysis for clinical studies was shown by the example of human T lymphocytes.

Keywords: adrenergic receptors, [^{125}I]cyanopindolol, radioligand assay
DOI: 10.1134/S1068162019020134

INTRODUCTION

β-Adrenergic receptors (β-ARs) have been intensively studied for over 50 years; tens of thousands of papers on this subject have been published in the world over the years and more than 7000 works have been published in the last ten years. The main reason for this interest is the involvement of β-ARs in the pathogenesis of a number of cardio-respiratory pathologies, which currently occupy leading positions in the structure of morbidity and mortality [1]. Administration of β-agonists and β-adrenergic blockers, which perform their action through β-ARs, to patients with this combined pathology is still an unresolved issue due to the possible development of side effects. The specificity of the interaction of β-agonists and β-blockers with β-ARs is one of the most important factors determining the possibility of their selective effects on the body, the duration and severity of action, as well as the likelihood of unwanted adverse reactions. The individual responses of a particular person to the action of β-agonists and β-blockers are of great importance, which leads to the need for a personalized approach in prescribing such drugs.

Investigations using radioligand assay have made a great contribution to the study of β-ARs [2, 3]. It was used to obtain the most important information about the nature and types of adrenergic receptors, the nature of interaction with various ligands, antagonists, and agonists, as well as the content of various types of adrenergic receptors in organs and tissues [4]. All these works were of a fundamental research or an applied character, and although their importance for medicine was declared in each work, radioligand methods of assay were not used in practical health care. Among the many reasons for this situation, two main points should be highlighted: firstly, when working in real clinical practice, only blood is available for radioligand assay, but the number of adrenergic receptors on the surface of blood cells is very limited. Despite the high sensitivity of the radioligand method, the determination of classical parameters such as ligand binding constants and the number of active cellular receptors requires a significant amount of blood. Secondly, the β-adrenoreceptor system is very dynamic; there is a constant exchange of information between cells. As a result, the number of adrenergic receptors on the cell surface and ligand binding constants are changed

Abbreviations: cAMP, cyclic adenosine monophosphate; BSA, bovine serum albumin; PBS, 5 mM potassium phosphate buffer solution with 0.14 M NaCl, pH 7.0.

[1] Corresponding author: phone +7 (916) 737-86-97; e-mail: smolyakovak@mail.ru.

under the influence of many different factors. Therefore, the use of traditional radioligand analysis in real clinical practice is complicated and unlikely.

We have previously proposed a method for assessing the receptor activity of β1 and β2 adrenergic receptors. The method is based on the displacement of iodine-125-labeled cyanopindolol that nonspecifically binds to receptors of several types by CGP-20712 and ICI 118551 ligands with high specificity for adrenergic receptors [5]. Using this approach, it was possible to begin studies of the dynamics of changes in β-AR activity and to obtain interesting data on the effect of β-agonist (salbutamol) on the activity of human T lymphocyte β2-ARs in healthy people and patients with respiratory pathology [6]. Moreover, in these works, the relationship of β2-AR and other clinical and laboratory parameters with proven clinical significance was observed. At the same time, the lymphocyte β1-AR activity in the same samples, which we determined, was at an extremely low level; about 10–20 times lower than the activity of β2-ARs of these cells. These data are in good agreement with studies on the distribution of β1- and β2-ARs in organs and tissues [7]. Therefore, we did not comprehensively analyze our results of the β1-AR activity in T lymphocytes due to the low reliability of the results.

However, in ongoing studies with T lymphocytes of patients with combined cardio-respiratory disorders, we have found that the extremely high activity of β1 adrenergic receptors in T lymphocytes is characteristic of some types of cardiac pathologies. This forced us to go back to the question of the specificity and reliable selectivity of the analysis we proposed, as well as the need to develop a modification of the method in order to increase the reliability of the results obtained for the activity of β1 adrenergic receptors in T lymphocytes.

RESULTS AND DISCUSSION

Determination of the β-AR activity of living cells is a rather complicated task. Despite the huge number of publications on this topic, there is still no simple and unambiguous method for solving this problem. This is partly due to the ambiguity of the problem itself and the terminology uncertainty. Various authors use the term β-adrenoreceptor activity to imply slightly different effects. Some authors believe that AR activity is the amount of ligand that binds to the receptor during the measurement [8]. This view to the radioligand approach corresponds more likely to a chemical point of view. Other authors believe that the activity of cellular receptors should be defined as the biological function of the receptor and more correct is the use of indirect methods, i.e., determination of secondary messengers (cAMP or protein kinase A) [9, 10]. This point of view corresponds to the medical and biological ideas of the object being measured.

We consider the chemical approach, in which the activity of cellular β-ARs is defined as the specific binding of a labeled ligand under certain standardized conditions, to be more correct [8]. Since when determining the receptor activity, the main measured parameter is the amount of radioactive ligand bound to the receptor, we believe that for a quantitative assessment it is more convenient to use the value of cpm (counts per minute) per 1 million cells. This allows quantitative comparisons of changes in receptor activity both under the influence of various external factors and in dynamics.

One of the problems of determining the β-AR activity of the cells using [^{125}I]cyanopindolol is the specificity of the detected adrenergic receptors. The use of specific tritium-labeled ligands makes it possible to solve this problem [11]. However, the sensitivity of the radioligand assay with the tritium ligand is about 50 times lower than that of the assay using [^{125}I]cyanopindolol, which is a critical indicator for possible medical application.

Unfortunately, [^{125}I]cyanopindolol does not have high specificity for different β-ARs. According to various authors, binding constants for β1 and β2 adrenergic receptors from different sources vary in the range of 7–40 pM (10^{-12} M) [12]. This led to the idea of using specific CGP-20712 and ICI 118551 ligands for the selective determination of β1 and β2 adrenergic receptors, which was first published in 1989 [13]. Later, the idea was developed, and data on the selective determination of the β1- and β2-AR activity for various types of cells, including horse lymphocytes, were obtained [14, 15]. Very interesting results on the ratio of the number of active β1- and β2-ARs on the cell surface were obtained, and the ligand binding constants were determined. But all these works were carried out on the objects that could be used in significant amounts for analysis. Taking into account the extremely limited amount of material available for analysis that can be taken from a real patient (especially during successive serial examinations), these methods could not be transferred to clinical practice without serious modification.

The method we proposed previously for determining the β2-AR activity in T lymphocytes consisted in the displacement of [^{125}I]cyanopindolol by a β2-specific ligand, namely *erythro*-(*S**,*S**)-1-[2,3-(dihydro-7-methyl-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)-amino]-2-butanol hydrochloride, better known by the abbreviated name ICI 118551 [16]. The ligand has a high degree of selectivity; the affinity of ICI 118551 for β2 exceeds the affinity for β1 by a factor of 550 [17]. In fact, we determined the cellular β2-AR activity as the [^{125}I]cyanopindolol amount replaced by the ICI 118551 specific ligand under certain fixed conditions.

Unfortunately, this approach is difficult to use for determination of the β1-AR activity in lymphocytes. The amount of β1-ARs on the surface of lymphocytes is too small, and the β1-specific ligand (2-[(3-carbamoyl-4-hydroxy)phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)phenoxy]-2-propanol

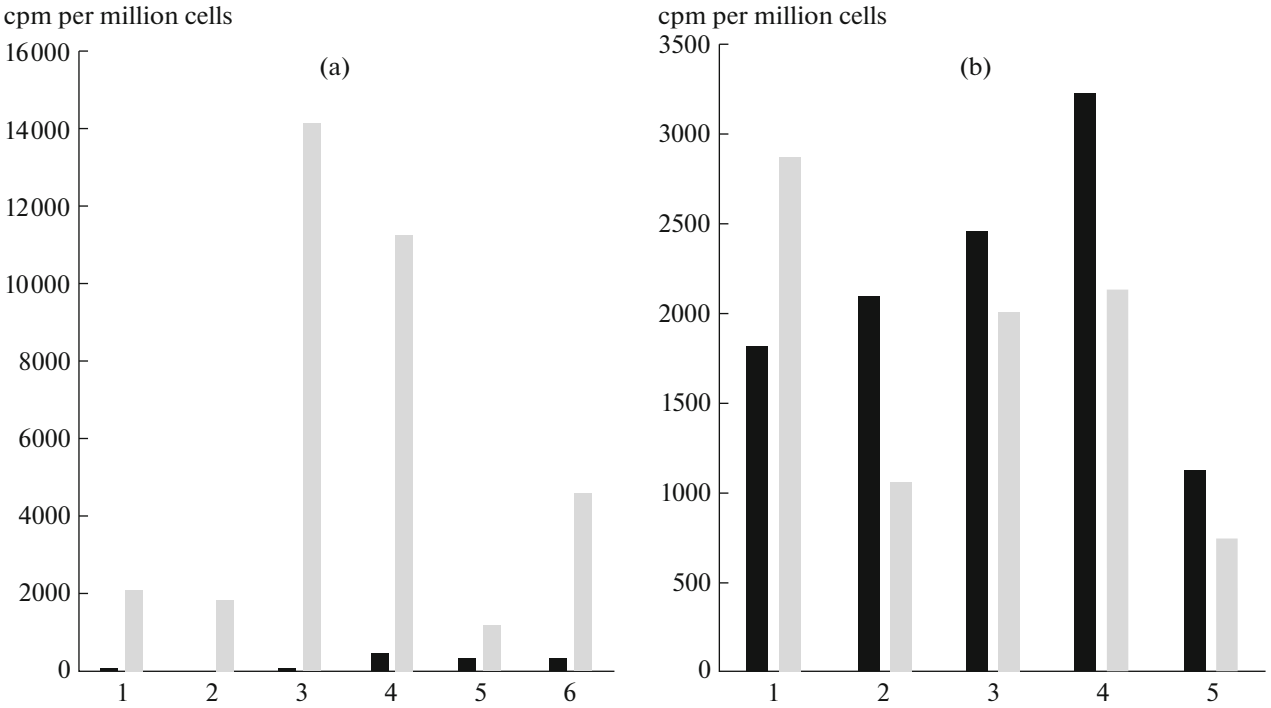

Fig. 1. Comparative activity of β1 (black bars) and β2 (light bars) adrenergic receptors in T lymphocytes. The activity of adrenergic receptors is given in cpm per million cells (T lymphocytes). *A*, adrenoreceptor activity of (1, 2, 3) healthy volunteers and (4, 5, 6) patients with bronchopulmonary pathology; *B*, adrenoreceptor activity in patients with idiopathic arrhythmia. The analysis was carried out in three parallel runs; standard deviations did not exceed 10%.

dihydrochloride) more commonly known as CGP-20712 [18] could not specifically displace [^{125}I]cyanopindolol from β1-ARs simply because of their too-small amount (see Fig. 1a). Moreover, the specificity of CGP-20712 towards β1-ARs is also very high: the affinity of CGP-20712 for β1 exceeds the affinity for β2 by a factor of 501 [17]. From the data we obtained on the comparative activity of β1- and β2-APs in the same samples of healthy volunteers (samples 1, 2, 3) and patients with bronchopulmonary pathology (samples 4, 5, 6) it follows that the activity of β1-ARs is very low and does not exceed 0.1 fmol (10^{-16} mol) receptors per 1 million T lymphocytes, or about 60 receptors per cell.

However, when determining the adrenoreceptor activity in T lymphocytes in patients with idiopathic cardiac rhythm disorders, it turned out that in some patients the β1-adrenoreceptor activity is significantly higher (1–1.5 fmol per 1 million cells) and is comparable to the β2-AR activity (see Fig. 1b). These results forced us to reanalyze the specificity and selectivity of the previously proposed method for determining the β-AR activity.

To verify the specificity of our proposed method, we used transfected ADL-7A or A2R9 cell lines expressing β1-ARs or β2-ARs, respectively. The ADL-7A cell line carries the recombinant human β1-AR gene under the control of a constitutive promoter P_{CMV} of the early cytomegalovirus genes; more than 2 million active β1-ARs are exposed on the surface of 1 cell [19]. The line of transfected A2R9 cells contains the β2-adrenoreceptor gene with the green fluorescent protein EGFP attached at the *C*-terminus also under the control of P_{CMV}. There are more than 1.5 million active β2-ARs on the surface of 1 cell. It has been previously shown that the attachment of green protein to the *C*-terminus does not practically alter their biological properties [20].

To confirm the specificity of the proposed method, we conducted experiments to displace labeled [^{125}I]cyanopindolol from cell receptors of the ADL-7A line with CGP-20712 ligand at various concentrations (see Fig. 2). A similar series of experiments to displace labeled [^{125}I]cyanopindolol by ICI 118551 ligand at various concentrations was carried out for the A2R9 cell line (Fig. 3).

Similar studies were previously carried out in CHO (Chinese hamster ovary) cell cultures, in which human β1- or β2-AR genes were expressed after appropriate gene-engineering transformations [17, 21]. These studies allow obtaining the data on the specificity of a number of adrenoreceptor ligands and their ability to affect the activity of intracellular adenylate cyclase. However, accurate quantitative estimates of the ligand specificity should be treated with caution, since these values depend on the specific method and the analysis conditions. For example, the selectivity coefficient for ICI 118551 determined in

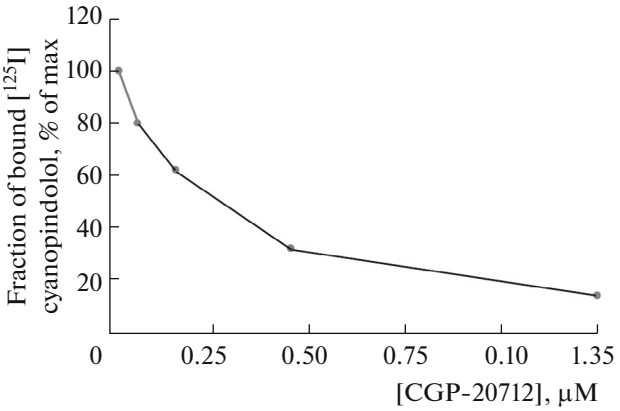

Fig. 2. Binding of [^{125}I]cyanopindolol to ADL-7A cells in the presence of CGP-20712. In the reaction mixture, there were 6000 ADL-7A cells. Details of the analysis see the Experimental section. Maximum binding 53 390 cpm per million cells from 100000 cpm per million cells. The analysis was carried out in three parallel runs; standard deviations did not exceed 10%.

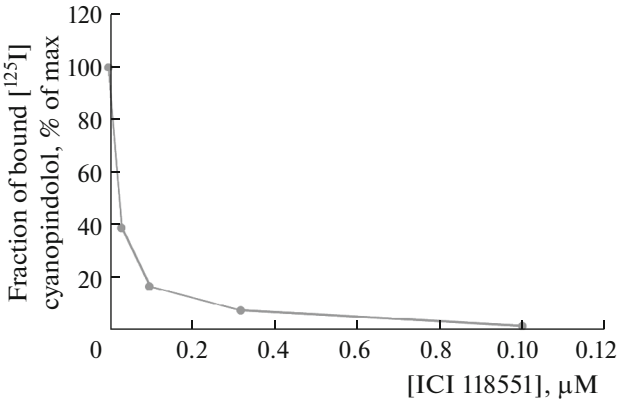

Fig. 3. Binding of [^{125}I]cyanopindolol to A2R9 cells in the presence of ICI 118551. In the reaction mixture, there were 7500 A2R9 cells. Details of the analysis see the Experimental section. Maximum binding 55980 cpm per million cells from 100000 cpm per million cells. The analysis was carried out in three parallel runs; standard deviations did not exceed 10%.

horse lymphocytes [14] differs from data obtained in cell cultures.

It should be noted that according to our data (see Table 1), CGP-20712, even at very high concentrations (0.44 μM), barely displaces [^{125}I]cyanopindolol from cellular β2-ARs (displacement is about 3%). This confirms the specificity of the proposed analysis scheme, since the selected concentration of CGP-20712 will have a minimum effect on the binding of a radioactive ligand to β2-ARs in T lymphocytes. At the same time, ICI 118551 ligand at a high concentration (0.32 μM) partially (about 20%) displaces [^{125}I]cyanopindolol from cellular lymphocytic β1-ARs. However, for the selective determination of the activity of T lymphocyte β1-ARs, this distortion of the quantitative assessment will not exceed 10% even for individuals with extremely high (rarely pathological) activity of T lymphocyte β1-ARs.

We carried out a modification of the proposed method, which consists in changing the order of the reagent addition in an incubation mixture. It was suggested the cells were first incubated with specific CGP-20712 or ICI 118551 ligands, and then a labeled cyanopindolol was added. The results of these experiments in ADL-7A and A2R9 cells are presented in Table 1. Interestingly, preincubation of cells with CGP-20712 (β1-specific ligand) activates cellular β1-ARs, while preincubation with ICI 118551 (β2-specific ligand) does not make significant changes in the activity of β2-ARs.

Analysis of β-Adrenoreceptor Activity in T Lymphocytes with Preincubation

To test this modified analysis scheme, we used several samples of T lymphocytes of real patients with combined cardio-respiratory pathology. Comparative data on the activity of β1- and β2-APs are presented in

Table 1. Binding of [^{125}I]cyanopindolol to ADL-7A and A2R9 cells under various conditions

Cells	Amount of bound radioactivity, cpm/million cells					Binding, % of maximum	
	without ligand	with CGP-20712		With ICI 118551			
		without preincubation	preincubation	without preincubation	preincubation	without preincubation	preincubation
ADL-7A	53392					100	
ADL-7A		17059	11846			32	22
ADL-7A				41335	52391	78	98
A2R9	55978					100	
A2R9		54296	52391			97	92
A2R9				7293	12445	13	22

The error of the presented experimental data does not exceed ±10%.

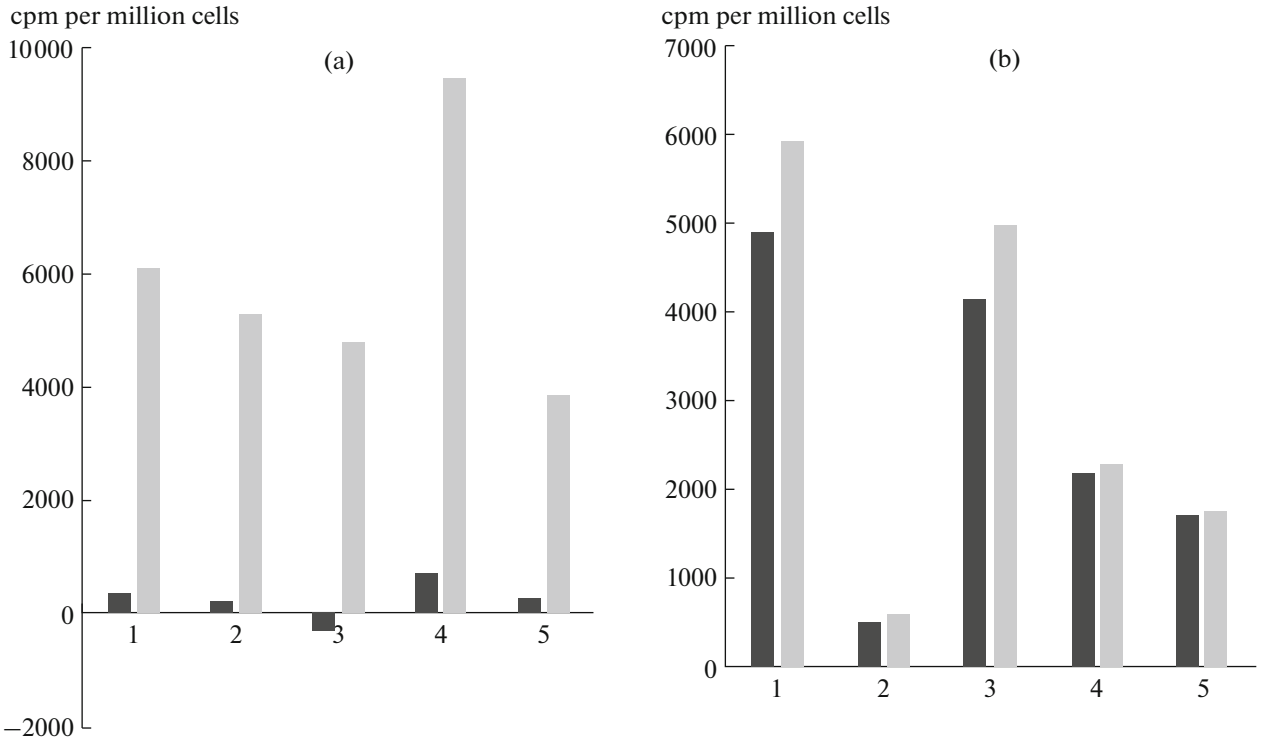

Fig. 4. Activity of (a) β1-ARs and (b) β2-ARs in T lymphocytes without preincubation (black bars) and with preincubation (light bars). The activity is given in cpm per million cells (T lymphocytes). 1, 2, 3, 4, 5 are real patients with combined cardio-respiratory pathology. The analysis was carried out in three parallel runs; standard deviations did not exceed 10%.

Fig. 4. It should be noted that the modification of the method does not lead to significant changes in the β2-AR activity in three (patients 1, 4, and 5) of five samples. At the same time, for β1-ARs, changes in the method give a radical increase in the activity. In this case, there is no doubt that in fact a slight correction of the procedure, namely, a change in the order of reagent addition to the cells under study, produces new information on the possible response of the cellular β-ARs to preincubation with specific ligands, which characterizes the functional state of the receptor apparatus. The mechanisms of the observed phenomenon are not yet clear; therefore, it is necessary to accumulate more observations in patients with various cardio-respiratory diseases (and their combination) to interpret them and evaluate the possible clinical significance of the data obtained.

Thus, on the basis of these results, we can conclude that our proposed method that is based on the displacement of labeled [^{125}I]cyanopindolol by specific CGP-20712 and ICI 118551 ligands makes it possible to selectively determine the activity of both β2- and β1-ARs in human T lymphocytes, and preincubation with the above ligands allows obtaining additional information characterizing the state of the adrenoreceptor apparatus.

EXPERIMENTAL

Specific CGP-20712 and ICI 118551 ligands, as well as cyanopindolol (Sigma, United States) were used in the work. Radioactive Na[^{125}I] with a molar activity of more than 2000 Ci/mmol was provided by Izotop (Russia). [^{125}I]Cyanopindolol was synthesized as previously described [5]. The rest of the reagents used in the work were not lower than analytical grade.

Cell Cultures

The ADL-7A cells obtained by transfecting the parental line of human embryo kidney HEK293 cells with the *ADRB1* gene of β1-ARs were kindly provided by T. N. Vlasik (Institute of Experimental Cardiology, National Medical Research Center, Ministry of Healthcare of the Russian Federation, Moscow). The A2R9 cells expressing human β2-ARs fused with a green fluorescent protein were provided by MonA (Russia). Cells were cultured in vials in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 0.1 mg/mL streptomycin (all reagents were from Invitrogen, United States) in a 5% CO_2 atmosphere at 37°C. For the analysis of adrenoreceptor activity, HEK293, ADL-7A, and A2R9 cells were removed from the surface of the vials with trypsin treatment; the proteolysis reaction was quenched by adding a culture medium

with 10% FBS; cells were precipitated by centrifugation at 1100 rpm (200 *g*) for 15 min, washed twice with PBS at room temperature, and suspended in a PBS solution with 0.1 mg/mL bovine serum albumin (BSA, Sigma-Aldrich, United States). In the specificity verification experiments, cell suspensions were mixed so that the sample for analysis contained 5×10^5 HEK293 cells performing the function of a carrier, 2000 ADL-7A cells or 3500 A2R9 cells. In experiments with preincubation, we used 3.5×10^5 HEK293 cells, 6000 ADL-7A cells, and 7500 A2R9 cells, respectively.

Radioligand Assay of β-Adrenoreceptor Activity

Peripheral blood sampling, T lymphocyte isolation, and adrenoreceptor activity analysis were performed as described previously [5]. For experiments with preincubation, a cell suspension was diluted with PBS supplemented with 0.1% BSA to a concentration of 10 million cells/mL; 100 μL of the cell suspension were incubated at 37°C for 30 min followed by the addition of 10 μL of a solution of unlabeled ligand (CGP 20712 or ICI 118551) with gentle stirring on a shaker at 100 rpm. Then, 100 μL of a solution of [^{125}I]cyanopindolol in PBS at a concentration of 1000 cpm/μL was added to the reaction mixture and incubated for 30 min. The process was quenched by adding 400 μL ice-cold water to each sample. To wash off unbound radioactivity, cells were centrifuged at 2000 *g* for 10 min; the pellet was washed three times by suspending in 200 μL ice-cold PBS solution and centrifuging under the same conditions and analyzed on a Wallac Wizard 1470 gamma counter (PerkinElmer, United States). All measurements were performed in three parallel runs.

ACKNOWLEDGMENTS

The work was carried out in the framework of the State Contract no. 19.001.18.14 of April 13, 2018, "Analysis of the Prevalence of Bronchial Hyperresponsiveness in Athletes in Cyclic Sports with a Personalized Justification of the Use of Inhaled β2-Agonists" (Code "Bronkh-18") (Research Institute of Pulmonology of the Federal Medical and Biological Agency of Russia). The authors are grateful to T.N. Vlasik for providing ADL-7A cells, I.N. Rybalkin for providing A2R9 cells, and A.Ya. Shevelev for assistance in analyzing the results.

COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants performed by any of the authors.

REFERENCES

1. Chazova, I.E., Chuchalin, A.G., Zykov, K.A., and Ratova, L.G., *Sist. Gipertenz.*, 2013, vol. 10, no. 1, pp. 5–35.
2. Perkins, J.P., *The beta-Adrenergic Receptors,* Perkins, J.P., Ed., Springer Science and Business Media, 2012.
3. Manukhin, B.N., Nesterova, L.A., Smurova, E.A., and Kichikulova, T.P., *Biol. Membr.*, 1999, vol. 16, no. 5, pp. 541–554.
4. Minneman, K.P., Pittman, R.N., Yeh, H.H., Woodward, D.J., Wolfe, B.B., and Molinoff, P.B., *Brain Res.*, 1981, vol. 209, no. 1, pp. 25–34.
5. Agapova, O.Yu., Skoblov, Yu.S., Zykov, K.A., Rvacheva, A.V., Beilina, V.B., Masenko, V.P., and Chazova, I.E., *Russ. J. Bioorg. Chem.*, 2015, vol. 41, no. 5, pp. 529–535.
6. Agapova, O.Yu., Skoblov, Yu.S., Tkachev, G.A., Mironova, N.A., Golitsyn, S.P., Masenko, V.P., Chazova, I.E., and Zykov, K.A., *Mol. Biol.* (Moscow), 2016, vol. 50, no. 6, pp. 880–886.
7. Abraham, G., Broddet, O.E., and Ungemach, F.R., *Equine Vet. J.*, 2001, vol. 33 no. 5, pp. 487–493.
8. Bundkirchen, A., Brixius, K., Bölck, B., Nguyen, Q., and Schwinger, R.H., *Eur. J. Pharmacol.*, 2003, vol. 460, pp. 19–26.
9. Abraham, G., Shibeshi, W., and Ungemach, F.R., *Pulm. Pharmacol. Ther.*, 2011, no. 1, pp. 174–181.
10. Guereschi, M.G., Araujo, L.P., Maricato, J.T., Takenaka, M.C., Nascimento, V.M., Vivanco, B.C., Reis, V.O., Keller, A.C., Brum, P.C, and Basso, A.S., *Eur. J. Immunol.*, 2013, vol. 43, pp. 1001–1012.
11. Krasnikova, T.L., Korichneva, I.L., and Radyukhin, V.A., *Biokhimiya*, 1989, vol. 54, no. 2, pp. 235–243.
12. Engel, G., Hoyer, D., Berthold, R., and Wagner, H., *Naunyn Schmiedebergs Arch. Pharmacol.*, 1981, vol. 317, no. 4, pp. 277–285.
13. Nagatomo, T. and Imai, S., *J. Pharmacobiodyn.*, 1989, vol. 12, no. 9, pp. 509–516.
14. Flugge, G., Ahrens, O., and Fuchs, E., *Cell Mol. Neurobiol.*, 1997, vol. 17, no. 4, pp. 401–415.
15. Abraham, G., Broddet, O.E., and Ungemach, F.R., *Equine Vet. J.*, 2001, vol. 33, no. 5, pp. 487–493.
16. Bilski, A.J., Halliday, S.E., Fitzgerald, J.D., and Wale, J.L., *J. Cardiovasc. Pharmacol.*, 1983, vol. 5, no. 3, pp. 430–437.
17. Baker, J.G., *Br. J. Pharmacol.*, 2005, vol. 144, no. 3, pp. 317–322.
18. Dooley, D.J. and Bittiger, H., *J. Pharmacol. Methods*, 1987, vol. 18, no. 2, pp. 131–136.
19. Shevelev, A.Ya., Kashirina, N.M., Kuznetsova, I.B., et al., *Vestn. Biotekhnol. Fiz.-Khim. Biol. im. Yu.A. Ovchinnikova*, 2015, vol. 11, pp. 5–14.
20. McLean, A.J. and Milligan, G., *Br. J. Pharmacol.*, 2000, vol. 130, no. 8, pp. 1825–32.
21. Baker, J.G., Hall, I.P., and Hill, S.J., *Mol. Pharmacol.*, 2003, vol. 63, pp. 1312–1321.

Translated by G. Levit