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β-Adrenoceptor subtype expression and function in rat white adipocytes

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1 The pharmacological features of rat white adipocyte β-adrenoceptor subtypes were investigated by saturation and β-agonist competition studies with [^{3}H]-CGP 12177 and by lipolysis induced by β-agonists as well as their inhibition by CGP 20712A (selective β_1-antagonist) and ICI 118551 (selective β_2-antagonist) in an attempt to establish a relationship between the functionality and binding capacity of β-adrenoceptor subtypes.

2 Two populations of binding sites were identified on adipocyte membranes, one with high affinity (0.22 ± 0.07 nM) and the other with low affinity (23 ± 7 nM). The low affinity binding sites constituted 90% of the total binding sites.

3 The competition curves, with 15 nM [^{3}H]-CGP 12177, for the β-agonists, isoprenaline (Iso), noradrenaline (NA) and adrenaline (Ad), and the selective β_3-agonist, BRL 37344 (BRL), were clearly biphasic ($P<0.001$). The rank orders of agonist potency (pK_i) in competing for [^{3}H]-CGP 12177 high affinity and low affinity binding sites, respectively, were Iso (9.28 ± 0.24) > NA (8.90 ± 0.12) > Ad (8.65 ± 0.12) > > BRL (4.53 ± 0.17) and BRL (7.38 ± 0.19) > > Iso (2.96 ± 0.26) $\geqslant$ NA (2.80 ± 0.17) > Ad (2.10 ± 0.11) indicating the expression of β_1- and β_3-adrenoceptor subtypes on rat white adipocytes, respectively. Inversely, competition studies with the selective β_1-agonist, xamoterol (Xam), provided evidence for a single homogeneous population of binding sites with low density (81 ± 9 fmol mg^{-1}) and high pK_i value (7.23 ± 0.26) confirming the presence of β_1-adrenoceptors.

4 To assess a possible contribution of the β_2-subtype, procaterol (Proc), a selective β_2-agonist, was used to compete with 2 nM [^{3}H]-CGP 12177. A single low affinity (4.61 ± 0.07) population of binding sites was identified. The density of these sites (71 ± 12 fmol mg^{-1}) was similar to the one obtained with Xam, suggesting that Proc displaced [^{3}H]-CGP 12177 from the β_1-subtype.

5 The functional potency (pD_2) order with BRL (9.07 ± 0.20) and catecholamines (Iso: 7.26 ± 0.06, NA: 6.89 ± 0.02 and Ad: 6.32 ± 0.07) was the same as that found for the low affinity binding sites in competition studies. Xam induced lipolysis with greater potency than dobutamine (Dob), 6.31 ± 0.06 and 5.66 ± 0.10, respectively. Proc stimulated lipolysis with a low potency (5.59 ± 0.21).

6 The lipolytic response to 0.001 μM BRL was inhibited by both, selective β_1- and β_2-antagonist, in a monophasic manner with low potencies (CGP 20712A pK_i: <4.5 and ICI 118551 pK_i: 5.57 ± 0.13). Similar monophasic profiles were obtained for inhibition of Xam- and Dob-induced lipolysis. In this case, CGP 20712A was more potent (>10 times) than ICI 118551. The monophasic inhibition was also observed with ICI 118551 in the presence of 0.05 μM Iso or 0.13 μM NA. In contrast, two populations of sites were identified with CGP 20712A in the presence of Iso as well as NA. The pK_i values for the first sites were 8.41 ± 0.09 and 8.58 ± 0.17, respectively, and for the second population of sites 4.73 ± 0.22 and 4.27 ± 0.27, respectively. The proportion of the first sites was low: 19 ± 4 and 22 ± 5%, respectively. Biphasic curves were obtained with both antagonists using 2.5 μM Proc (CGP 20712A: pK_i1: 8.17 ± 0.08, site1: 23 ± 6%, pK_i2: 4.77 ± 0.14; ICI 118551: pK_i1: 7.78 ± 0.03, site1: 37 ± 2%, pK_i2: 5.35 ± 0.25).

7 Our results show that the radioligand [^{3}H]-CGP 12177 allows the characterization of β_1- and β_3-adrenoceptor subtypes on rat white adipocytes. Lipolysis is highly dependent on β_1- and β_3-adrenoceptors. Finally, binding and functional studies confirm that lipolysis is mainly driven by the β_3-subtype.

Keywords: Rat white adipocytes; β_1-, β_2-, β_3-adrenoceptor subtypes; [^{3}H]-CGP 12177; lipolysis; BRL 37344; xamoterol; dobutamine; procaterol; catecholamines; selective β-antagonists

Introduction

The lipolytic effects of catecholamines (noradrenaline, adrenaline) on adipocytes are primarily mediated by β-adrenoceptors. Adipocyte β-adrenoceptors were first subclassified as the β_1-subtype by relative lipolytic potencies of several classical β-agonists (Lands *et al*., 1967) as well as by radioligand binding studies (Bojanic & Nahorski, 1983; Bahout & Malbon, 1988). Pharmacological analysis of the β-adrenoceptor-mediated metabolic responses of rat adipocytes suggested the existence of a third β-adrenoceptor, often called atypical β-adrenoceptor according to its metabolic and pharmacological features (De Vente *et al*., 1980; Wilson *et al*., 1984; Bojanic *et al*., 1985). Three pharmacological characteristics are well established (Arch & Kaumann, 1993). First, usual β-adrenoceptor antagonists display a weak capacity to inhibit lipolysis in rat adipocytes (De Vente *et al*., 1980; Wilson *et al*., 1984; Langin *et al*., 1991; Van Liefde *et al*., 1992). Second, the rodent adipocytes are more responsive to the novel β-agonists, such as BRL 37344, compared to classical β-agonists acting on β_1- and β_2-adrenoceptor subtypes (Wilson *et al*., 1984; Hollenga & Zaagsma, 1989; Langin *et al*., 1991). Finally, some antagonists of β_1- and β_2-receptors, such as CGP 12177, are, at higher concentrations, partial agonists of lipolytic function (Langin *et al*., 1991; Van Liefde *et al*., 1992).

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The β-adrenoceptor classification has been, at last, modified since the gene coding for the human (Emorine *et al*., 1989), rat (Granneman *et al*., 1991; Muzzin *et al*., 1992) and mouse (Nahmias *et al*., 1991) β_3-receptor was cloned and expressed in Chinese hamster ovary cells. Currently, the coexistence of three β-adrenoceptor subtypes is sustained by the identification of three subtypes of β-adrenoceptor mRNA in rodent white adipocytes (β_1-, β_2- and β_3-receptor mRNA), with β_3-adrenoceptor mRNA being expressed predominantly (Muzzin *et al*., 1991; Collins *et al*., 1994). However, functional studies have demonstrated that only the β_1- and β_3-receptor subtypes can stimulate lipolysis in rat (Hollenga & Zaagsma, 1989; Van Liefde *et al*., 1992; Murphy *et al*., 1993). Furthermore, catecholamine-induced lipolysis is mediated predominantly by β_3-receptors in rat white adipocytes (Hollenga & Zaagsma, 1989; Van Liefde *et al*., 1992; Murphy *et al*., 1993; Bousquet-Mélou *et al*., 1994).

While functional studies on rat white adipocytes revealed a major role for β_3-adrenoceptors and a minor role for β_1-receptors, the β_3-subtype has not been detected by binding studies on these cells. Although the ligands commonly used until now for β-adrenoceptor identification ([^{3}H]-CGP 12177 and [^{125}I]-iodocyanopindolol) possess weaker affinity for β_3- than for β_1/β_2-adrenoceptors (Arch & Kaumann, 1993), one of them ([^{3}H]-CGP 12177, β_1/β_2-antagonist and β_3-partial agonist) permitted the characterization of the β_3-subtype by binding studies on human and garden dormouse white adipocytes (Revelli *et al*., 1993; Carpéné *et al*., 1994), in homogenates of rat brown adipose tissue (Muzzin *et al*., 1992), of rat brown adipocytes (D'Allaire *et al*., 1995) and 3T3-F442A adipocytes (Fève *et al*., 1991). Although comparable studies were not successful for rat white adipocytes (Langin *et al*., 1991), several binding studies previous to 1990 had shown a heterogeneity of β-adrenoceptors on these cells suggesting the coexistence of two populations of sites with differing affinities for some β-adrenoceptor radioligands and for several β-adrenoceptor competitors (Giudicelli *et al*., 1979; Bahout & Malbon, 1988). The first population was identified as a β_1-subtype, but no explanation was given for the second population of binding sites. Up to date, no investigation has established clearly a relationship between lipolytic function and binding studies on rat white adipocytes. Another point of uncertainty is the role of β_2-adrenoceptors in the lipolysis of rat white adipocytes.

The aim of the present study was to characterize the β-adrenoceptor subtypes on rat white adipocytes by saturation and β-agonist competition studies with the radioligand [^{3}H]-CGP 12177. We also assessed the lipolytic activity of the β-agonists used for competition studies and the inhibition of their activity by CGP 20712A (selective β_1-antagonist) or ICI 118551 (selective β_2-antagonist). The binding affinities of β-agonists for β-adrenoceptor subtypes were compared with their potencies in activating β-adrenoceptor-mediated lipolysis.

Methods

Isolation of adipocytes

Adipocytes were prepared according to the method of Rodbell (1964) with minor modifications. Male Wistar rats (200–250 g) were fed *ad libitum* and killed by a blow on the head. Epididymal fat pads were removed, cut into small pieces and incubated with 0.1% collagenase in Krebs-Ringer buffer supplemented with 20 mM HEPES, 1.4 mM $CaCl_2$ and 3% bovine serum albumin, pH 7.4, for 60 min at 37°C in a shaking waterbath. After collagenase digestion, the adipocytes were separated by filtration through nylon cloth (mesh = 200 μm) and then washed 3 times with a supplemented Krebs-Ringer buffer by centrifugation (400 *g*, 5 min). The adipocyte suspension was divided into two aliquots for lipolysis measurement and binding studies.

Crude membrane preparation

Adipocytes were lysed at room temperature in hypotonic medium containing 2 mM Tris-HCl, 2.5 mM $MgCl_2$, 1 mM $KHCO_3$, 100 μM EGTA, pH 7.5, and the following protease inhibitors: 10 μg ml^{-1} leupeptin and 300 μM phenylmethylsulphonyl fluoride, then centrifuged for 15 min at 40 000 *g* at 15°C. The pellet was resuspended in 40 ml of binding buffer (50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.5) and again centrifuged (40 000 *g* for 15 min at 4°C). The resulting pellet was resuspended in binding buffer to a final concentration of 2–3 mg protein ml^{-1} and stored at −80°C for subsequent binding studies. Protein concentration was determined by the dye-binding assay with a commercial kit (Bio-Rad, München, Germany) and bovine serum albumin was used as standard.

Binding studies

For saturation studies, [^{3}H]-CGP 12177 was used at concentrations ranging from 0.3 to 100 nM. To the binding buffer containing 100 μM GTP, 50 μl ligand and 50 μl crude membranes (equivalent to 50–80 μg protein) were added to obtain a final volume of 200 μl. Competition studies were performed with 15 nM [^{3}H]-CGP 12177 in the presence of catecholamines, isoprenaline (Iso), noradrenaline (NA) and adrenaline (Ad), the selective β_3-agonist, BRL 37344 (BRL) and a selective β_1-agonist, xamoterol (Xam) as well as a β-antagonist, propranolol. With the selective β_2-agonist, procaterol (Proc), [^{3}H]-CGP 12177 was used at 2 nM. The mixture was incubated in a shaking waterbath at 37°C for 30 min and the reaction was stopped by the addition of 4.5 ml cold binding buffer immediately followed by filtration under vacuum through Whatman GF/C glass-fibre filters (Maidstone, U.K.) by use of a Millipore apparatus (Bedford, MA, U.S.A.). Filters were rapidly washed with 15 ml cold binding buffer. The radioactivity trapped by filters was measured with a liquid scintillation counter (LS6000SC, Beckman, U.S.A.). In saturation experiments, non specific binding was determined in the presence of 100 μM propranolol. This concentration was generally used to characterize all β-adrenoceptor subtypes on adipocytes other than rat white adipocytes (Muzzin *et al*., 1992; Sillence *et al*., 1993; Revelli *et al*., 1993).

Lipolysis measurements

Adipocytes ($2-3\times10^5$ cells) were incubated with pharmacological agents at suitable dilutions in a final volume of 500 μl. Lipolysis was assessed by use of selective β-agonists and the catecholamines, most of which were also used in competition studies (Iso, NA, Ad, BRL, CGP 12177, Dob, Xam and Proc). When the selective β_1-antagonist CGP 20712A and the selective β_2-antagonist ICI 118551 were used, cells were preincubated for 15 min with the antagonist before addition of the β-agonist. In first set of experiments, the high agonist concentrations used of Iso (1 μM), BRL (0.01 μM), Xam (10 μM), Dob (10 μM) and Proc (10 μM), allowed the recruitment of all β-adrenoceptor subtypes implicated in lipolysis stimulation (Granneman, 1992; Carpéné *et al*., 1993; Bousquet-Mélou *et al*., 1994). Hollenga *et al*. (1990) showed that CGP 20712A did not antagonize the BRL-induced lipolysis between 10 nM to 10 μM whereas it induced a small but consistent rightward shift of the Iso concentration-response curve, indicating that this antagonist had no affinity for the β_3-adrenoceptor subtype up to 10 μM; at 10 μM, only β_1-adrenoceptor-mediated stimulation was inhibited. Investigation of the accumulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) and lipolytic activity in rat brown adipocytes gave similar results (Nicoli *et al*., 1995). The β_2-antagonist, ICI 118551 (10 μM and higher), antagonized the Iso- and BRL-induced lipolysis; thus, at these concentrations, this antagonist counteracted the responses induced by activation of all β-adrenoceptor subtypes (Hollenga *et al*., 1990). Langin *et al*.

(1991) made the same observations with BRL induced-lipolysis. Thus, agonist-induced lipolysis was determined with 10 μM CGP 20712A and 1 μM and 5 μM ICI 118551. In a second run of experiments, the effects of concentrations of agonists close to their EC_{50} values (Iso 0.5 μM, NA 1.3 μM, BRL 0.001 μM, Xam 5 μM, Dob 2.0 μM and Proc 2.5 μM), were counteracted by both antagonists in the concentration range 10^{-10} to 10^{-4} M. Incubations were carried out at 37°C in a shaking waterbath. After a 90 min incubation, the reaction was stopped by plunging the tubes into an ice bath. After centrifugation (2000 *g* at 4°C for 10 min), an aliquot of the infranatant was taken to evaluate enzymatically the concentration of glycerol released during the incubation (Glycerol assay kit; Boehringer, Mannheim, Germany).

Data and statistical analysis

Results are expressed as means ± s.e.mean. The binding parameters (K_d, B_{max} and K_i) were determined by using LIGAND, a non-linear curve-fitting programme (Munson & Rodbard, 1980). The statistical method given by the LIGAND programme was used to determine whether the saturation and displacement curves were best fitted by a one- or two-site model. Concentration-response curves for glycerol release as a function of agonist and antagonist concentration were analysed by computer-assisted iteration with GraphPad PRISM (San Diego, CA, U.S.A.). For other data treatments, statistical significance was determined by analysis of variance (ANOVA). A two-tailed P value <0.05 was considered to be statistically significant.

Drugs and chemicals

Collagenase type II, bovine serum albumin (fraction V), GTP, (±)-isoprenaline hydrochloride, (±)-adrenaline hydrochloride, (±)-propranolol hydrochloride, procaterol hydrochloride were purchased from Sigma Chemicals (St. Lous, MO, U.S.A.). (±)-Noradrenaline was provided by Fluka Chemica-Biochemica (Buchs, Switzerland). Dobutamine hydrochloride was obtained from Tocris Cookson Ltd (Bristol, U.K.). Xamoterol fumarate was a gift from Zeneca (Cergy, France). BRL 37344 (sodium-4-{2′-[2-hydroxy-2-(3-chlorophenyl)-ethylamino]-propyl}phenoxyacetate sesquihydrate (RR.SS diastereoisomer)) was provided by Smith Kline Beecham Pharmaceutical (Epsom, UK). CGP 20712A (±)-(2-(3-carbomyl-4-hydroxyphenoxy)-ethylamino)-3-[4-(1-methyl(-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethane sulphonate), CGP 12177 ((-)-4-(3-t-butyl amino-2-hydroxy-propoxy) benzimidazole-2-one) and ICI 118551 (erythro-(±)-1-(7-methylindar-4-yloxy)-3-isopropyl-aminobutan-2-ol) were gifts from Ciba-Geigy (Basel, Switzerland) and ICI Pharmaceuticals (Macclesfield, UK) respectively. [^{3}H]-CGP 12177 (specific activity: 46 Ci mmol^{-1}) was obtained from Amersham (Les Ulis, France).

Results

Saturation binding study

Experiments on rat white adipocyte membranes generated a saturation binding curve for [^{3}H]-CGP 12177 that was clearly biphasic (Figure 1a). Although linear with increasing concentrations of [^{3}H]-CGP 12177 (data not shown), the non specific binding defined in the presence of 100 μM propranolol reached about 60% of total binding for 100 nM radioligand. A Scatchard plot of these data was curvilinear indicating the presence of two binding sites (Figure 1b). Indeed, analysis by LIGAND confirmed that the data fitted a two-site model ($P<0.001$). The binding characteristics are shown in Table 1. Low affinity binding sites were mainly expressed on rat white adipocytes (about 90% of total β-adrenoceptors).

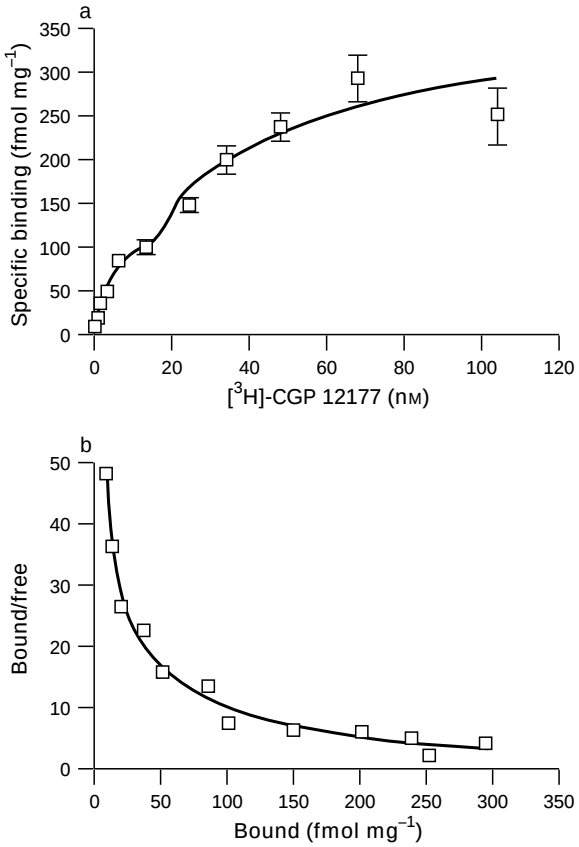

Figure 1 Specific [^{3}H]-CGP 12177 binding to rat white adipocyte membranes. Saturation curve (a) is representative of an experiment which was performed in triplicate. Scatchard plot of the same data (b) is curvilinear and was best fitted by a two-site model ($P<0.05$).

Table 1 Pharmacological characteristics of [^{3}H]-CGP 12177 binding sites in white adipocyte membranes

Binding sites	K_D (nM)	B_{max} (fmol mg^{-1})
High affinity	0.22 ± 0.07	27 ± 5
Low affinity	23 ± 7	334 ± 55

The values are means ± s.e.mean of 5 separate experiments performed in triplicate. The K_D values are expressed in nM and B_{max} in fmol of [^{3}H]-CGP 12177 bound mg^{-1} membrane protein.

Competition studies with β-adrenoceptor ligands

All competition and saturation studies were performed in the presence of 100 μM GTP to shift all receptor subtypes into the low affinity state for agonists (Kent *et al*., 1980). To label both, high and low affinity binding sites, the studies were performed with 15 nM [^{3}H]-CGP 12177. The competition curves for propranolol, Iso, NA, Ad and BRL were clearly biphasic (Figures 2a–c) and best described by a two-site model ($P<0.001$). Propranolol 100 μM inhibited the [^{3}H]-CGP 12177 binding to all β-adrenoceptor subtypes (Table 2, Figure 2a). It was a more potent competitor (× 10000) for the high affinity than for the low affinity [^{3}H]-CGP 12177 binding sites. The concentration of 100 μM represented about 50 times the K_i of low affinity binding sites. Thus, 100 μM propranolol was suitable to estimate the non specific binding in saturation studies.

The capacity of the catecholamines (NA, Ad and Iso) to inhibit [^{3}H]-CGP 12177 binding to β-adrenoceptors was compared with that of the selective β_3-agonist BRL (Table 2, Figure 2b and c). At a low concentration (10^{-6} M), BRL

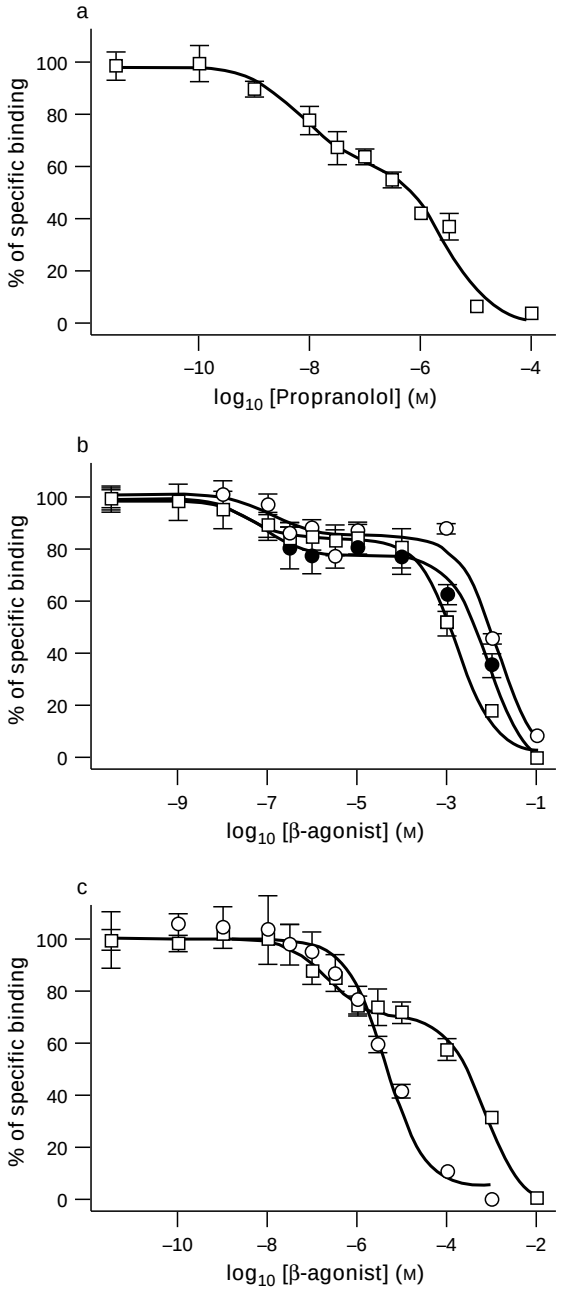

Figure 2 Competition curves of propranolol and the β-agonists for [^{3}H]-CGP 12177 (15 nM) binding to rat white adipocyte membranes. Inhibition of specific binding by (a) propranolol, by (b) isoprenaline (□), noradrenaline (○) and adrenaline (●), and by (c) BRL 37344 (□) and xamoterol (○). The results illustrated are means (vertical lines show s.e.mean) of 3–5 experiments performed in triplicate and are expressed as percentages of specific binding in the absence of the competitor. s.e.means not shown are within the size of the symbol.

competed with high efficiency for the predominant population of [^{3}H]-CGP 12177 binding sites, whereas at the same concentration NA, Ad and Iso inhibited radioligand binding from only a minor population of sites. The relative order of potency for competition with [^{3}H]-CGP 12177 from its high affinity binding sites was: Iso > NA > Ad > > BRL, and from its low affinity binding sites was: BRL > > Iso ≥ NA > Ad. These rank orders suggest that the high and low affinity β-adrenoceptors on rat white adipocytes are β_1- and β_3-subtypes, respectively.

With regard to the selective β_1-agonist Xam, only one population of sites was identified (Figure 2c) since the competition curve was best fitted to a one-site model ($P<0.001$). The B_{max} value indicated that the binding sites were the minor population of β-adrenoceptors and thus probably represented the β_1-subtype (Table 2).

The capacity of Proc, a selective β_2-agonist, to inhibit [^{3}H]-CGP 12177 binding was evaluated in order to assess a possible presence of the β_2-subtype among the population of high affinity binding sites (Figure 3). To label preferentially the high affinity binding sites the competition experiments were performed in the presence of 2 nM [^{3}H]-CGP 12177. The competition curve was best fitted to a one-site model ($P<0.001$), indicating a single homogeneous population of β-adrenoceptors. As Proc inhibited [^{3}H]-CGP 12177 binding at very high concentrations with a low pK_i value (4.61 ± 0.07, $n=3$), it seems that only β_1-adrenoceptors were detected in this experiment.

Lipolytic activity of β-agonists

To investigate the contribution of each β-adrenoceptor subtype to the induction of lipolysis, the functional effect of different β-agonists was examined. The dose-dependent stimulating effect of various β-agonists on glycerol release by

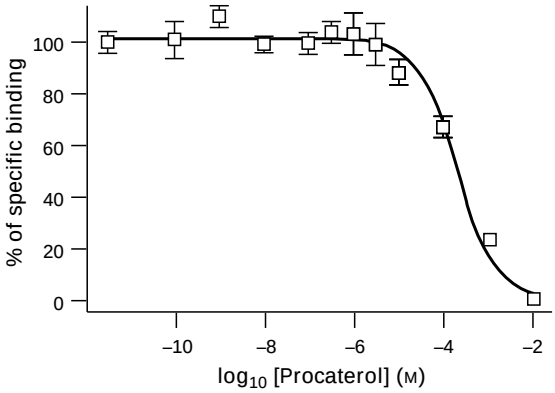

Figure 3 Competition curves of procaterol for [^{3}H]-CGP 12177 (2 nM) binding to rat white adipocyte membranes. The results illustrated are means (vertical lines show s.e.mean) of 3 experiments performed in triplicate and are expressed as percentages of specific binding in the absence of competitor. s.e.means not shown are within the size of the symbol.

Table 2 Binding parameters of various β-adrenoceptor competitors

	High affinity binding sites			*Low affinity binding sites*		
	B_{max}	*Inhibition constant*		B_{max}	*Inhibition constant*	
β-Adrenoceptor compounds	(fmol mg^{-1})	pK_i	K_i (nM)	(fmol mg^{-1})	pK_i	K_i (μM)
Propranolol	58±8	9.74±0.23	0.18	244±18	5.73±0.13	1.79
Isoprenaline	31±11	9.28±0.24	0.32	253±75	2.96±0.26	1099
Noradrenaline	48±2	8.90±0.12	1.26	359±73	2.80±0.17	1580
Adrenaline	51±10	8.65±0.12	2.22	509±26	2.10±0.11	7932
BRL 37344	61±9	4.53±0.17	29279	100±11	7.38±0.19	0.042
Xamoterol	81±9	7.23±0.26	59			

The values are means±s.e.mean of 3–5 separate experiments performed in triplicate with 15 nM [^{3}H]-CGP 12177. B_{max} values are expressed in fmol [^{3}H]-CGP 12177 bound mg^{-1} membrane protein, pK_i, K_i in nM and μM for β_1- and β_3- subtypes, respectively.

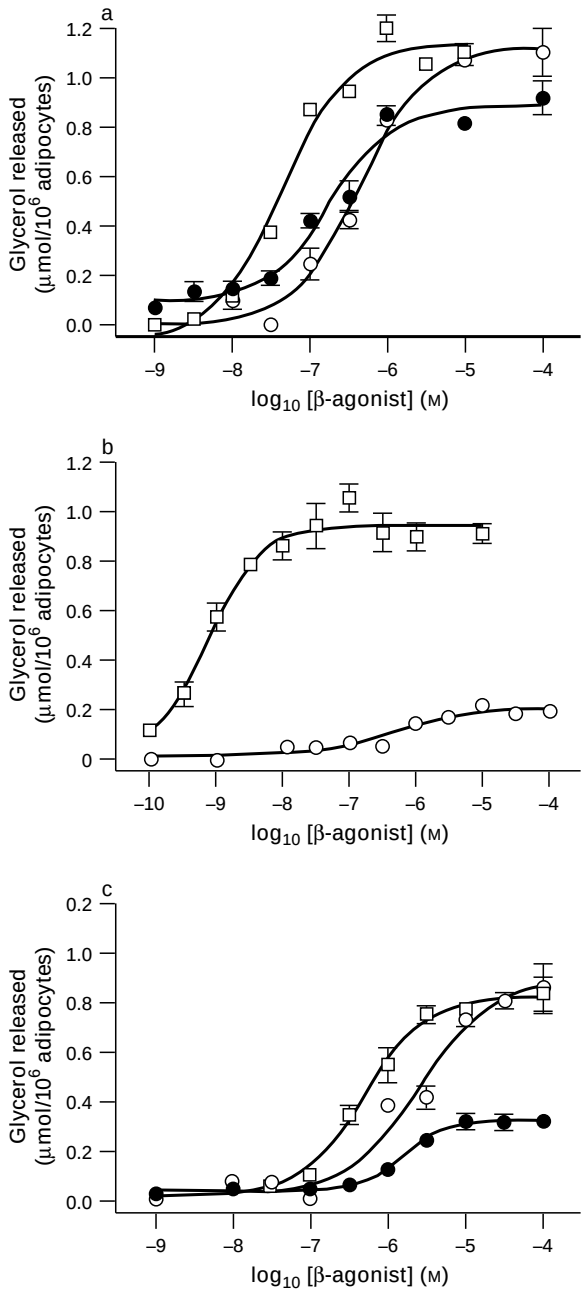

Figure 4 Concentration-response curves for stimulation of glycerol release from white adipocytes elicited by (a) isoprenaline (□), noradrenaline (○) and adrenaline (●), (b) BRL 37344 (□) and CGP 12177 (○), and (c) procaterol (□), dobutamine (○) and xamoterol (●). Each curve is a representative experiment performed in triplicate. s.e.means not shown are within the size of the symbol.

rat white adipocytes is shown in Figure 4. The pD_2 and E_{max} values of lipolytic function are given in Table 3.

The most potent inducer of lipolysis was the selective β_3-agonist, BRL. It was 65, 150 and 560 times more potent than Iso, NA and Ad, respectively (Figure 4a and b; Table 3). The rank order of agonist potency to induce lipolysis was BRL > > Iso > NA > Ad, which paralleled the ability of these agonists to compete with [^{3}H]-CGP 12177 for its low affinity binding sites. CGP 12177 (β_1- and β_2-antagonist) showed (Figure 4b, Table 3) features of a partial β_3-agonist inducing lipolysis with lower efficacy than BRL and the β-agonist, Iso.

Xam is known to be a partial selective β_1-agonist (Nuttall & Snow, 1982; Malta *et al*., 1985). We also used another selective β_1-agonist, Dob. These two selective β_1-agonists elicited different lipolytic activities. Xam exhibited the functional features of a partial but more potent agonist than Dob on white adipocytes (Figure 4c, Table 3).

The selective β_2-agonist, Proc, stimulated lipolysis less potently than the catecholamines, the selective β_3-agonists and Xam (Figure 4c, Table 3). The potencies of Proc and Dob were similar.

Effect of β-antagonists on lipolysis induced by different β-agonists

To characterize further the β-adrenoceptor subtypes involved in lipolysis, we studied the inhibition of the functional response by the selective β_1-antagonist CGP 20712A and the selective β_2-antagonist ICI 118551 (Figure 5, Tables 4 and 5). In Table 4 we show the results with agonists at concentrations promoting a nearly full lipolytic response (70–100%). The data obtained with lower concentrations of the agonists (about

Table 3 Lipolytic potency and maximal lipolytic responsiveness of selective and non-selective β-agonists

β-Adrenoceptor compounds	*EC_{50}* pD_2	(nM)	*E_{max}* (μmol/10^{6}adipocytes)
Isoprenaline	7.26±0.06	(55)	1.01±0.25
Noradrenaline	6.89±0.02	(129)	0.95±0.21
Adrenaline	6.32±0.07	(479)	1.01±0.20
BRL 37344	9.07±0.20	(0.85)	1.11±0.08
CGP 12177	6.20±0.16	(631)	0.25±0.04*
Dobutamine	5.66±0.10	(2187)	1.01±0.14
Xamoterol	6.31±0.06	(490)	0.22±0.05*
Procaterol	5.59±0.21	(2570)	0.93±0.11

The values are means±s.e.mean of 3–5 separate experiments performed in triplicate. The potencies of lipolytic agonists were evaluated by their EC_{50}, concentration of lipolytic agonists inducing 50% of maximal lipolysis expressed in pD_2: $-\log_{10}$ EC_{50} and in parentheses in nM. E_{max} is the maximal responsiveness minus basal lipolysis (0.44±0.01 μmol glycerol released/10^6 adipocytes) and is expressed in μmol glycerol released/10^6 adipocytes. *$P<0.05$ versus isoprenaline.

Table 4 Comparative antagonistic effects of CGP 20712A 10 μM, ICI 118551 1 μM and 5 μM on rat white adipocyte lipolysis induced by various agonists

β-Adrenoceptor compounds	*Lipolytic response* (μmol/10^6 adipocytes)	*Antagonistic effect (% of lipolytic response)* *CGP 20712A* (10 μM)	*ICI 118551* (1 μM)	*ICI 118551* (5 μM)
Isoprenaline (1 μM)	1.04±0.09	77±4*	86±3	70±1*
BRL 37344 (0.01 μM)	0.93±0.04	91±3	93±4	81±3*
Dobutamine (10 μM)	0.83±0.07	32±8*	88±7	
Xamoterol (10 μM)	0.16±0.07	5±5*	98±3	
Procaterol (1 μM)	0.43±0.08	50±7*	41±6*	9±5*
Procaterol (10 μM)	0.77±0.03	72±5*	61±1*	

The values are means±s.e.mean of 3–5 separate experiments performed in triplicate. The lipolytic response represents the lipolysis induced by the agonists without antagonist expressed in μmol/10^6 adipocytes minus basal lipolysis (0.44±0.01 μmol/10^6 adipocytes). *$P<0.05$ versus lipolytic response.

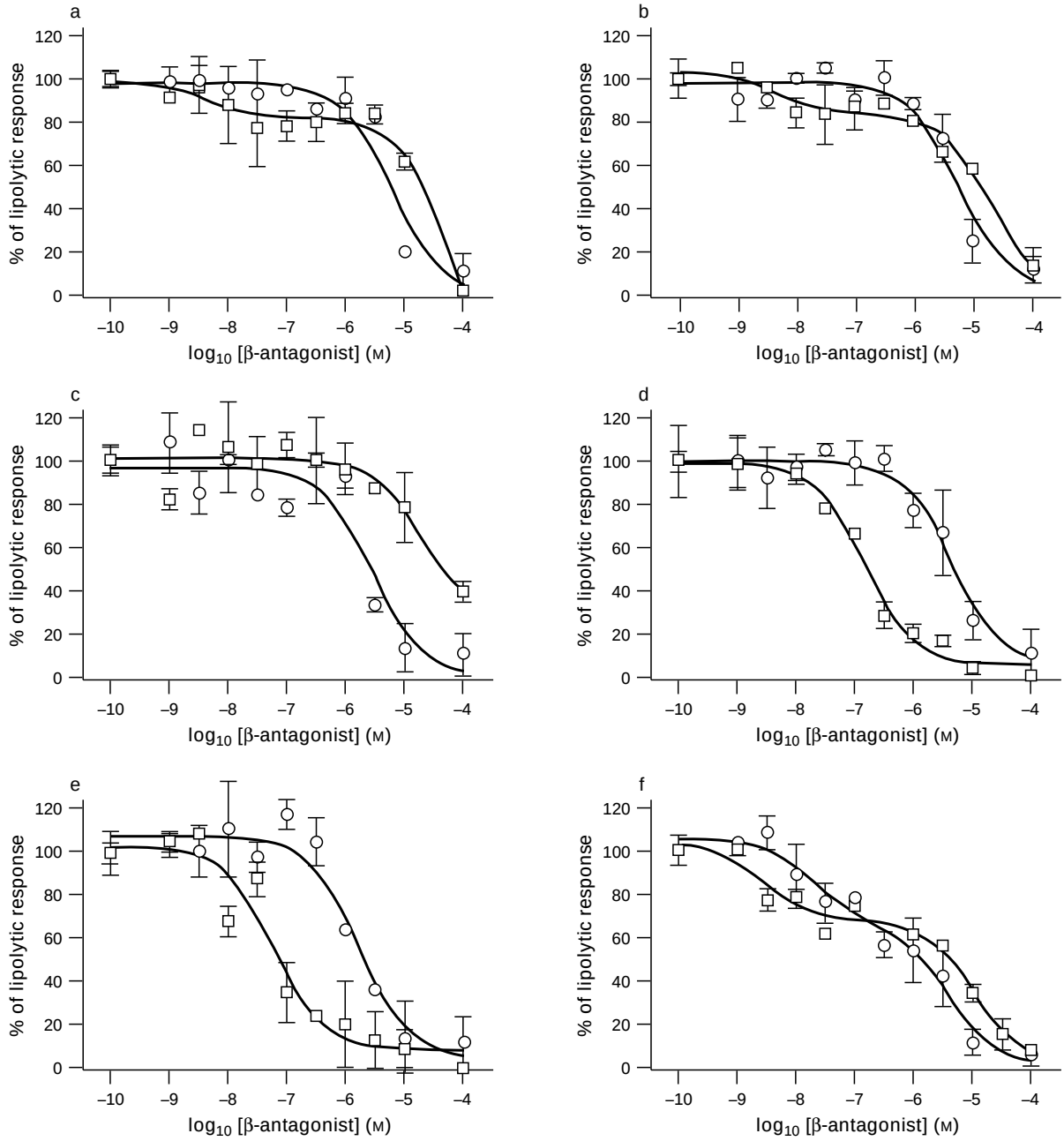

Figure 5 Concentration-response curves for inhibition of glycerol release by CGP 20712A (□) and ICI 118551 (○) induced by (a) 0.05 μM isoprenaline, (b) 0.13 μM noradrenaline, (c) 0.001 μM BRL 37344, (d) 0.5 μM xamoterol, (e) 2.0 μM dobutamine and (f) 2.5 μM procaterol from white adipocytes. The results are expressed as percentage of lipolytic response without antagonist. Each curve is a representative experiment performed in triplicate. s.e.means not shown are within the size of the symbol.

the EC_{50} value), which induced a partial lipolytic response (50–60%), are presented in Figure 5. The use of lower doses minimized the lipolytic response mediated by the second and more abundant sites when the response was produced by two populations of sites. Analysis of the inhibition curves (Figure 5) showed that a two site model fitted the data better than a one site model for the results with Iso (Figure 5a) and NA (Figure 5b) with CGP 20712A as antagonist and Proc (Figure 5f) with both antagonists ($P<0.001$). The IC_{50} values are shown in Table 5.

Catecholamines Iso-induced lipolysis was significantly but slightly (23%) decreased by the selective β_1-antagonist CGP 20712A at 10 μM but not significantly affected by β_2-antagonist, ICI 118551 1 μM (Table 4). This suggests that the majority (77%) of lipolysis was mediated by β_3-adrenoceptors. CGP 20712A exhibited biphasic dose-response curves for inhibition of lipolysis induced by two β-agonists, Iso and NA (Figure 5a and b). The first sites implicated in the induction of lipolysis represented 22 ± 5% and 19 ± 4%, respectively. CGP 20712A was at least 5000 times more potent as an inhibitor of the stimulating response mediated by the first population than by the second population of sites (Table 5). In contrast, the inhibition curves obtained with ICI 118551 were monophasic and the potency of ICI 118551 was low (Figure 5a and b; Table 5). These data indicate that, in rat white adipocytes, the catecholamines, Iso and NA, induce lipolysis predominantly by stimulation of the β_3-subtype and weakly by activation of β_1-adrenoceptors.

Selective β_3-agonist No significant antagonistic effects of the selective antagonists CGP 20712A (10 μM) and ICI 118551

Table 5 Antilipolytic potency of CGP 20712A and ICI 118551 on rat white adipocyte lipolysis induced by various agonists

β-adrenoceptor compounds	*Lipolytic response* (μmol/10^6 adipocytes)	*CGP 20712A* $-log\,IC_{50}$	(μM)	*ICI 118551* $-log\,IC_{50}$	(μM)
Noradrenaline (0.13 μM)	0.50±0.13	(**1**) 8.41±0.09	(0.0039)	5.24±0.27	(5.75)
		(**2**) 4.73±0.22	(18.6)		
Isoprenaline (0.05 μM)	0.65±0.09	(**1**) 8.58±0.17	(0.0026)	5.50±0.18	(3.16)
		(**2**) 4.25±0.27	(56)		
BRL 37344 (0.001 μM)	0.65±0.03	<4.5	(>32)	5.57±0.13	(2.69)
Dobutamine (2.0 μM)	0.53±0.05	6.81±0.38	(0.155)	5.74±0.07	(1.82)
Xamoterol (0.5 μM)	0.11±0.01	7.13±0.23	(0.074)	5.25±0.24	(5.62)
Procaterol (2.5 μM)	0.57±0.06	(**1**) 8.17±0.08	(0.0068)	7.78±0.03	(0.017)
		(**2**) 4.77±0.14	(16.9)	5.35±0.25	(4.77)

The values are means±s.e.mean of 3–4 separate experiments performed in triplicate. The lipolytic response represents the lipolysis induced by the agonists without antagonist expressed in μmol/10^6 adipocytes minus basal lipolysis (0.39±0.04 μmol/10^6 adipocytes). The potency of lipolytic antagonists were evaluated by their IC_{50}, concentration of antagonists inhibiting 50% of lipolytic response expressed in $-\log IC_{50}$ and in parentheses in μM. (**1**) $-\log IC_{50}$ of the first population of sites and (**2**) $-\log IC_{50}$ of the second population of sites.

(1 μM) on BRL (0.01 μM)-induced response were observed (Table 4). ICI 118551 5 μM inhibited significantly the BRL-induced lipolysis by 19%, raising the possibility that this antagonist can act on β_3-adrenoceptors (Table 4). When the concentration of BRL was lower (close to the EC_{50} value) CGP 20712A had a partial inhibitory effect with weak potency (Figure 5c and Table 5). The IC_{50} value for ICE 118551 inhibition was similar to the value obtained when lipolysis was stimulated by Iso or NA. The concentration-response curves for inhibition of BRL-induced lipolysis by both antagonists were monophasic. These results suggest strongly that BRL has a specific action on lipolysis through activation of β_3-adrenoceptors only.

Selective β_1-agonist CGP 20712A 10 μM inhibited Dob- and Xam-induced lipolysis powerfully (68% and 95%, respectively) whereas 1 μM ICI 118551 had no significant effect (Table 4). While studying the inhibition of the partial lipolytic response (Dob and Xam at lower concentration) by a full range of antagonist concentrations, the β_1-selective antagonist was found to be more potent than the β_2-selective antagonist (Figure 5d and e; Table 5). The IC_{50} values for CGP 20712A were 10 and 70 times lower than those for ICI 118551 against the Dob- and Xam-induced responses, respectively. These results, and the fact that only one population of sites was implicated in the stimulation of lipolysis by these agonists, indicate a high selectivity of Xam and Dob for the β_1-adrenoceptor subtype and this selectivity seems to be greater for Xam than for Dob.

Selective β_2-agonist The results obtained with Proc were unexpected. The lipolysis induced by Proc (10 μM and 1 μM) was antagonized not only by ICI 118551 but also by CGP 20712A, a β_1-antagonist (Table 4). ICI 118551 1 μM inhibited the lipolysis produced by 1 μM Proc by 59%, whereas this inhibition was 39% with 10 μM Proc. The selective β_1-antagonist suppressed the lipolysis induced by Proc 1 μM and 10 μM by 50% and 28%, respectively. The study of the inhibition of the lipolytic response by a range of concentrations of the antagonists showed that Proc-induced lipolysis was similarly antagonized not only by ICI 118551 but also by CGP 20712A (Figure 5f, Table 5). The heterogeneity of the inhibition curves indicates that at least two populations of sites were involved in the induction of lipolytic effect by Proc. Quantitative determination of percentage of the first population to induce functional response represented 23±6% and 37±2% when CGP 20712A and ICI 118551 were used, respectively. The potency of CGP 20712A and ICI 118551 to inhibit lipolysis was 2500 and 250 times greater for the first sites than for the second sites (Table 5). The CGP 20712A and ICI 118551 IC_{50} values for inhibition of lipolysis *via* the second sites were of the same order as the values obtained to counteract Iso and NA-induced responses for the same population of sites, as well as the values determined with BRL for which only one site was observed. These results suggest that Proc probably induces lipolysis *via* β_1- and β_2-adrenoceptor subtypes as well as *via* β_3-adrenoceptors in light of the functional inhibition by CGP 20712A.

Discussion

Previous studies performed on rat white adipocyte membranes before the existence of the β_3-adrenoceptor was accepted, showed that the binding of [^{3}H]-dihydroalprenolol ([^{3}H]-DHA) yields a Hill slope of <1 and a curvilinear Scatchard plot which is upwardly concave. Giudicelli *et al*. (1979) provided some evidence in favour of the heterogeneity of β-adrenoceptor agonist binding sites on these cells. Further, Bahout & Malbon (1988) showed the existence of two populations of sites with different affinities for [^{3}H]-DHA, including a minor population of β-adrenoceptors of high affinity. To confirm this finding they studied the competition between [^{125}I]-iodocyanopindolol ([^{125}I]-CYP), β-agonists and selective β-antagonists. More recently, Van Liefde *et al*. (1994) also with [^{125}I]-CYP, concluded that two β-adrenoceptor binding sites exist on rat white adipocytes, one with high and the other with low affinity for CGP 12177 (β_1/β_2-antagonist and β_3-partial agonist). The present study provides evidence that the low affinity sites can be detected by saturation studies with the radioligand [^{3}H]-CGP 12177 on rat white adipocytes (Figure 1; Table 1). They represent 90% of the total β-adrenoceptor population and correspond to the β_3-subtype. This subtype mediates predominantly lipolysis in white adipocytes. The population of high affinity [^{3}H]-CGP 12177 binding sites is mainly, if not totally, made up of the β_1-subtype. However, our functional studies suggest that the β_2-adrenoceptors can weakly induce lipolysis in some pharmacological conditions.

The potency order of β-agonists to inhibit [^{3}H]-CGP 12177 binding to high affinity sites was characteristic of the β_1-subtype, Iso>NA>Ad>>BRL (Figure 2; Table 2) (Bojanic & Nahorski, 1983; Bahout & Malbon, 1988). Likewise, the same agents inhibited radioligand binding to the low affinity sites with a potency order corresponding to that of the β_3-subtype: BRL>>Iso≥NA>Ad (Emorine *et al*., 1989; Muzzin *et al*., 1991; Nahmias *et al*., 1991; Liggett, 1992). All of our binding studies are in good agreement with the work of other laboratories. The K_D values of high affinity (0.22±0.07 nM) and low affinity binding sites (27±5 nM) were in the same range as the corresponding K_Ds observed in human and garden dormouse white adipocytes (Revelli *et al*., 1993; Carpéné *et al*., 1994), in homogenates of rat brown adipose tissue (Muzzin *et al*., 1992) as well as in rat brown adipocytes (D'Allaire *et al*., 1995) and in 3T3-F442A adipocytes (Fève *et al*., 1991). Thus, the use of [^{3}H]-

CGP 12177 allows the characterization of the β_3-subtype in rat white adipocytes as effectively as in other adipocyte types.

In our study the functional stimulations exhibited a similar potency order with agonists (BRL > > Iso > NA > Ad) as previously described for rat and garden dormouse white adipocytes, and 3T3-F442A adipocytes (Figure 4; Table 3) (Hollenga *et al*., 1990; Langin *et al*., 1991; Fève *et al*., 1991; Carpéné *et al*., 1993; Bousquet-Mélou *et al*., 1994). Furthermore, the pD_2 values were in the same potency order as those found by the authors cited. In our experiments BRL was highly selective for β_3-adrenoceptors on rat white adipocytes, since the response induced by a high concentration of this agonist was not blunted by 10 μM CGP 20712A (β_1-antagonist) or 1 μM ICI 118551 (β_2-antagonist) (Table 4). In addition, the inhibition curves for both antagonists were monophasic and their activities were low (Figure 5c; Table 5). These observations provide strong evidence that lipolysis is mainly mediated by the β_3-subtype in rat white adipocytes. Consequently, CGP 12177 (β_1- and β_2-antagonist, partial β_3-agonist) induced a partial lipolytic response in these cells as previously described (Hollenga & Zaagsma, 1989; Langin *et al*., 1991; Murphy *et al*., 1993; Van Liefde *et al*., 1993; Bousquet-Mélou *et al*., 1994).

The β_1-subtype was identified, not only by the potency order of BRL and catecholamines, but also by competition experiments conducted with Xam (selective β_1-agonist) at a [^{3}H]-CGP 12177 concentration which labels all β-subtypes (Figure 2c; Table 2). Our data show the presence of a single homogeneous population of high affinity binding sites. The K_i value was similar to those values obtained for human cardiac and guinea pig left atria β_1-adrenoceptors with [^{125}I]-CYP (Malta *et al*., 1985; Brodde *et al*., 1990), and for rat lung β_1-adrenoceptors determined with [^{3}H]-DHA (Cook *et al*., 1984). As Xam and Dob induced lipolysis in rat white adipocytes, the involvement of β_1-adrenoceptors in this biological response is indicated (Figure 4c; Table 3). Accordingly, Xam-induced lipolysis was blocked by the β_1-adrenoceptor-antagonist, CGP 20712A (Table 4). In contrast, ICI 118551 (selective β_2-antagonist) did not counteract the response to Xam. The result obtained with Dob was similar, although the lipolytic response was not completely attenuated by CGP 20712A, suggesting the possible recruitment of other β-adrenoceptor subtypes. It is also possible that Dob, being a selective β_1-agonist *in vivo*, lost this selectivity *in vitro* as described by Minneman *et al*. (1979). The biphasic inhibition of Iso- and NA-induced lipolysis by CGP 20712A showed that the minor population of β-adrenoceptors was counteracted potently by this β_1-antagonist (Figure 5a and b; Table 5). Results from functional studies agree well with those from competition studies; they indicate a minor role for the β_1-subtype in lipolysis activation similar to previous conclusions (Hollenga & Zaagsma, 1989; Van Liefde *et al*., 1992; Murphy *et al*., 1993; Bousquet-Mélou *et al*., 1994).

To evaluate the presence of the β_2-subtype, the inhibition of [^{3}H]-CGP 12177 binding was assessed with the selective β_2-agonist, Proc (Figure 3; Table 2). A single homogeneous population of β-adrenoceptors with low affinity was identified. The density of these sites was similar to that obtained with Xam, a selective β_1-agonist. The Proc K_i value for human cardiac β_1-adrenoceptors was 10.3 μM, whereas for human lung and lymphocyte β_2-receptors this constant was 0.122 μM and 0.079 μM, respectively (Brodde *et al*., 1990), indicating a 100 times greater potency for β_2- than for β_1-receptors. Thus, we believe that Proc competed only with β_1-adrenoceptors in rat white adipocytes and β_2-receptors were not detectable. The same result was obtained on rat brown adipocytes (D'Allaire *et al*., 1995). Bojanic and Nahorski (1983) using [^{125}I]-CYP compared the presence of β_1- and β_2-subtypes on rat whole fat pad and isolated adipocyte membranes. They concluded that, in view of the proportion of β_2-adrenoceptors on whole fat pads (62%) and adipocytes (15%), their presence might reflect contamination from other cell types, especially as the rank order of β-agonist potencies (Iso > NA > Ad) indicated the presence of the β_1-subtype.

Our functional studies suggest that β_2-adrenoceptors could play a minor role in mediating lipolysis since Proc, β_2-agonist, was able to induce the lipolytic response with a potency (2570 nM) close to that (3330 nM) observed by Bousquet-Mélou *et al*. (1994) in the same cells (Figure 4c; Table 3). The potency of Proc in white adipocytes was very low in comparison to the tissues containing high amounts of the β_2-adrenoceptors, approximately 1 nM (Yabuuchi, 1977; Cook *et al*., 1993). In our study, performed on rat white adipocytes, 1 μM ICI 118551 partially inhibited Proc-induced lipolysis, whereas on dog white adipocytes, at the same Proc concentration, the lipolytic response was totally abolished and the Proc EC_{50} value was 62 nM (Table 4) (Galitzky *et al*., 1993). These observations indicate a species difference in the expression of β-adrenoceptor subtypes.

The inhibition curves of Proc-induced lipolysis at a concentration close to the EC_{50} value by both antagonists were biphasic (Figure 5f; Table 5). Lipolysis mediated by the first population of sites represented 23% when CGP 20712A antagonized the response induced by this β_2-agonist as a similar result was obtained when Iso and NA were used as agonists. These data indicate the recruitment of the β_1-subtype in the functional response produced by Proc. The similar inhibition of Proc-, Iso- and NA-induced lipolysis by CGP 20712A *via* the second and major population of β-adrenoceptors suggests the participation of the β_3-subtype in the functional response produced by this β_2-agonist. ICI 118551 inhibited by 37% and with high potency the functional response of Proc mediated by the first population of sites. In contrast, the functional inhibition of the effects of NA and Iso by this antagonist was monophasic with low potency. These data suggest that this β_2-antagonist did not distinguish between β_1- and β_3-adrenoceptors. We conclude that Proc triggered lipolysis by stimulation of the three β-subtypes. All these observations indicate that, in rat white adipocytes, the β_2-subtype represents a very small population of β-adrenoceptors and that in physiological conditions the β_2-subtype is probably not implicated in the stimulation of lipolysis.

In conclusion, the radioligand [^{3}H]-CGP 12177 can be used to characterize two β-adrenoceptor subtypes, β_1 and β_3 on rat white adipocytes, as found for other types of adipocytes. [^{3}H]-CGP 12177 is, therefore, a suitable tool for the exploration of β-subtypes in various physiological or pathological situations, and it has been used to assess the effect of triiodothyronine on rat white adipocytes (Germack *et al*., 1996). Moreover, the three β-adrenoceptor subtypes are functional in rat white adipocytes, whereas the β_2-subtype represents the smallest population of β-adrenoceptors which was not detectable by binding studies. The overall response is essentially dependent on β_1- and β_3-receptors. Finally, the good relationship between functionality and identity of β-subtypes confirmed that lipolysis is mainly driven by the β_3-subtype in rat white adipocytes. We propose that the rank order of expression and capacity to induce lipolysis is $\beta_3 > > \beta_1 > \beta_2$.

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