Biochimica et Biophysica Acta, 673 (1981) 203—216

BBA 29519

β-ADRENERGIC ACTIVITY OF (±)-HYDROXYBENZYLISOPROTERENOL IN ISOLATED RAT FAT CELLS AND HEPATOCYTES

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(Received July 23rd, 1980)
(Revised manuscript received November 4th, 1980)

Key words: Hydroxybenzylisoproterenol; β-Adrenergic activity; β-Adrenergic receptor; Adenylate cyclase

Summary

The pharmacology of (±)-hydroxybenzylisoproterenol with respect to stimulation of cyclic AMP accumulation by isolated rat fat cells and liver cells was examined. (±)-Hydroxybenzylisoproterenol was found to be a full agonist and twice as potent as (—)-isoproterenol in liver cells, and equipotent to (—)-isoproterenol in fat cells with regard to stimulating cyclic AMP accumulation. A study of the ability of this catecholamine to stimulate adenylate cyclase activity of broken-cell preparations revealed that (±)-hydroxybenzylisoproterenol was equipotent to (—)-isoproterenol in liver cell homogenates, while 3- to 4-fold more potent than (—)-isoproterenol in fat cell ghost membranes. (±)-Hydroxybenzylisoproterenol was also found to be as potent as (—)-isoproterenol in stimulating cyclase activity of S49 mouse lymphoma cell membranes. Competition studies of specific [^{125}I]iodohydroxybenzylpindolol binding to liver cell membranes revealed a K_d of 10 nM for (±)-hydroxybenzylisoproterenol and 25 nM for (—)-isoproterenol binding to the liver β-adrenergic receptor. Competition studies of specific (—)-[^{3}H]dihydroalprenolol binding to fat cell membranes indicated a similar affinity of these sites for both (±)-hydroxybenzylisoproterenol and (—)-isoproterenol. The guanyl nucleotide Gpp(NH)p induced a shift in the curve for competition of (—)-[^{3}H]dihydroalprenolol binding by (—)-isoproterenol to the right, but failed to do so when (±)-hydroxybenzylisoproterenol was the competing agonist. Properties of (±)-[^{3}H]hydroxybenzylisoproterenol binding to fat cell or liver cell membranes were inconsistent with those expected of adenylate cyclase coupled β-adrenergic receptors.

Abbreviation: Mes, 3-(*N*-morpholino)ethanesulfonic acid.

Introduction

β-Adrenergic hormone responsive adenylate cyclase systems have been extensively studied in the quest to define the fundamental aspects of at least one mode of catecholamine action [1]. Direct radioligand binding techniques employing labelled high-affinity β-adrenergic antagonists such as [^{3}H]propranolol [2], [^{125}I]iodohydroxybenzylpindolol [3], [^{3}H]dihydroalprenolol [4], and [^{3}H]carazolol [5] have greatly facilitated the study of putative β-adrenergic hormone receptors. However, careful analysis of the data derived from studies using these radioligands indicates that the interaction of antagonists with the β-adrenergic receptor differs in several respects from the interaction of agonists with this receptor. Occupancy of the receptor by a β-adrenergic antagonist as compared to agonist fails to elicit an activation of the receptor-coupled adenylate cyclase (by definition), reflects a receptor state that displays no apparent sensitivity to guanine nucleotides [1,6 and 7], and fails to induce the phenomenon of receptor-mediated desensitization [8].

In view of the above considerations, it is clear that the ability to probe the β-receptor with a radioactive-labelled agonist of sufficient affinity to permit direct radioligand binding studies would be especially informative. Lefkowitz and Williams [9] first reported the successful development of a potent radioactive-labelled β-adrenergic agonist, (±)-[^{3}H]hydroxybenzylisoproterenol. This catecholamine is about 10-times more potent than (—)-isoproterenol in stimulating the adenylate cyclase activity of frog erythrocyte membranes [10]. Although (±)-[^{3}H]hydroxybenzylisoproterenol is commercially available, detailed information concerning the pharmacology of hydroxybenzylisoproterenol in systems other than the frog erythrocyte is sparse [11,12]. The present study seeks to explore the pharmacology of hydroxybenzylisoproterenol in fat cells and hepatocytes isolated from the rat.

Materials and Methods

Sources of the material used for these studies are as follows: (—)-isoproterenol hydrochloride, Trizma base, Mes and AMP were obtained from Sigma; 1-methyl-3-isobutyl zanthine was obtained from Aldrich; the (—)-, (+)- and (±)-propranolol hydrochlorides were gifts from Ayerst; guanyl-5′-yl imidodiphosphate, ATP, creatine phosphate, creatine phosphokinase and GTP from Boehringer Mannheim; crude collagenase (type CLS II 4176, lot no. 40B015) for isolation of liver cells from Millipore Corp.; crude collagenase (type II, lot no. 119C-6800) and bovine serum albumin (fraction V) for fat cell isolation from Sigma; cyclic[^{3}H]AMP (35 Ci/mmol), [^{125}I]iodohydroxybenzylpindolol (2200 Ci/mmol), (—)-[^{3}H]dihydroalprenolol (32.6 Ci/mmol), (±)-[^{3}H]hydroxybenzylisoproterenol (19 Ci/mmol), [α-^{32}P]-ATP (30 Ci/mmol), and Aquasol-2 from New England Nuclear; the unlabelled hydroxybenzylisoproterenol was kindly supplied by Dr. Christ Filer, New England Nuclear. All other chemicals were obtained from standard commercial sources.

Heptocytes were isolated from 175—200 g fed-female Sprague-Dawley rats (Charles River, CD strain) following the procedure of Berry and Friend [13] as previously described [14]. Following isolation the rat hepatocytes were incu-

bated in Krebs-Ringer bicarbonate buffer, pH 7.4, containing 22 mM glucose for 30 min, then washed and resuspended in fresh medium for 20 min for the experiments. The liver cells (approx. $7.0 \cdot 10^6$ cells/tube were incubated in plastic tubes (17 × 100 mm, Falcon 2017) in a total volume of 1 ml and were constantly shaken in an orbital water bath operating at 150—200 rev./min.

To measure total cyclic AMP content the hepatocytes were incubated as described above, the incubation was then stopped 1 min after addition of hormones by the addition of 0.1 ml of 2 M HCl. Stimulation of rat hepatocyte cyclic AMP accumulation by hormones has been shown to be maximal at 1 min following the addition of the hormone [15]. The incubation tubes were then heated in a boiling water bath for 1 min and, after cooling, were neutralized with NaOH. A 20-μl aliquot was taken from the tube and the cyclic AMP content was assayed by a modification of the method of Gilman [16]. The free cyclic AMP was separated from the bound cyclic AMP by charcoal adsorption, as suggested by Brown et al. [17].

Subcellular fractions were prepared from freshly isolated hepatocytes by a modification of the method of Neville [18]. Hepatocytes were homogenized in 6 vol. of ice-cold 1 mM $NaHCO_3$ buffer using a Willems Polytron (Brinkman Instruments) at setting No. 5 for 30 s and were then further homogenized by 16 strokes of a precision bore glass Potter-Elvehjem homogenizer (Arthur Thomas Co.) fitted with a Teflon pestle operated on a Eberback Con-Torque unit set at maximum running speed. All steps from homogenization to preparation of other subcellular fractions were performed in an ice bath. The homogenate was centrifuged at 1500 × *g* for 10 min at 4°C to yield a supernatant, which was discared, and a pellet ('Pellet 1). This pellet was resuspended in 30 ml of 1 mM $NaHCO_3$ and homogenized again with 16 strokes of the Potter-Elvehjem homogenizer. This fraction was centrifuged at 1500 × *g* for 10 min at 4°C. The resulting supernatant was discarded and the pellet (Pellet 2) was was resuspended in sufficient 69% (w/w) sucrose/1 mM $NaHCO_3$ to yield precisely 44% sucrose (w/w). 10 ml of this mixture were placed into a 13 ml polycarbonate ultracentrifuge tube and gently overlaid with 3 ml of a 42.3% surcose (w/w)/1 mM $NaHCO_3$ solution. The sucrose solutions were checked and adjusted to the indicated density by use of a Zeiss refractometer. The tubes were allowed to stand for 30 min at 4°C and then centrifuged at 100 000 × *g* for 2 h at 4°C in a Beckman L-65 ultracentrifuge fitted with an SW41-Ti rotor. Following centrifugation the float (Purified membranes) was aspirated, resuspended in 1 mM $NaHCO_3$, centrifuged at 12 000 × *g* for 10 min at 4°C, and then resuspended at approx. 2 mg protein/ml in the same buffer. Detailed information concerning protein and adenylate cyclase activity recoveries in this membrane fraction are reported elsewhere [19]. The fractions were immediately frozen and stored at —90°C. These fractions were stored for up to 4 weeks with no significant loss of adenylate cyclase activity or specific [^{125}I]-iodohydroxybenzylpindolol binding.

Binding of [^{125}I]iodohydroxybenzylpindolol to the subcellular fraction was performed according to a modification of the method of Wolfe et al. [20]. A study of β-adrenergic receptors of hepatocyte subcellular fractions was also examined with [^{125}I]iodohydroxybenzylpindolol under conditions identical to those used in the assay of adenylate cyclase activity [19]. However, since the

assay conditions developed by Wolfe et al. [20] yield higher levels of specific binding and greater reproducibility, they were adopted for these studies [19]. Binding assays were routinely performed in triplicate at 37°C for 40 min in a final volume of 0.2 ml containing 50 mM potassium phosphate, pH 7.5/4 mM $MgSO_4$/0.1 nM [^{125}I]iodohydroxybensylpindolol/70—100 μg of membrane protein, and with or without the indicated β-adrenergic agonists or antagonists. At the end of the incubation period 5 ml of 20 mM potassium phosphate, pH 7.5 at 37°C/1 mM $MgSO_4$ buffer were added to each tube and the contents of the tube filtered under vacuum onto a Whatman GFC filter (2.4 cm). The filter was washed with 15 ml of the same buffer to remove non-specific binding. Specific binding was defined as the radioligand binding which was inhibited in the presence of $3 \cdot 10^{-7}$ M (—)-propranolol [19]. This concentration of (—)-propranolol was approx. 2 orders of magnitude higher than its K_d, a condition where 99% of the β-adrenergic receptors would be occupied by this ligand [19]. Variation among the triplicates about the mean values was routinely less than 5%. Non-specific binding (i.e. binding retained in the presence of $3 \cdot 10^{-7}$ M (—)-propranolol at 0.1 nM radioligand was 30—45% of bound radioligand for purified membranes.

White fat cells were obtained by enzymatic digestion of parametrial adipose tissue according to the procedure of Rodbell [21]. Pooled adipose tissue (approx. 100 g) from 15—20 rats was minced with scissors and placed in small plastic bottles. Each bottle, containing 8—10 g of tissue and 10 ml of Krebs-Ringer phosphate buffer containing 3% albumin and 1 mg/ml of crude collagenase was incubated for 60 min at 37°C. The Krebs-Ringer phosphate buffer contained 128 mM NaCl/1.4 mM $MgSO_4$/5.2 mM KCl/10 mM Na_2HPO_4. The albumin buffer was freshly prepared each day and the pH adjusted to 7.4 with NaOH after addition of the bovine serum albumin Fraction V powder (Armour, lots N10101 and P56607). At the end of 60 min digestion, cells were filtered through one layer of nylon chiffon and washed twice with the albumin buffer.

Cyclic AMP accumulation was measured in cells plus medium after a 0.2 ml aliquot of cells plus medium, in duplicate, was extracted from 1 ml of incubation volume and added to tubes on ice containing 20 μl of 2 M HCl. The tubes were then placed in a boiling water bath for 1 min. The tubes were allowed to cool before 10 μl of 4 M NaOH was added. The contents of the tubes were mixed and centrifuged prior to removal of 20-μl aliquots for determination of cyclic AMP. In each experiment, no more than 50 mg of fat cells were incubated/ml of medium which means that the 20-μl aliquots taken for cyclic AMP analysis, represented less than 1 mg of fat cells. Cyclic AMP release to the medium was analyzed by taking 20-μl aliquots of the medium at the end of the incubation just prior to removing the 0.2-ml aliquots of cells plus medium. The cyclic AMP standards were prepared in incubation medium containing albumin which was treated in the same manner as the unknown samples by adding acid and then boiling and neutralizing. The assay for cyclic AMP was done by a modification of the Gilman [16] protein kinase binding procedure using the adrenal binding protein. The free cyclic AMP was separated from the bound cyclic AMP by charcoal absorption [17].

Fat cell membranes were prepared essentially according to Williams et al. [22]. Briefly, fat cells, following isolation, were washed twice with the Krebs-

Ringer phosphate buffer containing 3% albumin (pH 7.4) and once with 0.25 M sucrose/1 mM EDTA/10 mM Tris-HCl buffer, pH 7.5. The fat cells were then resuspended in this same buffer (1 ml of buffer/g, wet weight, of packed cells) and homogenized at room temperature by six strokes of a glass Potter-Elvehjem homogenizer (Arthur Thomas Co.) fitted with a Teflon pestle operated on a Dyna-Mix (Fisher Scientific) at setting No. 4. The homogenate was centrifuged at 15 000 × *g* for 15 min at 4°C. The pellet was washed by resuspension in a 50 mM Tris-HCl (pH 8.0, 4°C)/10 mM $MgCl_2$ buffer (which was ice-cold) and then centrifuged at the same speed and time. This was repeated once. The final pellet was resuspended in a 50 mM Tris-HCl, pH 7.4 at 22°C, 10 mM $MgCl_2$ buffer to a final protein concentration of 2.5 mg/ml. This membrane fraction was used directly in the binding assay or frozen and maintained at −140°C until assayed. No loss of specific binding was noted in membranes stored in this fashion. Protein concentrations were determined by the method of Lowry et al. [23] using crystalline bovine serum albumin as a standard.

Binding assays were performed in a 50 mM Tris-HCl, pH 7.4, at 22°C, 10 mM $MgCl_2$ buffer at a final volume of 0.2 ml containing 200—400 μg of membrane protein and 10 nM (−)-[^{3}H]dihydroalprenolol [24]. Incubations of the radioligand, competitors and membranes were initiated by the addition of membranes into the incubation vessel or, alternatively, by the addition of radioligand and competitors to the membrane suspension. The incubation vessels (Falcon 17 × 100 mm, No. 2017 tubes) were incubated at 22°C with constant shaking for up to 20 min. At the end of incubation, 7 ml of ice-cold incubation buffer was added to each tube to terminate the reaction. The contents of the tube was rapidly filtered under vacuum through a single Whatman GFC filter (24 mm in diameter). The filters were then washed with two 7-ml aliquots of ice-cold incubation buffer to minimize the non-specific binding. The filters were placed directly into glass scintillation vials and then dried in a 50°C oven for 10—15 min. A 4-ml aliquot of a solution containing Triton X-100 and 5% Omnifluor in toluene (1 : 2) was added to each vial and then counted. Assay tubes were performed in duplicate or triplicate. Nonspecific binding was determined by incubating corresponding tubes with 10 μM (±)-propranolol. This concentration of (±)-propranolol was selected to differentiate specific binding (stereospecific) by inhibition studies utilizing increasing concentrations of (+)- and (−)-propranolol. Specific binding of (−)-[^{3}H]dihydroalprenol is thus defined as the total binding less binding remaining in presence of 10 μM (±)-propranolol. Nonspecific binding was generally between 20 and 30% of the total counts bound. Less than 0.5% of the total counts filtered bound to the filters and this binding was virtually insensitive to competition by β-adrenergic agonists or antagonists.

Adenylate cyclase activity was assayed in a final volume of 0.1 ml containing 25 mM Tris-HCl, pH 7.5/5 mM $MgCl_2$/20 mM creatine phosphate/100 U/ml creatine phosphokinase/0.5 mg/ml bovine serum albumin/1 mM cyclic AMP/0.1 mM ascorbic acid/0.5 mM [α-^{32}P]ATP (35—50 cpm/pmol)/subcellular fractions (50—150 μg protein), and hormones, plus the inclusion of 1 μM GTP in all assays. The low specific activity and relatively small response of the liver homogenate adenylate cyclase to catecholamine stimulation necessitated higher amounts of protein (0.5—1.2 mg) for some assays. The results were verified

with assays employing lower amounts of protein (100—200 μg) and higher specific activity ATP. The assays were initiated by addition of the enzyme preparation to reaction tubes and were performed in duplicate or triplicate at 30°C for 10 min. The reaction was terminated, and the adenylate cyclase activity determined by the method of Solomon et al. [25]. Membranes from S49 mouse lymphoma cells were prepared by a modification [26] of the method described by Ross et al. [27] and were kindly supplied by Dr. Gary Johnson, Brown University.

The hypothyroid rats used in these studies were rendered hypothyroid by maintenance on an iodine-deficient diet (No. 17700, United States Biochemical Corp.) and drinking water containing 0.00625% 6-*N*-propyl-2-thoiuracil for 21 days.

Membrane-bound adenylate cyclase activity of fat cells was assayed in ghosts prepared by hypotonic lysis of fat cells [28]. These ghost preparations were used in the cyclase assay directly from preparation without prior freezing and storage.

(±)-[^{3}H]Hydroxybenzylisoproterenol binding to fat and liver cell membranes was performed in 50 mM Tris-HCl, pH 7.5 at 22°C/10 mM $MgCl_2$/0.3 mM catechol and 0.8 mM ascorbic acid. The incubation period and assay of binding was performed, as indicated above, with the labelled antagonist binding assays.

Results

Effects of (±)-hydroxybenzylisoproterenol on cyclic AMP accumulation of isolated rat fat cells and liver cells

As shown in Fig. 1 (±)-hydroxybenzylisoproterenol ((±)-HBI) stimulated cyclic AMP accumulation in both isolated liver and fat cells. In the presence of 50 μM 1-methyl-3-isobutylxanthine, (±)-HBI stimulated liver cell cyclic AMP accumulation to the same maximal level as achieved with the potent β-adrenergic agonist (—)-isoproterenol (Fig. 1, left panel). Half-maximal activation of cyclic AMP accumulation in liver cells was obtained at 27 ± 6 nM (—)-isoproterenol or 10 ± 3 nM (±)-HBI. Since these data were obtained with a racemic mixture of HBI, it would seem that the (—)-isomer of HBI is 3—4-fold more potent than (—)-isoproterenol in stimulating cyclic AMP accumulation in liver cells.

In fat cells incubated in the presence of 100 μM theophylline (±)-HBI also stimulated cyclic AMP accumulation to the same maxima as (—)-isoproterenol (Fig. 1, right panel). Half-maximal stimulation of cyclic AMP levels of fat cells occurred at 0.1 μM (±)-HBI or (—)-isoproterenol, suggesting that the (—) isomer of HBI is likely to be twice as potent as (—)-isoproterenol. The methid xanthines were included in the incubations to potentiate catecholamine stimulation of cyclic AMP levels and permit more accurate assessment of the β-adrenergic activity of (±)-HBI. An identical relationship between the relative potencies of (±)-HBI and (—)-isoproterenol was obtained in liver and fat cells when the xanthines were omitted (data not shown).

Stimulation of adenylate cyclase activity by (±)-HBI

The ability of (±)-HBI to stimulate adenylate cyclase activity of liver cells and fat cells was examined next. Catecholamine stimulation of liver cell ade-

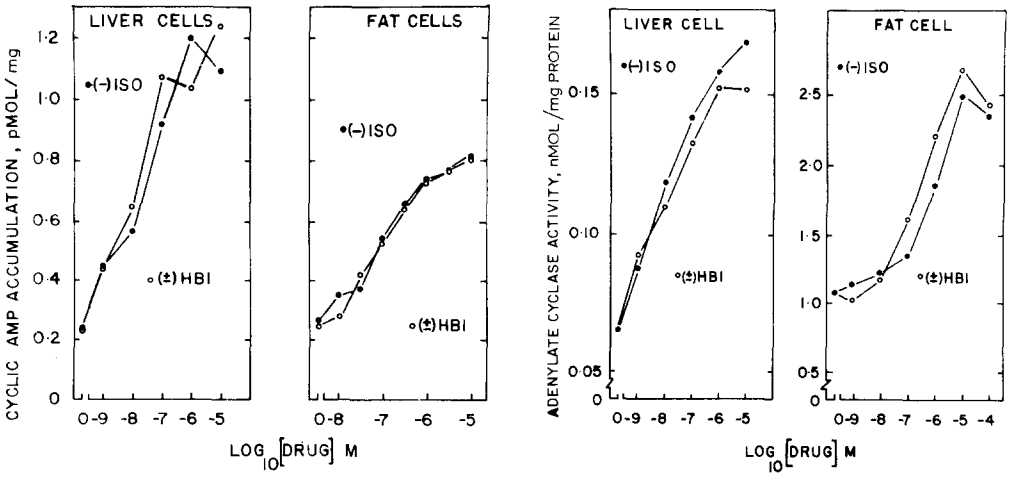

Fig. 1. (±)-Hydroxybenzylisoproterenol stimulation of cyclic AMP accumulation by isolated rat liver and fat cells. Rat hepatocytes (50 mg packed wet weight of cells/tube) were incubated at 37°C in a Krebs-Ringer bicarbonate buffer, pH 7.4, containing 22 mM glucose for 30 min, then resuspended in fresh medium for another 20 min for the experiment. The catecholamine was added 1 min before the end of the 20 min incubation and was added with 50 μM methyl isobutylxanthine. Cyclic AMP accumulation was measured as described in Materials and Methods. The data presented are the mean values of three separate experiments performed with different hepatocyte preparations. Fat cells were incubated in a Krebs-Ringer phosphate buffer, pH 7.4, containing 0.1 mM theophylline/3% bovine serum albumin, with and without the indicated catecholamine for a 2 min period at 37°C. At the end of the incubation the fat cells were treated with acid followed by base, and the cyclic AMP accumulation assayed. The data presented are the mean values from three separate experiments performed on separate days. ISO, isoproterenol.

Fig. 2. (±)-Hydroxybenzylisoproterenol stimulation of adenylate cyclase activity of rat liver and fat cells. Liver cells were isolated from hypothyroid rats. Adenylate cyclase activity was determined over a 10 min period at 30°C for an aliquot of a whole homogenate fraction in the absence or presence of the indicated catecholamine in the standard cyclase assay buffer. The reaction was terminated and the cyclic AMP generated was determined. The data presented are the mean value from three separate experiments performed with as many different hepatocyte homogenates. Fat cell ghosts were prepared and the adenylate cyclase activity measured. The data are expressed as the mean values from three separate experiments indicated catecholamine in the standard cyclase assay medium. The reaction was terminated and the cyclase activity measured. The data are expressed as the mean values from three separate experiments performed on separate days. ISO, isoproterenol.

nylate cyclase was assayed using whole hepatocyte homogenates obtained from rats rendered hypothyroid. This particular system was selected to optimize the sensitivity in comparing HBI to (−)-isoproterenol for the following reasons: (i) catecholamine sensitivity of liver adenylate cyclase is lost during preparation of washed particulate and purified plasma membranes [19,29] and (ii) the hypothroid rat liver hepatocyte preparation displays a 3—4-fold greater catecholamine response with respect to adenylate cyclase activation than the euthyroid rat preparation, with no discernable alteration in the character of the response [19].

As shown in Fig. 2 (left-panel), (±)-HBI stimulated the liver adenylate cyclase activity to higher maximal levels than (−)-isoproterenol, although the difference was not great. The half-maximal stimulation of adenylate cyclase was obtained with approx. 10 nM (±)-HBI or (−)-isoproterenol, again suggesting that the (−) isomer of HBI is more potent than that of isoproterenol (Fig. 2).

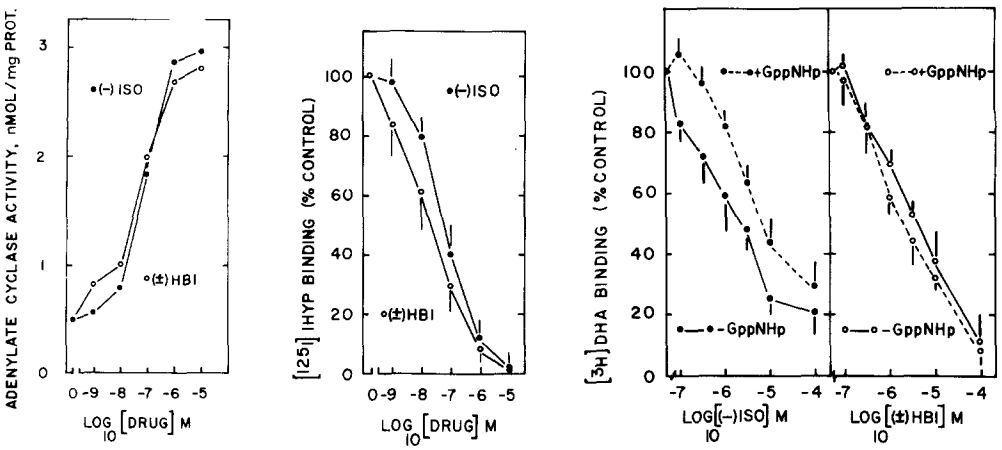

Fig. 3. (±)-Hydroxybenzylisoproterenol stimulation of S49 mouse lymphoma adenylate cyclase activity. (Left). Adenylate cyclase activity of plasma membranes prepared from S49 cells was assayed in the absence or presence of the indicated catecholamine as described in the legend to Fig. 2. The data are expressed as the mean values from three separate experiments performed on separate days. ISO, isoproterenol.

Fig. 4. (±)-Hydroxybenzylisoproterenol inhibition of specific [^{125}I]iodohydroxybenzylpindolol (IHYP) binding to purified membranes prepared from rat liver cells. (Center). The data are expressed as the mean values from three separate liver cell membrane preparations. Specific binding to these purified membranes was 2 fmol/mg of protein. ISO, isoproterenol.

Fig. 5. (—)-Isoproterenol and (+)-hydroxybenzylisoproterenol inhibition of specific (—)-[^{3}H]dihydroalprenolol binding to fat cell membranes: effects of Gpp(NH)p. (Right). Competition for specific (—)-[^{3}H]-dihydroalprernolol (DHA) binding sites by (—)-isoproterenol (filled circles) or (±)-HBI (open circles) was assayed in the absence (solid lines) or presence (dotted lines) of 50 μM Gpp(NH)p with 10 nM radioligand. The data are expressed as the mean values from three separate experiments performed on different occasions with different membrane preparations.

In fat cell membrane preparations (±)-HBI stimulated adenylate cyclase activity more potently than (—)-isoproterenol (Fig. 2, right panel). At 0.5 ± 0.1 μM, (±)-HBI stimulated half-maximal adenylate cyclase activity, while 0.8 ± 0.2 μM (—)-isoproterenol was needed to obtain this same level of activity. Although (±)-HBI is more potent than (—)-isoproterenol on the fat cell cyclase, this differential is apparently not transduced to the isolated cell cyclic AMP response (Fig. 1).

To aid in assessing the pharmacology of (±)-HBI, the ability of (±)-HBI to stimulate adenylate cyclase activity in plasma membranes prepared from S49 mouse lymphoma cells was also determined (Fig. 3). Half-maximal stimulation of activity was achieved at 20—30 nM concentrations of either (±)-HBI or (—)-isoproterenol. From this data it would appear that (±)-HBI is a full agonist with respect to adenylate cyclase activation in rat fat and liver cells, and in S49 mouse lymphoma cells. (±)-HBI also appears to be as potent as (—)-isoproterenol on the liver and S49 mouse cell adenylate cyclase activation, indicating the (—) isomer of HBI is likely to be 1—2-fold more potent than (—)-isoproterenol. In the fat cell cyclase system (—)-HBI is likely to be about 3—4-fold more potent than (—)-isoproterenol.

Binding of (±)-HBI to fat cell and hepatocyte membranes

The binding of (±)-HBI to fat and liver cell membranes was first examined indirectly via competition studies utilizing radioactively labelled potent β-adrenergic antagonists and membranes obtained from isolated cells. (±)-HBI binding to liver cell membrane β-receptors was assessed through competition studies with [^{125}I]iodohydroxybenzylpindolol (Fig. 4). The binding of this radioligand to purified membranes prepared from isolated liver cells has recently been characterized [19]. The K_d for the interaction of [^{125}I]iodohydroxybenzylpindolol with the β-adrenergic receptor of liver cell membranes was found to be 0.2 nM [19]. From these data and the point of half-maximal displacement of this radioligand by a competitor (Fig. 4), the K_d (obtained via the Cheng and Prusoff equation [20]) for the interaction of agonist with the β-receptor was calculated to be about 10 nM for (±)-HBI and about 25 nM for (−)-isoproterenol. These calculated values are in good agreement with those derived from data on the cyclic AMP response of intact hepatocytes (Fig. 1) and from the adenylate cyclase response (Fig. 2).

(±)-HBI binding to fat cell membranes was probed by competition studies using the potent β-adrenergic antagonist (−)-[^{3}H]dihydroalprenolol. The binding of (−)-[^{3}H]dihydroalprenolol to the fat cell β-receptor has been previously characterized in both particulate membranes [22,24] and with intact fat cells

TABLE I

INHIBITION OF (±)-[^{3}H]HYDROXYBENZYLISOPROTERENOL AND (−)-[^{3}H]DIHYDROALPRENOLOL BINDING TO FAT CELL MEMBRANES BY β-ADRENERGIC AGONISTS AND ANTAGONISTS

The results are the means of triplicate incubations. (±)-[^{3}H]HBI was present at 30 nM, (−)-[^{3}H]dihydroprenolol was present at 20 nm. The incubation mixture contained 0.25 mg of membrane protein, the assay was performed and binding determined.

Addition	(±)-[^{3}H]Hydroxybenzylisoproterenol bound (cpm)	
None	4993	
(±)-Hydroxybenzylisoproterenol		
10^{-8} M	5119	
10^{-7} M	4830	
10^{-6} M	3902	
10^{-5} M	2030	
(±)-Propranolol		
10^{-5} M	4956	
Addition	**(−)-[^{3}H]Dihydroalprenolol bound (cpm)**	**(±)-[^{3}H]HBI bound (cpm)**
None	1942	4798
(−)-Isoproterenol		
10^{-6} M	1427	3997
10^{-5} M	1112	2676
(±)-Hydroxybenzylisoproterenol		
10^{-6} M	1211	3617
10^{-5} M	1024	2028
(±)-Propranolol		
10^{-5} M	822	4615

TABLE II

RAT FAT CELL MEMBRANE ADENYLATE CYCLASE ACTIVITY IN RESPONSE TO STIMULATION BY (—)-ISOPROTERENOL AS COMPARED TO (±)-HYDROXYBENZYLISOPROTERENOL: EFFECTS OF PROPRANOLOL

Rat fat cell membrane adenylate cyclase was measured over 10 min at 30°C with varying concentrations of either (—)-isoproterenol or (±)-hydroxybenzylisoproterenol in the presence or absence of 10^{-5} M (—)-propranolol. The results are the mean values of triplicate determinations from a representative experiment.

Additions	Adenylate cyclase activity (pmol/mg of protein in 10 min)
None	460
(—)-Isoproterenol, 10^{-7} M	620
(—)-Isoproterenol, 10^{-7} M + (—)-Propranolol, 10^{-5} M	440
(—)-Isoproterenol, 10^{-6} M	830
(—)-Isoproterenol, 10^{-6} M + (—)-Propranolol, 10^{-5} M	380
(—)-Isoproterenol, 10^{-5} M	1260
(—)-Isoproterenol, 10^{-5} M + (—)-Propranolol, 10^{-5} M	420
(±)-Hydroxybenzylisoproterenol, 10^{-7} M	480
(±)-Hydroxybenzylisoproterenol, 10^{-7} M + (—)-Propranolol, 10^{-5} M	430
(±)-Hydroxybenzylisoproterenol, 10^{-6} M	860
(±)-Hydroxybenzylisoproterenol, 10^{-6} M + (—)-Propranolol, 10^{-5} M	460
(±)-Hydroxybenzylisoproterenol, 10^{-5} M	1230
(±)-Hydroxybenzylisoproterenol, 10^{-5} M + (—)-Propranolol, 10^{-5} M	620

[31]. Half-maximal inhibition of (—)-[^{3}H]dihydroalprenolol binding to fat cell membranes occurred at about 2 μM (±)-HBI or (—)-isoproterenol (Fig. 5). Due to the non-classical binding data (e.g., curvilinear Sctachard plots [22], pseudo-Hill coefficients less than unit [22,24]) noted with (—)-[^{3}H]dihydroalprenolol binding to fat cell membranes, accurate and reliable values for the K_d of these interactions cannot be calculated. Clearly the affinity of the racemate HBI is similar to (—)-isoproterenol, suggesting that (—)-HBI would likely have a

TABLE III

INHIBITION OF (±)-HYDROXYBENZYLISOPROTERENOL BINDING TO RAT HEPATOCYTE PURIFIED MEMBRANES BY β-ADRENERGIC AGONISTS AND ANTAGONISTS

Rat heptocyte purified membranes (0.15 mg of protein) were incubated with 20 nM (±)-[^{3}H]HBI for 20 min at 22°C in 50 mM Tris-HCl (pH 7.4 at 22°C), 10 mM $MgCl_2$ 0.3 mM catechol, 0.8 mM ascorbic acid and the binding assayed in triplicate. The results are the means of closely agreeing triplicates from a single experiment.

Addition	(±)-Hydroxybenzylisoproterenol bound (cpm)
None	2104
(—)-Isoproterenol 10^{-7} M	2520
(+)-Isoproterenol 10^{-7} M	2399
(—)-Isoproterenol 10^{-6} M	1817
(+)-Isoproterenol 10^{-6} M	1869
(—)-Isoproterenol 10^{-5} M	917
(+)-Isoproterenol 10^{-5} M	936
(—)-Propranolol 10^{-5} M	2241
(+)-Propranolol 10^{-5} M	2507
(±)-Propranolol 10^{-5} M	2198

higher affinity for the β-adrenergic receptor than (—)-isoproterenol.

The ability of guanine nucleotides to induce an agonist-specific reduction in the affinity of β-adrenergic receptors has been observed in a number of systems [1,7] including the fat cell [32,33]. As shown in Fig. 5 (left panel), the affinity of the receptor for (—)-isoproterenol is reduced in the presence of 50 μM Gpp(NH)p. What is most intriguing is the apparent inability of Gpp(NH)p to induce a similar effect when (±)-HBI is the competing agonist. In no experiment was a Gpp(NH)p-induced reduction of receptor affinity for (±)-HBI noted. In contrast, Gpp(NH)p induced a small but reproducible left-shift in the competition curve for (±)-HBI (Fig. 5, right panel).

The above data fostered our interest in probing the β-receptor of fat and liver cells via direct radioligand binding studies using (±)-[^{3}H]HBI. A dose-dependent inhibition of (±)-[^{3}H]HBI binding to fat cell membranes was observed with (±)-HBI, yet 10 μM (±)-propranolol failed to compete with this binding (Table I, top). To further explore this observation agonist competition curves for (—)-[^{3}H]dihydroalprenolol and (±)-[^{3}H]HBI binding were generated simultaneously using the same membrane preparation (Table I, bottom). Although both (—)-isoproterenol and (±)-HBI were each capable of competing for (—)-[^{3}H]dihydroalprenolol and (±)-[^{3}H]HBI binding to fat cell membranes, 10 μM (±)-propranolol competed for only the (—)-[^{3}H]dihydroalprenolol binding sites and not those for (±)-[^{3}H]HBI. Blocking studies of catecholamine-stimulated adenylate cyclase activity of fat cell membranes using (—)-propranolol indicated that this potent β-adrenergic antagonist was capable of blocking those sites transducing (±)-HBI occupancy into cyclase activation (Table II). Binding studies performed at 50 nM (±)-[^{3}H]HBI similarly displayed an insensitivity to competition by 10 μM (±)-or (—)-propranolol (data not shown).

(±)-[^{3}H]HBI binding to liver cell membranes was also examined (Table III). Although (±)-[^{3}H]HBI binding could be displaced in a dose-dependent fashion by (—)-isoproterenol, no stereospecific differentiation for the (—) versus (+) isomers of isoproterenol was observed. The (—) and (+) stereoisomers of propranolol failed (at 10 μM) to inhibit (±)-[^{3}H]HBI binding to these liver cell membranes. These properties of the (±)-[^{3}H]HBI binding sites noted in either fat or liver cell membranes are inconsistent with those expected of physiologically relevant β-adrenergic receptors.

Discussion

Elucidating the mechanism by which catecholamines activate adenylate cyclase has been the focal point of intense research. Study of the catecholamine receptors which are coupled to adenylate cyclase has been greatly facilitated by the development of high affinity radioligands which permit direct binding studies. Radioactively labelled antagonists such as hydroxybenzylpindolol [3] and dihydroalprenolol [4] have been employed extensively in the study of β-adrenergic receptors. As useful as these ligands have been, it is now clear that antagonists interact with the receptor in such a fashion as to give only a partial picture of the agonist-receptor interaction. Employing a labelled agonist catecholamine would seem appropriate. However, it was the high level of non-receptor ligand binding and labelling of sites with properties incompati-

ble with the known pharmacology of β-adrenergic responses noted in studies using labelled natural catecholamine agonists that fostered the development of radioactive labelled antagonists. The potential advantages of having a relatively high affinity agonist catecholamine which could be radioactively labelled for study of the β-receptor were obvious.

Lefkowitz and coworkers [10,34] first reported the agonist activity of (±)-hydroxbenzylisoproterenol on the activation of adenylate cyclase. Using membranes prepared from frog erythrocytes, these investigators demonstrated that (±)-HBI was at least 10-times more potent than (—)-isoproterenol and was the most potent full agonist tested in this system [10]. Insel and Stoolman [35] reported that the potency of (±)-HBI in stimulating cyclic AMP accumulation by intact S49 mouse lumphoma cells was 8-times greater than that of (—)-isoproterenol. In the present study (±)-HBI is shown to be a full agonist with respect to stimulation of cyclic AMP accumulation in both intact rat fat and liver cells (Fig. 1). (±)-HBI was slightly more potent than (—)-isoproterenol in liver cells and equipotent to (—)-isoproterenol in fat cells in stimulating cyclic AMP accumulation.

According to the classification scheme of Lands et al. [36] for β_1 and β_2 subtypes of β-adrenergic receptors, the receptors mediating the stimulation of cyclic AMP accumulation of rat fat cells are the β_1 type [22,24], whereas the receptors mediating the stimulation of cyclic AMP accumulation of rat liver cells [20] or S49 cells [35] appear to be β_2. Data from the present study suggest that the enhanced potency of (±)-HBI over (—)-isoproterenol is expressed to a greater extent in cells with β_2 as compared to β_1 type adrenergic receptors mediating the response. (±)-HBI is, perhaps, a β_2 type receptor super agonist. Curiously, (±)-HBI was a more potent stimulator of adenylate cyclase activity than (—)-isoproterenol in membranes from fat cells but only slightly more potent than (—)-isoproterenol in S49 and rat liver cell membranes (Figs. 2 and 3).

(±)-HBI and (—)-isoproterenol were nearly equipotent in competing for specific (—)-[^{3}H]dihydroalprenolol binding sites on fat cell membranes (Fig. 5). In similar studies using [^{125}I]iodohydroxybenzylpindolol to label putative β-adrenergic receptors in liver cell membranes these binding sites displayed a 2-fold higher affinity for (±)-HBI than (—)-isoproterenol (Fig. 4). Half-maximal inhibition of [^{3}H]hydroalprenolol binding to frog erythrocyte membranes was reported by Lefkowitz and coworkers to occur at 6—30-fold lower concentrations of (—)-HBI as compared to (—)-isoproterenol [10,34]. Insel and Stoolman [35] reported that the affinity of β receptors on intact S49 cells for (±)-HBI was more than 10-times greater than that for (—)-isoproterenol as determined by competition studies using either (—)-[^{3}H]dihydroalprenolol or [^{125}I]iodohydroxybenzylpindolol. The above data support the proposal (±)-HBI is more potent than isoproterenol in systems classified as β_2, such as the frog erythrocyte, S49 and rat liver cells as compared to β_1-type systems like the rat fat cell.

Guanyl nucleotides reduce the affinity of β-adrenergic receptors for agonists (but not antagonists) in membranes derived from a variety of cells, including rat fat cells [32,33]. The dose-response curve for the inhibition of specific (—)-[^{3}H]dihydroalprenolol binding to fat cell membranes by (—)-isoproterenol was displaced to the right (suggesting a lower receptor affinity) when 50 μM Gpp-

(NH)p was present in the assay (Fig. 5). This effect of Gpp(NH)p on the affinity of the receptor for an agonist was recently shown to be dose-dependent with respect to Gpp(NH)p (half-maximal effect at approx. 1 μM nucleotide) and reversible in this system [33]. Interestingly, this nucleotide-induced reduction of receptor affinity was not observed when (±)-HBI was the competing agonist in the (—)-[^{3}H]dihydroalprenolol binding assay (Fig. 5). In contrast, a small leftward shift in the position of the (±)-HBI competition curve was noted in the presence of Gpp(NH)p (Fig. 5). These data suggest that (±)-HBI is an agonist which, unlike (—)-isoproterenol, (i) cannot report the Gpp(NH)p-induced change in the β-adrenergic receptor affinity in this steady state assay, or (ii) does not permit this Gpp(NH)p-induced alteration in receptor affinity to occur.

Direct binding studies performed with (±)-HBI identified sites on fat cell membranes which were sensitive to competition by unlabelled (±)-HBI or (—)-isoproterenol yet insensitive to 10 μM (±)-propranolol (Table I). This concentration of propranolol could, however, block stimulation of the cyclase activity in these membranes in the response to (±)-HBI. (±)-[^{3}H]HBI binding sites identified on liver cell membranes were also insensitive to competition by 10 μM propranolol and failed to display stereospecific inhibition by the (—) versus (+) isomers of isoproterenol. The properties of the (±)-[^{3}H]HBI binding sites identified in fat and liver cell membranes are thus not consistent with those of physiological β-adrenergic receptors.

Lefkowitz and Williams [9] first characterized the properties of (±)-[^{3}H]HBI binding to putative β-adrenergic receptors of frog erythrocyte membranes. The properties of these binding sites including stereospecific inhibition of (±)-[^{3}H]-HBI binding by both β-adrenergic agonists and antagonists were consistent with those of physiological β_2-type adrenergic receptors [9]. (±)-[^{3}H]HBI binding in this system was slowly reversible and a major portion of this binding was rapidly dissociated by guanine nucleotides [37]. It is difficult to reconcile the present data with that derived in frog erythrocyte membranes. Clearly the fat and liver cell membranes possesed intact β-receptors which could be labelled with antagonist radioligand under conditions identical to those used for (±)-[^{3}H]HBI binding. Since several lots of freshly prepared (±)-[^{3}H]HBI were used in these studies, radioligand quality would not appear to be at fault. If (±)-[^{3}H]HBI binding was selective for β_2-type adrenergic receptors (as typified by the frog erythrocyte system), one would have predicted successful labelling of the liver β-receptors which are also β_2 in character. However, the nature of the (±)-[^{3}H]HBI sites in liver cells is not consistent with that of the receptors mediating activation of cyclase by catecholamines such as isoproterenol.

It appears that (±)-[^{3}H]HBI binds to sites in liver and fat cell membranes which (i) display an affinity of agonists but not antagonists, (ii) display little stereospecific inhibition by β-adrenergic agonists, (iii) demonstrate little change in affinity for HBI in the presence of guanyl nucleotides, and (iv) which bind HBI although not apparently involved in stimulating the cyclase. Further study of (±)-[^{3}H]HBI and its binding in other systems will be required before the basis for these observations is clarified.

Acknowledgements

This work was supported by United States Public Health Service Research Grant AM-25410 from the National Institute of Arthritis, Metabolism, and Digestive Diseases, National Institutes of Health. C.C.M. is the recipient of a Research Career Development Award (K04 AM00786) form the National Institutes of Arthritis, Metabolism, and Digestive Diseases, N.I.H.

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