β-ADRENERGIC RECEPTOR AND ADENYLATE CYCLASE OF RAT RETICULOCYTES AS A TEST SYSTEM FOR DRUG SCREENING

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UDC 612.111.7.015:577.152.633]:615.21.3.07

KEY WORDS: β-adrenoreceptor; adenylate cyclase; reticulocytes; thioproperazine, trifluperidol

The creation of highly specific drugs entails the development of test systems which will adequately reflect the mechanism of action of the particular substance. The primary receptor of the signal created by neurohumoral stimuli in the body, and also the target for a large number of drugs, is the adenylate cyclase system [2]. This transmembrane oligomeric complex consists of hormonal receptors, GTB-binding proteins of two types (activatory and inhibitory), and the catalytic subunit of adenylate cyclase (AC) proper [1]. The fact that β-adrenergic receptors interact with the AC-system [1, 2] means that, by the use of the same membrane preparation, it is possible to measure both binding of the ligand with the receptor and AC activity, modified in the presence of the ligand.

In this paper a biochemical test system is suggested for pharmacologic screening of physiologically active substances that influence β-adrenergic receptors.

EXPERIMENTAL METHOD

Reticulocytosis was induced as described in [4, 11], with certain modifications. Male Wistar rats weighing about 300 g were given single subcutaneous injections of phenylhydrazine in a concentration of 36 mg/ml and a dose of 0.5 ml daily for 3 days. The rats were anesthetized with ether or chloroform, fixed, and subjected to thoractomy. Blood was taken directly from the heart into a syringe containing 2 ml of anticoagulant: 5 mM EDTA in 0.9% NaCl solution. The blood was centrifuged 4 times, for 5 min each time, at 1500-2000g, and the supernatant was carefully decanted. The cell residue was lysed with a solution containing 30 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol, and 0.5 mM EDTA, the ratio of lytic solution to residue being 1:10 (1:15). The lysed product was allowed to stand for 30 min and then homogenized with a glass-Teflon homogenizer. The resulting suspension was centrifuged at 14,000g for 30 min. The residue was washed twice with lytic solution. For the last time centrifugation was carried out in a solution containing 30 mM Tris-HCl, pH 7.5, and 0.25 M sucrose. The membranes were suspended in the above solution and kept at the temperature of liquid nitrogen. The protein concentration in the membranes thus obtained was 8 mg/ml. AC activity was measured as described in [7]. The membrane preparation (5-50 μg protein) was incubated for 20 min at 30°C in 50 μl of incubation medium of the following composition: 50 mM Tris-HCl, pH 7.5, at 30°C, 5 mM $MgCl_2$, 0.5 mM cAMP, 0.5 mM isobutylmethylxanthine, 0.1 mM ATP (α-^{32}P-ATP, 0.5 μCi per sample, from Amersham International, England), 20 mM creatine phosphate, and 0.2 mg/ml crea-

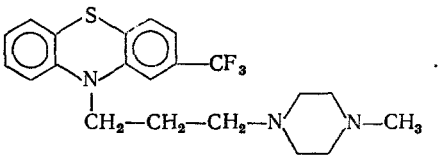
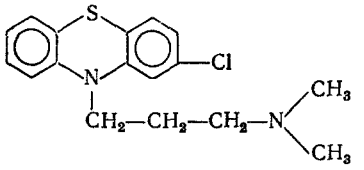
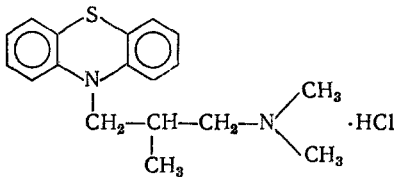
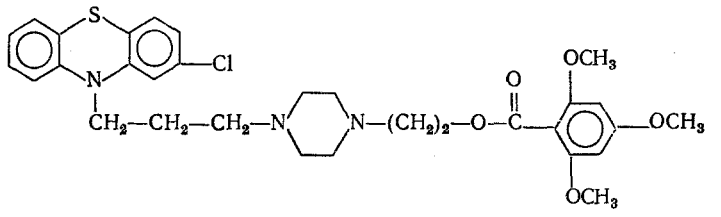
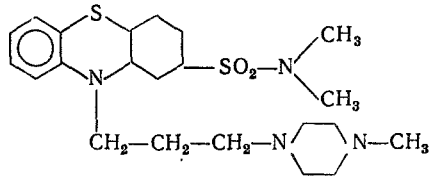
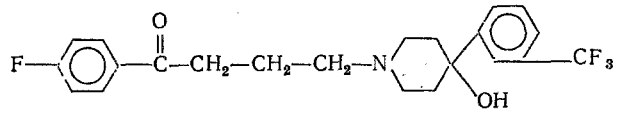
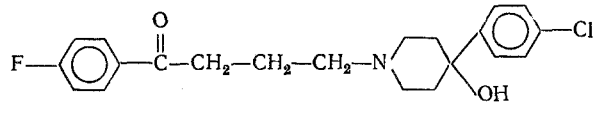
TABLE 1. Regulatory Properties of AC of Rat Reticulocyte Membranes (M ± m)

Experimental conditions	AC activity, pmoles cAMP/mg protein/min	Degree of activation
Control	4,5±0,5	1
GTP, 10^{-4} M	4,0±0,4	1
GIDP, 10^{-4} M	58,0±5	13
ISO, 5×10^{-5} M	57,0±5	12,6
ISO + GTP	208,5±15	44
ISO + GIDP	189,0±10	42

Institute of Experimental Cardiology, All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR, Moscow. (Presented by Academician of the Academy of Medical Sciences of the USSR V. N. Smirnov.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 104, No. 11, pp. 590-594, November, 1987. Original article submitted January 21, 1987.

 0007-4888/87/0011-1578$12.50

TABLE 2. Effect of Various Drugs on Basal AC Activity of Rat Reticulocytes (M ± m)

Drug, 10^{-4} M	Structural formula	Basal activity, %
Control		100
Phenothiazine trifluoroperazine		78±15
chlorpromazine		95±13
levomepromazine (tisercin)		93±9
metaphenazine (frenolone)		49±4
thioproperazine (majeptil)		49±7
Butyrophenones trifluperidol (trisedil)		75±7
haloperidol		68±4

Legend. Reaction carried out at 30°C for 15 min. Incubation medium contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.5 mM cAMP, 0.1 mM ATP, 0.5 μCi [α-^{32}P]-ATP, 10^{-4} M GTP, and ATP-regenerating system.

tine phosphokinase. All values of AC activity are presented in the form of the average of three parallel measurements.

Binding of (–)-[^{3}H]-dihydroalprenolol (DHA) with β-adrenergic receptors of the reticulocytes was determined as described in [8], with certain modifications. The membranes (25 μg per sample) were incubated with DHA (40 nM) in medium with a total volume of 1 ml, containing 20 mM Tris-HCl, pH 7.5, at 4°C, and 5 mM $MgCl_2$, with subsequent filtration in vacuo through filters of GF/C type (Whatman, England). The filters were dried, put into flasks with scintillation fluid, and their radioactivity was measured on a liquid scintillation counter. To determine nonspecific binding, (–)-propranolol was added to special samples in a concentration of 10^{-5} M. Specific binding of DHA with β-adrenergic receptors was determined by subtracting nonspecific from total binding. The value of K_i and the number of receptors were calculated by the method of [14], using a program for linear regression, on a HP-67 microcalculator (Hewlett-Packard, USA). The protein concentration was determined as in [12].

TABLE 3. Effect of Drugs on Isoprotenernol-Stimulated AC Activity of Reticulocytes (M ± m)

Drug, 10^{-4} M	AC activation in the presence of 3×10^{-6} M ISO,* %	IC_{50}, μM
Control ISO 3×10^{-6} M	100	—
Phenothiazine		
trifluoperazine	80±16	>100
chlorpromazine	73±13	>100
levomepromazine (tisercin)	79±8	>100
metaphenazine (frenolone)	128±19	—
thioproperazine (majeptil)	24±4	56,2
Butyrophenones		
trifluperidol (trisedil)	30±2	46,4
haloperidol	89±12	>100

Legend. Reaction carried out at 30°C for 15 min. Incubation medium contained 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM ATP, 0.5 μCi [α-^{32}P]-ATP, 0.5 mM cAMP, 10^{-4} M GTP, $3\cdot10^{-6}$ M isoproterenol, and ATP-regenerating system. *) [Activity of AC + ISO]/[Basal activity] × 100% in presence (absence) of drug.

EXPERIMENTAL RESULTS

Unlike mature erythrocytes, reticulocytes have hormone-sensitive AC [11]. To obtain reticulocyte-enriched rat blood we used phenylhydrazine, which induces reticulocytosis [6]. According to the literature, rat reticulocytes contain only one β_2-subtype of adrenergic receptors [9]. Moreover, there is no information on the presence of α-adrenoreceptors on reticulocytes. The regulatory properties of the AC preparation which we obtained were virtually indistinguishable from those described in the literature [13] (Table 1). According to our data, GTC had virtually no effect on AC activity, but GIDP increased its activity about 13-fold. Isoproterenol in a concentration of $5\cdot10^{-5}$ M activated AC about 13-fold. Addition of the hormone together with the guanyl nucleotides led to marked activation of AC, the degree of which rose to 40-fold (Table 1).

Of the series of cardiotropic and neurotropic drugs investigated (Table 2) metaphenazine and theoproperazine (phenothiazine derivatives) had an inhibitory action on basal AC activity. However, thioproperazine (phenothiazine derivatives) and trifluoperidol (butylrophenone derivatives) had a blocking effect on hormonal stimulation of AC by isoproterenol (Table 3). Metaphenazine had no effect on hormonal sensitivity of AC (Table 3). The fact that basal AC activity was inhibited by metaphenazine and thioproperazine can be explained by the presence of a nonspecific membrane effect in the action of these substances in high concentrations.

Besides the drugs listed in Table 3, we also tested the (+)- and (–)-isomers of butaclamol, and also imipramine, vinblastine, and verapamil. These substances, in a concentration of 10^{-4} M, did not affect the sensitivity of AC to isoproperenol, although they inhibited basal AC activity very slightly.

We determined the 50% of maximal inhibition constants (IC_{50}) of isoproterenol-stimulated AC. The thioproperazine $IC_{50} = 5.62\cdot10^{-5}$ M (Table 3) and for trifluperidol $IC_{50} = 4.64\cdot10^{-5}$ (Table 3). For a more detailed study of the mechanism of inhibition, their effect on stimulation of AC by isoproteronol was investigated in the presence of two concentrations of the blockers. It was found (Fig. 1a) that under the influence of thioproperazine in a concentration of $5\cdot10^{-5}$ M the curve of AC activation by the hormone was shifted to the left. With a concentration of 10^{-4} M a marked shift of the curve was observed with a decrease in the degree of AC activation by isoproterenol. Transformation of these data to Dixon's coordinates (Fig. 1b) enabled the inhibition constant of hormone-stimulated AC activity to be determined for thioproperazine: $K_i = 4.0\cdot10^{-5}$ M. Similar investigations were undertaken for trifluperidol. This compound also reduced the affinity of the β-receptor for isoproterenol (Fig. 2a) and reduced the degree of AC activation by the hormone. The inhibition constant determined by Dixon's method was $4.5\cdot10^{-4}$ M (Fig. 2b). On the basis of these experimental results a blocking effect of thioproperazine and trifluoperazine on the β-adrenergic receptor coupled with

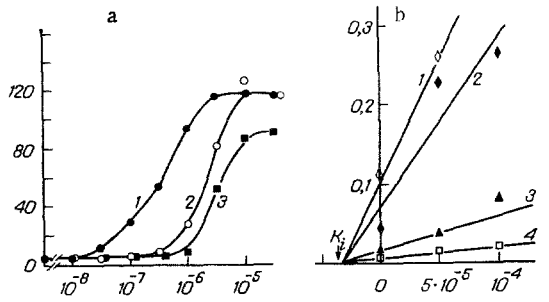


Fig. 1

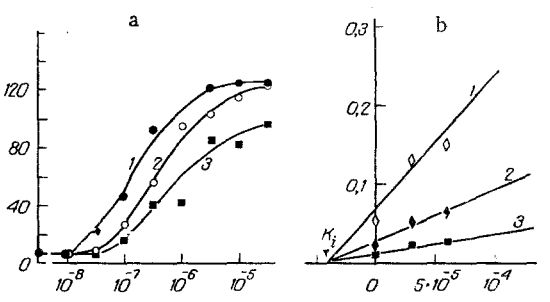


Fig. 2

Fig. 1. Effect of thioproperazine on isoproterenol-stimulated AC activity of reticulocytes (a) and analysis of data between Dixon's coordinates (b). a: Abscissa, isoproterenol concentration, M; ordinate, AC activity, pmoles cAMP/mg protein/min. 1) without additives; 2) in presence of $5\cdot10^{-5}$ M thioproperazine; 3) in presence of 10^{-4} M thioproperazine; b: abscissa, thioproperazine concentration, M; ordinate, ratio of AC activity in presence of thioproperazine to AC activity without additives, and with isoproterenol in concentrations of $3\cdot10^{-8}$ M (1), 10^{-7} M (2), 10^{-6} M (3), and $3\cdot10^{-6}$ M (4). $K_i = 4.01\cdot10^{-5}$ M.

Fig. 2. Effect of trifluperidol on isoproterenol-stimulated AC activity of reticulocytes (a) and analysis of data between Dixon's coordinates (b). 1) Without additives; 2) in presence of $3\cdot10^{-5}$ M trifluperidol; 3) in presence of $6\cdot10^{-5}$ M trifluperidol; b: abscissa, trifluperidol concentration, M; ordinate, ratio of AC activity in presence of trifluperidol to AC activity without additives, with isoproterenol in concentrations of $3\cdot10^{-8}$ M (1), 10^{-7} M (2) and $3\cdot10^{-7}$ M (3). $K_i = 4.5\cdot10^{-5}$ M.

AC also could be postulated. These compounds had no effect on the catalytic subunit (no action on basal activity on the enzyme). The only remaining suggestion was that these two compounds either affect the β-adrenergic receptor of the reticulocytes directly or they disturbed the process of coupling of the receptor with AC.

Direct proof of the β-blocking action of thioproperazine and trifluperidol on β-adrenoreceptors was obtained by a study of the action of these drugs on binding of DHA with reticulocyte membranes (Fig. 3). Both drugs displaced the labeled ligands about equally, with a 50% maximal displacement constant of $IC_{50} = 1.3\cdot10^{-5}$ M for thioproperazine and $IC_{50} = 2.3\cdot10^{-5}$ M for trifluperidol. Inhibition constants of ligand binding were calculated for these substances by the method of Cheng and Prusoff. Their values were $K_i = 5\cdot10^{-6}$ M for thioproperazine and $K_i = 9\cdot10^{-6}$ M for trifluperidol. Inhibition constants of hormonal sensitivity of AC coincided within the limits of an order of magnitude, with the displacement constants of the labeled ligand. On the basis of these results it can be postulated that the biological effect of a change in activity of isoproteronol-stimulated AC is connected with the direct action of thioproperazine and of trifluperidol on β-adrenoreceptors.

No direct proof of the β-blocking effect of butyrophenone derivatives has yet been published in the literature. We succeeded in demonstrating directly the indirect data obtained

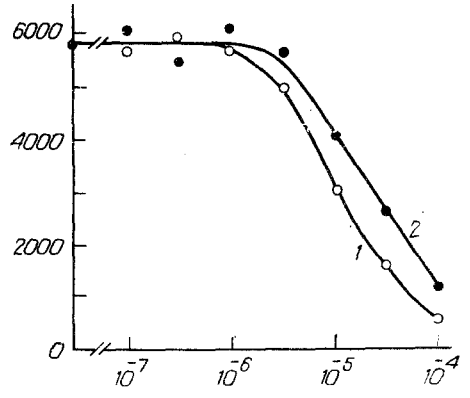


Fig. 3. Displacement of [^{3}H]-dihydroalprenolol, bound with β-receptor of rat reticulocytes, by thioproperazine (1) and by trifluperidol (2). Abscissa, concentration of drugs (in M) on logarithmic scale; ordinate, specific binding of [^{3}H]-dihydroalprenolol, in cpm.

previously on the adrenolytic properties of thioproperazine [8]. The β-blocking effect of trifluperidol, which we found, is evidence that the rat reticulocyte membrane preparation can be effectively used as a biochemical test system for estimating the effect of drugs on the β-adrenergic receptor coupled with AC. Affinity constants of thioproperazine for trifluperidol are 3 orders of magnitude lower than those of the known β-blockers propranolol and alprenolol [3], but the doses of these drugs used in clinical practice suggests that the adrenolytic action of trifluperidol and thioproperazine may play a significant role in the manifestation of their side effects.

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EFFECT OF NONACHLAZINE ON THE ADENYLATE CYCLASE SYSTEM OF THE RABBIT HEART

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UDC 615.224:547.869.2].015.4:[612.173.1.015.1:577.152.633

KEY WORDS: adenylate cyclase; β-adrenoreceptors; myocardium; nonachlazine, ischemic heart disease

The drug nonachlazine — 10-β-[1,4-diazobicyclo-(4,3,0)-nonanyl-4-]-propionyl-2-chlorophenothiazine dihydrochloride — synthesized in the Institute of Pharmacology, Academy of Medical Sciences of the USSR, is used in the treatment of ischemic heart disease. The pharmacologic properties of nonachlazine have been investigated in detail [1]. The study of the effect of nonachlazine on cardiac activity (cardiac output, contractility) has shown that this compound possesses not only β-adrenostimulating properties, but also the ability to induce a partial β-adenoblockage [1]. We know that the β-adrenergic effect in animal cells is realized through the adenylate cyclase system (ACS) [3]. The molecular mechanisms of action of nonachlazine on adrenergic structures of the myocardium have not been elucidated.

The aim of this investigation was to study the effect of nonachlazine on the ACS of the rabbit heart, on regulation of adenylate cyclase (AC) activity by isoproterenol and also on binding of the β-adrenoreceptor antagonist [^{3}H]-dihydroalprenolol (DHA).

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Translated from Byulleten' Éksperimental Biologii i Meditsiny, Vol. 104, No. 11, pp. 594-596, November, 1987. Original article submitted December 18, 1986.

0007-4888/87/0011-1582$12.50