0026-895X/88/030318-09$02.00/0

MOLECULAR PHARMACOLOGY, 34:318-326

Subclassification of β-Adrenergic Receptors of Rat Fat Cells: A Re-Evaluation

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Received September 8, 1987; Accepted June 10, 1988

SUMMARY

Catecholamines regulate lipolysis in rat fat cells via β-adrenergic receptors. Recently, it has been proposed that β-adrenergic receptors of rat fat cells are neither β_1 nor β_2 in character but rather an 'isoreceptor,' 'hybrid,' or 'β_3' [*Br. J. Pharmacol.* **84**:131–137 (1985)]. This putative receptor subtype has been envisioned as possessing an alkanolamine side-chain interaction site of β_1 nature and an aromatic moiety interaction site of β_2 nature. These proposals were evaluated in the present work through a reexamination of the nature of the fat cell β-adrenergic receptor using four radioligands that differ chemically in one or both of these regions of the molecule as well as in their hydrophobicity. Equilibrium binding of agonist and of β_1- and β_2-subtype-selective high affinity antagonist ligands to fat cell membranes was detailed. The binding sites labeled by these ligands had the characteristics of β_1-adrenergic receptors. The rank order of subtype-selective antagonists in competing for radioligand binding to fat cell membranes was the same as that for inhibition of agonist-stimulated cyclic AMP accumulation by these ligands. At equimolar concentrations, the β_1-selective antagonist CGP-20712A provided a greater degree of inhibition of catecholamine-stimulated lipolysis than the β_2-selective antagonist ICI-118,551. These results document the character of the rat fat cell β-adrenergic receptor as *solely* β_1.

The lypolytic effects of catecholamine hormones on fat cells are primarily mediated by β-adrenergic receptors. These receptors were subclassified as β_1-adrenergic receptors based upon the rank order of various sympathomimetic amines in stimulating lipolysis (1). Recent studies using β-adrenergic ligands that are considered to be selective for β_1- or β_2-subtypes have called into question, however, the subclassification of fat cell receptors as strictly β_1 in nature (2–9). It has been speculated that β-adrenergic receptors mediating lipolysis possess both β_1- and β_2-adrenergic character, appearing 'dualistic' in nature (4, 5). Furthermore, it has been suggested that rat fat cell β-adrenergic receptors may in fact represent a 'hybrid' subtype of receptor that is neither β_1 or β_2 (6–10). This 'hybrid' receptor is envisioned as possessing an interaction site for the alkanolamine side-chain of ligands that is β_1 in nature and an interaction site for the aromatic moiety of arylethanolamines and aryloxypropanolamines that is β_2-like (5). These provocative proposals were evaluated in the present work by analysis of equilibrium binding of [^{125}I]ICYP, [^{3}H]DHA, [^{3}H]CGP-12177, and [^{125}I]*p*ABC to rat fat cell membranes. The subtypes of receptors labeled by each radioligand were characterized and quantified using both the β_2-selective antagonist ICI-118,551 (11) and a novel, high selective β_1-adrenergic receptor antagonist ligand, CGP-20712A (12, 13). The β-adrenergic receptor mediating catecholamine-stimulated cyclic AMP accumulation and lipolysis in rat fat cells was subclassified by comparing the potency of β_1- and β_2-selective antagonist ligands for inhibition of both responses.

Experimental Procedures

Materials. (−)-[^{3}H]DHA (90 Ci/mmol), (−)-[^{125}I]ICYP (2200 Ci/mmol), and [^{125}I]*p*ABC (2200 Ci/mmol) were obtained from New England Nuclear (Boston, MA). [^{3}H]CGP-12177 (50 Ci/mmol) was obtained from Amersham (Arlington Heights, IL). ICI-118,551 and CGP-20712A were a generous gift from ICI Pharmaceuticals (Wilmington, DE) and Ciba-Geigy Pharmaceuticals (Basel, Switzerland), respectively. Crude bacterial collagenase (type I) was purchased from Worthington (Freehold, NJ). The enzymes used in the determination of glycerol were obtained from Boehringer Mannheim (Indianapolis, IN). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of fat cell membranes. Rat white fat cells were prepared by collagenase digestion of parametrial adipose tissue according to the procedure of Rodbell (14), as modified by Cabelli and Malbon (15). These cells were used for the measurement of cyclic AMP accumulation and glycerol release. Particulate plasma membranes were

This work was supported by United States Public Health Service Grants DK30111 and DK25410 from the National Institutes of Health. C.C.M. is recipient of Career Development Award K04 AM00786 from the National Institutes of Health.

ABBREVIATIONS: [^{125}I]ICYP, (−)-[^{125}I]iodocyanopindolol; [^{3}H]DHA, (−)-[^{3}H]dihydroalprenolol; [^{125}I]*p*ABC, [^{125}I]*p*-azidobenzylcarazolol; CGP-20712A, (±)-(2-hydroxy-5-[2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1*H*-imidazole-2-yl-)phenoxy)propyl)amino)ethoxy]-benzamide monomethane sulfonate; [^{3}H]CGP-12177, (±)-[^{3}H]4-(3-terttiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride;ICI-118,551,erythro-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol.

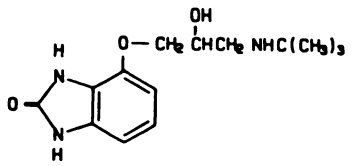
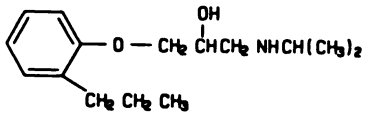
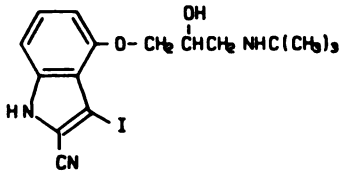
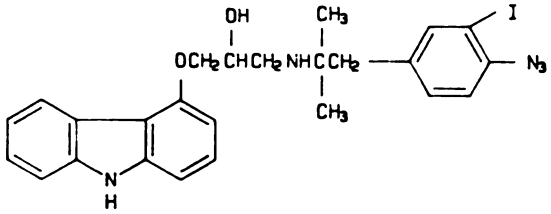
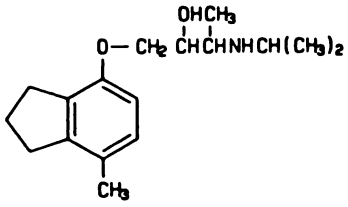
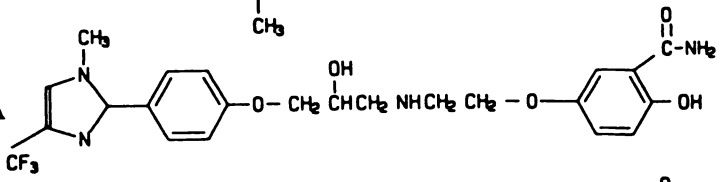
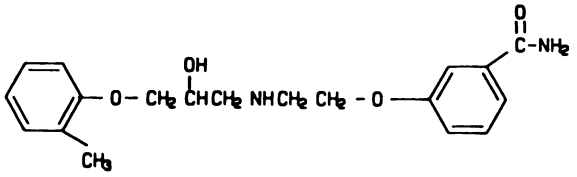
Ligand	Structure	Adrenergic selectivity
Radioactive		
CGP-12177		non-selective
DHA		non-selective
ICYP		non-selective
pABC		non-selective
Non-Radioactive:		
ICI-118,551		β_2-subtype
CGP-20712A		β_1-subtype
Tolamolol		β_1-subtype

Fig. 1. Structure of β-adrenergic ligands used in these studies. The term 'aromatic moiety of arylethanolamine and arylpropanolamine side-chain' that appears in the text refers to the chemical groups that are attached to the β-carbon of the ethylamine moiety. The term 'alkanolamine substituent' in the text refers to the chemical groups that are attached to the amino group of ethylamine.

prepared from these cells as described (16). Protein content of the membranes was determined by the method of Lowry *et al.* (17).

Radioligand binding assays. Rat fat cell membranes were prepared and resuspended in a buffer composed of 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, leupeptin (10 μg/ml), and aprotinin (10 μg/ml). Incubations with [^{3}H]DHA and [^{125}I]ICYP were performed at 22° for 20 and 60 min, respectively. When [^{3}H]CGP-12177 was used, the assays were performed at 35° for 45 min (18). The amounts of [^{3}H]DHA and [^{125}I]ICYP bound to cell membranes were quantified after filtering the incubation mixture on a single Whatman GF/C filter and washing the filter with ice-cold 50 mM Tris·HCl (pH 7.4), 10 mM $MgCl_2$. For binding of [^{3}H]CGP-12177 the procedure was essentially the same except that the filters were washed with ice-cold 10 mM sodium phosphate, 4 mM $MgSO_4$ (pH 7.4). [^{125}I] *p*ABC binding to fat cell membranes was performed in a buffer composed of 25 mM Tris·HCl (pH 7.4), 2 mM $MgCl_2$, protease inhibitors, and radioligand. The incubation was maintained in the dark at 22° for 60 min. The incubation mixture was then filtered on a single GF/C

filter and the filter was washed with 20 ml of buffer composed of either 25 mM $MgCl_2$ and 75 mM Tris·HCl (pH 7.4) or ice-cold 25 mM sodium acetate buffer (pH 5.1). Nonspecific binding was defined as the radioligand binding that was insensitive to competition by 100 μM (−)-isoproterenol.

Analysis of the binding data. All receptor binding parameters were analyzed by the use of a computer operating a nonlinear, least-squares, curve-fitting procedure and the modified LIGAND program (Biosoft-Elsevier, Cambridge, England). Pooled data are presented as means ± standard errors of at least three separate experiments that were analyzed simultaneously.

Measurement of cyclic AMP accumulation and glycerol release from rat fat cells. Cyclic AMP accumulation and glycerol release from rat fat cells were determined as described (19). The determinations of cyclic AMP and glycerol were performed at 5 and 60 min, respectively, after the addition of β-adrenergic ligands to cell suspensions. In each experiment, the values for glycerol and cyclic AMP are the mean values of triplicate determinations. The data are expressed in units per 10^6 fat cells and were calculated from the number of cells used in each assay.

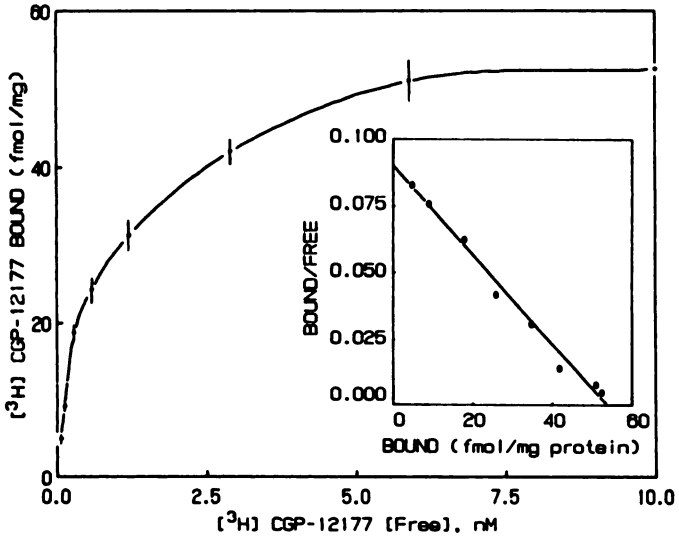


Fig. 2. [3H]CGP-12177 binding to rat fat cell membranes. Rat fat cell membranes were incubated for 45 min at 35° with the indicated concentrations of [3H]CGP-12177 in the absence or presence of 100 μM (−)-isoproterenol. *Inset*, a Scatchard plot of these same data. Each *point* is the mean of triplicate determinations of a single representative experiment. The dissociation constant (K_d) and the maximal concentration of binding sites B_{max} were calculated from three separate experiments using the LIGAND computer program. The binding parameters were as follows: K_d = 0.4 ± 0.07 nM and B_{max} = 53 ± 7 fmol of receptor/mg of protein.

Results

The chemical structures of the radiolabeled and unlabeled ligands used in the analysis of fat cell β-adrenergic receptors are shown in Fig. 1. The rank order of hydrophobicity for the radioligands is as follows: *p*ABC > ICYP > DHA > CGP-12177 (20). The ability of each radioligand to display saturable specific binding to rat fat cell membranes was examined (data not shown). As shown for [3H]CGP-12177 (Fig. 2), the binding properties of each of these radioligands were established under equilibrium conditions (Table I). The affinities (K_d) of the β-adrenergic receptor for three of these ligands, [125I]ICYP, [3H]CGP-12177, and [3H]DHA, were 0.03, 0.4, and 2.2 nM, respectively. Specific binding of [3H]CGP-12177 and [125I]ICYP displayed Hill coefficients (n_H) of unity and B_{max} values of 50 and 150 fmol/mg of protein, respectively. Saturation of specific binding of [3H]DHA, in contrast, required somewhat higher concentrations of radioligand (>70 nM). The B_{max} for [3H]DHA was estimated to be 400–600 fmol/mg of membrane protein. Transformation of these data by the method of Scatchard (21) yielded curvilinear plots with upward concavity, as previously reported (19, 22). Analysis of the data by the LIGAND computer program (23) revealed the existence of two populations of sites with differing affinities for [3H]DHA (Table 1). The B_{max} for the highest affinity component of the binding was ~80 fmol/mg of protein.

Equilibrium binding studies were attempted using the photoaffinity reagent [125I]*p*ABC. Like [125I]ICYP, [3H]DHA, and [3H]CGP-12177, [125I]*p*ABC has a bulky substitution on the aromatic moiety of the aryloxypropanolamine side-chain (Fig. 1). [125I]*p*ABC was unique in this series of radioligands in that it also possesses a bulky addition to the alkylamino side-chain. Binding studies with [125I]*p*ABC were conducted in the absence of light. Under the conditions used, more than 90% of the ligand (0.3 nM) incubated with fat cell membranes (50–100 μg) was unexpectedly found to be associated with the membranes ('bound'). This nearly quantitative 'binding' of [125I]*p*ABC to fat cell membranes was insensitive to competition by isoproterenol at concentrations as high as 100 μM (see below).

TABLE 1

Radioligand binding properties of fat cell membranes

The binding of [125I]ICYP (0.005–1 nM) to rat fat cell membranes (15 μg) and the binding of [3H]DHA (0.5–100 nM) and [3H]CGP-12177 (0.02–10 nM) to fat cell membranes (200 μg) was assayed as described in Experimental Procedures. Each experimental point was determined from triplicates. All four radioligands were examined on the same preparation of freshly prepared rat fat cell membranes. For each experiment the concentration range over which specific radioligand binding was determined varied by at least 2 orders of magnitude. The data from three such experiments were pooled and analyzed by the LIGAND computer program. Data represents the mean ± standard error of the pooled results. Hill coefficients were calculated from the slope of the linear regression analysis by plotting log ($B/B_{max} - B$) versus free ligand concentration, where B represents the concentration of the bound ligand. The terms K_d, B_{max}, and n_H represent the equilibrium dissociation constant, maximal concentration of binding sites, and Hill coefficient, respectively.

Ligand	K_d	B_{max}	K_{dL}[a]	B_{maxL}[b]	n_H
	nM	fmol/mg of membrane protein	nM	fmol/mg of membrane protein	
[3H]CGP-12177	0.4 ± 0.1	50 ± 7			0.97 ± 0.03
[3H]DHA	2.2 ± 0.3[c]	80 ± 10[c]	26 ± 8	500 ± 130	0.77 ± 0.05
[125I]ICYP	0.03 ± 0.01	150 ± 24			1.0 ± 0.02
[125I]pABC	ND[d]	<20[e]			

[a] K_{dL}, the equilibrium dissociation constant for the low affinity binding site.
[b] B_{maxL}, the maximal concentration of low affinity binding sites.
[c] Values for the high affinity binding site of [3H]DHA.
[d] ND, not determined.
[e] Binding performed at 0.1 nM radioligand.

Competition of radioligand binding to fat cell membranes by unlabeled β-adrenergic agonists and β-subtype-selective antagonist ligands was probed in order to more fully characterize the binding sites. The rank order of potencies of β-adrenergic agonist competition for [^{125}I]ICYP binding was (−)-isoproterenol ≫ (−)-norepinephrine ≥ (−)-epinephrine (Fig. 3A), indicative of a β_1-character as originally proposed by Lands *et al.* (1). The Hill coefficient for binding for all three agonist ligands was determined to be less than unity (see legend to Fig. 3), suggesting the possibility that multiple populations of binding sites with differing affinities for agonist ligands exist in rat fat cell membranes.

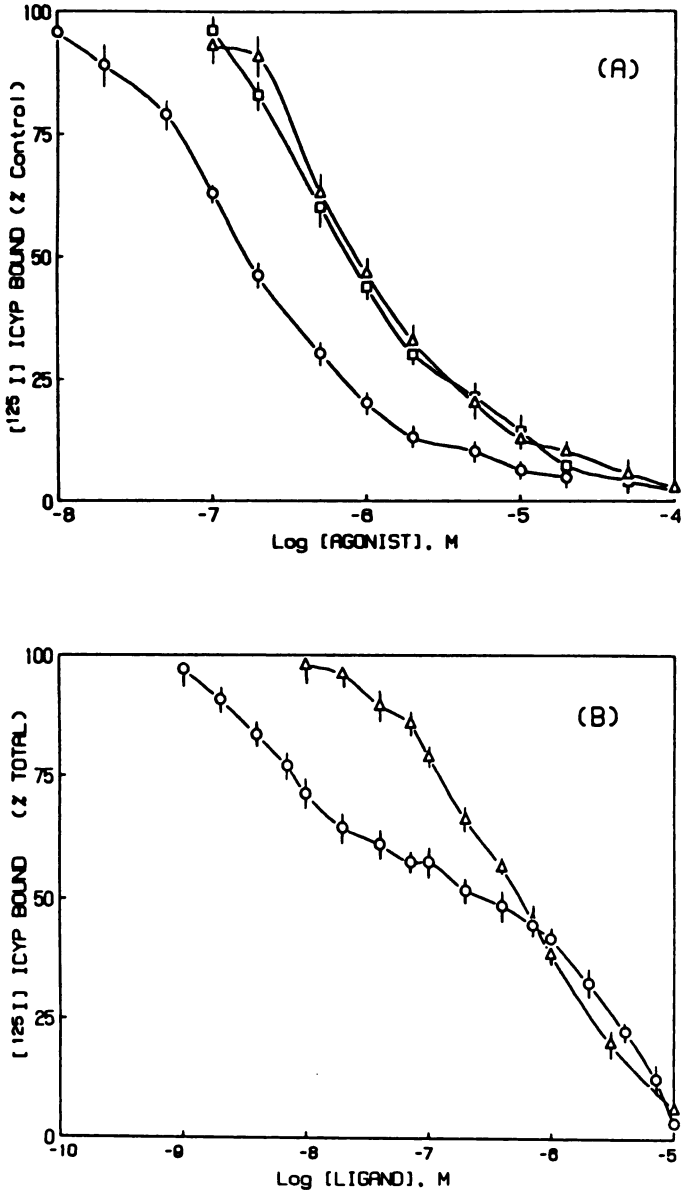


Fig. 3. Competition of specific [^{125}I]ICYP binding to rat fat cell membranes by β-adrenergic agonist and antagonist ligands. A, Rat fat cell membranes (15 μg) were incubated for 60 min at 22° with [^{125}I]ICYP (0.1 nM) and the indicated concentrations of (−)-isoproterenol (○), (−)-norepinephrine (□), and (−)-epinephrine (△). B, Competition of [^{125}I]ICYP binding to rat fat cell membrane using the indicated concentrations of ICI-118,551 (△) and CGP-20712A (○). Binding assays were performed as outlined under Experimental Procedures. In A, the amount of specific binding (percentage of control) is plotted against the concentrations of competing agonist ligand. In A, the concentrations at which the agonist ligands competed for 50% of radioligand binding (IC_{50}) are as follows: isoproterenol, 0.15 μM; norepinephrine and epinephrine, 1 μM. The Hill coefficient for competition of radioligand binding by isoproterenol, epinephrine, and norepinephrine is 0.71, 0.81, and 0.68, respectively. For B, the data from four separate experiments were pooled and analyzed simultaneously using the LIGAND computer program. The data are mean values ± standard errors from the four separate experiments. In all cases, determination of binding in each experiment was performed in triplicate.

The antagonists selected for defining the receptor subtypes in competitions with the radioligands were the β_2-selective ligand ICI-118,551 and the β_1-selective ligand CGP-20712A. Competition curves of ICI-118,551 and CGP-20712A against [^{125}I]ICYP binding were found to be rather shallow for both ligands (Fig. 3B). Similar curves were observed for competition studies performed with β-adrenergic agonists (Fig. 3A). The Hill coefficients for ICI-118,551 and CGP-20712A were less than unity, 0.76 and 0.41, respectively (Table 2). For CGP-20712A competition of [^{125}I]ICYP binding the curves were biphasic. High (K_d = 2 nM) and low (K_d = 1.1 μM) affinity populations of sites were observed in nearly equal proportions. The K_d of the high affinity population for CGP-20712A was in close agreement with that of β_1-adrenergic receptors reported in a variety of systems (12, 13). The affinity of the second component for CGP-20712A was nearly 3 orders of magnitude lower than that of the high affinity component. The high affinity component of the binding that was sensitive to 0.1–0.5 μM CGP-20712A displayed properties in close agreement with those of the β_1-adrenergic component in tissues that express both receptor subtypes (12, 13). Thus, only half of the [^{125}I]ICYP binding to fat cell membranes (or approximately 75 fmol/mg of protein) displays the properties expected of true β_1-adrenergic receptors as defined by competition studies with *both* agonist and antagonist ligands (Table 2).

The rank order of potencies of β-adrenergic agonists for competition of [^{3}H]DHA binding was found to be (−)-isoproterenol ≫ (−)-norepinephrine ≥ (−)-epinephrine (data not shown), in agreement with the results obtained using the [^{125}I]ICYP ligand. Competition studies of [^{3}H]DHA binding by ICI-118,551 and CGP-20712A were performed at 2 and 20 nM radioligand. At 2 nM [^{3}H]DHA (Fig. 4A), approximately half of the high affinity sites would be expected to be occupied (Table 1). At 20 nM [^{3}H]DHA (Fig. 4B), all of the high affinity population and half of the low affinity population would be expected to be occupied (Table 1). Competition curves of [^{3}H]DHA binding by ICI-118,551 were steep, with Hill coefficients of 0.9–1.0 at 2 or 20 nM radioligand, suggesting the existence of a single homogeneous population of receptors with a K_d for ICI-118,551 of 0.5 μM (Table 2). Competition of CGP-20712A, unlike that of ICI-118,551, was found to be biphasic at both 2 and 20 nM [^{3}H]DHA. The Hill coefficients for CGP-20712A-sensitive binding of [^{3}H]DHA to fat cell membranes were calculated to be 0.4–0.5. Analysis of these data by the LIGAND computer program suggested the existence of two populations of sites with differing affinities for CGP-20712A (Table 2). For competition studies performed at 2 nM [^{3}H]DHA, more than 70% of the sites were found to display high affinity (K_d = 0.5 nM) for this β_1-selective ligand.

The rank order of potencies of agonists competing for the binding of the more hydrophilic radioligand [^{3}H]CGP-12177 to fat cell membranes was (−)-isoproterenol ≫ (−)-norepinephrine ≥ (−)-epinephrine (Fig. 5A). This rank order agrees well with that observed for competitive binding assays that used [^{125}I]ICYP (Fig. 3A) and [^{3}H]DHA (19, 24). For the competition

TABLE 2

Affinities of ICI-118,551 and CGP-20712A for the specific binding sites of radioligands to fat cell β-adrenergic receptors

K_{dH} and K_{dL} represent the dissociation constants of the high affinity and low affinity sites of radioligand binding for the unlabeled antagonists, respectively. R_H and R_L values represent the percentage of the binding sites having the higher and lower affinities, respectively. The values were calculated from the competition curves generated using the radioligands indicated below, as outlined in Experimental Procedures. The Hill coefficient (n_H) was calculated as described in the legend to Table 1. All determinations were performed in triplicate for a given experiment. Each value is the mean ± standard error of a pooled number of separate experiments (numbers in parentheses).

β-Antagonist	Radioligand	Radioligand concentration	n_H	K_{dH}	R_H	K_{dL}	R_L
		nM		nM	%	nM	%
ICI-118,551 (4)	[^{125}I]ICYP	0.1	0.76	24 ± 4	16 ± 5	317 ± 54	84 ± 7
CGP-20712A (4)	[^{125}I]ICYP	0.1	0.41	2 ± 0.2	48 ± 4	1116 ± 200	52 ± 8
ICI-118,551 (3)	[^{3}H]DHA	20	0.96			488 ± 40	~100
CGP-20712A (3)	[^{3}H]DHA	20	0.47	6 ± 2	34 ± 7	1072 ± 200	66 ± 10
ICI-118,551 (3)	[^{3}H]DHA	2	0.92			600 ± 80	~100
CGP-20712A (3)	[^{3}H]DHA	2	0.44	0.5 ± 0.2	70 ± 12	250 ± 24	30 ± 9
ICI-118,551 (4)	[^{3}H]CGP-12177	2.5–4	1.0			300 ± 35	~100
CGP-20712A (4)	[^{3}H]CGP-12177	2.5–4	0.88	3.4 ± 0.6	85 ± 7	540 ± 60	15 ± 5

studies against ICI-118,551 and CGP-20712A, 3 nM [^{3}H]CGP-12177 was used in order to obtain sufficient amounts of radioligand binding to quantify accurately the subtype character of these receptors. The slopes of competition curves of ICI-118,551 for [^{3}H]CGP-12177 binding were found to be steep (Fig. 5B) and displayed Hill coefficients of unity (Table 2). The affinity of the sites for ICI-118,551 was approximately 0.3 μM, characteristic of the labeling of a β_1 site with this β_2-selective ligand (11). Thus, both [^{3}H]CGP-12177 and ICI-118,551 appear to recognize a single homogeneous population of binding sites with β_1-adrenergic character.

The Hill coefficient for the binding of CGP-20712A to sites labeled with [^{3}H]CGP-12177 (Fig. 5B) was less than unity (n_H = 0.88). The best fit of these data suggested the existence of two populations of binding sites, one with high (K_d = 3.4 nM) and the other with low (K_d = 0.54 μM) affinity for the CGP-20712A ligand (Table 2). The high affinity sites for CGP-20712A constituted 85–90% of the binding sites and their K_d agreed well with those of β_1-adrenergic receptors identified in several other systems (12, 13).

[^{125}I]*p*ABC, a β-adrenergic ligand with bulky group substituents in both the aromatic moiety and alkanolamine side-chain of the molecule, was a particularly attractive ligand for evaluation of the hypothesis of the 'hybrid' nature of the fat cell β-adrenergic receptor. As mentioned above, the association of [^{125}I]*p*ABC with fat cell membranes was essentially quantitative, raising as likely the possibility that this hydrophobic ligand was partitioning into, rather than 'binding' to, the fat cell membranes. To further evaluate this possibility, the binding of a fixed concentration of [^{125}I]*p*ABC to a constant amount of fat cell membranes was performed at 1-, 5-, and 10-fold the normal assay volume (Table 3). The binding of the three other radioligands was evaluated also in these experiments. As one would predict, the nonspecific binding component at a fixed concentration of [^{125}I]ICYP, [^{3}H]DHA, or [^{3}H]CGP-12177 was found to increase with increasing volume of incubation. Specific binding of these radioligands remained essentially constant. The amount of [^{125}I]*p*ABC associated with the membranes, in sharp contrast, was observed to increase as the assay volume increased. A total of 90 to 98% of the ligand was removed from the bulk solution in incubations containing 0.1 mg of membrane protein. Only at relatively high incubation volumes with low amounts (0.1 mg of protein) of membranes was a small (<20 fmol of specific binding /mg of membrane protein), isoproterenol-sensitive component of the [^{125}I]*p*ABC binding observed. These data highlight the formidable complication in detailing the binding of a hydrophobic ligand to membranes into which the ligand may partition.

Inhibition of agonist-stimulated cyclic AMP accumulation and lipolysis of rat fat cells by CGP-20712A and ICI-118,551 were examined in order to more fully characterize the β-adrenergic receptor subtype mediating these responses. Cyclic AMP accumulation in response to epinephrine was assayed in freshly prepared fat cells. Inhibition of catecholamine-stimulated cyclic AMP accumulation by the β_1-adrenergic receptor antagonist CGP-20712A was apparent at 0.01 μM (Fig. 6A). Inhibition by the β_2-subtype-selective antagonist, in contrast, was first observed at concentrations of ICI-118,551 that were 30-fold higher than that of CGP-20712A.

The ability of equimolar concentrations of ICI-118,551 and CGP-20712A to inhibit catecholamine-stimulated lipolysis were assessed (Fig. 6B). Basal glycerol release was 0.3 ± 0.05 μmol/10^6 cells/hr. In the presence of 2 μM (−)-epinephrine, glycerol release increased to 2 ± 0.4 μmol/10^6 cells/hr. At equimolar concentrations, the β_1-selective antagonist CGP-20712A provided a greater degree of inhibition of epinephrine-stimulated lipolysis than did its β_2-selective counterpart ICI-118,551 (Fig. 6B). These data demonstrate that, in addition to their greater potency in competitive binding assays, β_1-selective antagonist ligands are more potent than β_2-subtype selective ligands in blocking epinephrine-stimulated cyclic AMP accumulation and lipolysis.

Discussion

The goal of the present study was to evaluate recent proposals that the β-adrenergic receptor mediating stimulation of cyclic AMP accumulation and lipolysis in rat fat cells was neither β_1 nor β_2 (2–9), but rather a novel 'β_3' (8), 'isotype' (3, 4), 'hybrid' subtype (5, 6), or a receptor with 'dualistic' character (4, 5). These proposals are at odds with studies from several other laboratories in which the pharmacology of β-adrenergic receptors of rat fat cells has been detailed in membranes, detergent extracts of membranes, and highly purified preparations and shown to be β_1 in character (15, 16, 19, 22, 24–26). The new data provided herein fail to support the tenet that the fat cell β-adrenergic receptor is a novel β_3-subtype but rather demonstrate convincingly that the fat cell receptor is β_1 in nature. In

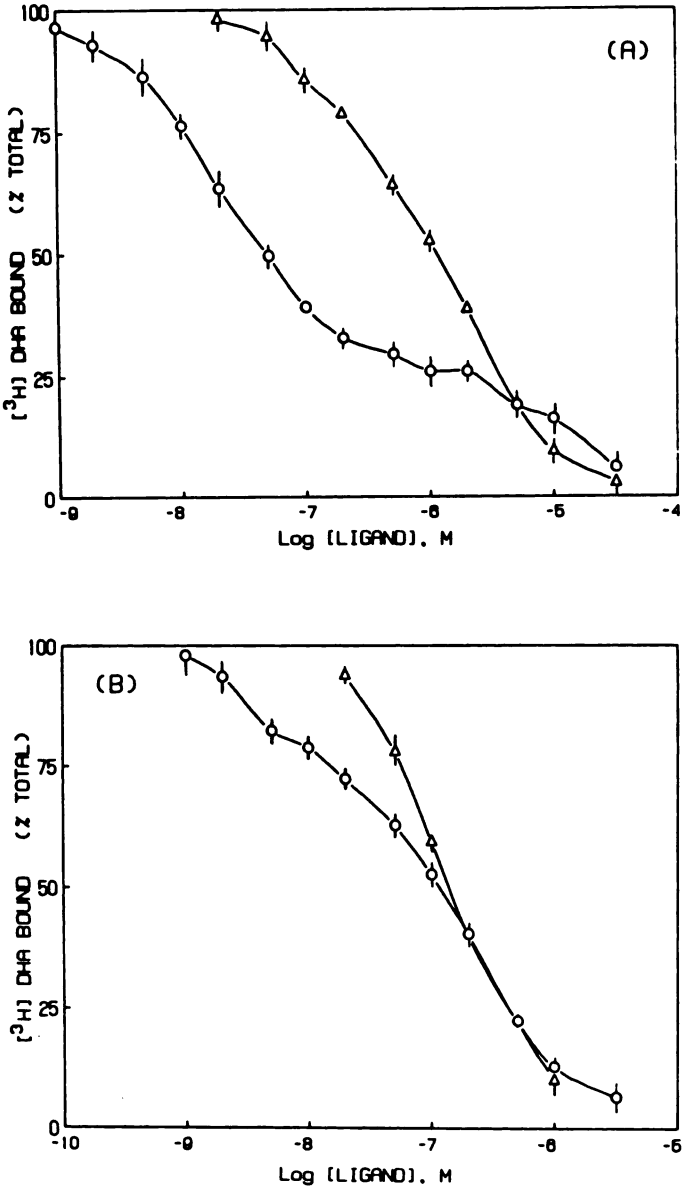


Fig. 4. Competition of specific [^{3}H]DHA binding to rat fat cell membranes by increasing concentrations of the β-subtype selective ligands ICI-118,551 and CGP-20712A. Rat fat cell membranes (0.2 mg) were incubated for 20 min at 22° with 2 nM [^{3}H]DHA (A) or 20 nM [^{3}H]DHA (B) and the indicated concentrations of the β_2-selective ligand ICI-118,551 (Δ) or the β_1-selective ligand CGP-20712A (○). For panels A and B, specific binding of [^{3}H]DHA is plotted against the concentration of the competing ligands. The data from three separate experiments were analyzed simultaneously using the LIGAND computer program and are expressed as mean values ± standard errors. In each separate experiment the binding was determined in triplicate.

addition, alternative explanations for the data on which the proposals for a new β-adrenergic receptor subtype were based are offered that support the position that the receptors are β_1.

Comparison of the B_{max} values for the equilibrium binding studies performed with four chemically diverse β-adrenergic antagonist radioligands and two subtype-selective antagonist ligands establishes a common level of high affinity β-adrenergic receptor in rat fat cell membranes and establishes the subtype as strictly β_1. The B_{max} values established for β_1-adrenergic receptors radiolabeled with either [^{3}H]DHA or [^{125}I]ICYP and sensitive to competition by CGP-20712A were 50–60 fmol/mg of membrane protein. The B_{max} obtained with [^{3}H]CGP-12177, 50 fmol/mg of protein, agrees well with the results obtained with [^{3}H]DHA or [^{125}I]ICYP. Small discrepancies among the

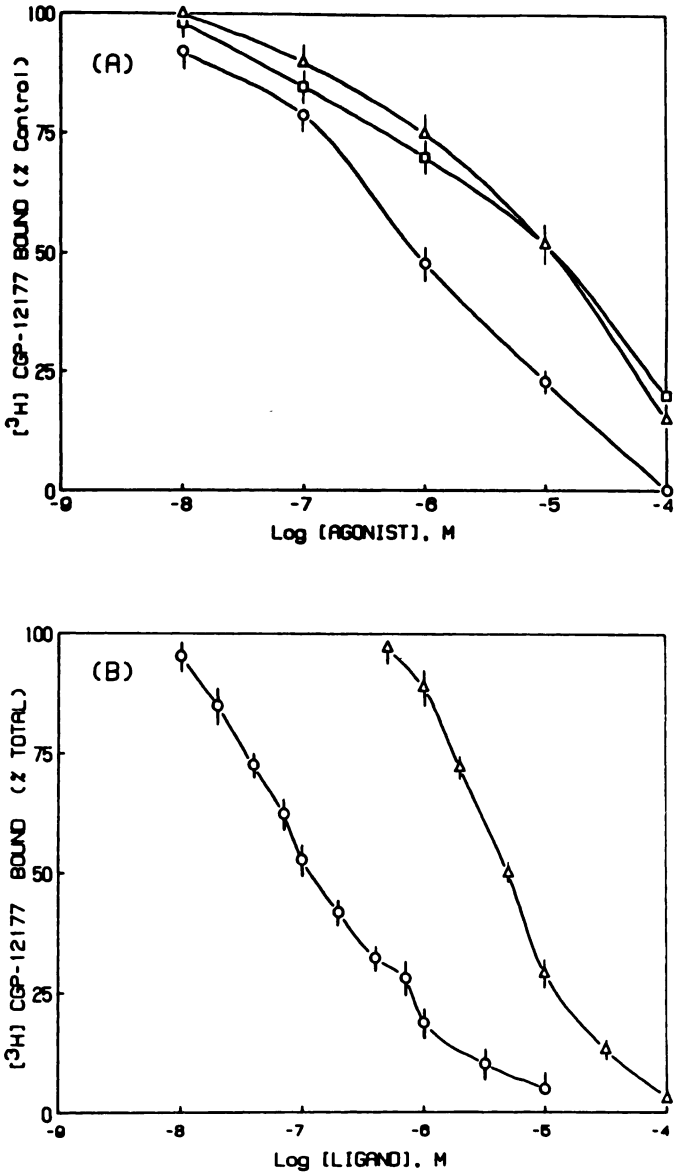


Fig. 5. Competition of specific [^{3}H]CGP-12177 binding to rat fat cell membranes by β-adrenergic agonist and antagonist ligands. A, Rat fat cell membranes (0.2 mg of protein) were incubated for 45 min at 35° with 2.5–4 nM [^{3}H]CGP-12177 and the indicated concentrations of the following β-adrenergic agonist ligands: (−)-isoproterenol (○), (−)-norepinephrine (□), and (−)-epinephrine (Δ). B, Rat fat cell membranes were incubated under the same conditions with [^{3}H]CGP-12177 and the indicated concentrations of either ICI-118,551 (Δ) or CGP-20712A (○). Binding assays were performed as outlined under Experimental Procedures. In A, the amount of specific binding (percentage of control) is plotted against the concentration of competing agonist ligand. The data are mean values ± standard errors from a single representative experiment. For B, the data from four separate experiments were pooled and analyzed simultaneously using the LIGAND computer program. The data are mean values ± standard errors from the four separate experiments. In all cases, determinations of binding in each experiment was performed in triplicate.

TABLE 3

Effect of varying the volume of incubation on the binding of β-adrenergic radioligands to rat fat cell membranes

Rat fat cell membranes (microgram quantity of membranes employed in each assay) were incubated with the following radioligands: [^{125}I]ICYP (15 μg); [^{3}H]DHA (200 μg); [^{3}H]CGP-12177 (300 μg), and [^{125}I]pABC (100 μg). The concentration of radioligand was fixed and volume of incubation varied as indicated below. The incubation conditions and the quantification of the binding are described in Experimental Procedures. The specific activity of each radioligand were as follows: [^{125}I]ICYP (1700 cpm/fmol); [^{3}H]DHA (102/cpm fmol); [^{3}H]CGP-12177 (60 cpm/fmol), and [^{125}I]pABC (570 cpm/fmol).

Radioligand	Radioligand concentration	Incubation volume	Bound radioligand			Specific binding[d]
			Total[a]	Nonspecific[b]	Specific[c]	
	nM	ml		cpm		fmol/mg
[^{125}I]ICYP	0.1	0.2	4136	766	3370	132
	0.1	1.0	5639	1494	4145	160
	0.1	2.0	6571	2433	4138	160
[^{3}H]CGP-12177	1.0	0.2	1725	982	743	41
	1.0	1.0	2052	989	1063	58
	1.0	2.0	2193	1255	938	51
[^{3}H]DHA	2.0	0.2	2144	804	1340	65
	2.0	1.0	3603	1862	1741	86
	2.0	2.0	5070	2741	2329	114
[^{3}H]DHA	20	0.2	9060	5130	3930	211
	20	1.0	17790	12837	4953	262
	20	2.0	23813	19840	3973	215
[^{125}I]pABC[e]	0.1	0.2	5881	5728	153	2
	0.1	1.0	20208	19673	653	11
	0.1	2.0	43130	42143	987	17

[a] Bound radioligand in the absence of isoproterenol.
[b] Bound radioligand in the presence of 100 μM (−)-isoproterenol.
[c] The difference between total and nonspecific binding.
[d] Concentration of binding sites calculated from the specific binding data.
[e] Incubation performed in the dark, filtered on a GF/C filter, and washed with 20 ml of 25 mM sodium acetate, pH 5.1, buffer.

values for the B_{max} obtained with these different radioligands and competing ligands were anticipated due to the marked differences in the hydrophobicity of the ligands. Membrane preparations are well known to contain a complement of 'inside out' vesicles, to which the more hydrophilic ligands may not gain ready access. Thus, the predominant high affinity binding species of rat fat cell membranes is a typical β_1-adrenergic receptor found in an abundance of 50–60 fmol/mg of protein of crude membranes.

Lands *et al.* (1) proposed the pharmacological character of the receptor mediating catecholamine stimulation of lipolysis to be β_1, based upon the relative lipolytic potencies of several agonist ligands. The character of the predominant binding species identified via radiolabeling with [^{3}H]DHA, [^{125}I]ICYP, or [^{3}H]CGP-12177 was shown to be *uniformly* β_1, with a rank order of potency for agonists of isoproterenol ≫ norepinephrine ≥ epinephrine. This is precisely the rank order for the stimulation of lipolysis first reported by Lands *et al.* (1) and the rank order for stimulation of cyclic AMP accumulation reported by several groups (27).

On what basis was an 'atypical' β-adrenergic receptor postulated to exist in the rat fat cell? Salbutamol, a ligand reported to be β_2-selective, was reported to stimulate lipolysis (2, 8, 9, 28). It was concluded from this observation that the receptor mediating lipolysis could not be β_1 in character. It should be pointed out, however, that the concentration of salbutamol required to stimulate lipolysis half-maximally was more than 2 orders of magnitude greater than that for isoproterenol (9, 28). In addition, the potency of the β_1-selective antagonist betaxolol was found to be greater than that of β_2-selective antagonists with respect to blocking lipolysis in response to stimulation by isoproterenol, norepinephrine, salbutamol, or sonoterol (6, 8). In the present work, the β_1-selective antagonist CGP-20712A is shown to inhibit agonist-stimulated cyclic AMP accumulation and lipolysis at concentrations that, for the β_2-selective antagonist ICI-118,551, are without effect. Taken together, these data provide compelling evidence that the high affinity β_1-adrenergic receptors identified by radiolabeling with [^{3}H]DHA, [^{125}I]ICYP, and [^{3}H]CGP-12177 mediate catecholamine-stimulated cyclic AMP accumulation and lipolysis in rat fat cells.

CGP-20712A is 10,000-fold more selective for β_1- as compared with β_2-adrenergic receptors in tissues containing mixed populations of β_1- and β_2-adrenergic receptors (12, 13). CGP-20712A was used in the present report to characterize the β-adrenergic receptor in rat fat cells because, unlike most β_1- and β_2-adrenergic antagonists, CGP-20712A contains bulky constituents both on the alkanolamine side-chain and on the aromatic moiety of the oxypropanolamine side-chain (Fig. 1). The chemical structure of the alkanolamine side-chain of CGP-20712A is similar to that of the tolamolol derivatives (Fig. 1) used by DeVente *et al.* (5) to evaluate the subtype specificity of the rat fat cell β-adrenergic receptor. From their studies on the ability of tolamolol derivatives to antagonize isoproterenol-stimulated lipolysis, DeVente *et al.* (5) proposed that the rat fat cell β-adrenergic receptors mediating lipolysis were not β_1. DeVente *et al.* (5) proposed that for the rat fat cell β-adrenergic receptor the interaction site for the aromatic moiety of arylethanolamines and aryloxypropanolamines was β_2-like, whereas the interaction site for the alkanolamine side-chain has β_1 character. The following data fail to support this tenet: (i) the radioligands [^{3}H]DHA and [^{125}I]ICYP both contain bulky constituents in the aromatic moiety of their oxypropanolamine termini but bind with high affinity to β_1-adrenergic receptors of rat fat cells; (ii) catecholamine agonist ligands containing structurally distinct substituents at the aromatic moiety of the arylethanolamine and alkanolamine side-chains have been used to define a β_1-character for the lypolytic response (10); (iii) analysis of [^{3}H]DHA, [^{125}I]ICYP, and [^{3}H]CGP-12177 equilibrium binding data with the use of the LIGAND computer

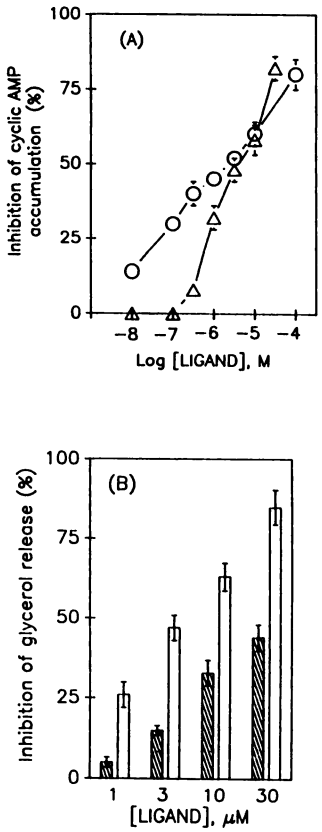


Fig. 6. Inhibition of epinephrine-mediated cyclic AMP accumulation and glycerol release from rat fat cells by CGP-20712A and ICI-118,551. A, Fat cells (20–25 mg/tube) incubated in Krebs-Ringer's phosphate buffer (120 mM NaCl, 4.75 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, and 10 mM sodium phosphate, pH 7.4), 3% albumin, and 1 μg/ml adenosine deaminase were added to epinephrine (2 μM) and the indicated concentrations of CGP-20712A (○) or ICI-118,551 (△). Cyclic AMP accumulation was measured at 5 min. Cyclic AMP accumulation in the absence or presence of epinephrine was 0.1 and 0.67 nmol/10^6 cells/min, respectively. The plot represents the inhibition of epinephrine-mediated cyclic AMP accumulation by the indicated concentrations of antagonists. B, Fat cells (30–40 mg/tube) incubated in Krebs-Ringer's phosphate and 3% albumin were added to epinephrine (2 μM) and the indicated concentrations of antagonists. Glycerol release was measured 60 min after the incubation. Glycerol release in the absence or presence of epinephrine was 0.3 and 2 μmol/10^6 cells/hr. Inhibition of epinephrine-mediated glycerol release by CGP-20712A (□) and ICI-118,551 (▨) is shown. The data represents mean ± standard error of triplicate determinations from a representative experiment that was replicated three times with similar results.

program identified a homogeneous population of high affinity β-adrenergic receptors in rat fat cell membranes that were β_1 in character (25, 29, 30); (iv) the character of these receptor sites was established not only with β_1-selective ligands but also with β_2-selective ligands that displayed discrete, albeit lower affinity, binding to the fat cell receptors typically observed with β_1-adrenergic receptors in other tissues (26, 31, 32); (v) previous work has shown that practolol (β_1-selective) was more potent than butoxamine (β_2-selective) at inhibiting isoproterenol-stimulated adenylate cyclase in rat fat cell membranes (26); and (vi) betaxolol (β_1-selective) was more potent than ICI-118,551 (β_2-selective) in antagonizing isoproterenol-stimulated lipolysis (26, 31, 32). It should be clear from the discussion above that the conclusions of the study by DeVente *et al.* (5) are at odds with the data presented here and elsewhere.

Bojanic and Nahorski (33), too, concluded that the β-adrenergic receptor of fat cells mediating lipolysis was 'atypical' based upon its relatively low affinity for a photoaffinity ligand, *p*-aminobenzyl-carazolol. However, the results of the present study using a structurally analogous ligand, [^{125}I]*p*ABC, reveal a grave complication to the interpretation of such experiments. Nearly all of the [^{125}I]*p*ABC added to the incubation media was found in association ('bound') with the membranes, whereas little binding to β-adrenergic receptors was detected even when the concentration of radioligand was held constant and the incubation volumes were expanded an order of magnitude (Table 3). Based on these data, the 'free' concentration of [^{125}I]*p*ABC (or *p*-aminobenzyl-carazolol) would be expected to be extremely low, difficult to quantify with any accuracy, and likely to afford limited receptor occupancy. Both the inhibition of radioligand binding and the inhibition of isoproterenol-stimulated adenylate cyclase activity by the photoaffinity ligand reported in the work of Bojanic and Nahorski (33) was found to be irreversible, even in the *absence* of photolysis. The nonclassical behavior of this ligand would appear to preclude analysis of equilibrium binding by classical techniques. Dax and Paritilla (34) first highlighted the potential problems associated with the use of hydrophobic ligands to study β-adrenergic receptors. The lower apparent affinity of propranolol for binding to β-adrenergic receptors of rat fat cell membranes was shown by Dax and Partilla to be a reflection of the liposolubility of this antagonist ligand (34).

Careful analysis of the data from the present study and the literature provides strong evidence that the β-adrenergic receptors characterized by radioligand binding are indeed those receptors that mediate catecholamine-stimulated lipolysis in rat fat cells. Alprenolol, DHA, and propranolol display similar affinities for fat cell β-adrenergic receptors (19, 24) and for antagonism of isoproterenol-stimulated lipolysis (25, 35). Thus, it seems improbable that the β-adrenergic receptors mediating lipolysis would be 'cryptic' to radiolabeling with ligands with this chemical structure. The β-adrenergic receptors isolated and purified from rat fat cells have been shown to be homogeneous by structural and pharmacological criteria and display β_1 character (16). These receptors, isolated in part by affinity chromatography on Sepharose-alprenolol matrices, have been shown to be structurally and immunologically related to their pharmacologically distinct counterparts, β_2-adrenergic receptors (36, 37). Frielle *et al.* (38) recently reported the tissue distribution of β_1-adrenergic receptor mRNA in rats finding a close correlation between the levels of mRNA and the levels of β_1-adrenergic receptors expressed in these tissues. Interestingly, the proposal of Arch *et al.* (39) that the β-adrenergic receptor of rodent brown fat was 'atypical' has also been challenged (40). The subtype of the brown adipose tissue β-adrenergic receptor has now been shown to be β_1 also (40). Thus, the present study and data gathered from a number of other laboratories support the tenet that the β-adrenergic receptor coupled to adenylate cyclase and mediating lipolysis in rat fat cells is β_1 in nature.

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