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Radioligand binding studies of the atypical β_3-adrenergic receptor in rat brown adipose tissue using [^{3}H]CGP 12177

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Two populations of [^{3}H]CGP 12177 binding sites exist in rat interscapular brown adipose tissue (IBAT) plasma membranes. The majority of binding sites are of low affinity with a K_d of 31 nM, a value in close agreement with that for the K_d of [^{3}H]CGP 12177 binding to a cloned rat β_3-adrenergic receptor (AR) expressed in CHO cells (44 nM). Competition binding studies demonstrate that the K_i values of the cloned rat β_3-AR and of the low affinity sites in IBAT are 45 and 29 nM, respectively, for BRL 37344 and 1.4 and 1.0 μM, for (−)-propranolol. These findings strongly suggest that the low affinity [^{3}H]CGP 12177 binding site measured in IBAT plasma membranes represents the atypical β_3-AR in this tissue.

Atypical β-adrenergic receptor; Brown fat; Binding

1. INTRODUCTION

Brown adipose tissue (BAT) is the main effector of cold- and diet-induced thermogenesis in rodents [1,2]. The metabolism of this tissue is primarily regulated by norepinephrine released from sympathetic nerves which acts through β-adrenergic receptors (-AR) [3,4]. Although β_1 and β_2-ARs are present in BAT, considerable evidence has suggested that the β-AR which mediates lipolysis in this tissue displays pharmacological properties inconsistent with the β_1/β_2-AR subclassification scheme [5]. Recently, we isolated and characterized a cDNA from a rat interscapular BAT (IBAT) library which encodes a β_3-AR that displays low affinity for classical β-AR antagonists such as propranolol and alprenolol, and high affinity for thermogenic β-AR agonists such as BRL 37344 [6]. The rank order of agonist potency for stimulation of cAMP accumulation in CHO cells transfected with the rat IBAT β_3-AR correlates with that previously reported for stimulation of lipolysis in rat IBAT [5].

It has been difficult to directly measure the β_3-AR in BAT in radioligand binding studies because the available radiolabeled antagonists display a markedly lower affinity for the β_3-AR than for β_1- and β_2-ARs. It was shown that the large component of non-specific binding of [^{125}I]iodocyanopindolol (ICYP) to BAT plasma membranes at the high concentrations necessary to detect specific binding to the β_3-AR prevented accurate characterization of the binding parameters of this receptor [7,8]. The hydrophilic compound CGP 12177 [9], generally classified as a β-AR antagonist, was recently found to behave as a thermogenic agonist in BAT [10]. Blum-Kaelin et al. [11] used [^{3}H]CGP 12177 to demonstrate the existence of two populations of binding sites in rat IBAT plasma membranes, one with a high affinity which was presumed to represent the β_1/β_2-AR subtypes present in this tissue and one with a low affinity, presumed to represent the β_3-AR.

The aim of this study was to compare the binding characteristics of [^{3}H]CGP 12177 binding to β-ARs in isolated IBAT plasma membranes and in CHO cells transfected with the rat β_3-AR in order to gain more information on the identity of the two populations of [^{3}H]CGP 12177 binding sites described in IBAT plasma membranes. Herein, we describe conditions that allow for the measurement of specific [^{3}H]CGP 12177 binding to the β_3-AR in IBAT.

2. MATERIALS AND METHODS

All organic and inorganic chemicals were of analytical or molecular biology grade. [^{3}H]CGP 12177 (30 Ci/mmol) was purchased from Amersham. CHO-K1 cells were from the American Type Culture Collection. Tissue culture reagents were from Gibco/BRL. BRL 37344 was a gift from Beecham Pharmaceuticals (Epsin, UK). Sprague–Dawley male rats (9 weeks old) were kept at room temperature (about 21°C) with 12 h of illumination per day and fed ad libitum with Provimi Lacta chow (Cossonay, Switzerland). The IBAT plasma membranes were prepared from pools of IBAT from six rats as previously described [12]. Protein concentrations were determined by the method of Lowry et al. [13].

The cloning of the rat cDNA encoding the β_3-AR, its stable expression in CHO-K1 cells and the preparation of CHO-K1 cell membrane fractions have been described [6].

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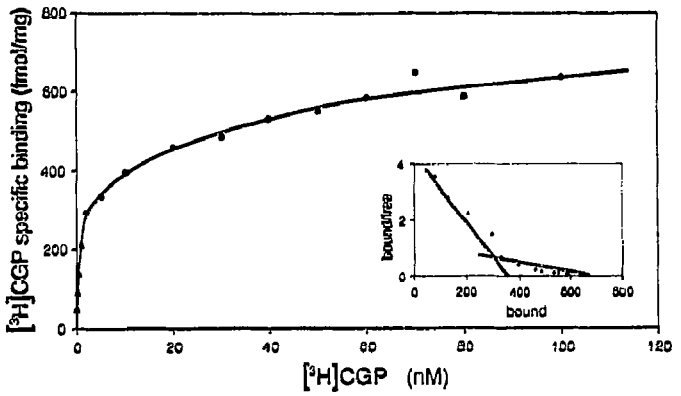

Fig. 1. Specific binding of [³H]CGP 12177 to IBAT plasma membranes as a function of increasing concentrations of the ligand. The results illustrated are from one representative experiment and are expressed in fmol of ligand bound per mg of plasma membrane proteins. (Inset) Scatchard analysis of the data of the representative experiment.

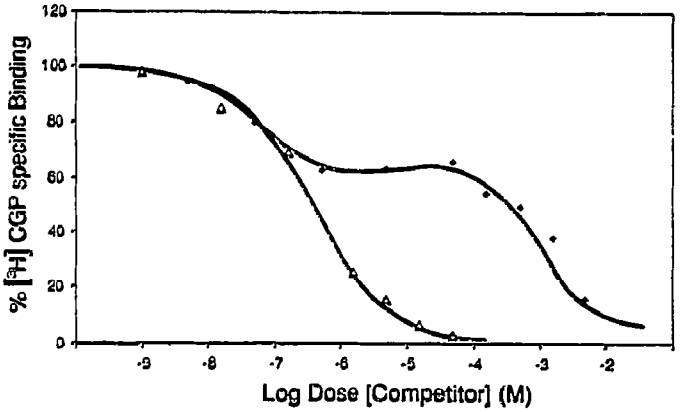

Fig. 2. Displacement of [³H]CGP 12177 (50 nM) binding to IBAT plasma membranes by increasing concentrations of BRL 37344 (+) or (−)-propranolol (▽). The results illustrated are from one representative experiment and are expressed as percent of the specific binding value in the absence of competitor.

In radioligand binding studies, membranes were incubated for 30 min at 37°C in Tris–HCl 50 mM, pH 7.4, $MgCl_2$ 10 mM (total volume 0.5 ml) containing GTP 50 μM and the indicated amount(s) of [³H]CGP 12177. The binding of the radioligand to the membranes was determined by filtration using a Brandel M-24 R apparatus. Specific binding was defined as the difference between the total binding obtained in the absence of competing ligand and the non-specific binding obtained in the presence of (−)-propranolol (100 μM). Each assay was performed in duplicate.

3. RESULTS AND DISCUSSION

Fig. 1 shows the radioligand binding studies performed on rat IBAT plasma membranes. Scatchard analysis of saturation isotherms indicate the presence of two populations of [³H]CGP 12177 binding sites. Their K_d values were found by a non-linear regression analysis using the LIGAND program [14] to be 0.64 and 31 nM (Table I). These values are in excellent agreement with those reported by Blum-Kaelin et al. [11] of 0.42 and 48 nM obtained from similar experiments. The K_d value for [³H]CGP 12177 binding to the rat IBAT β_3-AR in transfected CHO cell membranes was 44 nM (Table I), a value essentially identical to that for the low affinity binding site in IBAT plasma membranes. Our data are consistent with the hypothesis that the population of low affinity binding sites in IBAT represents the β_3-AR whereas the population of high affinity sites represents the β_1/β_2-AR subtypes.

As summarized in Table I, the low affinity [³H]CGP 12177 binding sites in IBAT plasma membranes represent 70% of the total [³H]CGP 12177 binding sites in this tissue. The distribution of low and high affinity sites in IBAT correlates with the relative proportion of β_3-AR- and β_1/β_2-AR-specific mRNA, respectively, in IBAT [6]. Together, these data suggest that the β_3-AR, which displays low affinity for [³H]CGP 12177, represents the predominant β-AR subtype in IBAT.

In order to examine the binding of other β-AR ligands to the IBAT β-ARs, competition studies with [³H]CGP 12177 and BRL 37344 or (−)-propranolol were performed (Fig. 2). K_i values for BRL 37344 and propranolol binding to the high affinity [³H]CGP 12177 sites in IBAT were calculated by an Hofstee analysis using the LIGAND program [14] to be 12.2 and 0.003 μM, respectively (Table II). These values are similar to those reported for the binding of these compounds to β_1- or β_2-ARs [7,11]. The K_i value for propranolol binding to the high affinity [³H]CGP 12177 sites in IBAT is

Table I

[³H]CGP 12177 binding to IBAT and CHO-K1 β_3 cell membranes

	High affinity binding sites		Low affinity binding sites	
	K_d (nM)	B_{max} (fmol per mg)	K_d (nM)	B_{max} (fmol per mg)
IBAT	0.64 ± 0.17 (3)	330 ± 44 (3)	31 ± 9 (3)	770 ± 85 (3)
CHO-K1 β_3	–	–	44 ± 4 (3)	1180 ± 140 (3)

The results are the mean ± S.E.M. of the number of experiments in parentheses. Isolation of cell membranes and assays are described in Materials and Methods. Concentrations of [³H]CGP 12177 used varied from 125 pM to 100 nM. The K_d are expressed in nM and the B_{max} in fmol of [³H]CGP 12177 bound per mg of membrane proteins.

Table II

Displacement of [^{3}H]CGP 12177 binding to IBAT and CHO-K1 β_3 cell membranes by (−)-propranolol and BRL 37344

	High affinity binding sites		Low affinity binding sites	
	K_i of (−)-propranolol (μM)	K_i of BRL 37344 (μM)	K_i of (−)-propranolol (μM)	K_i of BRL 37344 (μM)
IBAT	0.003 ± 0.001 (3)	12.1 ± 3.6 (3)	1.0 ± 0.1 (3)	0.029 ± 0.008 (3)
CHO-K1 β_3	–	–	1.4 ± 1.7 (4)	0.045 ± 0.009 (5)

The results are the mean ± S.E.M. of the number of experiments in parentheses. Isolation of cell membranes and assays are described in Materials and Methods. The concentration of [^{3}H]CGP 12177 used was 50 nM. The results are expressed in inhibition constants (K_i) in μM.

4100-fold lower than that for BRL 37344. In contrast, the K_i of BRL 37344 (0.029 μM) binding to the low affinity [^{3}H]CGP 12177 site in IBAT is 34-fold lower than that for propranolol (1.0 μM). The K_i values for BRL 37344 and propranolol binding to the low affinity [^{3}H]CGP 12177 sites in IBAT membranes are very similar to those obtained in competition experiments with membranes prepared from CHO cells expressing the rat β_3-AR (0.045 and 1.4 μM, respectively). These findings confirm that the low affinity [^{3}H]CGP 12177 binding site in IBAT plasma membranes represents the β_3-AR subtype, whereas the high affinity binding site represents β_1- and β_2-ARs.

In summary, using [^{3}H]CGP 12177 as a radioligand, the β_3-AR subtype of rat IBAT plasma membranes can be directly measured. This can be accomplished by using a concentration of [^{3}H]CGP 12177 equal to the K_d of the β-AR in the absence or presence of a concentration of BRL 37344 which would displace only the binding to the β_3-AR (10 μM). Specific binding to the β_3-AR is then defined as the difference between total binding obtained in the absence of competing ligand and the binding obtained in the presence of BRL 37344. Specific binding to β_1/β_2-AR subtypes in IBAT can be quantitated as the difference between [^{3}H]CGP 12177 in the presence of 10 μM BRL 37344 and in the presence of 100 μM (−)-propranolol, a concentration sufficient to displace [^{3}H]CGP 12177 binding to all β-AR subtypes in IBAT membranes.

It has been suggested that the population of low affinity binding sites in IBAT mediates the metabolic effects of CGP 12177 [10,11]. This would imply that CGP 12177 exerts its effects on IBAT metabolism only via the β_3-AR. More studies are necessary to determine the respective biological importance of β-AR subtypes in the response of BAT to catecholamines.

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