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HORMONE ACTION AT THE MEMBRANE LEVEL

V. BINDING OF (±)-[^{3}H]ISOPROTERENOL TO INTACT CHICKEN ERYTHROCYTES AND ERYTHROCYTE GHOSTS

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SUMMARY

The binding of (±)-[7-^{3}H]isoproterenol to intact chicken erythrocytes has been investigated by a rapid centrifugation technique. The binding is displaceable by a one thousand-fold excess of cold isoproterenol and consists of two fractions, only one of which is inhibitable by the beta antagonist (−)-propranolol. The total displaceable binding to intact cells amounts to 80 or 127 molecules per cell at a (±)-isoproterenol concentration of 0.4 μM depending on the method employed to analyze the binding. Under similar conditions, the total displaceable binding to isolated membrane ghosts is 12 600 molecules per cell. The propranolol-inhibitable binding to intact cell reaches saturation within 5 min at 4 °C and gives by Scatchard analysis a maximum binding of 108 molecules per cell and with a K_D of 0.4 μM. 50 % inhibition of binding is obtained with 0.3 μM unlabeled (−)-isoproterenol as compared to 20 μM unlabeled (±)-isoproterenol. The binding of isoproterenol thus shows a marked stereospecific preference for the (−)-isomer.

INTRODUCTION

Catecholamine binding to membranes of avian red cells has been done nearly exclusively on isolated ghosts rather than on intact cells [1–4]. Radioactive-labeled beta adrenergic agonists have been used in attempts to elucidate the properties of the receptor. These reports have shown large amounts of agonist binding per milligram of membrane protein equivalent, in one case to 84 000 sites per cell at saturation [2].

Recently, studies with high-affinity radioactive beta antagonists on isolated erythrocytes ghosts have shown that the number of beta receptors per red cell is much lower than the number obtained with beta adrenergic agonists. Using propranolol, 550 beta receptors were found per turkey erythrocyte [3] and with alprenolol, 1400 beta receptors have been found per frog erythrocyte [4].

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In this study we compare the binding of the beta adrenergic agonist (±)-[7-^{3}H]isoproterenol to intact chicken erythrocytes to that of erythrocyte ghosts. This nucleated red cell possesses a catecholamine-sensitive adenylate cyclase [5]. We report a very marked difference in isoproterenol binding to intact cells as compared to ghosts.

MATERIALS AND METHODS

Chicken blood was obtained fresh from a local slaughterhouse. The cells were washed three times and stored at 4 °C in isotonic saline containing 1 % bovine serum albumin, 1.6 % glucose, 0.14 % NaH_2PO_4 plus 0.025 % streptomycin. The (±)-[7-^{3}H]-isoproterenol (4.9 Ci/mmol) was obtained from New England Nuclear and shown to be > 95 % pure by thin-layer chromatography, using butanol/acetic acid/water (12 : 3 : 5 v/v). The following reagents were used: (−)-propranolol hydrochloride (Ayerst Labs), phenoxybenzamine hydrochloride (Smith, Kline and French), (−)-isoproterenol-(+)-bitartrate (Sigma), and (+)-isoproterenol-(+)-bitartrate (Sterling-Winthrop).

1. Preparation of red cell ghosts

The chicken blood was washed three times at 4 °C with isotonic saline by centrifugation on a table-top International Centrifuge at 3000 rev./min and the buffy coat aspirated from the top of the erythrocyte pellet. 4 ml of packed cells were mixed with 25 ml of lysing buffer (30 mM $NaHCO_3$/0.05 mM EDTA/5 mM mercaptoethanol, pH 7.4) at 4 °C and homogenized with 20 strokes in a Dounce homogenizer. The homogenate was spun in an International PR-2 centrifuge at 1500 rev./min for 15 min at 4 °C to remove the nuclei and unlysed cells. The supernatant containing red cell ghosts was centrifuged in a Spinco centrifuge for 15 min at 4 °C at 40 000 × g. The ghosts were homogenized with a Thomas teflon homogenizer in a buffer containing 7.5 mM $NaHCO_3$/0.05 mM EDTA/5 mM mercaptoethanol, pH 7.4 and centrifuged at 15 000 × g for 15 min. The homogenization and centrifugation were repeated two more times, and a light-yellow ghost pellet was obtained. The ghosts were stored at −15 °C in Krebs-Ringer bicarbonate buffer to give a final protein concentration of 5 mg/ml. Protein was determined by the method of Lowry et al. [6].

2. Binding to erythrocyte ghosts

Binding of (±)-[7-^{3}H]isoproterenol to chicken erythrocyte ghosts was assayed by rapid centrifugation. Aliquots containing 1 mg of ghost protein were incubated for 4 min at 4 °C with isoproterenol in 1 ml of Krebs-Ringer bicarbonate buffer containing 25 μM phenoxybenzamine. The latter was added to minimize agonist uptake [7]. Incubation was carried out in 1.5 ml polyethylene centrifuge tubes. The ghosts were centrifuged at 14 000 rev./min for 2 min on an Eppendorf microcentrifuge (model 3200), the supernatant discarded, and the top of the pellet washed three times with 1 ml of cold isotonic saline. The pellet was dissolved overnight in 0.5 ml of 1 % sodium dodecyl sulfate (SDS), transferred to a counting vial containing 10 ml of Beckman-Biosolv Formula TLA scintillation cocktail and counted in a Packard Tri Carb spectrometer. Trapping and non-displaceable binding were estimated by measuring the binding in the presence of a 1000-fold excess of unlabelled (−)-isoproterenol. This value was subtracted from the total binding to obtain displaceable binding.

3. Binding to intact erythrocytes

The binding of (±)[^{3}H]-isoproterenol to intact erythrocytes was done on cells which were washed three times at 4 °C with isotonic saline and then incubated for 1 h at 20 °C in Krebs-Ringer bicarbonate buffer containing 1 % albumin. This was done to remove endogenous catecholamines. The cells were spun and washed once in Krebs-Ringer bicarbonate buffer. The cell number was determined by counting an appropriate diluted aliquot with a Coulter Electronic Particle Counter (Model ZBI).

Aliquots of 0.5 ml of packed erythrocytes (about $2 \cdot 10^9$ cells) were added to siliconized glass tubes and 0.5 ml of Krebs-Ringer bicarbonate buffer containing 25 μM phenoxybenzamine was layered on top. The labeled isoproterenol was added and the two phases were mixed to begin the binding assay. Except where otherwise noted, all steps were carried out at 4 °C and binding done for 4 min. After the 4-min incubation, 2.0 ml of cold isotonic saline were added. Three, 0.6 ml aliquots were immediately layered on top of 2.0 ml of 20 % sucrose (w/w) solution and spun on an IEC table-top centrifuge at 3000 rev./min for 90 s. Trapping and nondisplaceable binding were estimated by assaying the binding in the presence of a 1000-fold excess of unlabeled (−)-isoproterenol. The (±)-[^{3}H]isoproterenol bound to the membrane of the cell was measured either by the dissociation technique or by isolating the membranes as described below.

Method A. Dissociation technique for measuring bound (±)-[^{3}H]isoproterenol. At the end of the 4-min binding and centrifugation through sucrose described in Section 3, the top of the erythrocyte pellet was washed twice with 3 ml of isotonic saline at 4 °C. The cells were then lysed at 20 °C in 1.0 ml of 2 mM SDS containing 250 μM cold (−)-isoproterenol and the bound (±)-[^{3}H]isoproterenol was allowed to dissociate from the membrane for 2 h at 20 °C. 0.20 ml of 70 % perchloric acid was then added to each tube, and the tubes were spun in an IEC table-top centrifuge for 10 min at 3000 rev./min. From each tube an 0.80 ml aliquot of the supernatant was added to 10 ml of the Beckman Biosolv-Formula TLA scintillation cocktail and counted on a Packard Tri-Carb Spectrometer.

Method B. Measurement of membrane-bound (±)-[^{3}H]isoproterenol. At the end of the 4-min binding described in Section 3, three, 0.20 ml samples were immediately layered onto 1.0 ml of 20 % sucrose and spun down for 1 min at 14 000 rev./min on the Eppendorf microfuge. The top of the pellet was washed twice with 1 ml of isotonic saline. The cells were then lysed for 90 s at 4 °C in 1.0 ml of 10 mM Tris buffer, pH 7.4 containing 250 μM cold (−)-isoproterenol. The ghosts were centrifuged at 14 000 rev./min for 4 min on the microfuge. The top of the pellet was washed three times at 4 °C with 1 ml of isotonic saline. The lysing step was originally done in the presence of cold isoproterenol to prevent any further binding of labeled free isoproterenol which might have been taken up by the cell. However, the same amount of binding was obtained without the addition of the cold isoproterenol under these experimental conditions of low temperature and short time of incubation.

The pink ghosts were decolorized by a modification of the method of Mahin and Lofberg [8]. 0.20 ml of 30 % H_2O_2 was added to the ghosts after they have been allowed to dissolve overnight at 30 °C in 0.50 ml of ethanol/Biosolv (2 : 1). The samples were then transferred to counting vials containing 10 ml of Beckman Biosolv-Formula TLA scintillation cocktail and mixed. 0.10 ml of 0.5 M HCl was then added and the samples were allowed to equilibrate overnight and counted.

RESULTS

The binding of (±)-[7-^{3}H]isoproterenol to chicken erythrocyte ghosts and to intact chicken erythrocytes is shown in Table I. In both cases, the binding is displaceable by 1000-fold excess of cold hormone and inhibitable by (−)-propranolol. For the erythrocyte ghosts there are 10.5 pmol of isoproterenol bound per mg of membrane protein at a non-saturating level of 0.4 μM isoproterenol. This is similar to the data obtained by Bilezekian and Aurbach [4] on (±)-[^{3}H]isoproterenol binding to turkey erythrocyte ghosts. This non-saturating binding to ghosts is equivalent to 12 600 molecules of isoproterenol bound per red cell. However, with intact chicken

TABLE I

BINDING OF (±)-[^{3}H]ISOPROTERENOL TO INTACT CHICKEN ERYTHROCYTES AND ERYTHROCYTE GHOSTS

TABLE IA. 0.50 ml of packed cells was incubated in 0.50 ml of Krebs-Ringer bicarbonate buffer containing 25 μM phenoxybenzamine, (±)-[^{3}H]isoproterenol at a concentration of 0.4 μM (1 μCi) in the presence and absence of 80 nM (−)-propranolol. The binding assay was done for 4 min at 4 °C and assayed by Methods A and B as described in the text. Trapping was estimated by doing the binding in the presence of a 1000-fold excess of unlabelled isoproterenol. Each value represents the mean ± S.D. of three experiments performed in triplicate and has been corrected for trapping.

Intact cells	Isoproterenol bound (molecules/cell)
Method A	
0.4 μM isoproterenol	127 ± 18
0.4 μM isoproterenol plus 80 nM (−)-propranolol	66 ± 23
propranolol-inhibitable binding	61 ± 20
Method B	
0.4 μM isoproterenol	80 ± 13
0.4 μM isoproterenol plus 80 nM (−)-propranolol	37 ± 7
propranolol-inhibitable binding	43 ± 10

TABLE IB. 5 mg of ghost protein were incubated in 1.0 ml of Krebs-Ringer bicarbonate buffer containing 25 μM phenoxybenzamine and (±)-[^{3}H] isoproterenol at concentrations of 0.1, 0.4 and 1.0 μM in the presence and absence of 4 μM (−)-propranolol. Following a 4 min incubation at 4 °C the ghosts were centrifuged by a 2 min spin in a Brinkman microfuge. The ghosts were washed, dissolved and counted as described in the text. The trapping blanks were estimated by doing the binding in the presence of 2 mM unlabeled (−)-isoproterenol. Each value represents the mean ± S.D. of one experiment done in triplicate, and has been corrected for trapping.

Erythrocyte ghosts	pmol per mg ghost protein	Molecules bound per cell
0.1 μM isoproterenol	3.5 ± 1	4200 ± 1200
0.1 μM isoproterenol plus 4 μM (−)-propranolol	2.5 ± 0.4	3010 ± 480
0.4 μM isoproterenol	10.5 ± 4	12600 ± 4800
0.4 μM isoproterenol plus 4 μM (−)-propranolol	4.3 ± 1.2	5180 ± 1440
1 μM isoproterenol	32 ± 4	38500 ± 4800
1 μM isoproterenol plus 4 μM (−)-propranolol	15 ± 4.8	18100 ± 5800

cells (Table I) at the same isoproterenol concentration of 0.4 μM, only 127 or 80 molecules of isoproterenol are bound per cell using Methods A and B. The isoproterenol binding to intact cells is inhibited to the extent of 48–54 % by 80 nM (−)-propranolol. Higher concentrations of (−)-propranolol did not give any further inhibition, indicating the existence of two binding fractions. A much higher concentration of (−)-propranolol (4000 nM) is required to give 60 % inhibition of binding to ghosts (at 0.4 μM isoproterenol).

Since the dissociation technique used to measure the binding of isoproterenol to the membrane measures the radioactive label in the supernatant following lysis of the cell, it does not distinguish binding from uptake. The procedure was modified so that only the isoproterenol directly bound to the membrane was assayed. With this technique (Method B) the agonist was first incubated with intact cells, the cells were washed, lysed and the ghosts isolated and counted. The results are shown in Table I. The propranolol-inhibitable binding at 0.4 μM isoproterenol is 43±10 molecules per cell using Method B as compared to 61±20 molecules per cell seen using the dissociation technique of Method A. This difference in propranolol-inhibitable binding is not statistically significant. However, there is a significant decrease in total binding (from 127 to 80 molecules per cell) when Method B is used. This is believed to represent dissociation of some isoproterenol during preparation of the ghosts. This loss occurs mainly with the binding sites not inhibited by propranolol.

The propranolol-inhibitable fraction of the isoproterenol binding to whole cells was investigated further. Fig. 1 demonstrates that this binding (using Method A) is saturable. Analysis by Scatchard plots [9] gives a dissociation constant of 0.4 μM and 109 binding sites per cell. The characteristics of the binding is that of a beta receptor. In Fig. 2 it is seen that the propranolol-inhibitable binding is rapid at 4 °C and reaches equilibrium within 5 min.

To check for degradation of (±)-[^{3}H]isoproterenol during the binding assay, the hormone was incubated at 4 °C for 4 min with and without chicken erythrocytes in the Krebs-Ringer bicarbonate buffer. The cells were centrifuged and the iso-

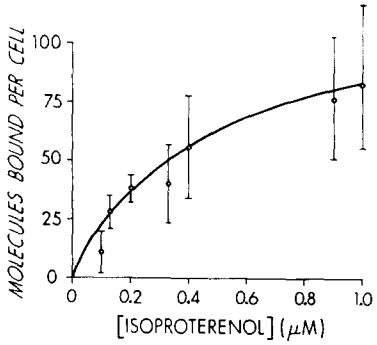

Fig. 1. Dose response of the propranolol-inhibitable binding of (±)-[^{3}H]isoproterenol to intact chicken erythrocytes. 0.50 ml of packed erythrocytes were incubated in 0.50 ml o. Krebs-Ringer bicarbonate buffer containing 25 μM phenoxybenzamine and different amounts of (+)-[^{3}H]isoproterenol for 4 min at 4 °C. Binding (Method A) was also done in the presence of 8 μM (−)-propranolol. The values represent the difference in binding with and without the propranolol. Each point represents the mean ±S.D. of two experiments done in triplicate.

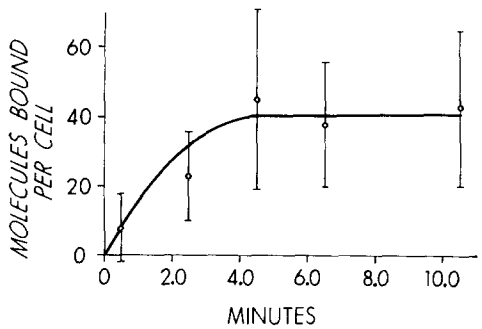

Fig. 2. Time course of the propranolol-inhibitable isoproterenol binding to intact chicken erythrocytes. 1.5 ml of packed erythrocytes were incubated for different time intervals in 1.5 ml of Krebs-Ringer bicarbonate buffer containing 25 μM phenoxybenzamine and 0.33 μM (±)-[^{3}H]isoproterenol at 4 °C. Binding (Method A) was also done in the presence of 8 μM (−)-propranolol. At the indicated times two 0.20 ml samples were removed, spun through a 20 % sucrose solution and assayed according to the dissociation technique (Method A) for the amount of isoproterenol bound. Each point is the mean ±S.D. of two experiments done in duplicate.

proterenol in the supernatant fluid was examined by thin-layer chromatography using silica gel plates and two different solvent systems. In both the solvent systems (butanol/acetic acid/water, 12 : 3 : 5, v/v and water saturated with phenol) over 85 % of the counts migrated with the standard isoproterenol. This represents a minimal recovery due to unavoidable loss and degradation during the chromatography.

The stereospecificity of isoproterenol binding to intact cells was also examined. As shown in Fig. 3, the propranolol-inhibitable binding is highly stereospecific since (−)-isoproterenol was nearly 100 fold more potent in inhibiting binding than (+)-isoproterenol.

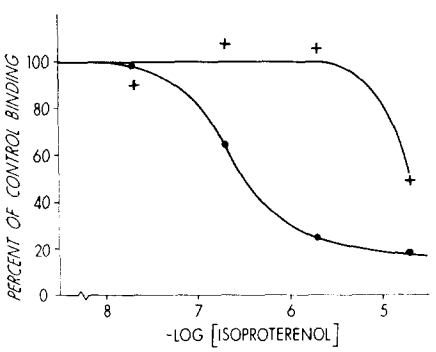

Fig. 3. Inhibition of (±)-[^{3}H]isoproterenol binding to intact chicken erythrocytes by isoproterenol stereoisomers. 0.50 ml of packed erythrocytes were incubated in 0.50 ml of the Krebs-Ringer bicarbonate buffer containing 25 μM phenoxybenzamine, 0.4 μM (±)-[^{3}H]isoproterenol, and different concentrations of the unlabeled (+)- and (−)-stereoisomers of isoproterenol. Binding was also done in the presence of 8 μM propranolol and in both cases analyzed by Method B. The values represent the difference in binding with and without propranolol, with 100 % binding equal to 46.4 molecules per cell. Each point represents the mean of two experiments done in triplicate. +–+, (+)-isoproterenol; ●–●, (−)-isoproterenol.

DISCUSSION

The binding of (±)-[^{3}H]isoproterenol to intact chicken erythrocytes is rapid, saturable, dissociable, stereospecific and inhibited by propranolol. The number of total sites per cell is small. The important observation is that the total displaceable binding of (±)-[7-^{3}H]isoproterenol to the intact chicken erythrocytes (127 sites/cell by Method A) is only 0.9 % of the binding to isolated erythrocyte ghosts (12 600 sites/cell) at an isoproterenol concentration of 0.4 μM. However, the extrapolated maximum number of propranolol-inhibitable sites at saturation is 109 molecules per cell as determined by Scatchard analysis. Bilezikian and Aurbach [2] demonstrated that the binding of isoproterenol to turkey ghosts reaches a maximum of 70 pmol per mg of membrane protein. Since there are approx. 10 mg of membrane protein per $5 \cdot 10^9$ cells, this amounts to 84 000 sites per cell. They found that propranolol at 1 mM concentration inhibited about 75 % of the binding. In our study the isoproterenol binding to intact chicken erythrocytes is inhibited 46–52 % at a propranolol concentration of only 80 nM.

Intact erythrocytes represent a more physiological system than do erythrocyte ghosts. This raises the question of the significance of catecholamine binding to erythrocyte ghosts. The large amount of binding of (±)-isoproterenol to the ghosts may be an artifact produced by lysing the cell, or may represent binding to non-specific sites on the inner surface of the membrane. However, one cannot eliminate the possibility that a binding inhibitor is present in the intact erythrocyte, but not in the ghost preparations, or that a large number of cryptic sites are exposed during the preparation of ghosts.

Working with intact erythrocytes made it necessary to distinguish between hormone uptake and binding. Since binding is done for 4 min at 4 °C in the presence of the uptake inhibitor, phenoxybenzamine [7], it is unlikely that appreciable uptake was being measured. In addition, isolation of the membrane following the binding to intact cells (Method B) demonstrated that the propranolol-sensitive isoproterenol binding was associated primarily with the membrane.

Chromatographic analysis of the (±)-[^{3}H]isoproterenol following the incubation with the intact chicken erythrocytes demonstrated that the isoproterenol was not degraded.

The binding of isoproterenol to the intact erythrocyte consists of two fractions, only one of which is inhibitable by propranolol. The propranolol-inhibitable fraction is usually considered to represent binding to the beta receptor. This binding site demonstrates a high degree of stereospecificity. The other fraction may represent significant binding to another catecholamine receptor or it may represent "non-specific binding". In any case, it also represents a very small number of sites per cell.

The total isoproterenol binding as assayed by isolating the ghosts following binding to intact cells was significantly lower than the binding assayed by the dissociation technique. It is likely that during the time required to prepare the ghosts some of the labelled isoproterenol dissociated from the membrane. It is noteworthy that the propranolol-inhibitable binding fraction is not significantly lower when Method B is used.

By Scatchard analysis the maximum value of the isoproterenol binding inhibitable by propranolol amounts to 109 molecules per cell. Using beta antagonists

and binding to red-cell ghosts, 550 propranolol-binding sites per cell have been observed in turkey erythrocytes [3], and 1400 alprenolol-binding sites per cell have been found in frog erythrocyte [4]. The chicken erythrocyte adenylate cyclase system is less responsive to catecholamines than that of the turkey or frog erythrocyte, and therefore may have fewer hormone receptors.

Preliminary studies have been done to compare the binding of the beta antagonist (−)-[^{3}H]alprenolol (New England Nuclear Corp., spec. act. 32 Ci/mM) to intact chicken erythrocytes and ghosts. The binding of alprenolol to ghosts is inhibited by propranolol and is saturable having a K_D of about 1.5 nM. At saturation the binding is equivalent to approx. 130 sites per cell. However, although the binding of alprenolol to intact cells is also inhibited by propranolol, it does not show saturation over the concentration range 0.5–10 nM. It is of interest that in the concentration range of 0.5–2.0 nM the binding of alprenolol to ghosts and cells is identical. Above 2 nM alprenolol the binding to ghosts has reached a plateau whereas the binding to cells continues to rise. It is apparent that the binding of alprenolol has a much lower dissociation constant than the binding of isoproterenol (1.5 nM for alprenolol as compared to 400 nM for isoproterenol). Moreover, isoproterenol show a much higher binding to ghosts as compared to cells whereas alprenolol appears to bind more to cells than to ghosts. Studies are underway to clarify these observations.

The beta adrenergic antagonists [^{3}H]propranolol [3] and [^{125}I]hydroxybenzylpindolol [10] have been observed to bind stereospecifically to turkey erythrocyte ghosts. In addition, the binding of [^{3}H]alprenolol, another beta antagonist, to frog erythrocyte ghosts has been shown to be stereospecific [4]. To our knowledge the work presented in this paper provides the first evidence for the stereospecific binding of an adrenergic agonist to intact erythrocytes.

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