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Hydroxybenzylpindolol and Hydroxybenzylpropranolol: Partial *Beta* Adrenergic Agonists of Adenylate Cyclase in the Rat Adipocyte

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SUMMARY

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Hydroxybenzylpindolol stimulates adenylate cyclase activity in purified plasma membranes from the rat adipocyte. The intrinsic activity of hydroxybenzylpindolol is about 35% less than that of isoproterenol and is inhibited stereoselectively by (−)-propranolol. Thus hydroxybenzylpindolol is both a partial agonist and an antagonist of catecholamine activation of adenylate cyclase. Hydroxybenzylpropranolol, the corresponding derivative of the antagonist propranolol, is also a partial agonist, although considerably weaker than hydroxybenzylpindolol and about equivalent in intrinsic activity to pindolol. Based on the relative inhibitory effects of propranolol, the affinities of the *beta* receptor for hydroxybenzylpindolol and hydroxybenzylpropranolol are equivalent but are about 10 times that of pindolol or propranolol and 25–40 times that of isoproterenol. The stimulatory effects of the partial agonists are mediated by the same guanine nucleotide regulatory process observed with catecholamines. The potency and intrinsic activity exhibited by hydroxybenzylpindolol on adenylate cyclase in isolated membranes correlated with its stimulatory effect on cyclic 3′,5′-AMP accumulation and lipolysis in intact fat cells. Hydroxybenzylpindolol increased lipolysis and cyclic AMP accumulation by intact fat cells at a concentration of 10 nM, while isoproterenol was without effect at this concentration. However, at higher concentrations hydroxybenzylpindolol gave a maximal activation of either parameter which was about one-third that due to isoproterenol.

INTRODUCTION

Activation of adenylate cyclase systems by catecholamines is initiated through the *beta* adrenergic receptor (1). This receptor interacts preferentially with the levorotatory isomers of both agonists and antagonists. Numerous compounds have been tested recently for their actions on catecholamine-sensitive adenylate cyclase systems in isolated membranes (2, 3). The general conclusion was that the most important structural requirements for stimulation involve critical substitutions of po-

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lar groups on the phenyl ring. Bulky substitutions on the amine group tend to increase the affinity of adrenergic compounds but seem not to be essential for activity. *Beta* receptor antagonists fall into two categories, those that are partial agonists (i.e., have lower intrinsic activities at saturating concentrations) and those which are purely antagonistic, such as propranolol. Because of their relatively higher affinity than catecholamines for the *beta* receptor, appropriately labeled antagonists have been employed recently for studies of binding to the *beta* receptor (4–6).

Aurbach and associates (6) showed that hydroxybenzylpinolol[2] binds stereoselectively and with high affinity to the *beta* receptor of the turkey erythrocyte plasma membrane and competitively inhibits catecholamine action on adenylate cyclase. As a preliminary to studying the binding of hydroxybenzylpindolol to plasma membranes of rat fat cells, we investigated the effects of the compound on adenylate cyclase activity. To our surprise, we found that hydroxybenzylpindolol, rather than acting as a pure antagonist, was a potent partial agonist on this system. Incorporation of the hydroxybenzyl group into propranolol converted this compound from a pure antagonist to a weak, partial agonist. Hydroxybenzylpindolol was subsequently found to stimulate cyclic 3′,5′-AMP production and lipolysis in intact fat cells via the same *beta* receptor-mediated process occurring in isolated membranes, and with similar relative potency and efficacy.

MATERIALS AND METHODS

[α-^{32}P]ATP and Gpp(NH)p[3] were obtained from International Chemical and Nuclear Corporation; cyclic [^{3}H]AMP was purchased from New England Nuclear Corporation. ATP, GTP, cyclic AMP, phosphocreatine, creatine phosphokinase, dithiothreitol, *dl*(±)-isoproterenol HCl, and (±)-propranolol were obtained from Sigma; epinephrine, from Parke, Davis; and 1-methyl-3-isobutylxanthine, from Aldrich. Hydroxybenzylpindolol, pindolol, hydroxybenzylpropranolol, and (+)- and (−)-propranolol were synthesized by Sandoz and kindly supplied by Dr. Gerald Aurbach. All other materials were obtained from previously reported sources (7).

Preparation of fat cells and plasma membranes. Fat cells were prepared as described previously (8), using collagenase treatment of epididymal adipose tissue from 150–170-g Sprague-Dawley rats. Fat cell membranes were prepared by a simplification of the method of Avruch and Wallach (9) as described previously (10). The final membrane pellet was suspended in 2 mM EDTA and 20 mM Tris-HCl (pH 7.5) to give a concentration of about 1 mg of membrane protein per milliliter. Aliquots were frozen and stored in liquid nitrogen.

Assay of adenylate cyclase. Adenylate cyclase activity was assayed using the method of Salomon *et al.* (11). In all experiments the assay medium (0.1 ml) contained 1 mM dithiothreitol, 1 mM cyclic AMP, 0.05% bovine serum albumin, 30 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM [α-^{32}P]ATP (50–200 cpm/pmole), 5 mM creatine phosphate, and 50 units/ml of creatine phosphokinase. Except where indicated, the incubation medium also contained 0.1 μM GTP and various concentrations of agonists and antagonists. Incubations were initiated by the addition of membranes (to give a final protein concentration of 10 μg/ml) and were carried out, usually for 6 min, at 37°. Reactions were stopped by the addition of 0.1 ml of stopping solutions (11).

Cyclic AMP and glycerol determinations in intact fat cells. Fat cells, prepared as above, were incubated at 37° in medium consisting of Krebs-Ringer-phosphate buffer (pH 7.4) containing 3% bovine serum albumin. For cyclic AMP determinations, the cells were incubated for 5 min and the cyclic AMP content of medium plus cells was determined by procedures described elsewhere (12). For glycerol determinations, fat cells were incubated for 60 min and the amount of glycerol was

[2] Hydroxybenzylpindolol is the trivial name for *l*-(1-*p*-hydroxyphenyl-2-methyl-2-propylamino)-3-(4-indoloxy)-2-propanol.

[3] The abbreviation used is: Gpp(NH)p, guanyl-5′-yl imidodiphosphate.

determined fluorometrically, using glycerokinase and α-glycerophosphate dehydrogenase (13).

Other determinations. Protein was determined by the method of Lowry *et al.* (14), using bovine serum albumin as standard. The quantity of fat cells used for cyclic AMP and glycerol determinations was estimated from the total triglyceride content (8).

All experiments were carried out with at least two preparations of plasma membranes or cells. Because of variations in levels of activity with different preparations, representative data are reported.

RESULTS

Effects of hydroxybenzylpindolol on adenylate cyclase activity in fat cell membranes. Figure 1 shows the effects of hydroxybenzylpindolol in the absence and presence of isoproterenol (10 μM). At its maximal effective concentration (1 μM) hydroxybenzylpindolol stimulated adenylate cyclase activity to about 65% of that given by isoproterenol; half-maximal activation was obtained at 80 nM. Isoproterenol-stimulated activity was reduced in the presence of hydroxybenzylpindolol to that given by the latter at its maximal effective concentration. These findings suggested that hydroxybenzylpindolol stimulates adenylate cyclase through the catecholamine receptor site and, because of its weaker stimulatory effects, inhibits the actions of catecholamines.

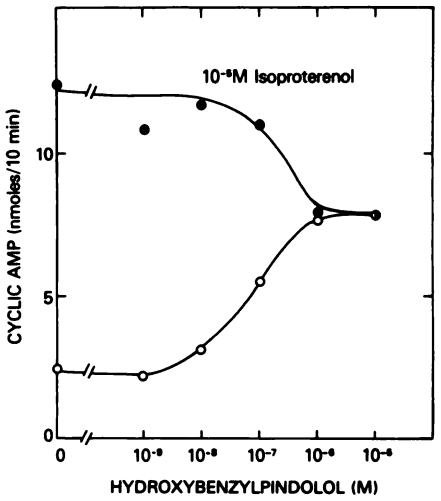

FIG. 1. *Effects of hydroxybenzylpindolol on adenylate cyclase activity in the absence and presence of isoproterenol*

Adenylate cyclase activity in fat cell membranes was assayed under standard assay conditions described in MATERIALS AND METHODS as a function of hydroxybenzylpindolol concentration in the absence (○) and presence (●) of isoproterenol (10 μM).

Effects of propranolol. The *beta* adrenergic receptor reacts specifically with the (−) congeners of catecholamines or propranolol. Accordingly, if hydroxybenzylpindolol indeed activates adenylate cyclase through the *beta* receptor, its action also should be preferentially inhibited by (−)-propranolol. As shown in Fig. 2, (−)- and (±)-propranolol inhibited the stimulatory effects of both hydroxybenzylpindolol and isoproterenol, whereas the (+) congener exhibited only weak inhibitory effects. (−)-Propranolol was twice as effective as the racemate; half-maximal inhibition of the effects of 1 μM hydroxybenzylpindolol were obtained with 5 μM (−)-propranolol and 10 μM (±)-propranolol. These findings provided strong evidence that hydroxybenzylpindolol interacts stereoselectively with the *beta* adrenergic receptor in the process of adenylate cyclase activation.

Effects of other beta blockers. Since hydroxybenzylpindolol is a partial agonist

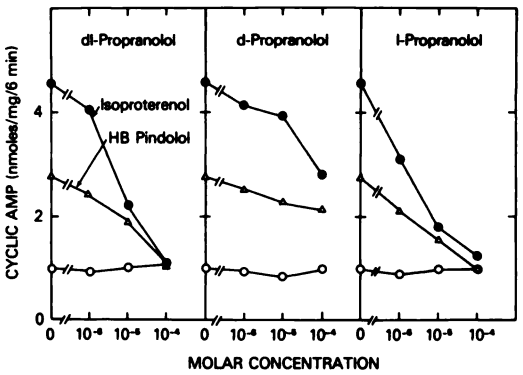

FIG. 2. *Effects of dl-, d-, and l-propranolol on activation of adenylate cyclase by hydroxybenzylpindolol and isoproterenol*

Fat cell membranes were incubated under standard assay conditions without agonists (○), with 10 μM isoproterenol (●), and with 1 μM hydroxybenzylpindolol (HB pindolol) (△).

and propranolol acts as a pure antagonist on the fat cell system, it was of interest to compare the effects of pindolol, the parent compound of hydroxybenzylpindolol, and the corresponding hydroxybenzyl derivative of propranolol. Both pindolol and hydroxybenzylpropranolol inhibited the actions of isoproterenol and hydroxybenzylpindolol (Fig. 3). The concentrations of propranolol, pindolol, and hydroxybenzylpropranolol required for half-maximal inhibition of the stimulatory effects of 10 μM isoproterenol and 1 μM hydroxybenzylpindolol are given in Table 1; the relative affinities of hydroxybenzylpindolol and hydroxybenzylpropranolol were essentially identical and were 25–40 times the affinity of isoproterenol and 10 times that of pindolol and propranolol. Thus introduction of the hydroxybenzyl group increases to an equivalent extent the affinity of both pindolol and propranolol for the *beta* receptor. However, hydroxybenzylpindolol is considerably more effective as an agonist than its parent compound and the corresponding hydroxybenzyl derivative of propranolol (see Fig. 4).

Effects of GTP. Catecholamines and peptide hormones do not activate the mul-

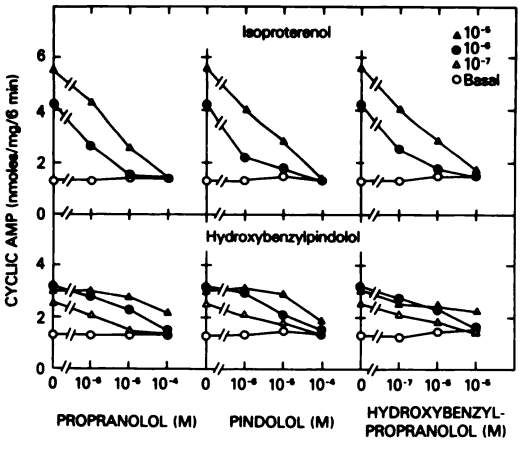

FIG. 3. *Inhibition by l-propranolol, pindolol, and hydroxybenzylpropranolol of adenylate cyclase activation by hydroxybenzylpindolol and isoproterenol*

Fat cell membranes were assayed for adenylate cyclase activity under standard assay conditions without agonists (isoproterenol or hydroxybenzylpindolol) (○), or with 10 μM agonist (▲), 1 μM agonist (●), or 0.1 μM agonist (△).

TABLE 1

Concentrations of hydroxybenzylpropranolol, propranolol, and pindolol required for half-maximal inhibition (IC_{50}) of stimulatory effects of hydroxybenzylpindolol and isoproterenol

The IC_{50} values were estimated from the data in Fig. 4.

	IC_{50}	
	Isoproterenol (10 μM)	Hydroxybenzylpindolol (1 μM)
	μM	μM
Propranolol	4	9
Pindolol	4	8
Hydroxybenzylpropranolol	0.4	1

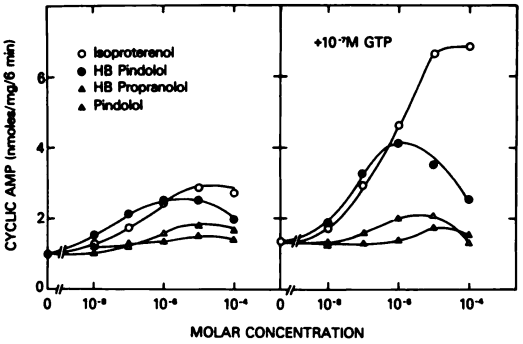

FIG. 4. *Effects of hydroxybenzylpindolol, pindolol, hydroxybenzylpropranolol, and isoproterenol on adenylate cyclase in the absence and presence of GTP*

Fat cell membranes were assayed for adenylate cyclase activity in the absence (left) or presence (right) of 0.1 μM GTP, but otherwise under standard assay conditions (see MATERIALS AND METHODS). Hydroxybenzylpindolol (HB pindolol) (●), hydroxybenzylpropranolol (△), pindolol (▲), and isoproterenol (○) were present at the indicated concentrations.

tireceptor fat cell adenylate cyclase system in the absence of a purine nucleotide triphosphate; GTP acts at considerably lower concentrations than does ATP in the activation process (10). The data in Fig. 4 show that 0.1 μM GTP enhances the stimulatory effects of both isoproterenol and hydroxybenzylpindolol observed in the presence of 0.1 mM ATP. The major effect of GTP was on V_{max}. Maximal effects of hydroxybenzylpindolol were obtained at 1 μM in both the absence and presence of GTP. Higher concentrations of the com-

pound resulted in inhibition of adenylate cyclase activity. GTP also increased the weak agonist effects of pindolol and hydroxybenzylpropranolol (Fig. 4), indicating that the same nucleotide-dependent process applies to the agonist effects of these compounds; a bell-shaped dose-response relationship was also observed with hydroxybenzylpropranolol.

Effects of Gpp(NH)p. Gpp(NH)p, a nucleotide phosphohydrolase-resistant analogue of GTP, stimulates adenylate cyclase activity in membranes prepared from a variety of eukaryotic cells (15, 16). Gpp(NH)p activates the fat cell system in the absence of hormones by a time-dependent or transient process; the time dependency is abolished or diminished in the presence of peptide hormones or catecholamines (7). Epinephrine or hydroxybenzylpindolol diminished the transient phase of Gpp(NH)p activation (Fig. 5); the final steady-state rate obtained with Gpp(NH)p alone was increased by approximately 20% in the presence of these compounds, as previously observed with catecholamines and peptide hormones (7). For comparison, Fig. 5 shows that GTP (0.1 μM) increased the initial rate of activity given with epinephrine or hydroxybenzylpindolol without altering basal activity.

Effects on cyclic AMP production and lipolysis in intact cells. In view of the stimulatory effects of hydroxybenzylpindolol on adenylate cyclase in isolated membranes, it was anticipated that the compound would stimulate cyclic AMP accumulation and increase lipolysis by intact fat cells. Table 2 describes the results obtained when fat cells were incubated in the presence of various concentrations of hydroxybenzylpindolol, isoproterenol, and combinations of hydroxybenzylpindolol and isoproterenol in both the absence and presence of methylisobutylxanthine, a phosphodiesterase inhibitor. Hydroxybenzylpindolol alone did not elevate cyclic AMP but did produce a 7-fold stimulation of glycerol production (as a measure of lipolysis). The rate of lipolysis even with 1 μM hydroxybenzylpindolol was about 30% of the rate seen in the presence of 1 μM isoproterenol (Table 2 and Fig. 6). Thus

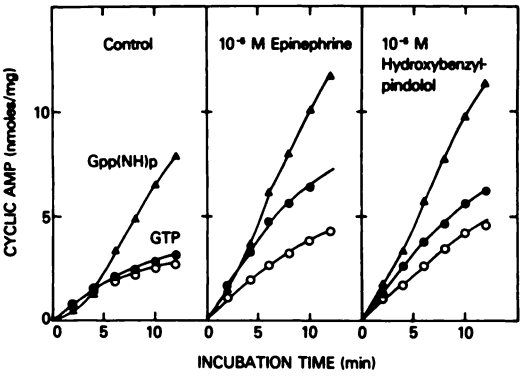

FIG. 5. *Effects of epinephrine and hydroxybenzylpindolol on time course of adenylate cyclase activity in the absence and presence of GTP or GPP(NH)p*

Fat cell membranes (4 μg of protein) were incubated in 400 μl of standard assay medium (see MATERIALS AND METHODS) without guanine nucleotides (○), or with 0.1 μM GTP (●) or 10 μM Gpp(NH)p (▲). At each time point a 50-μl aliquot was withdrawn and assayed for cyclic AMP content.

hydroxybenzylpindolol acts as a partial agonist on lipolysis and, as shown below, on cyclic AMP production.

In the presence of methylisobutylxanthine, it was possible to observe a large effect of hydroxybenzylpindolol on cyclic AMP accumulation (Table 2). Under these conditions the rise in cyclic AMP due to hydroxybenzylpindolol was about 50% of that due to isoproterenol (Table 2). The stimulatory effect of hydroxybenzylpindolol on cyclic AMP production and lipolysis was through the *beta* adrenergic receptor, since the compound inhibited, in a competitive manner, the stimulatory effect of isoproterenol. Furthermore, propranolol inhibited the activation of lipolysis by 1 μM hydroxybenzylpindolol to the same extent as did isoproterenol (data not shown). Thus, as observed with the adenylate cyclase system in isolated fat cell membranes, hydroxybenzylpindolol acts as a partial *beta* adrenergic agonist on the adenylate cyclase system in intact cells as well as on the enzyme in isolated membranes.

The data obtained with the cyclase system in isolated membranes indicated that hydroxybenzylpindolol binds to the *beta* receptor with a 25–40-fold higher affinity

TABLE 2

Inhibition of cyclic AMP accumulation and lipolysis due to isoproterenol by hydroxybenzylpindolol

Isolated fat cells equivalent to about 20 mg of triglyceride were incubated for 5 min for cyclic AMP determinations or 60 min for glycerol release in 1 ml of Krebs-Ringer-phosphate buffer (pH 7.4) containing 3% bovine serum albumin. Glycerol release in the presence of 0.1 mM methylisobutylxanthine is not shown, since it was about 35 μmoles/g of fat cells and no further elevations were seen in the concomitant presence of hydroxybenzylpindolol or isoproterenol. The values are the means of two paired experiments.

Additions	Basal	Change due to isoproterenol		
		0.1 μM	0.3 μM	1 μM
		nmoles cyclic AMP/g		
None	0.13	+0.15	+0.54	+0.62
Hydroxybenzylpindolol, 0.1 μM	0.15	+0.09	+0.06	+0.76
Hydroxybenzylpindolol, 1.0 μM	0.15		+0.07	+0.37
Methylisobutylxanthine, 0.1 mM	0.16	+6.6	+10.1	+9.0
+ Hydroxybenzylpindolol, 0.1 μM	2.2	+2.9	+6.4	+8.4
+ Hydroxybenzylpindolol, 1.0 μM	4.4		+1.2	+3.6
		μmoles glycerol released/g		
None	1.4	+14.8	+25.3	+28.4
Hydroxybenzylpindolol, 0.1 μM	6.0	+3.3	+9.7	+21.3
Hydroxybenzylpindolol, 1.0 μM	9.3		−1.0	+1.5

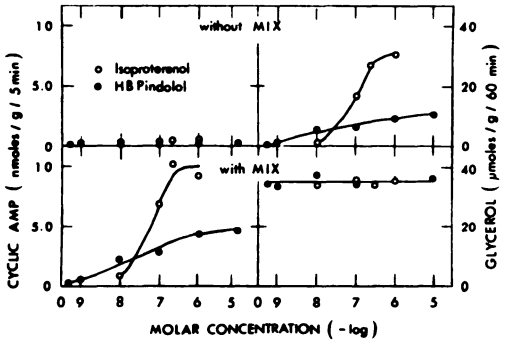

FIG. 6. *Activation of lipolysis and cyclic AMP accumulation by hydroxybenzylpindolol*

Isolated fat cells equivalent to about 20 mg of triglyceride were incubated for 5 min for cyclic AMP determinations or 60 min for glycerol release in 1 ml of Krebs-Ringer-phosphate buffer containing 3% bovine serum albumin. Values in the absence of methylisobutylxanthine (MIX) are shown in the top half of the figure, and those in the presence of 0.1 mM methylisobutylxanthine, in the lower half. The values are from two paired experiments for cyclic AMP and one for lipolysis. ○, isoproterenol; ●, hydroxybenzylpindolol.

than isoproterenol (Table 1). This higher affinity was also reflected both in cyclic AMP production (with the phosphodiesterase inhibitor) and in glycerol production (without the inhibitor) (Fig. 6). Hydroxybenzylpindolol at 10 nM increased both cyclic AMP and lipolysis, whereas isoproterenol at this concentration did not.

As observed by a number of investigators [for review, see Fain (17)], the levels of cyclic AMP in fat cells do not necessarily correlate with the degree of lipolysis. In part this is due to the fact that basal levels of cyclic AMP are high relative to the concentrations required for stimulation of lipolysis and may reflect compartmentation, binding to tissue proteins, or the presence of factors that antagonize the effects of basal cyclic AMP on protein kinase [see also Beavo *et al.* (18) for further discussion of this point]. In any event, a plot (Fig. 7) of the relationship between cyclic AMP levels and glycerol production (taken from the data in Fig. 6) indicates that maximal production of lipolysis occurs when the total concentration of cyclic AMP reaches about 1.0 nmole/g of fat cells, measured at 5 min. Further increases in cyclic AMP production do not lead to further change in the rate of lipolysis. Thus the cyclic AMP-generating capacity of the fat cell is far in excess of that required to satisfy the lipolysis-stimulating mechanism.

DISCUSSION

This study shows that hydroxybenzylpindolol stimulates the fat cell adenylate cyclase system via a receptor having the

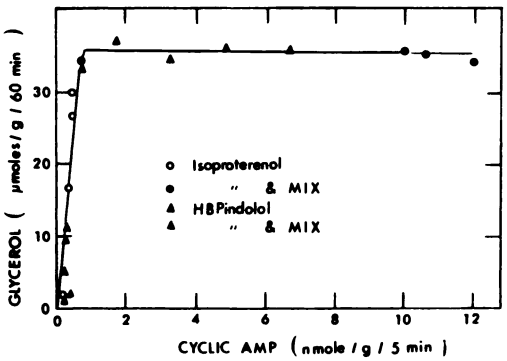

FIG. 7. *Correlation between cyclic AMP accumulation at 5 min and lipolysis at 1 hr in the presence of isoproterenol, hydroxybenzylpindolol (HB pindolol), and methylisobutylxanthine (MIX)*

Isolated fat cells equivalent to about 20 mg of triglyceride were incubated for 5 min for cyclic AMP determinations or 60 min for glycerol release in 1 ml of Krebs-Ringer-phosphate buffer containing 3% bovine serum albumin. The data are taken from Fig. 6. The different points for each symbol represent varying concentrations of isoproterenol or hydroxybenzylpindolol.

stereospecific characteristics of the *beta* adrenergic receptor. The compound competitively inhibited both the actions of catecholamines on adenylate cyclase in isolated membranes and their effects on lipolysis and cyclic AMP production in intact fat cells. With isolated membranes, stimulation of adenylate cyclase activity by hydroxybenzylpindolol (or the corresponding derivative of propranolol) also displayed the same guanine nucleotide-dependent effects described previously for the actions of both catecholamines and peptide hormones on the multireceptor fat cell system (7, 10). In the presence of Gpp(NH)p, which stimulates adenylate cyclase activity in membranes from a variety of eukaryotic cells (15), hydroxybenzylpindolol decreased the lag phase of Gpp(NH)p activation and increased activity to the same extent observed with isoproterenol (see Fig. 5). The lag phase and the effects of hormones thereon have been suggested to reflect the rates of transition between states having different kinetic parameters at the catalytic site (7). In the presence of GTP, considered to be a natural regulatory nucleotide for adenylate cyclase systems (15), the stimulatory effect of hydroxybenzylpindolol was limited to about 60% that given by isoproterenol. In the intact cell, the efficacy of hydroxybenzylpindolol action was limited to between 30% and 50% of that given by isoproterenol.

To our knowledge, hydroxybenzylpindolol is the first example of a *beta* adrenergic *agonist* having a higher affinity than catecholamines for the *beta* receptor. Pindolol, the parent compound, is a considerably weaker agonist and has lower affinity for the *beta* receptor, which suggests that the hydroxybenzyl group is largely responsible for both increased efficacy and potency. Interestingly, serotonin (5-hydroxytryptamine), like pindolol, contains an indole nucleus and has been reported to be a very weak *beta* adrenergic agonist in fat cells, with considerably less affinity than catecholamines (19, 20). Hydroxybenzylpropranolol, unlike its parent compound propranolol, also exhibited weak agonist effects and had an affinity for the *beta* receptor identical with that of hydroxybenzylpindolol. As noted in other studies (2, 3), the catechol nucleus is not required for *beta* receptor recognition or action; substitutions of bulky aryl or alkyl functions increase the affinity of both agonists and antagonists. It would appear that the binding domains of the *beta* receptor are quite flexible in structure and that function (or action) derived from ligand interaction cannot be ascribed simply to a particular functional group, but rather to a combination of properly ordered domains of both the ligand and the receptor. However, additional factors must be postulated to account for the behavior of partial agonists like hydroxybenzylpindolol, which bind with high affinity to the receptor but with lower efficacy than catecholamines.

Aurbach *et al.* (6) were the first to show that iodinated hydroxybenzylpindolol binds to the *beta* receptor in turkey erythrocyte membranes with considerably higher affinity than do catecholamines. Recently they have reported (21) that the compound also stimulates adenylate cyclase activity in these membranes, provided that Gpp(NH)p is present in the assay medium. Under these conditions excellent correlations between the binding and actions of iodohydroxybenzylpindolol were

found. Thus, because of its high affinity for the *beta* receptor and its ability to stimulate adenylate cyclase activity, hydroxybenzylpindolol should prove to be a valuable compound for evaluating the content and properties of *beta* receptors coupled to adenylate cyclase systems. Such studies on the fat cell system are in progress.

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