Ligand binding properties of putative β_3 -adrenoceptors compared in brown adipose tissue and in skeletal muscle membranes

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1 The β -adrenoceptor population was characterized in membrane preparations from rat brown adipose tissue (BAT) and from soleus muscle by use of the radioligand [¹²⁵I]-iodocyanopindolol ([¹²⁵I]-ICYP). In addition, atypical binding sites for [¹²⁵I]-ICYP found in both tissues were examined, and the relationship between these sites and the putative rat β_3 -adrenoceptor is discussed.

2 It was established that BAT membranes host a mixed population of β_1 - and β_2 -adrenoceptors. Of these two sites, 55% showed a high affinity for the β_1 -selective ligand CGP 20712A (pK 8.5), and 45% showed a high affinity for the β_2 -selective antagonist ICI 118551 (pK 8.6). Soleus muscle membranes were found to host a population of β_2 -adrenoceptors, characterized by a high affinity for ICI 118551 (pK 9.1), but β_1 -adrenoceptors could not be detected in this preparation. 5-Hydroxytryptamine receptors were not detected in either preparation.

3 In addition to β_1 - and β_2 -adrenoceptors, atypical binding sites were identified in both tissues using high concentrations of radioligand (0.5–0.6 nM) and in the presence of 1 μ M (–)-propranolol. The atypical sites were abundant, representing 80 and 81% of the total [¹²⁵I]-ICYP binding sites in BAT and soleus muscle respectively. When the pK values for 11 ligands were compared, the correlation coefficient for atypical sites in BAT and soleus muscle was 0.94.

4 The atypical binding sites showed a moderate affinity for (±)-cyanopindolol (pK 7.3–7.7), poor stereoselectivity for the (+)- and (–)-enantiomers of alprenolol (<10 fold), and a low affinity for β -adrenoceptor antagonists and partial agonists in the order: (±)-cyanopindolol > (–)-alprenolol > (–)-propranolol = (±)-ICI 118551 > (±)-CGP20712A. The affinity of these ligands for the atypical sites reflects their behaviour in functional studies of putative β_3 -adrenoceptors in rat BAT, white adipose tissue, intestine and colon.

5 The atypical sites labelled by [¹²⁵I]-ICYP were resistant to agonist binding, and while the order of affinity of the agonists BRL 37344 > isoprenaline > noradrenaline matches their order of potency at putative β_3 -adrenoceptors, none of these compounds caused displacement of the radioligand at concentrations below 10 μ M.

6 It is concluded that the atypical binding sites for [¹²⁵I]-ICYP found in rat BAT and soleus muscle membranes are the same, and that these sites show some relationship to the putative rat β_3 -adrenoceptor identified in functional studies using antagonists. However, under the conditions used in the present study, pK values obtained for β_3 -agonist binding are not useful.

Keywords: Brown adipose tissue; skeletal muscle; putative β_3 -adrenoceptors

Introduction

In addition to standard β_1 - and β_2 -adrenoceptors, there is strong evidence for the existence of a third β -adrenoceptor in brown and white adipose tissue (BAT and WAT) (Arch *et al.*, 1984; Hollenga & Zaagsma, 1989). This discovery was based initially on the observation that functional responses of rat BAT and WAT were not typical of those seen by the activation of standard β -adrenoceptors, and was later supported by the identification of human and rat β_3 -adrenoceptor genes (Emorine *et al.*, 1989; Granneman *et al.*, 1991). As a result of this work, novel, selective β_3 -agonists have been developed, and these compounds offer a new therapeutic opportunity for the treatment of obesity (Arch *et al.*, 1991; Holloway *et al.*, 1991). However, while β_3 -agonists may become useful medicinal agents, their success or failure will depend in part on whether they cause any adverse or desirable effects in tissues other than fat. Thus, it would be useful to know the distribution of putative β_3 -adrenoceptors in organs and tissues throughout the body.

There is evidence from studies involving a number of animal species that β_3 -adrenoceptors may not be found exclusively in adipose tissue. In the rat, the strongest evidence emanates from studies of atypical β -adrenoceptors in smooth muscle of the small intestine (Van Der Vliet *et al.*, 1990), colon (Bianchetti & Manara, 1990; McLaughlin & MacDonald, 1990; Kirkham & Kelly, 1992), gastric fundus (McLaughlin & MacDonald, 1991) and oesophagus (Buckner & Christopherson, 1974). Skeletal muscle is a tissue which may host β_3 -adrenoceptors also, and this could be important for two reasons. First, muscle tremor is a common response to β_2 -agonist treatment and is a factor which may limit the usefulness of certain β_3 -agonists as anti-obesity agents (Holloway *et al.*, 1991). This problem has been overcome partly by the development of agents with increased β_3 -/ β_2 -adrenoceptor selectivity (Holloway *et al.*, 1991). However, the identification of β_3 -adrenoceptors in skeletal muscle would suggest that alternative strategies might be needed to limit this unwanted side-effect. The second reason for determining whether skeletal muscle hosts β_3 -adrenoceptors, is to predict whether β_3 -agonists are likely to increase muscle growth. Because of the large amount of water which accompanies each gram of muscle protein deposited, such an effect would have a con-

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siderable impact on the pattern of weight change in an obese individual being treated with these agents.

Compared with other tissues hosting β -adrenoceptors, skeletal muscle has not been studied intensively. Challiss *et al.* (1988) proposed that an atypical β -adrenoceptor may mediate the metabolic responses to a novel β -agonist in rat soleus muscle, while others have identified an atypical binding site in this muscle using autoradiography (Molenaar *et al.*, 1991). Recently it was proposed that this atypical site could be characterized by competitive ligand binding experiments using [125 I]-iodocyanopindolol ([125 I]-ICYP) as the radioligand (Roberts *et al.*, 1991).

The radioligand binding technique offers the ability to screen rapidly many tissues for a given receptor subtype. Studies can be performed on small biopsy samples, and by this method receptors may be quantified and temporal changes in their density can be examined. Unfortunately, previous attempts to characterize the putative β_3 -adrenoceptor using the ligand binding technique have not been as successful as functional experiments. Although [125 I]-ICYP is the radioligand with highest affinity for standard β -adrenoceptors, some workers have suggested that this compound is unsuitable for labelling atypical β -adrenoceptors (Van Der Vliet *et al.*, 1990), while other workers (Ahmad *et al.*, 1990) have described an atypical binding site which we believe could be an enantiomeric artefact caused by the unusually high affinity of (+)[125 I]-ICYP for binding to β_2 -adrenoceptors (Moore *et al.*, 1991). A number of different factors which have led to the incomplete characterization of the β -adrenoceptor population in adipose tissue by ligand binding techniques have been outlined by Arch (1989) and by Zaagsma & Nahorski (1990).

There were three aims of the present study: first, to characterize the β -adrenoceptor populations in rat soleus muscle and in BAT using the ligand binding technique; second, to compare the binding properties of atypical sites found in these two tissues; and third to determine how drug affinity constants obtained for the atypical sites compare with published values for putative rat β_3 -adrenoceptors obtained from functional experiments.

Methods

Preparation of tissue homogenates

Female Wistar rats (200–250 g) were killed by cervical dislocation. Interscapular BAT was removed and the soleus muscles were dissected from each hind limb. Tissue from batches of ten rats was pooled and minced with scissors. The BAT and soleus muscles were homogenized separately; each in 10 ml of ice-cold buffer (50 mM Tris 7.0, 0.25 M sucrose, 1 mM EGTA; pH 7.4 at 4°C) using an Ultra-Turrax tissue homogenizer set at half-maximum speed and run for 10–20 s.

All centrifugations were performed at 4°C. The homogenates were first centrifuged for 10 min at 1,000 g. The pellets were discarded and each supernatant was filtered through a triple layer of surgical gauze. The gauze was washed with a further 2 ml of buffer and the filtrates and washings were centrifuged for 15 min at 10,000 g. Next, the supernatants were transferred to ultracentrifuge tubes and centrifuged for a further 30 min at 100,000 g. The supernatants were discarded and the pellets were each resuspended in 3 ml of ice-cold buffer (50 mM Tris 7.0, 5 mM MgCl₂, 1 mM EGTA; pH 7.4 at 4°C). After a further centrifugation again at 100,000 g for 30 min, the supernatants were discarded and the pellets were each resuspended in 1 ml of ice-cold Tris assay buffer (50 mM Tris 7.7, 10 mM MgCl₂, 0.15 M NaCl; pH 7.4 at 37°C) and stored at –80°C. Membrane protein concentration was determined by the Bradford Protein Assay (Bio-Rad, Richmond, U.S.A.) with bovine serum albumin standards.

Radioligand binding assays and data analysis

The same procedure was applied to BAT and soleus muscle membranes. Assays were performed in triplicate by incubating 100 μ l of cell membrane suspension (incubation concentration 0.1–0.2 mg protein ml^{–1}) with 50 μ l [125 I]-ICYP, 50 μ l of GppNHp (incubation concentration 100 μ M) and 50 μ l of various drugs, in polystyrene tubes (12 mm $\times$ 75 mm). Assays were initiated by the addition of cell membranes, and tubes were incubated for 90 min in a shaking water bath set at 37°C and 120 cycles min^{–1}. All components of the assay were in Tris assay buffer. Ascorbic acid (2.8 mM) was added to the Tris buffer when catecholamines were used in the assay to reduce the rate of oxidation of these ligands. Separation of bound from free radioligand was achieved by filtering through Whatman GF-C glass fibre filter papers using a Brandel cell harvester (Beckman Instruments, Brisbane, Australia). Each tube was rinsed 5 times with 5 ml ice-cold Tris assay buffer. Radioactivity remaining on the filters was determined with a Gammamaster gamma counter (Linbrook, Brisbane, Australia) at a counting efficiency of 68.3%.

For saturation studies, tubes were incubated with [125 I]-ICYP at increasing concentrations. A range of concentrations from 1–200 pM was used to determine the K_D value for binding to standard β_1 - and β_2 -adrenoceptors, with nonspecific binding fixed at the level defined by 1 μ M (–)-propranolol. The K_D value for the binding of [125 I]-ICYP to atypical sites was determined using a radioligand concentration range of 5–2500 pM, with nonspecific binding defined by 0.5 mM (–)-propranolol. Specific binding to both standard and atypical sites increased linearly over the protein concentration range 0.04–0.3 mg protein ml^{–1}. Results were analysed using the Ligand computer programme (Munson & Rodbard, 1980).

Competition studies were performed with 0.5–0.6 nM [125 I]-ICYP. From the results of a preliminary competition study (Figure 1a), 0.5 mM (–)-propranolol was selected for use in obtaining an initial estimate of non-specific binding, while 1 μ M (–)-propranolol was used to estimate binding not accounted for by β_1 - or β_2 -adrenoceptors. Final estimates for non-specific binding, for binding site density (B_{max}) and pK values, were obtained using the Ligand programme, allowing each parameter to float. A comparison of pK values obtained for the atypical binding sites in BAT and soleus muscle was made with the Spearman Rank Correlation Test.

Drugs

(–)-Isoprenaline, ($\pm$)-pindolol, (–)-propranolol hydrochloride, 5-hydroxytryptamine hydrochloride and all buffer salts were purchased from Sigma Chemical Co (St. Louis, U.S.A.). Bovine serum albumin was from Armour (Kanakee, U.S.A.). (–)-[125 I]-iodocyanopindolol was from Dupont (North Ryde, Australia). Guanylylimidodiphosphate (GppNHp) was from Boehringer Mannheim (New Farm, Australia). Alprenolol hydrogen tartrate ((+) and (–)-enantiomers) was purchased from Labkemi (Tyresol, Sweden). The following drugs were gratefully received as gifts: ($\pm$)-cyanopindolol (Sandoz, Basle, Switzerland); CGP20712A (1-[2-((3-carbamoyl-4-hydroxy)-phenoxy)ethylamino]-3-[4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy]-propan-2-ol) Ciba Geigy, Basel, Switzerland); ICI 118551 (erythro-1-(7-methylindan-4-yloxy)-3-(isopropylamino)-butan-2-ol) (ICI, Macclesfield, England); BRL 37344 (4-[2-[(2-hydroxy-2-(3-chlorophenyl)ethyl)amino]-propyl]-phenoxyacetic acid) (SmithKline Beecham, Epsom, England); (–)-noradrenaline bitartrate and (–)-adrenaline bitartrate (Sterling Winthrop, Rensselaer, U.S.A.).

Results

Brown adipose tissue membranes

Saturation studies using BAT cell membranes revealed the presence of two binding sites for [125 I]-ICYP. A high-affinity/

Figure 1 Representative curves showing the displacement of [125 I]-iodocyanopindolol ([125 I]-ICYP) (0.5–0.6 nM) from brown adipose tissue membranes by various competing ligands. Upper and lower horizontal lines show [125 I]-ICYP binding in the presence of 1 μ M and 0.5 mM (–)-propranolol respectively. Competing ligands were: (a) (–)-propranolol; (b) ICI 118551 (○) and CGP20712A (●); (c) cyanopindolol (○) and pindolol (●); (d) (+)-alprenolol (○) and BRL 37344 (●).

low-density site was observed (pK 10.9 ± 0.04 , B_{max} 109 ± 12 fmol mg^{-1} protein) as well as a lower-affinity/high-density site (pK 8.77 ± 0.11 , B_{max} 430 ± 61 fmol mg^{-1} protein) (Figure 2).

When competition studies were performed, the high affinity/low-density [125 I]ICYP binding site in BAT behaved in a manner characteristic of a mixed population of β_1 - and β_2 -adrenoceptors. The ligands (–)-propranolol (pK 8.69 ± 0.03), cyanopindolol (pK 10.14 ± 0.20), and pindolol (pK 8.41 ± 0.07) could not distinguish between the two adrenoceptors, but their presence was revealed with the ligands CGP 20712A and ICI 118551 (Figure 1b) which are selective for β_1 - and β_2 -adrenoceptors respectively. CGP 20712A bound to the larger population of β_1 -adrenoceptors with a high affinity (pK 8.47 ± 0.18) and to the smaller population of β_2 -adrenoceptors with a lower affinity (pK 5.45 ± 0.12). Conversely, ICI 118551 bound to β_2 -adrenoceptors with a high affinity (pK 8.58 ± 0.09) and to β_1 -adrenoceptors with a lower affinity (pK 5.79 ± 0.23). ICI 118551 was among the compounds which revealed the presence of a third binding site in BAT, and because of the dominance of this third site it was difficult to assess accurately the relative proportions of β_1 - and β_2 -adrenoceptors present. Nevertheless, the mean proportion estimated from six competition studies using ICI 118551 and CGP 20712A was 55% β_1 -adrenoceptors, 45% β_2 -adrenoceptors. Competition experiments using 5-hydroxytryptamine (5-HT) failed to reveal any significant displacement of radioligand at concentrations up to 12 μ M, indicating the absence of 5-HT binding sites.

In addition to standard β -adrenoceptors, the presence of a high density atypical binding site in BAT was apparent when (–)-propranolol and pindolol were used as the competing ligands. Displacement curves for these compounds were clearly bi-phasic (Figure 1a,c) and were best described by models having two binding sites ($P < 0.01$). Cyanopindolol and ICI 118551 produced shallow displacement curves (Figure 1b,c) which, although not distinctly bi-phasic, were best described by models of two binding sites also ($P < 0.001$). Other ligands such as (+)-alprenolol and BRL 37344 showed little selectivity between the standard β -adrenoceptors and the atypical binding site, and produced displacement curves which were steep and monophasic (Figure 1d). However, when these data are modelled, the Ligand programme calculated a level of non-specific binding which was much

lower than that defined by 1 μ M (–)-propranolol, but similar to that defined by 0.5 mM (–)-propranolol. In order to determine the affinity constants of (+)-alprenolol, BRL 37344, and other ligands not selective against the atypical site, displacement experiments were performed in the presence of 1 μ M (–)-propranolol to block [125 I]-ICYP binding to β_1 - and β_2 -adrenoceptors. The pK values for the binding of all ligands tested to the high density atypical site are presented in Table 1.

Skeletal muscle membranes

Saturation studies using soleus muscle membranes produced results very similar to those described above for BAT. Binding parameters for [125 I]-ICYP to the high affinity/low density site, and to the low affinity/high density atypical site were pK 11.01 ± 0.02 , B_{max} 48 ± 11 fmol mg^{-1} protein and pK 9.16 ± 0.30 , B_{max} 203 ± 70 fmol mg^{-1} protein respectively (Figure 2).

In contrast to BAT, the high affinity/low density in soleus muscle behaved in a manner consistent with a homogeneous population of β_2 -adrenoceptors. Binding sites which could be saturated with 1 μ M (–)-propranolol (pK 8.12 ± 0.10) were occupied by the β_2 -adrenoceptor-selective ligand ICI 118551 with similarly high affinity (pK 9.09 ± 0.25). Displacement studies using the β_1 -selective antagonist, CGP20712A, failed to reveal any binding sites with high affinity for this ligand, consistent with the absence of β_1 -adrenoceptors from the preparation. No displacement of the radioligand was observed by 5-HT at concentrations up to 1 mM, consistent with the absence of labelled 5-HT binding sites.

As with BAT membranes, the presence of high-density atypical binding site in soleus muscle was identified with (–)-propranolol, and was confirmed using the ligands cyanopindolol, pindolol and ICI 118551. All of these ligands yielded displacement curves which were best described by models having two binding sites ($P < 0.05$). The pK values for other ligands were determined in the presence of 1 μ M (–)-propranolol, and the results for all ligands tested are presented in Table 1.

Correlations

When the pK values for all ligands were compared, results obtained for the atypical binding site in soleus muscle correlated well with those obtained in BAT ($r = 0.944$, $P < 0.01$, $n = 11$). A plot of these data is shown in Figure 3. Because the true affinity of β_3 -adrenoceptor agonists might not be represented under the experimental conditions used, the data were also analysed after excluding the pK values for the compounds BRL 37344, isoprenaline, adrenaline and noradrenaline. A significant correlation between BAT and soleus muscle pK values was still observed ($r = 0.905$, $P < 0.05$, $n = 8$).

Table 1 Dissociation constants for atypical ligand binding sites in brown adipose tissue (BAT) and soleus muscle membranes

Ligand	BAT	pK^a Soleus muscle
(–)-[125 I]-iodocyanopindolol	8.77 ± 0.11	9.16 ± 0.30
(±)-Cyanopindolol	7.66 ± 0.19	7.33 ± 0.04
(±)-Pindolol	3.94 ± 0.09	4.03 ± 0.22
(–)-Propranolol	4.93 ± 0.10	5.00 ± 0.18
(±)-ICI 118551	5.00 ± 0.10	5.05 ± 0.09
(±)-GCGP 20712A ^b	< 4.00	< 4.00
(–)-Alprenolol ^b	5.37 ± 0.21	5.83 ± 0.04
(+)-Alprenolol ^b	5.02 ± 0.19	5.45 ± 0.21
(±)-BRL 37344 ^b	3.87 ± 0.03	4.19 ± 0.09
(–)-Isoprenaline ^b	3.14 ± 0.16	2.32 ± 0.37
(–)-Adrenaline ^b	2.20 ± 0.07	2.61 ± 0.37
(–)-Noradrenaline ^b	1.99 ± 0.03	2.09 ± 0.07

^aNegative log of the dissociation constant.

^bLigands tested in the presence of 1 μ M (–)-propranolol. Values are means $\pm$ s.e.mean ($n = 3$).

Figure 2 Specific binding of [¹²⁵I]-iodocyanopindolol ([¹²⁵I]-ICYP) to brown adipose tissue (a,c,e) and soleus muscle (b,d,f) membranes studied under different experimental conditions. (a,b) Biphasic saturation curves from preliminary experiments using low concentrations of radioligand with non-specific binding defined by use of 0.5 mM (–)-propranolol. Inset shows Scatchard analysis of these data with regression lines fitted to the two distinct binding sites. (c,d) Typical saturation studies used to determine the affinity of [¹²⁵I]-ICYP for β_1/β_2 -adrenoceptors using low concentrations of radioligand with non-specific binding defined by use of 1 μ M (–)-propranolol. Inset shows a single binding site by Scatchard analysis. (e,f) Typical saturation studies used to determine the affinity of [¹²⁵I]-ICYP for atypical binding sites using high concentrations of radioligand with non-specific binding defined by use of 0.5 mM (–)-propranolol. Inset shows Scatchard analysis.

Discussion

The results obtained in the present study for the identification of standard β -adrenoceptors in soleus muscle and BAT agree with those of earlier reports. Rat skeletal muscle is a well-known source of β_2 -adrenoceptors (Williams *et al.*, 1984; Rothwell *et al.*, 1987; Kim *et al.*, 1991), and some workers have suggested that soleus muscle hosts a minor population of β_1 -adrenoceptors (Rothwell *et al.*, 1987; Kim *et al.*, 1991). However, as in a previous study (Sillence *et al.*, 1991), we failed to detect any β_1 -adrenoceptors in this tissue.

By using the highly sensitive technique of autoradiography, Molenaar *et al.* (1991) found that [¹²⁵I]-ICYP labels a small number of 5-HT receptors in soleus muscle; however, these workers were unable to detect labelled 5-HT binding sites in competitive ligand binding experiments (R.J. Summers, personal communication) and the results of the present study confirm this latter observation. The present observation that BAT hosts a mixed population of β_1 - or β_2 -adrenoceptors also agrees with a number of earlier findings (for review see Arch, 1989), and the proportion of these two sites observed in the present study is in particularly close agreement with

Figure 3 The relationship between atypical [125 I]-iodocyanpindolol binding sites in soleus muscle and brown adipose tissue. Values for pK (negative log dissociation constant) were obtained for eleven compounds from competitive ligand binding experiments.

the proportion of 59% β_1 - and 41% β_2 -adrenoceptors reported by Rothwell *et al.* (1985).

Previous reports of atypical binding sites being present in rat skeletal muscle are relatively few (Moore *et al.*, 1990; 1991; Kim *et al.*, 1991; Roberts *et al.*, 1991). Furthermore, when Molenaar *et al.* (1991) reported the localization of a propranolol-resistant [125 I]-ICYP binding site in soleus muscle, they found certain discrepancies between their autoradiography data and pA_2 values reported in the literature for the atypical receptor in rat adipocytes. This prompted Molenaar *et al.* (1991) to suggest that a family of atypical receptors may exist rather than a single β_3 -adrenoceptor. To address this question we have made a direct comparison between the atypical [125 I]-ICYP binding sites present in soleus muscle and those in BAT. The strong correlation of binding affinities observed for the 11 ligands tested supports the conclusion that the atypical binding sites in these two tissues are the same. Furthermore, the high density atypical binding sites in both tissues ($\sim 80\%$ of the total β -adrenoceptor population) agrees with the results of binding studies conducted recently by Fève *et al.* (1991) who reported that β_3 -adrenoceptors form about 90% of the total β -adrenoceptor population in cultured 3T3-F442A adipocytes.

The final aim of this study was to determine the relationship between the atypical binding sites observed in BAT and soleus muscle and those putative β_3 -adrenoceptors thought to be present in rat adipose tissue and gut from the results of functional studies. When comparing ligand binding data with the results of functional experiments, comparisons made using antagonists provide the most useful information, and this has been noted particularly for the β_3 -adrenoceptor (Arch, 1989). The results published previously for antagonists and for partial agonists of the rat β_3 -adrenoceptor show some relationship to those obtained in the present study in terms of stereoselectivity, order of potency and in absolute pK values.

The low stereoselectivity observed for alprenolol binding in the present study is consistent with the suggestion of Harms *et al.* (1977) that low stereoselectivity for standard β -adrenoceptor antagonists is a characteristic of atypical rat β -adrenoceptors. While this feature is useful to distinguish atypical β -adrenoceptors from standard β -adrenoceptors, it also increases the difficulty for workers using ligand binding techniques to discriminate between authentic β_3 -adrenoceptors and nonspecific lipophilic binding sites. Indeed, when two inde-

pendent groups observed similar atypical binding sites in smooth muscle, the observed lack of stereoselectivity for propranolol caused these workers to reject the hypothesis that these sites were related to β -adrenoceptors (Blue *et al.*, 1990; Van Der Vliet *et al.*, 1990).

If differences in pK values of less than 0.1 log units are ignored, then the order of affinity observed in the present study from the binding of partial agonists and antagonists to atypical sites is: cyanopindolol > (-)-alprenolol > (-)-propranolol = ICI 118551 >> CGP20712A. This order is similar to that obtained in functional studies of the β_3 -adrenoceptor when different combinations of these drugs were tested in rat WAT (Hollenga & Zaagsma, 1989; Kirkham & Kelly, 1992), colon (Kirkham & Kelly, 1992) and BAT (Arch *et al.*, 1984), guinea-pig ileum (Blue *et al.*, 1990) and cultured adipocytes (Fève *et al.*, 1991).

When comparing ligand binding data with pK values obtained from functional studies it is important to consider the experimental conditions used, and in particular to be aware of the possible contribution of multiple receptors to the response being studied. For example, the pK value observed for (-)-propranolol in the present work agrees with the value of 5.0 reported by Roberts *et al.* (1991) using ligand binding techniques. However, this value is lower than published values from functional studies against isoprenaline, which range from 6.1 in rat small intestine (Van Der Vliet *et al.*, 1990) to 7.3 for BAT lipolysis (Arch *et al.*, 1984). We suggest that the latter pK values are over-estimates which reflect the contribution of both β_1 - and β_3 -adrenoceptors to the functional response. This hypothesis is supported by the finding that when isoprenaline is replaced by an agonist more selective for β_3 -adrenoceptors, a fall in the pK value for propranolol of between 0.4 and 0.5 log units is seen in WAT (Wilson *et al.*, 1984) and BAT (Arch *et al.*, 1984).

In contrast to the results obtained with propranolol, studies in adipose tissue which have employed ICI 118551 against isoprenaline probably give a more accurate estimate of the antagonist pK value because β_2 -adrenoceptors do not contribute to the functional response, and because ICI 118551 has a relatively low affinity for β_1 -adrenoceptors. This was illustrated in WAT when a difference in the pK values for ICI 118551 of less than 0.2 log units was observed when isoprenaline was replaced by the β_3 -selective agonist BRL 37344 (Hollenga & Zaagsma, 1989). The pK values observed for ICI 118551 in the latter study (5.33), and in a similar study using BAT (5.7) (Arch *et al.*, 1984) are acceptably close to those of the present work. The third antagonist for which there are published data from functional studies is ($\pm$)-cyanopindolol. In 1992, Kirkham & Kelly reported the results of a study of both WAT lipolysis and colon relaxation. These workers used isoprenaline as the agonist, but carried out their experiments in the presence of 0.1 μM propranolol to attenuate the contribution of β_1 - and β_2 -adrenoceptors to the response. The pK values for cyanopindolol obtained by Kirkham & Kelly (1992) for lipolysis (7.5) and colon relaxation (7.0) agree well with the results of the present study.

Data for agonist binding obtained in the present study are difficult to interpret. It is a concern that extremely high drug concentrations were required to displace the radioligand in the case of all agonists tested, and these data suggest the possibility that the atypical binding sites observed in the present study are purely lipophilic sites, unrelated to atypical β -adrenoceptors. An alternative explanation is that antagonists and agonists may bind differently to the β_3 -adrenoceptor, and that the active site for agonists was not occupied by the radioligand under the conditions we employed. It has been argued that the only meaningful pK values for agonists are those obtained for an induced high-affinity state, whereas under binding conditions which employ GppNHp and high concentrations of Na^+ ions, low affinity states of the receptor are strongly favoured (Molenaar *et al.*, 1991). In this regard it is noteworthy that Fève *et al.* (1991), who conducted

binding studies under slightly different conditions, using 3T3-F422A adipocytes and Chinese hamster ovary (CHO) cells transfected with the human β_3 -adrenoceptor gene, observed pK values for the agonists BRL 37344, isoprenaline and noradrenaline in the same rank order as that seen in the present study, but which were higher by two orders of magnitude. In contrast, the pK values observed by Fève *et al.* (1991) for the binding of [125 I]-ICYP to adipocytes (8.82) and β_3 -CHO cells (8.72) are strikingly similar to the pK values for this antagonist seen in the present study. It is also of interest that Fève *et al.* (1991) observed binding of similarly high affinity to both tissues by the hydrophilic radioligand (–)-[3 H]-CGP12177. Thus, although agonist pK values seen in the present work do not support the hypothesis that the atypical binding site is the putative β_3 -adrenoceptor, it would seem unwise to reject this possibility based on agonist data alone, when the data obtained with a range of antagonists lend the hypothesis such strong support.

In summary, ligand binding studies have shown that mem-

branes from rat BAT and soleus muscle host identical binding sites which are present in abundance in each tissue. From antagonist and partial agonist binding data these sites show a resemblance to the rat β_3 -adrenoceptor described by previous workers. If it can be confirmed that these sites are indeed β_3 -adrenoceptors, then other tissues could be screened for these receptors using the binding conditions described, to help predict the effects of β_3 -agonists *in vivo*. In addition, rat cell membranes might provide a useful primary screen for new β_3 -adrenoceptor antagonist drugs. However, we suggest that this system is not useful to obtain valid data for the binding of β_3 -agonists.

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