Preponderance of α_2- over β_1-adrenergic receptor sites in human fat cells is not predictive of the lipolytic effect of physiological catecholamines

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Abstract Adrenergic control of human fat cell lipolysis is mediated by two kinds of receptor sites that are simultaneously stimulated by physiological amines. To establish a correlation between the binding characteristics of the receptor and biological functions, the ability of physiological amines to stimulate or inhibit isolated fat cell lipolysis *in vitro* was compared to the β- and α_2-adrenoceptor properties of the same fat cell batch. The β-selective antagonist (−)[^{3}H]dihydroalprenolol ([^{3}H]DHA) and the α_2-selective antagonists [^{3}H]yohimbine ([^{3}H]YOH) and [^{3}H]rauwolscine ([^{3}H]RAU) were used to identify and characterize the two receptor sites. Binding of each ligand was rapid, saturable, and specific. The results demonstrate *1*) the weaker lipolytic effect of epinephrine compared with norepinephrine. This can be explained by the equipotency of the amines at the β_1-sites and the higher affinity of epinephrine for α_2-adrenergic receptors. *2*) The preponderance of α_2-adrenergic receptor sites labeled by [^{3}H]YOH (B_{max}, 586 ± 95 fmol/mg protein; K_D, 2.7 ± 0.2 nM) or [^{3}H]RAU (B_{max}, 580 ± 100 fmol/mg protein; K_D, 3.7 ± 0.1 nM). These two ligands can be successfully used to label α_2-adrenergic receptor sites. *3*) The β_1-adrenergic receptor population labeled by [^{3}H]DHA (B_{max}, 234 ± 37 fmol/mg protein; K_D, 1.8 ± 0.4 nM), although a third as numerous as the α_2-adrenergic population, is responsible for the lipolytic effect of physiological amines and is weakly counteracted by simultaneous α_2-adrenergic receptor stimulation under our experimental conditions. It is concluded that, in human fat cells, the characterization of β_1- and α_2-adrenergic receptors by saturation studies or kinetic analysis to determine affinity (K_D) and maximal number of binding sites (B_{max}) is not sufficient for an accurate characterization of the *functional* adrenergic receptors involved in the observed biological effect.—**Lafontan, M., M. Berlan, and A. Villeneuve.** Preponderance of α_2- over β_1-adrenergic receptor sites in human fat cells is not predictive of the lipolytic effect of physiological catecholamines. *J. Lipid Res.* 1983. **24:** 429–440.

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It is now well accepted that lipid mobilization is modulated by the sympathetic nervous system and that catecholamines control the lipolytic activity of the adipose tissue of various species. The human fat cell is known to have β- and α_2-adrenoceptors in the plasma membrane (1–6). Physiological studies have shown that epinephrine and norepinephrine, although they are able to stimulate both kinds of receptor, exert, when infused intravenously, a predominant lipolytic effect (7) that is ascribed to an action on the β-adrenoceptors. *In vitro* studies on isolated fat cells have confirmed the physiological observations. Generally, the stimulating effect of norepinephrine on lipolysis, mediated by the β-adrenoceptors, is seen to predominate over the inhibitory effects, involving α_2-adrenoceptors. However under certain physiological conditions the balance between these effects can be modified, leading to the occurrence of antilipolytic effects for physiologic amines. Evidence of an increased α-adrenergic effect has been reported in humans during starvation (8–10), caloric restriction (11), and hypothyroidism (12). Moreover this α-effect seems to be predominant in the subcutaneous adipose tissue at certain locations (13–15). The changes between α- and β-receptor-mediated responses require a great deal of additional data, taking the characteristics (number and affinity) of the two families of receptors and various post-receptor events into account.

The direct identification of adrenergic receptors by binding of radiolabeled ligand has improved the knowledge of fat cell adrenoreceptor properties considerably. In the past few years a number of laboratories have reported studies of β- and α-adrenoceptor sites on fat cell membrane extracts from humans, hamsters, and rats using various radiolabeled ligands. The tritiated antagonist (−)[^{3}H]dihydroalprenolol ([^{3}H]DHA) has

Abbreviations: [^{3}H]DHA, (−)[^{3}H]dihydroalprenolol; [^{3}H]YOH, [^{3}H]yohimbine; [^{3}H]RAU, [^{3}H]rauwolscine; [^{3}H]CLO, [^{3}H]clonidine; [^{3}H]PAC, [^{3}H]paraaminoclonidine; [^{3}H]DHEC, [^{3}H]dihydroergocryptine; KRBA buffer, Krebs Ringer bicarbonate-albumin buffer.

been widely used for the identification of fat cell β-adrenoceptors (6, 16–18). A number of radioligands have been developed to permit a better insight into the α-adrenoceptor subtypes. Some studies have used [^{3}H]dihydroergocryptine ([^{3}H]DHEC), a non-subtype selective α-antagonist (19, 20). The biological definition of the fat cell α-adrenoceptor modulating lipolysis as an α_2-subtype (3, 4) promoted research focused on more selective radioligands. Alpha$_2$-sensitive agonists such as clonidine (21) or paraaminoclonidine (4) have been used to label human or hamster fat cell (22) adrenoceptors. More recently, the α_2-antagonist, [^{3}H]yohimbine ([^{3}H]YOH) has been shown to be an excellent ligand for the characterization of human fat cell α_2-adrenoceptors (23, 24).

Most of the data concerning the β- and α-adrenoceptors of the adipose tissue given by use of radiolabeled ligands were focused on the direct demonstration of the sites. This information did not allow the results in the literature to be used easily to interpret the biological effects previously described. Numerous discrepancies exist in the α_2-receptor number and affinities proposed by various investigators (4, 21, 23). Possible explanations for the apparent heterogeneity of the data include poorly selected donors, differences in adipose tissue origin or treatment, and membrane storage. The most striking fact is that important differences exist in agonist- or antagonist-binding properties to α_2-adrenoceptors that can be summarized as follows: two affinity states of the receptor are apparently involved in the binding of α_2-agonists (23, 25). Furthermore, the affinity of the α_2-receptor for the agonists is decreased in the presence of guanine nucleotides (23, 25), and lastly, the antagonist [^{3}H]YOH labels two to three times as many sites as the agonists [^{3}H]clonidine or [^{3}H]paraaminoclonidine (24, 26).

The above considerations led us to undertake a study in which we attempted to circumvent the factors causing conflicting results and to compare binding studies and biological effects. In this report, we present the results of experiments that were carried out to delineate, on the same human fat cell preparation, the β- and α_2-adrenoceptor activity of physiological amines (epinephrine and norepinephrine) and the number and affinity of the β- and α_2-adrenoceptor binding sites determined using selective adrenergic antagonists. In this way, the β-adrenergic receptors were identified by [^{3}H]DHA and the α_2-adrenoceptors were identified by using [^{3}H]YOH and its diastereoisomer [^{3}H]rauwolscine ([^{3}H]RAU), which are potent antagonists of α_2-adrenoceptors. We found that the number of β-binding sites is lower than that of α_2-binding sites. In order to assess our data on α_2-antagonist binding, we also present additional data on [^{3}H]YOH binding characteristics to fat cell α_2-adrenoceptors.

MATERIALS AND METHODS

Selection of patients

Subcutaneous abdominal adipose tissue was collected at the end of the surgical procedure from obese females (aged 21–50 years old) undergoing surgical lipectomy. General anesthesia was induced with thiopental and the patients had not received drugs active on the autonomic nervous system. The patients fasted overnight before tissue removal and had no identified metabolic or endocrinological disorder. Immediately after removal, the tissue was placed in cooled, sterile physiological saline and quickly transferred to the laboratory. For investigation under the best conditions with a limited incidence of inter-subject variations, all the assays of one experiment were carried out on the same batch of tissue. The complete investigation requires a mass of at least 100 g of adipose tissue which can be easily obtained after abdominal lipectomy.

Human fat cell preparation

Isolated fat cells were obtained as previously described (3) by collagenase digestion of adipose tissue fragments in Krebs Ringer bicarbonate buffer containing 2 g/100 ml bovine serum albumin at pH 7.4. Under our experimental conditions (1.5 mg/ml of collagenase and 37°C with strong shaking), isolated fat cells were obtained after 30–50 min of incubation (the collagenase contact being as short as possible). Then the fat cells were filtered on a silk screen and washed twice with the incubation buffer.

Measurements of lipolysis

White fat cells (≅20 mg of total lipid) obtained by collagenase digestion were incubated in 1 ml of Krebs Ringer bicarbonate buffer, pH 7.4, containing bovine serum albumin (3.5 g/100 ml) and glucose (0.6 mmol/100 ml) at 37°C with air as the gas phase with gentle shaking (≅60 cycles per min) in a water bath. After 90 min incubation, the tubes were placed on ice and 200-μl aliquots of infranatant were removed for determination of glycerol release. Glycerol was measured by the enzymatic method of Wieland (27) and total lipid was determined gravimetrically after extraction according to Dole and Meinertz (28). Ascorbic acid (0.1 mM) was included in the incubation medium to prevent catecholamine oxidation. Epinephrine and norepinephrine were added just before the beginning of the assay in 10-

μl portions to obtain the desired final concentration. When necessary, the antilipolytic effects of catecholamines were studied in the incubation buffer supplemented with 2 μg/ml adenosine deaminase associated with 1.6 mM theophylline, which are well known lipolytic agents and allow more accurate investigation of α_2-mediated antilipolytic effects (3, 29, 30).

Preparation of human adipocyte membranes

The isolated fat cells were washed four times at 8–10°C in a lysing medium (2.5 mM $MgCl_2$; 1 mM $KHCO_3$; 1 mM ATP; 2 mM Tris-HCL, pH 7.6). Crude adipocyte ghosts were pelleted by centrifugation (10 000 *g*, 10 min) at 4°C, washed twice in buffer (5 mM Tris-HCl; 1 mM EDTA, pH 7.6) and finally resuspended in the incubation buffer commonly used for binding studies (10 mM Mg Cl_2; 50 mM Tris-HCl, pH 7.5) at a final concentration of 2–2.5 mg protein/ml and immediately frozen. Some preparations were used immediately (mentioned in text). The membrane preparation was stored at −80°C. When specified, some ghosts were also obtained after homogenization of fat cell suspensions in a Potter apparatus with a Teflon pestle (8–10 strokes) in buffer (0.25 M sucrose; 5 mM Tris-HCl; 1 mM EDTA, pH 7.6) and treated as described above. Generally the treatment of the fat cells gave six to ten crude fat cell membrane extracts, all containing the same protein concentration. The membranes were generally used within 1–2 weeks; longer storage (5–6 weeks) revealed a decrease in the total number of binding sites although the affinity was less modified (not shown). Protein was assayed by the method of Lowry et al. (31) using bovine albumin as standard.

Binding assays

Radioactive ligands, (−)[^{3}H]dihydroalprenolol (a β-antagonist), and [^{3}H] yohimbine and [^{3}H]rauwolscine (α_2-antagonists) were used. Thawed frozen membranes were rehomogenized with four pestle strokes in a Potter apparatus immediately before use. Binding studies were conducted in a final volume of 400 μl as previously reported (21, 24). The incubation mixture consisted of 100 μl of aqueous radioligand solutions ranging in final concentration from 0.4 to 20 nM and 100 μl of the membrane suspension (200–250 μg protein) made up to the final volume of 400 μl with 50 mM Tris-HCl, pH 7.5, containing 10 mM $MgCl_2$. Separate incubations were carried out in the presence of 0.1 mM propranolol or 0.1 mM yohimbine for the evaluation of nonspecific binding. In the competition experiments, the agonists or antagonists were dissolved in water or buffer, diluted, and added to the assay (100 μl) just prior to the experiments along with a mixture containing 750 μM ascorbic acid, 25 μM pargyline, and 0.3 mM catechol. Incubations were carried out at 25°C in a water bath for 20 min under constant shaking at 150 cycles per min. At the end of incubation duplicate 150-μl aliquots were diluted in 4 ml ice-cold buffer and vacuum filtered immediately through Whatman GF/C glass fiber filters placed on a Millipore manifold. The filters were washed twice with 10-ml portions of ice-cold incubation buffer. Filters were placed in minivials, dried at 60°C for 30–40 min and counted in 4 ml of liquid scintillation medium (ACS-Amersham Centre) in an Intertechnique scintillation spectrometer at an efficiency of 40–45%. Specific binding was taken as the amount of radioactivity bound to the membranes defined as total binding minus binding in the presence of 0.1 mM cold ligand (propranolol or yohimbine). Specific binding ranged from 70–80% of the total binding for [^{3}H]DHA and 90–95% for [^{3}H]YOH and [^{3}H]RAU at final incubation concentrations of 2–10 nM. The binding data in this report refer to specifically bound labeled ligands. In a typical experiment run between concentrations of 0.4 and 20 nM, [^{3}H]DHA specifically bound counts ranged from 195 to 2000 cpm. For an equivalent range of concentrations of [^{3}H]YOH or [^{3}H]RAU, specifically bound counts ranged from 450–700 cpm to 5000–6500 cpm for equivalent protein concentrations (2.5 mg/ml).

Data analysis

All experiments were performed in duplicate and repeated 5–8 times unless otherwise specified. Half-maximal inhibitory concentrations (EC_{50} values) and slope factors (*pseudo* Hill coefficients) for each competitor were calculated from log-logit regression analysis. The number (B_{max}) and affinity (K_D) of the binding sites were analyzed according to the method of Scatchard as described in the Williams and Lefkowitz monograph (32). The use of selective antagonist radioligands did not require the use of computer modeling of the data (33).

Materials

[^{3}H]Dihydroalprenolol (90.0 Ci/mmol), [^{3}H]yohimbine (89.7 Ci/mmol), and [^{3}H]rauwolscine (85.7 Ci/mmol) were obtained from New England Nuclear (Boston MA). (−)Epinephrine bitartrate, (−)norepinephrine bitartrate, (−)isoproterenol chlorhydrate, ascorbic acid, pargyline, pyrocatechol, theophylline, bovine serum albumin (fraction V), and crude collagenase were obtained from Sigma Chemical Co; (+)epinephrine and (+)norepinephrine came from Sterling-Winthrop Company. The following drugs were kindly given by the indicated companies: propranolol (ICI Pharma); phentolamine (Ciba Geigy); prazosin (Pfizer); clonidine

TABLE 1. Comparison of the lipolytic and antilipolytic effects of epinephrine and norepinephrine in human adipocytes

		$-\log_{10}$[Epinephrine or Norepinephrine] M				
	Control	8	7	6	5	4
A. Glycerol release of human isolated fat cells in response to increasing doses of catecholamines[a]						
(−)Epinephrine	0.200 ± 0.038[b]		0.181 ± 0.012	0.245 ± 0.032	0.457 ± 0.149	0.554 ± 0.151
(−)Norepinephrine	0.200 ± 0.038		0.222 ± 0.037	0.415 ± 0.100*[c]	0.519 ± 0.150***	0.614 ± 0.170*
B. Inhibition by epinephrine and norepinephrine of lipolysis stimulated by theophylline in the presence of adenosine deaminase[d]						
(−)Epinephrine	0.670 ± 0.090	0.589 ± 0.116	0.315 ± 0.043	0.203 ± 0.032	0.196 ± 0.037	
(−)Norepinephrine	0.670 ± 0.090	0.614 ± 0.117	0.552 ± 0.053***	0.320 ± 0.083*	0.265 ± 0.039**	

[a] Human fat cells (20–25 mg total lipid) were incubated in 1 ml of KRBA buffer containing 3.5% bovine serum albumin and 0.6 mmol% glucose as described in Materials and Methods.

[b] Lipolysis is expressed as μmol of glycerol released per 100 mg total lipid over 90 min; the values are means ± SEM.

[c] *, $P < 0.05$; **, $P < 0.02$; ***, $P < 0.01$; indicates a significant difference between epinephrine- and norepinephrine-stimulated glycerol release at the same concentration of catecholamines according to Student's paired t test.

[d] Human fat cells were incubated as in (A) with 1.6 mM theophylline and 2 μg/ml adenosine deaminase in the incubation buffer to promote an increase in basal lipolytic activity of the cells. The inhibition experiments were performed in the presence of 10^{-5} M propranolol (beta-adrenergic antagonist).

(Boehringer Ingelheim); guanfacine (BS 100-141, N-amidino 2-(2.6 dichlorophenyl) acetamide (Sandoz); methoxamine (Wellcome); nicergoline (Specia). Piperoxane and tramazoline were kindly given by Dr. H. Schmitt (Department of Pharmacology–Paris V). Adenosine deaminase, guanyl-5′-yl-imidodiphosphate (Gpp (NH)p), and enzymes were obtained from Boehringer Mannheim. All other chemicals were reagent grade.

RESULTS

β- and α_2-Adrenergic responses initiated by physiological catecholamines

We compared simultaneously the effect of the two putative physiological agonists with regard to their relative ability to stimulate or inhibit the lipolytic activity of isolated human fat cells (**Table 1**). Dose–response studies demonstrated that the two agents were able to stimulate lipolysis, as previously known. However it is noticeable that epinephrine is a weaker lipolytic agent than norepinephrine. The order of potency of the drugs eliciting a lipolytic response was isoproterenol > norepinephrine > epinephrine; the maximal response was with isoproterenol as previously shown (1, 2, 11). However, when the α_2-adrenoceptors were blocked by the α_2-adrenergic antagonist yohimbine (10^{-5} M) or the mixed alpha-antagonist phentolamine (10^{-5} M), epinephrine and norepinephrine-stimulated lipolysis were potentiated to similar levels (0.870 ± 95 vs 0.910 ± 108 μmol glycerol/100 mg lipid per 90 min, with 10^{-5} M epinephrine or norepinephrine, respectively). Such data suggest the equipotency of the two catecholamines in the stimulation of β-adrenoceptors and lipolysis. Since the catecholamines are mixed beta/alpha-agonists, their capacity to elicit antilipolytic actions through α_2-adrenoceptor stimulation was studied concomitantly. As the inhibiting effect promoted by α_2-adrenoceptor stimulation is mainly dependent on the level of basal lipolytic activity, two well-known lipolytic agents, acting by a mechanism unrelated to β-receptor sites, were included in the incubation buffer to promote an increase in human fat cell lipolysis (29, 30). Adenosine deaminase (2 μg/ml) in the presence of theophylline (1.6 mM) strongly stimulated lipolysis (0.670 ± 0.090 vs. 0.200 ± 0.038 μmol/100 mg lipid per 90 min in the absence of lipolytic agents). The ability of epinephrine and norepinephrine to inhibit lipolysis was measured in the presence of 10^{-5} M propranolol (in order to suppress the β-adrenergic effect of the amines). The two catecholamines inhibited in a dose-dependent manner the lipolytic activity of the adipocytes (Table 1). Epinephrine was the most potent antilipolytic agent; the equilibrium dissociation constant (K_i) for (−) epinephrine and norepinephrine, estimated from inhibition of lipolysis, was 35 ± 9 nM for epinephrine and 130 ± 20 nM for norepinephrine. Thus, the order of potency at the α_2-adrenoceptor sites, defined by the biological studies, was epinephrine > norepinephrine.

The comparative study of the physiological catecholamines demonstrated significant differences in their lipolytic or antilipolytic potencies although, under our experimental conditions, their predominant effect on subcutaneous human fat cells was always lipolytic when basal (unstimulated) lipolysis was taken as a reference.

Identification of β-adrenoceptor sites by [^{3}H]DHA binding

The comparison of the two types of adrenergic receptors was conducted on the same fat cell batch in parallel with lipolysis studies. The use of unselected populations of adipocytes can hamper binding results by misleading measurements, owing to differences in tissue localization or to variations in membrane recovery from one experiment to another. Saturation binding experiments performed with (−)[^{3}H]dihydroalprenolol on human fat cell membrane preparations demonstrated that [^{3}H]DHA binding sites appeared to be saturable at concentrations of 8–10 nM of radioactive ligand (Fig. 2). The calculated total number of binding sites from Scatchard analysis of the data was 234 ± 37 fmol/mg protein and the dissociation constant of the binding site (K_D) was 1.80 ± 0.4 nM (**Table 2**). The Scatchard plot was linear (r = 0.98) and the Hill coefficients (n_H) of 1 indicated the absence of any significant cooperative interactions among the binding sites. Inhibition of [^{3}H]DHA binding to human fat cell membranes by isoproterenol and the two physiological amines was in the order of potency (−)isoproterenol > (−)epinephrine ≅ (−)norepinephrine, which is a typical binding for β_1-adrenergic receptors. Epinephrine and norepinephrine were equipotent in inhibiting [^{3}H]DHA binding (5 nM) to human fat cell β_1-adrenoceptors; EC_{50} values were 5.1 ± 1.2 μM for epinephrine and 5.6 ± 1.6 μM for norepinephrine. Since this result fits previously reported data on human fat cell β-adrenergic receptors, a more detailed study of the kinetics of binding and inhibition of [^{3}H]DHA binding by competing antagonists is not reported in this study.

Identification of α_2-adrenoceptor sites by α_2-antagonists

Kinetics of [^{3}H]YOH and [^{3}H]RAU binding. The careful determination of the characteristics of α_2-adrenergic receptor sites requires the labeling of the total population of fat cell membrane α_2-adrenoceptors. The use of α_2-antagonists instead of the previously used α_2-agonists (4, 21) seemed to be more accurate for this purpose (33). Two yohimbine diastereoisomers were used. Yohimbine and rauwolscine (α-yohimbine) are known to be practically equipotent at α_2-adrenoceptor sites. The main difference between the two drugs is that rauwolscine is less potent than yohimbine at α_1-adrenoceptor sites of the central nervous system (34).

The results obtained from the study of the kinetics of binding of the two drugs are presented in **Fig. 1.** The binding characteristics of [^{3}H]YOH and [^{3}H]RAU are not noticeably different. At 25°C, specific binding of 5–9 nM of the drugs was rapid (the half maximal specific binding was obtained in 3–4 min and was monophasic). The equilibrium was reached within the first 10 min and was stable for at least 30 min. Dissociation of binding at 25°C in the presence of 10^{-4} M phentolamine, added after the equilibrium had been reached, was rapid and represented a first order process. The similarity of kinetics of the two diastereoisomers of yohimbine suggests that they can be used independently on human fat cell membrane preparations.

TABLE 2. Comparison of adrenergic receptor binding sites from simultaneous determinations of [^{3}H]DHA, [^{3}H]YOH, and [^{3}H]RAU binding in human fat cell membranes of abdominal subcutaneous adipose tissue

Receptor	Radioligand	N	B_{max}[a]	K_D[b]	n_H[c]
			fmol/mg protein	*nM*	
Beta	[^{3}H]DHA	6	234 ± 37	1.8 ± 0.4	1.01 ± 0.02
$Alpha_2$	[^{3}H]YOH	6	586 ± 95	2.7 ± 0.2	0.98 ± 0.04
	[^{3}H]RAU	6	580 ± 100	3.7 ± 0.1	1.00 ± 0.03

[a] B_{max} is the total number of binding sites.
[b] K_D is the equilibrium dissociation constant of binding.
[c] n_H represents the slope of the Hill plot. In the Hill plot the log of radioligand concentration (L) is plotted against the ratio log(B/B_{max} − B) where (B) is the amount of ligand bound at various concentrations (L) and B_{max} is the maximum number of binding sites determined by Scatchard analysis. The slope of the regression line equals to n_H (Hill number). Hill analysis of full saturation curves was performed to determine the presence or absence of cooperativity among the binding sites.

Parameters were determined from equilibrium studies by the use of linear transformation described by Scatchard (32) of the saturation binding data in which the amount of ligand specifically bound is plotted against the ratio of bound to the free ligand as shown in a typical example in Fig. 2. The data are expressed as means ± SEM of (N) separate experiments.

Equilibrium studies and Scatchard analysis of [^{3}H]YOH and [^{3}H]RAU binding. **Fig. 2A** shows the steady state binding of [^{3}H]YOH and [^{3}H]RAU to human fat cell membranes as a function of radioligand concentration in a typical experiment. The binding was saturable and equivalent for the two drugs. Scatchard plots of the data (Fig. 2B) were linear, a single binding component was found with no cooperativity among the binding sites, and the Hill coefficients (n_H) were not significantly different from 1 (Table 2). The mean apparent dissociation constant (K_D) was 2.7 ± 0.2 nM and the mean receptor density (B_{max}) was 586 ± 95 fmol/mg protein for [^{3}H]YOH and K_D was 3.7 ± 0.1 nM with a B_{max} of 580 ± 100 fmol/mg protein for [^{3}H]RAU. The mean K_D for [^{3}H]YOH was significantly lower ($P < 0.05$) than that of [^{3}H]RAU. As clearly shown in Fig. 2, the most striking fact is the preponderance of α_2-adrenoceptor sites. The mean ratio of the number of [^{3}H]YOH binding sites to that of [^{3}H]DHA, calculated from each fat cell preparation from different patients, was 2.9 ± 0.1.

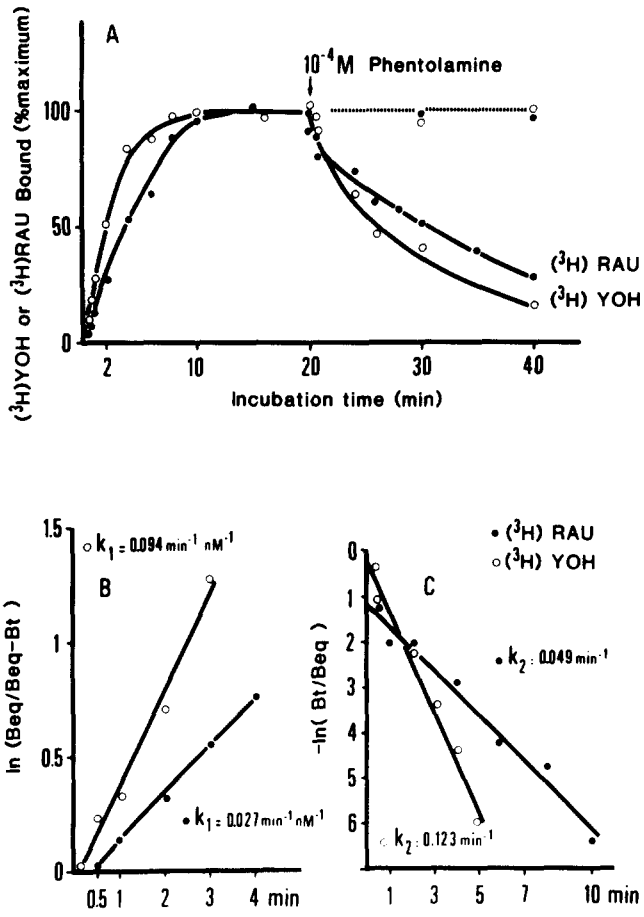

Fig. 1. Time-course of [^{3}H]yohimbine and [^{3}H]rauwolscine binding and dissociation to human fat cell membranes. Data shown are averages of duplicate determinations from one representative experiment. Panel A. [^{3}H]YOH (6 nM) and [^{3}H]RAU (9 nM) were incubated with fat cell membranes for the indicated times at 25°C. Specific binding was determined as described in Materials and Methods. In dissociation studies, membranes were incubated with [^{3}H]YOH and [^{3}H]RAU at 25°C for 20 min, followed by addition of an excess of phentolamine (10^{-4}M) corresponding to time 0. 100% bound corresponds to specific binding of [^{3}H]ligands at equilibrium. Dotted line represents the kinetics of binding in the absence of phentolamine. Panel B. Pseudo first order kinetic plot of initial [^{3}H]YOH and [^{3}H]RAU binding. B_{eq} and B_t represent the amount of ligands specifically bound at equilibrium and at time, respectively. The slope of the line (k_{ob}) determined by linear regression analysis (r = 0.99) provides an estimate of the observed rate constant for the pseudo first order reversible reaction, i.e., 0.45 min^{-1} for [^{3}H]YOH and 0.20 min^{-1} for [^{3}H]RAU. Panel C. First order kinetic plot of [^{3}H]YOH and [^{3}H]RAU dissociation from human fat cell membranes after addition of 10^{-4}M phentolamine. B_t is the amount of [^{3}H]ligand bound at the indicated time, while B_{eq} refers to maximal specific binding at 0 time. The slope of the line, determined by linear regression analysis (0.99), corresponds to the first order rate constant of dissociation (k_2). The second order constant of association (k_1) is determined from the equation $k_1 = (k_{ob} - k_2)/[\text{ligand}]$; [ligand] refers to the concentration of [^{3}H]YOH or [^{3}H]RAU used in kinetic studies.

The similarity of the results furnished by [^{3}H]YOH and [^{3}H]RAU led us to select [^{3}H]YOH for further investigations on α_2-adrenoceptor characteristics. The need for a further, more accurate characterization of [^{3}H]YOH binding sites was made evident by the large discrepancy between the number of sites we found and that previously reported by another group (23), which was only about 145 ± 34 fmol/mg protein, and thus lower than the β-receptor number we defined. Since the authors used Potter lysis of the cells in an isotonic medium, we carried out four experiments under similar conditions and did not find significant differences with our previous data; B_{max} values were similar to those reported using hypotonic lysis of the adipocytes.

Competition studies of [^{3}H]YOH binding. Adrenergic antagonists competed for [^{3}H]YOH binding sites in the order expected for an α-adrenergic receptor (**Fig. 3; Table 3**). The relative order of potency for the antagonists was: yohimbine > phentolamine > piperoxan > nicergoline > prazosin. The antagonist competition curves are characterized by slope factors that were not significantly different from 1.0, suggesting the antagonists interact in a homogenous manner at [^{3}H]YOH binding sites. The binding of [^{3}H]YOH was also inhibited by various families of agonists such as catecholamines, imidazolines, and a phenyl guanidine (guanfacine). Catecholamine binding displayed the expected stereospecificity (Table 3). The agonsists exhibited shallow competition curves with slope factors (n_{Hill} values) ranging between 0.45 and 0.75 (Fig. 3A; Table 3), suggesting that agonists interact in a heterogeneous manner at [^{3}H]YOH binding sites. The relative order of potency of the drugs used was: clonidine > tramazoline > guanfacine > adrenaline > noradrenaline > methoxamine. Clonidine and tramazoline displayed the highest affinities for [^{3}H]YOH sites, as expected for an α_2-adrenoceptor. One noticeable fact is the greater affinity of (−)epinephrine for [^{3}H]YOH sites compared with (−)norepinephrine (Table 3).

Effect of sodium ion and guanyl nucleotides on [^{3}H]YOH binding. It is well known that agonists', but not antagonists' affinities are modulated by cations and guanine nucleotides (35–38) at the α_2-adrenoceptors.

The inclusion of 100 mM NaCl plus the GTP analog Gpp(NH)p (10^{-4} M) in the binding assay had no effect on the antagonist affinities, while the binding affinities of α-adrenergic agonists to human fat cell α_2-adrenoceptors were decreased (**Table 4**). A 15 to 20-fold decrease in the average affinity was observed with (−)epinephrine and (−)norepinephrine while the decrease was weaker for clonidine and guanfacine (3–6 times). The displacement curves (not shown) shifted to the right; the slope factors were increased, but did not reach 1. The clear difference between physiological amines and the two other synthetic agonists (clonidine, guanfacine) should be noted.

Moreover, in five out of eight fat cell preparations we noticed that Gpp(NH)p (10^{-4} M) + 100 mM NaCl

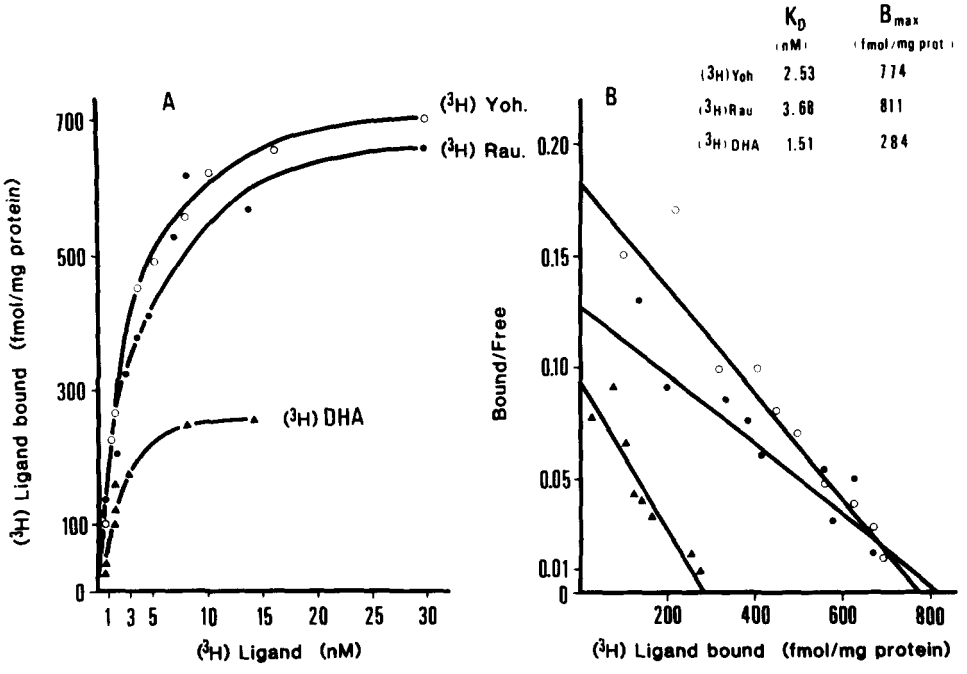

Fig. 2. Specific binding of [^{3}H]DHA, [^{3}H]YOH, and [^{3}H]RAU to human fat cell membranes from one representative experiment. Panel A. As a function of [^{3}H]ligands concentration. Fat cell membranes were incubated with the indicated concentration of [^{3}H]ligands for 20 min at 25°C and specific binding was determined as described in Materials and Methods. Panel B. Scatchard plot of specific binding of [^{3}H]ligands derived from the saturation curves (Panel A). The ratio (Bound/Free) of bound ligands (nM) to free ligands (nM) is plotted as a function of bound ligands. The slope of the plot ($-1/K_d$) was determined by linear regression analysis. The number of binding sites (B_{max}) is calculated from the x intercept of regression line. Hill plots of [^{3}H]ligands binding (not shown) gave a slope (n_H), determined by linear regression analysis which was respectively: 1.06 for [^{3}H]DHA, 1.01 for [^{3}H]YOH and 0.98 for [^{3}H]RAU.

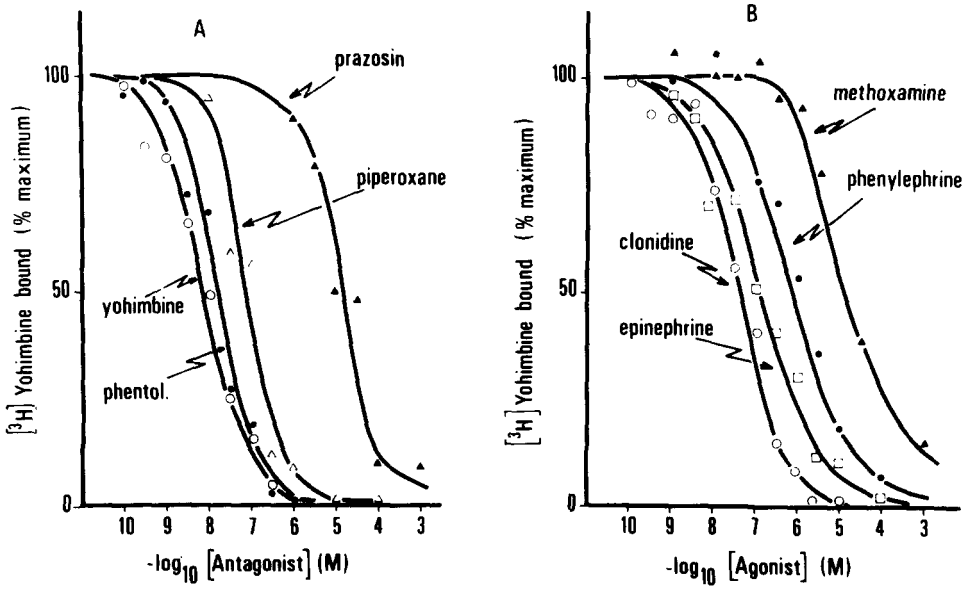

Fig. 3. Displacement of [^{3}H]YOH specifically bound to human adipocyte membranes by different alpha-adrenergic antagonists (A) and agonists (B). Membranes were incubated with (5–7 nM) [^{3}H]YOH and various concentrations of competing agents at 25°C for 20 min, then the amount of specifically bound ligand was determined as described in Materials and Methods. Results are expressed as percent of [^{3}H]YOH specifically bound in the absence of competing drugs. Number of experiments (n), EC_{50} values, and slope factors are given in Table 3.

TABLE 3. Inhibition of [^{3}H]YOH binding to fat cell membranes of subcutaneous adipose tissue by various alpha-adrenergic agonists and antagonists

Competing Drugs	N	EC_{50}[a]	n_{Hill}[b]
		nM	
Alpha-adrenergic antagonists			
Yohimbine	7	18 ± 4	0.97 ± 0.1
Phentolamine	6	24 ± 7	0.96 ± 0.2
Piperoxan	4	120 ± 20	0.91 ± 0.3
Nicergoline	5	440 ± 40	1.10 ± 0.1
Prazosin	5	9600 ± 2000	0.95 ± 0.3
Alpha-adrenergic agonists			
Clonidine	8	60 ± 7	0.74 ± 0.1
Tramazoline	3	110 ± 25	0.79 ± 0.1
Guanfacine	8	290 ± 70	0.48 ± 0.1
(−)Epinephrine	8	510 ± 60	0.48 ± 0.1
(+)Epinephrine	4	7000 ± 650	0.50 ± 0.2
(−)Norepinephrine	8	1750 ± 170	0.61 ± 0.1
(+)Norepinephrine	4	13500 ± 1200	0.55 ± 0.1
Methoxamine	3	55150 ± 10000	0.55 ± 0.1

[a] EC_{50}, concentration of competing agent causing 50% inhibition of specific [^{3}H]YOH binding.

[b] n_{Hill}, slope factors (pseudo Hill coefficients). The two parameters were determined from Hill plot transformations of drug competition curves (8–10 concentrations) and regression analysis of the data.

Fat cell membranes were incubated 20 min at 25°C with [^{3}H]YOH (7 nM) in the presence of increasing concentrations (10^{-9}–10^{-3} M) of competing drugs as explained in Materials and Methods and shown in Fig. 3. Each value is mean ± SEM of (N) separate experiments performed in duplicate.

increased the specific binding of [^{3}H]YOH without any noticeable effect on nonspecific binding. Scatchard analysis demonstrated that the increase in binding was essentially due to an increase in B_{max} (doubled) without any change in the affinity. Since this effect clearly did not occur in all the fat cell preparations, an interpretation of the fact would be premature since an undetected parameter will be involved in such an effect. More research is needed to assess this preliminary observation.

DISCUSSION

Our results indicate that the differences in the lipolytic potencies of physiological amines in isolated human fat cells can be explained by the significant difference in their relative affinities for α_2-adrenergic receptor sites. Furthermore, characterization of β- and α_2-adrenergic receptors by radioligand binding studies with selected antagonists reveals the preponderance of α_2-adrenoceptor sites over β-adrenoceptors. These results will be discussed in terms of the validity of radioligand binding data in the interpretation of the biological effects of physiological amines in human fat cells.

First of all, our findings demonstrate that epinephrine is less potent than norepinephrine as a lipolytic agent, while it is a most efficient antilipolytic agent (Table 1). Although there were some discrepancies among the various fat cell batches concerning maximal responses, the mean relationship quoted was found in each individual preparation and K_a values were always higher for epinephrine compared with norepinephrine (**Table 5**). The significant difference between lipolytic potencies of the two catecholamines could reflect either a higher affinity of norepinephrine for fat cell β_1-adrenergic receptors in comparison with epinephrine, or a higher affinity and efficiency of epinephrine for the α_2-adrenoceptor site compared to the lower affinity of norepinephrine. With regard to their comparative potencies at the β-adrenergic site, we observed that when α_2-adrenoceptors are blocked by yohimbine or phentolamine, norepinephrine and epinephrine exhibit similar lipolytic effects. This result fits in with the binding data which demonstrated that, as previously reported for β_1-adrenoceptors (39), human fat cell β-adrenoceptors that are of the β_1-subtype have approximately the same affinity for the two physiological catecholamines (Table 5); the K_Ds obtained by displacement of [^{3}H]DHA

TABLE 4. Parameters of the regulatory effects of Gpp(NH)p (10^{-4} M) associated with sodium (100 mM) on the competition of (−)epinephrine, (−)norepinephrine, clonidine, and guanfacine with [^{3}H]YOH binding sites in human fat cell membranes

		No Additions		With Gpp(NH)p and NaCl	
Competing Drugs	N[a]	EC_{50}[b]	n_{Hill}[c]	EC_{50}	n_{Hill}
		nM		*nM*	
(−)Epinephrine	8	510 ± 60	0.48 ± 0.06	10500 ± 2300	0.76 ± 0.04
(−)Norepinephrine	5	1850 ± 140	0.56 ± 0.07	24370 ± 3460	0.78 ± 0.03
Clonidine	8	60 ± 10	0.74 ± 0.06	350 ± 20	0.78 ± 0.04
Guanfacine	8	290 ± 70	0.48 ± 0.03	750 ± 130	0.72 ± 0.03

[a] Values are the means ± SEM of (N) experiments.

[b] EC_{50}, concentration of competing drug producing 50% inhibition of specific [^{3}H]YOH binding.

[c] n_{Hill}, slope factor (pseudo Hill coefficient). Slope factors and EC_{50} values were determined from Hill plot transformations of drug competition curves (8–10 concentrations) and regression analysis.

Fat cell membranes were incubated 20 min at 25°C with [^{3}H]YOH (5–7 nM) in the presence of various competing drugs with or without 10^{-4} M Gpp(NH)p and 100 mM NaCl as described in the text and Table 3.

TABLE 5. Comparison of dissociation constants for the physiological agonists (epinephrine and norepinephrine) estimated from in vitro studies on lipolysis in intact fat cells or from direct radioligand binding to plasma membranes

	Lipolysis		Direct Radioligand Binding		
	K_a[a]	K_i[b]	K_D ([^{3}H]DHA)[c]	K_D ([^{3}H]YOH)	K_D ([^{3}H]CLO)
			nM		
(−)Epinephrine	2600 ± 500 (8)	35 ± 9 (8)	2050 ± 700 (8)	240 ± 40 (8)	9 ± 2 (8)
(−)Norepinephrine	1350 ± 400*	130 ± 20*	1750 ± 530^{NS}	820 ± 110*	43 ± 16*

[a] K_a, Agonists concentration required for half maximal stimulation of lipolysis.

[b] K_i, Equilibrium dissociation constants for agonists estimated from experiments on inhibition of lipolysis (Table 1).

[c] K_D, Equilibrium dissociation constants (apparent dissociation constants) were calculated from the equation: $K_D = EC_{50} \Big/ \left(1 + \frac{[^3\text{H}]\text{ligand}}{K_D \text{ ligand}}\right)$. EC_{50} is the concentration of agonist causing a 50% inhibition of [^{3}H]ligand binding. [^{3}H]ligand is the concentration of [^{3}H]DHA, [^{3}H]YOH, and [^{3}H]CLO present in the assays (5–8 nM), while K_D ligand is the dissociation constant obtained in saturation studies for each ligand. Binding studies were performed as described in Materials and Methods.

Each value is the mean ± SEM of (n) determinations. *, $P < 0.01$, result significantly different from correspondent value (by Student's paired t test); NS, not statistically significant.

binding are similar. Thus, it seems reasonable to propose that the differences in the lipolytic effects of the physiological amines are linked to a greater affinity of epinephrine (as opposed to norepinephrine) for the α_2-adrenoceptors of fat cell membrane. Comparison of the K_i values estimated from inhibition of lipolysis and the K_D values estimated from inhibition of [^{3}H]YOH binding (Table 5) demonstrates that epinephrine exhibits a higher affinity than norepinephrine for the α_2-sites in *in vitro* assays and binding studies. However, when determined by competitive displacement of [^{3}H]YOH, the observed K_D values were about an order of magnitude higher than the K_i values. This data could be explained by heterogenous interaction of catecholamines with [^{3}H]YOH binding sites (23, 25). A better correlation between binding studies (K_D) and lipolytic studies (K_i) is obtained when the K_D values for epinephrine and norepinephrine are determined in displacement studies with agonist binding ([^{3}H]clonidine) (Table 5). Thus, under these conditions, a rather good correlation is obtained between the K_i values and the K_D values. This result suggests that the use of high affinity sites, labeled with [^{3}H]clonidine gave a better approximation of the K_Ds when correlations with biological effects are required.

Compared with norepinephrine, which mainly acts as a neurotransmitter, epinephrine is a circulating hormone which is known to act predominantly at β_2-adrenoceptor sites (40, 41). As is the case for β_2-adrenoceptors, human fat cell α_2-adrenoceptors react preferentially with the circulating hormone. Is there a correlation between such an observation and the fact that there is no direct innervation of adipocytes (42)? The physiological significance of such noticeable differences in fat cell α_2-adrenoceptor selectivity between the physiological amines is still unexplained and makes the adrenergic regulation of human fat cell function more complex to understand. Nevertheless, although the physiological significance of the antilipolytic α_2-adrenergic receptors is still subject to question (43), mainly because their efficiency is not immediately obvious and cannot generally be shown without special experimental conditions (Table 1), their existence explains the differences between the *in vitro* lipolytic potencies of physiological amines.

The present study also deals with receptor characterization. The specific binding of [^{3}H]DHA to human fat cell membranes. (Fig. 2; Table 2) was practically identical to that previously reported (4, 6, 26). Concerning α_2-adrenergic receptors, [^{3}H]YOH and [^{3}H]RAU labeled a similar family of binding sites and probably the same population of sites if we refer to K_D and B_{max} values (Fig. 2; Table 2). The B_{max} values were three to four times higher than those previously reported in a similar study (23). The authors used isoosmotic lysis instead of hypotonic lysis. Moreover we determined that the difference in the lysing method was without effect on the total number of binding sites. This discrepancy is still unexplained. However, two results mainly argue for the validity of our determinations. First, the total number of [^{3}H]DHA binding sites found in our experiments is similar to that previously reported (4, 6), and suggests that the fat cell membranes were unaltered. Second, during the formulation of this paper a report by Burns et al. (26) on [^{3}H]YOH binding on human fat cell membranes appeared to fit in with our

results. The authors found 543 ± 99 fmol/mg protein of [^{3}H]YOH binding sites in fat cell membranes of human subcutaneous adipose tissue. The binding sites identified with the particulate membrane preparations (fat cell *ghosts*) used in the studies of fat cell adrenoceptors contain a mixture of membranes from outer and inner structures of the cell. It seems likely that the receptor density is underestimated compared to that expected for the plasma membrane alone. In the present experiments, simultaneous investigations on lipolysis and [^{3}H]DHA or [^{3}H]YOH binding determinations were performed on the same fat cell batch, with the same membrane preparation and the same assay conditions. The significant difference in [^{3}H]DHA vs [^{3}H]YOH or [^{3}H]RAU maximum binding capacity (Table 2; Fig. 2), whatever the contaminating internal membrane proteins, is unquestionable.

Thus, after this sort of observation, one must consider whether the sites detected with [^{3}H]YOH or [^{3}H]RAU possess all the characteristics expected for α_2-adrenergic receptors. Competition for [^{3}H]YOH binding sites in fat cell particulates by unlabeled agonists or antagonists (Table 3; Fig. 3) gave results that are in complete agreement with the data reported in biological studies (2, 3, 24) or by α_2-agonist binding investigations (4, 21, 24). Moreover, these observations suggest that α-agonists bind to heterogeneous binding sites of more than one affinity as previously reported for human platelets (36, 37), rat liver (44), and hamster adipose tissue (38). This effect is observed whatever the chemical nature of the agonists (Table 3). Agonist affinity for α_2-adrenoceptors has been described to be modulated by cations and guanyl nucleotides in various tissues (35–38, 44), while antagonist binding is unaffected. The guanine nucleotide analog Gpp(NH)p in association with 100 mM NaCl was seen to decrease markedly the ability of epinephrine and norepinephrine to inhibit [^{3}H]YOH binding (Table 4). Clonidine and guanfacine, which are potent synthetic α_2-adrenergic agonists, gave similar results. However it is noticeable that the binding affinities of these agonists are less affected by Gpp(NH)p and Na^+ than are the binding affinities of the physiological amines. The significance of this observation is not fully understood although we tried to associate the binding characteristics of clonidine and (−)epinephrine with the results of adenylate cyclase activities in a preliminary paper (25). Another fact which still requires further studies concerns the total number of [^{3}H]YOH binding sites (B_{max}) which was seen to be increased by the addition of 100 mM sodium chloride plus Gpp(NH)p. A recent report by Woodcok and Murley (45) mentioned similar results for central and hypothalamic α_2-adrenergic receptors.

What do our studies tell us about the significance of human fat cell β_1- and α_2-adrenergic receptors identified by [^{3}H]DHA and [^{3}H]YOH, respectively? The use of radiolabeled ligands for α- and β-adrenergic receptor identification has been claimed to be a convenient tool for physiological studies, bringing to light correlations between variations in the biological effects of physiological amines and receptor number and affinity. This kind of correlation has been made for β- and α-adrenergic receptors in various tissues after desensitization experiments (6, 26) endocrine gland removal (46–48), or hormonal treatments (49, 50). Nevertheless, a recent report on the pancreatic islets of the rat mentioned that there are 2.7 times as many β-adrenergic binding sites as α-binding sites, while the inhibitory influence of the α-receptor predominates (51). Such a finding suggests that mechanisms other than receptor number can modify biological effects. The preponderance of α_2-adrenergic binding over β_1-adrenergic binding in human fat cells seems inadequate to interpret the effect of catecholamines on fat cell lipolysis. Several interpretations can be reasonably proposed. It is now well documented, in the adipose tissue and various other tissues, that β_1-adrenergic receptors are linked to adenylate cyclase in a stimulatory fashion while α_2-adrenergic receptors are coupled to the same enzyme in an inhibitory fashion (52–56). If we consider the biological effect promoted by circulating physiological amines (mainly epinephrine), the dissociation constants of the two receptors being similar, the excess number of α_2-adrenoceptors ([^{3}H]YOH binding sites) will be considered to dominate the lower β_1-adrenergic receptor population. Instead of this hypothesis, the β_1-adrenoceptor-mediated adenylate cyclase stimulation is always prominent and explains the lipolytic effects (Table 1). It is not counteracted by the inhibitory α_2-adrenoceptors. Such a result suggests that not all the [^{3}H]YOH binding sites, with α_2-adrenoceptor characteristics, are involved in adenylate cyclase inhibition. The hypothesis of uncoupling or loose coupling of α_2-adrenoceptors to adenylate cyclase can be proposed, the coupling systems being independent for stimulatory or inhibitory actions (55, 56). Such a hypothesis seems to be reasonable since it must be noticed that although homogeneous in binding studies, β_1-adrenoceptors of human fat cells display functional heterogeneity; 50% of the β_1-adrenoceptor population is coupled to adenylate cyclase while the remaining 50% is uncoupled (6). Saturation binding experiments and kinetics analysis, although commonly used for receptor definition, do not allow a demonstration to be made of heterogeneity or cooperative interactions among the β_1- or α_2-adrenoceptor population of human fat cell membranes (Fig. 2; Table 2) and do not give any information on the sites involved in biological effects. Functional heterogeneity of receptors and various af-

finity levels can be detected by agonist-induced conformational changes (25, 35–38) or desensitization studies (6, 26). High and low affinity states of the α_2-receptor for the agonists is revealed by competition studies of α_2-antagonist binding (Table 4) or when α_2-adrenoceptors are labeled by an α_2-agonist such as clonidine (21) or paraamino-clonidine (4).

In conclusion, our studies have *1*) provided an experimental design that permitted us to delineate selectively the physiological agonist of each kind of receptor and to focus on epinephrine efficiency at α_2-adrenoceptors; *2*) demonstrated the preponderance of α_2-adrenergic receptors (labeled by [^{3}H]YOH or [^{3}H]RAU); *3*) focused on the fact that, although a third as numerous as the α_2-sites, the β_1-adrenergic receptor population (labeled by [^{3}H]DHA) is responsible for the lipolytic effect of physiological amines and weakly counteracted by α_2-adrenoceptors. For human fat cells, determinations of affinity and maximal number of binding sites and the potency orders for agonists or antagonists in competing for the sites labeled by a tritiated ligand ([^{3}H]DHA, [^{3}H]YOH, [^{3}H]RAU) are not sufficient for an accurate characterization of adrenergic receptor efficiencies. One should focus on the distinction between [^{3}H]YOH, [^{3}H]CLO, or [^{3}H]DHA binding sites and the *true* β_1- or α_2-adrenergic receptors coupled with adenylate cyclase and involved in biological effects.

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