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Classical and atypical binding sites for β-adrenoceptor ligands and activation of adenylyl cyclase in bovine skeletal muscle and adipose tissue membranes

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1 The radioligand [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) was used under standard ligand binding conditions, to detect β_1- and β_2-adrenoceptors in membrane preparations from bovine skeletal muscle and adipose tissue. High concentrations of [^{125}I]-ICYP were also used, to identify an 'atypical' binding site in skeletal muscle. Finally, adenosine 3':5'-cyclic monophosphate (cyclic AMP) production was measured in the same membrane preparations, to determine the relationship between the β-adrenoceptor sub-types present and the production of this second-messenger.

2 According to the results of radioligand binding studies, both skeletal muscle and adipose tissue membranes have β_2-adrenoceptors, characterized by a high affinity for the β_2-selective antagonist, ICI 118551 (pK 8.3 and 8.6 respectively); and a low affinity for the β_1-selective antagonist CGP 20712A (pK 5.2 in both tissues). Antagonism of (−)-isoprenaline-stimulated cyclic AMP production by low concentrations of ICI 118551, yielded pseudo pA_2 values in muscle and adipose tissue of 7.6 and 8.7 respectively, confirming that β_2-adrenoceptors in these tissues are linked to the production of the second-messenger.

3 Although β_1-adrenoceptors could not be detected in either skeletal muscle or adipose tissue membranes by use of ligand binding techniques, high pseudo pA_2 values were obtained (8.0 and 8.2 respectively), when CGP 20712A was used to block the stimulation of cyclic AMP production by (−)-isoprenaline. This finding is consistent with the presence in both tissues of a population of β_1-adrenoceptors which is small, but efficiently coupled to the second-messenger.

4 In addition to identifying standard β_1- and β_2-adrenoceptors, it was also established that skeletal muscle membranes have an 'atypical' binding site which has a relatively low affinity for [^{125}I]-ICYP (pK 8.84), but which exists in abundance. At high concentrations of radioligand, the 'atypical' site accounted for 89% of the total [^{125}I]-ICYP binding sites present.

5 The results of second-messenger studies do not support the hypothesis that skeletal muscle or adipose tissue membranes contain functional β_3-adrenoceptors: based on the failure of a β_3-adrenoceptor-selective agonist (BRL 37344) to stimulate cyclic AMP production, the absence of a biphasic response to (−)-isoprenaline, and the observation that cyclic AMP production was not resistant to blockade by either ICI 118551 or CGP 20712A.

6 It is concluded that data from radioligand binding studies do not accurately reflect the contribution made by β_1- and β_2-adrenoceptors to cyclic AMP production in bovine skeletal muscle and adipose tissue membranes. Furthermore, the 'atypical' [^{125}I]-ICYP binding site identified in bovine skeletal muscle does not represent a functional bovine β_3-adrenoceptor.

Keywords: cyclic AMP; skeletal muscle; adipose tissue; β-adrenoceptor ligands; β_1 and β_2-adrenoceptors

Introduction

The role of classical β-adrenoceptors in controlling skeletal muscle and adipose tissue metabolism was thought to be well understood. There is a perception that in skeletal muscle, the β_2-adrenoceptor is the only β-adrenoceptor sub-type present (Ijzerman *et al.*, 1984; Liggett *et al.*, 1988; Disatnik *et al.*, 1990); whereas in adipose tissue, β_1-adrenoceptors are believed to govern lipolysis, with an additional contribution from β_2-adrenoceptors in some species (Arner, 1992). This perception is now changing.

Following the discovery that functional adrenoceptors of the putative β_3-sub-type are present in rat adipose tissue (Arch *et al.*, 1984; Wilson *et al.*, 1984), the control of lipolysis by β-adrenoceptors has been re-examined in a number of different laboratory animal species. A few comparative studies have been conducted on human adipose tissue (Hollenga *et al.*, 1991a; Langin *et al.*, 1991), but to our knowledge there have been no previous studies using adipose tissue from domestic animals.

Although most studies of putative β_3-adrenoceptors have focussed on adipose tissue, there is also some evidence that β_3-adrenoceptors may be found in skeletal muscle. Three years after a report that a propranolol-resistant, 'atypical' β-adrenoceptor might be involved in the control of glycogen synthesis in rat soleus muscle (Challiss *et al.*, 1988), a propranolol-resistant 'atypical' binding site for the radioligand [^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) was identified in the same tissue (Molenaar *et al.*, 1991). Subsequently, this site was shown to be identical to a putative β_3-adrenoceptor binding site found in rat brown adipose tissue (BAT) (Sillence *et al.*, 1993), and to display a strong resemblance to the cloned human β_3-adrenoceptor expressed in Chinese hamster ovary cells (Fève *et al.*, 1991). However, low pK values observed for the binding of β-adrenoceptor agonists to the 'atypical' site, caused uncertainty about whether the binding site was indeed an authentic β_3-adrenoceptor (Sillence *et al.*, 1993).

Establishing whether or not skeletal muscle contains putative β_3-adrenoceptors might help to improve our understanding of the control of skeletal muscle growth, and could be particularly important in relation to the clinical use of β_3-

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adrenoceptor-selective drugs. So far, the principal therapeutic benefit derived from using these compounds, seems to be an improvement in glucose tolerance, seen in patients suffering from non-insulin-dependent diabetes. This effect is thought to be mediated by an increase in the sensitivity of skeletal muscle to insulin-stimulated glucose uptake (Smith *et al.*, 1990).

In the present study, our aim was to examine the β-adrenoceptor population found in bovine skeletal muscle. By using both conventional and modified radioligand binding techniques, we aimed to identify both classical and 'atypical' β-adrenoceptor binding sites, and then determine which of the receptor sub-types present are linked to the production of the second-messenger adenosine 3':5'-cyclic monophosphate (cyclic AMP). The responsiveness of skeletal muscle membranes to a β_3-adrenoceptor-selective agonist was examined, as well as to (−)-isoprenaline, in the presence and absence of β_1- and β_2-adrenoceptor-selective antagonists. For comparative purposes, we report the results of similar experiments on bovine adipose tissue.

Methods

Preparation of tissue homogenates

Four young brahman (*Bos indicus*) heifers (150 kg) were stunned with a captive-bolt pistol, then exsanguinated. Samples (2 g) longissimus dorsi muscle and peri-renal white adipose tissue were quickly removed and placed in buffer (50 mM Tris 7.0, 0.25 M sucrose, 1 mM EGTA; pH 7.4 at 4°C) which was either ice cold (for skeletal muscle) or at room temperature (for adipose tissue). The tissues were minced with scissors, then homogenized separately; each in 10 ml of buffer with an Ultra-Turrax tissue homogenizer set at half-maximum speed and run for 10–20 s.

Cell membrane fragments were prepared by centrifugation at 4°C as described by Sillence *et al.* (1993). Briefly, the homogenates were centrifuged for 10 min at 1,000 *g*. The supernatants were filtered through gauze, then centrifuged for a further 15 min at 10,000 *g*. Next, the supernatants were centrifuged for 30 min at 100,000 *g* and the pellets obtained from this centrifugation were resuspended in 3 ml of ice-cold buffer (50 mM Tris 7.0, 5 mM $MgCl_2$, 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 stored dry at − 80°C. Cell membrane fragments prepared for adenylyl cyclase assays were used within 1 week of preparation, whereas those prepared for ligand binding assays were used within 4 weeks.

Radioligand binding assays

The same procedure was applied to adipose tissue and muscle membranes and was identical to that described previously for the characterization of rat brown adipose tissue and soleus muscle (Sillence *et al.*, 1993). Frozen cell membrane pellets were thawed, pooled and resuspended in 5 ml of assay buffer (50 mM Tris 7.7, 10 mM $MgCl_2$, 0.15 M NaCl; pH 7.4 at 37°C) using three strokes of the tissue homogenizer. Protein concentration was determined by the Bradford Protein Assay (Bio-Rad, Richmond, U.S.A.) with bovine serum albumin standards. The membrane suspension was diluted to a working strength of 0.1 mg protein ml^{-1} (incubation concentration 0.04 mg protein ml^{-1}) after preliminary experiments had shown that binding of the radioligand to both standard and atypical sites was linear over the protein concentration range of 0.05 to 0.3 mg protein ml^{-1} (working strength).

Assays were performed in triplicate by incubating 100 μl cell membrane suspension with 50 μl [^{125}I]-ICYP, 50 μl guanylylimidodiphosphate (GppNHp) and 50 μl of various drugs, in polystyrene tubes (12 mm × 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 (final concentration 2.8 mM) was added to the Tris buffer when isoprenaline was used in the assay. 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). Radioactivity remaining on the filters was determined in a Gammamaster gamma counter (Linbrook, Brisbane, Australia) at a counting efficiency of 68.3%.

To determine a p*K* value for the binding of [^{125}I]-ICYP to standard β_1- and β_2-adrenoceptors, a range of [^{125}I]-ICYP concentrations from 1 to 250 pM was used, and nonspecific binding was fixed at the level defined by 1 μM (−)-propranolol. The p*K* value for the binding of [^{125}I]-ICYP to putative β_3-adrenoceptors was determined using a radioligand concentration range of 5 to 2,000 pM, with nonspecific binding defined by 0.5 mM (−)-propranolol. Results were analysed by the Ligand computer programme (Munson & Rodbard, 1980).

Competition studies were performed under three sets of conditions, as described by Sillence *et al.* (1993). To characterize binding to β_1- and β_2-adrenoceptors, low concentrations of radioligand (30 pM) were used, and nonspecific binding was fixed at the level defined by 1 μM (−)-propranolol. To examine the binding of ligands to putative β_3-adrenoceptors, higher concentrations of radioligand were used (300 pM). In this case, nonspecific binding was defined using the Ligand curve-fitting programme, allowing this paramater to float about an initial estimate obtained in the presence of 0.5 mM (−)-propranolol. For ligands which were selective between standard adrenoceptors and putative β_3-adrenoceptors, p*K* values were obtained from multiple-site binding models generated by Ligand. A third set of binding conditions was necessary to characterize certain drugs which did not discriminate between standard β-adrenoceptors and putative β_3-adrenoceptors. These were the compounds which described a level of nonspecific binding similar to that defined by 0.5 mM (−)-propranolol, but gave displacement curves which were best described by one-site binding models. To determine p*K* values for these ligands at putative β_3-adrenoceptors, competition studies were repeated using 300 pM [^{125}I]-ICYP, but with all tubes containing 1 μM (−)-propranolol to block binding of the radioligand to β_1- and β_2-adrenoceptors.

Cyclic AMP assays

Frozen cell membranes were thawed, pooled and resuspended in 1 ml buffer (50 mM Tris 7.0, 5 mM $MgCl_2$, 1 mM EGTA; pH 7.0 at 25°C) using the tissue homogenizer. Following protein determination, the membrane suspension was diluted to a concentration of 0.6 mg protein ml^{-1}, and left at room temperature for approximately 30 min in order to prevent a lag-phase in cyclic AMP production, which would otherwise occur at the beginning of the incubation. Drugs were prepared in a buffer containing 50 mM Tris 7.7 and 100 μM ascorbic acid (pH 7.4 at 37°C). A third buffer was made immediately before the incubation, and contained: 50 mM Tris 7.7; 10 mM $MgCl_2$; 2 mM EGTA; 2 mM isobutylmethylxanthine; 0.4 mg bacitracin ml^{-1}; 6 mg bovine serum albumin ml^{-1}; 40 nM phosphocreatine, 2 mM ATP; 0.2 mM GTP; and 0.6 mg creatine phosphokinase ml^{-1}. A small volume (1 ml) of the latter 'incubation buffer' was reserved for the addition of 1.7 mg NaF.

All incubations were performed in triplicate. At the beginning of the incubation period 50 μl of incubation buffer was placed in 12 mm × 75 mm glass tubes along with 25 μl of drug solution. The incubation was initiated by the addition of 25 μl of membrane suspension and was carried out for 10 min in a shaking water bath set at 30°C. To determine the basal level of adenylyl cyclase activity, one set of tubes contained 25 μl drug-dilution buffer with no drug added. To determine maximum adenylyl cyclase activity, a second set of

tubes contained 50 μl NaF-incubation buffer, instead of standard incubation buffer.

At the end of the incubation period, the tubes were placed in a bath of boiling water for 4 min. Next, they were transferred to an ice-cold water bath and allowed to cool. The extraction of cyclic AMP from the samples was performed by adding 2 ml of ice-cold 65% ethanol to all tubes, mixing, then leaving the tubes to stand for 5 min at 4°C. The tubes were then centrifuged for 15 min at 2,000 *g* and 4°C. The supernatant was transferred to a second glass tube and a further 1 ml of cold ethanol was added to the pellet. The tubes containing pellet were vortexed and centrifuged again for 15 min at 2,000 *g*. Finally, the supernatants from both extractions were combined, and evaporated in a vacuum oven overnight at 60°C.

The samples were assayed in duplicate for cyclic AMP concentration with an assay kit (Amersham, North Ryde, Australia), after being resuspended in 3 ml of assay buffer supplied with the kit.

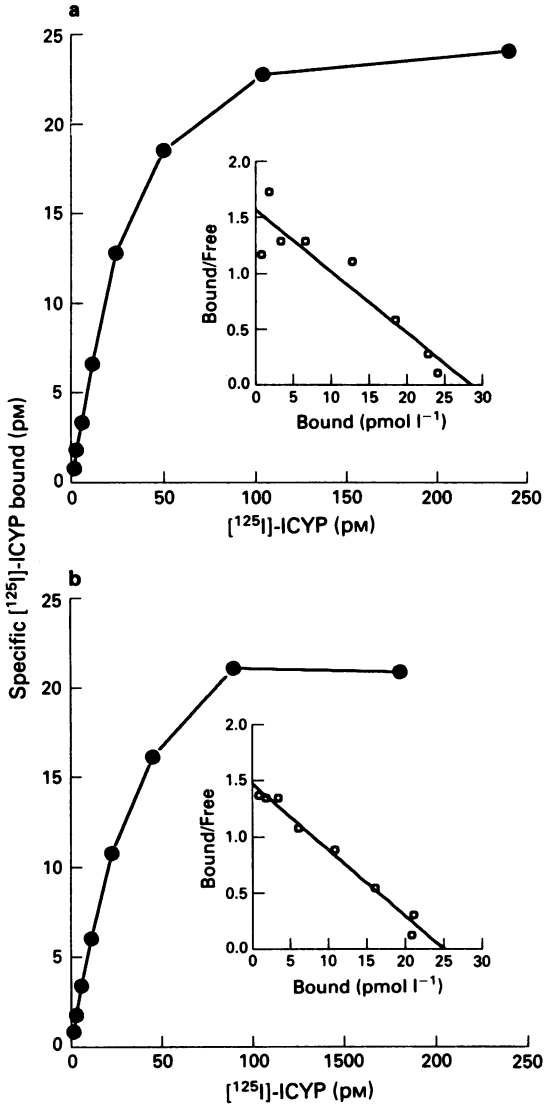

Figure 1 Specific binding of (−)-[^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) to bovine cell membranes prepared from skeletal muscle (a) and adipose tissue (b). Assay conditions were designed to assess binding to β_1- and β_2-adrenoceptors: a low range of radioligand concentration was used, and binding to non-β_1/β_2-adrenoceptor sites was defined by use of 1 μM (−)-propranolol. Inset shows a Scatchard plot of the data.

Table 1 Dissociation constants for β_2-adrenoceptor binding sites in bovine skeletal muscle membranes

Ligand	*pK*[a]
(−)-[^{125}I]-iodocyanopindolol	10.7 ± 0.02
(−)-Propranolol	8.58 ± 0.06
(±)-Oxprenolol	7.71 ± 0.03
(±)-BRL 37344	5.96 ± 0.04
(−)-Isoprenaline	5.84 ± 0.04
(+)-Isoprenaline	3.77 ± 0.02

[a]Negative log of the dissociation constant. Values are means ± s.e.mean (n = 3).

Data analysis

Data presented as means ± s.e.mean. Curves were fitted to the cyclic AMP concentration-response data by eye, to allow the estimates of EC_{50} values. Pseudo pA_2 values were calculated by the dose-ratio method according to: $pA_2 = -\log$ {[antagonist]/(DR − 1)} (Mackay, 1978). The non-additive nature of multiple receptor responses, combined with insufficient data, precluded more rigorous Schild analysis.

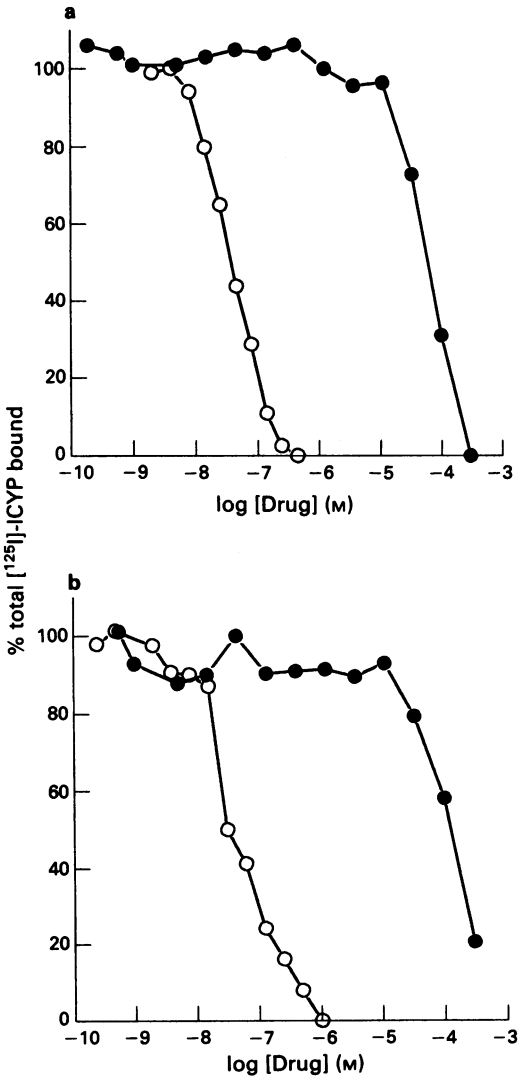

Figure 2 Representative curves showing the displacement of (−)-[^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) (30 pM) from bovine skeletal muscle (a) and white adipose tissue (b) membranes by ICI 118551 (○) and CGP 20712A (●). Binding to non-β_1/β_2-adrenoceptor sites was determined by use of 1 μM (−)-propranolol and has been subtracted from the data.

Table 2 Dissociation constants and pseudo pA_2 values for the antagonism of (−)-isoprenaline-stimulated cyclic AMP production in bovine cell membrane fragments

	Skeletal muscle		*Adipose tissue*	
	pK^a	pA_2^b	pK^a	pA_2^b
ICI 118551	8.26 ± 0.03	7.57 ± 0.17	8.56 ± 0.06	8.69 ± 0.37
CGP20712A	5.16 ± 0.07	8.03 ± 0.21	5.15 ± 0.07	8.20 ± 0.37

[a]Negative log of the dissociation constant.
[b]Pseudo pA_2 values determined from the shift in concentration-response curve caused by a low concentration of antagonist. Note that Schild plots with a slope of unity were not obtained.
Values are means ± s.e.mean ($n = 3$).

Drugs

(−)-Isoprenaline, (−)-propranolol hydrochloride, (±)-oxprenolol 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 (2200 Ci mmol^{-1}) was from Dupont (North Ryde, Australia). Guanylylimidodiphosphate (GppNHp) was from Boehringer Mannheim (New Farm, Australia). The following drugs were received as gifts: CGP 20712A (2-hydroxy-5-(2-[{2-hydroxy-3-(4-[1-methyl-4-trifluoromethyl-2-imidazolyl]phenoxy]propyl}amino]ethoxy)benzamide) (Ciba Geigy, Basel, Switzerland); ICI 118551 (erythro-1-(7-methylindan-4-yloxy)-3-(isopropylamino)-butan-2-ol) (ICI, Macclesfield, England); BRL 37344 (sodium-4-(2-[2-hydroxy-{3-chlorophenyl} ethylamino] propyl) phenoxyacetate) (SmithKline Beecham, Epsom, England); (+)-isoprenaline (Sterling Winthrop, Rensselaer, U.S.A.).

Results

Ligand binding studies in skeletal muscle membranes

In saturation studies using low concentrations of [^{125}I]-ICYP (Figure 1), a single saturable binding site was revealed which had a high affinity for the radioligand (Table 1) and a relatively low density (B_{max} 619 ± 35 fmol mg^{-1} protein), characteristic of a population of β_1- or β_2-adrenoceptors. In displacement studies, both agonists and antagonists yielded pK values consistent with binding to either β_1- or β_2-adrenoceptors, with greater than 100 fold stereoselectivity being demonstrated by the enantiomers of isoprenaline (Table 1). When the β_1-/β_2-adrenoceptor sub-type-selective ligands CGP 20712A and ICI 118551 were used, both compounds gave pK values consistent with binding to β_2-adrenoceptors (Table 2). Although the displacement of [^{125}I]-ICYP by low concentrations of CGP 20712A was erratic, neither drug could be modelled to reveal the presence of any β_1-adrenoceptors (Figure 2).

Next, the binding conditions were modified to detect putative β_3-adrenoceptors. Saturation studies using high concentrations of [^{125}I]-ICYP (Figure 3) revealed a binding site of high density (B_{max} 5 ± 0.48 pmol mg^{-1} protein) with relatively low affinity for the radioligand (Table 3), consistent with the putative β_3-adrenoceptor. Displacement studies using five different competing ligands also gave results characteristic of the putative β_3-adrenoceptor, as seen by a comparison of pK values with those from an earlier study of rat brown adipose tissue (Table 3).

Ligand binding studies in adipose tissue membranes

Although the characterization of adipose tissue membranes was less extensive than that of skeletal muscle, the results obtained in both tissues were strikingly similar (Figures 1 and 2). Saturation studies using low concentrations of [^{125}I]-ICYP revealed a single radioligand binding site (pK 10.78 ± 0.04, B_{max} 648 ± 43 fmol kg^{-1} protein) and pK values obtained using the sub-type-selective compounds CGP 20712A and ICI 118551 were also consistent with the presence of a homogeneous population of β_2-adrenoceptors (Table 2).

Table 3 Dissociation constants for atypical ligand binding sites in bovine skeletal muscle and rat brown adipose tissue (BAT)[a] membranes

	pK^b	
Ligand	*Bovine muscle*	*BAT*
(−)-[^{125}I]-iodocyanopindolol	8.84 ± 0.06	8.77 ± 0.11
(±)-ICI 118551	5.04 ± 0.11	5.00 ± 0.10
(−)-Propranolol	4.82 ± 0.02	4.93 ± 0.10
(±)-BRL 37344	3.47 ± 0.07	3.87 ± 0.03
(−)-Isoprenaline[c]	<3	3.14 ± 0.16
(±)-Phentolamine[c]	3.64 ± 0.01	3.58 ± 0.04

[a]Results taken from Sillence *et al.* (1993).
[b]Negative log of the dissociation constant.
[c]Ligands tested in the presence of 1 μM (−)-propranolol.
Values are means ± s.e.mean ($n = 3$).

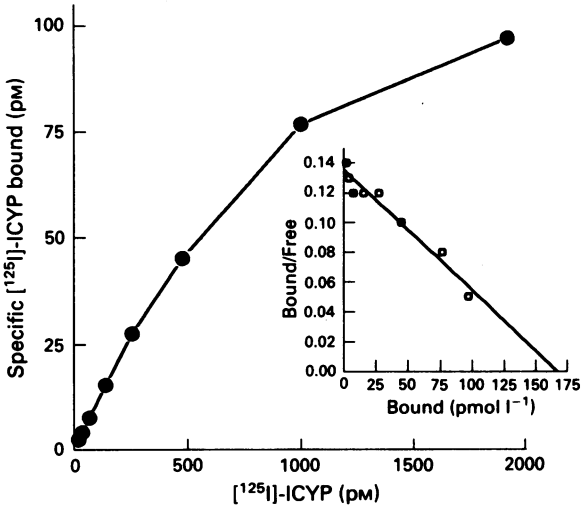

Figure 3 Representative curve showing the binding of (−)-[^{125}I]-iodocyanopindolol ([^{125}I]-ICYP) to skeletal muscle membranes. Assay conditions were designed to assess binding to putative β_3-adrenoceptors rather than to β_1- or β_2-adrenoceptors. Thus, high concentrations of radioligand were used, and nonspecific binding was determined in the presence of 0.5 mM (−)-propranolol. Inset shows a Scatchard plot of these data.

Second-messenger studies

The basal rates of cyclic AMP production were similar in skeletal muscle (1.71 ± 0.22 pmol mg^{-1} protein min^{-1}) and

adipose tissue membranes (2.07 ± 0.41 pmol mg^{-1} protein min^{-1}), whereas the stimulation of cyclic AMP production by NaF was markedly greater in adipose tissue membranes (6.8 ± 0.13 pmol mg^{-1} protein min^{-1}) than in skeletal muscle (4.55 ± 0.26 pmol mg^{-1} protein min^{-1}). The effects of (−)-isoprenaline and BRL 37344 are shown in Figure 4. (−)-Isoprenaline caused a dose-dependent stimulation of cyclic AMP production with mean pD_2 values ($-\log EC_{50}$) of 5.74 ± 0.14 and 5.7 ± 0.17 in skeletal muscle and adipose tissue membranes respectively. The pD_2 values for (−)-isoprenaline were in close agreement with the pK value determined in ligand binding studies, consistent with there being no receptor reserve for the response to this agonist. In contrast to (−)-isoprenaline, the β_3-adrenoceptor-selective agonist, BRL 37344, failed to stimulate cyclic AMP production in either tissue, and at high concentrations caused a slight fall in the output of second-messenger, most noticeable in skeletal muscle.

In both tissues the antagonists CGP 20712A and ICI 118551 caused a rightward shift of the concentration-response curve to (−)-isoprenaline (Figure 5). For CGP 20712A, the pseudo pA_2 values calculated from the shift

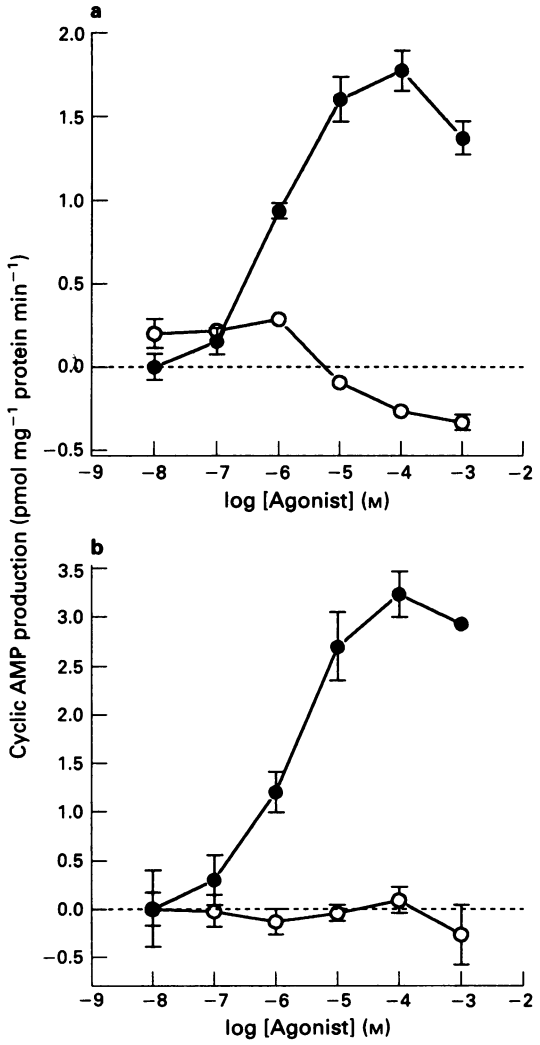

Figure 4 Effects of (−)-isoprenaline (●) and BRL 37344 (○) on the production of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) in bovine cell membrane fragments: from (a) skeletal muscle and (b) white adipose tissue. Incubations were for 10 min. Each point represents the mean (± s.e.mean) value for three observations. Where standard errors are not visible, they are less than the size of the symbols. The results are presented relative to the basal amount of cyclic AMP produced in the absence of drug.

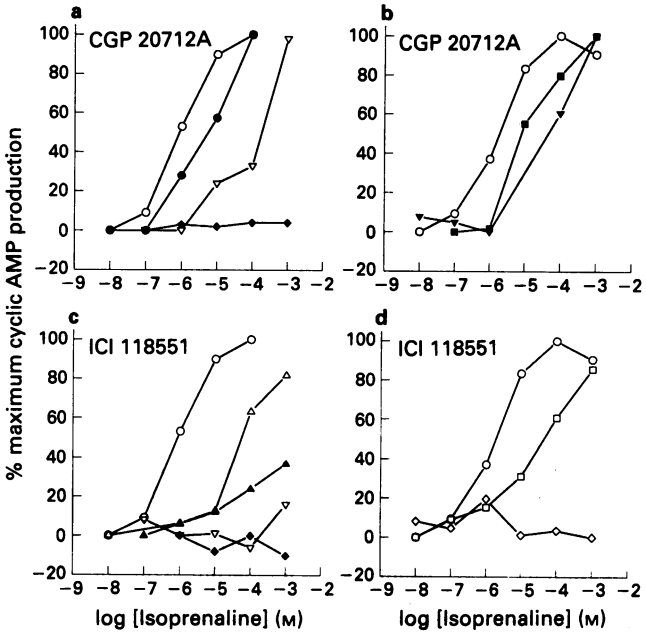

Figure 5 Effects of CGP 20712A and ICI 118551 on the stimulation of adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production in bovine cell membranes by (−)-isoprenaline (○). Results are expressed relative to the maximum response to (−)-isoprenaline, after the subtraction of basal rates of production determined in the absence of agonist. Each point represents the mean of three observations. Standard errors are omitted for the sake of clarity, but were always less than 10% of the mean. Concentrations of CGP 20712A used in skeletal muscle (a) were 36 nM (●), 10 μM (▽), and 1 mM (◆); concentrations used in adipose tissue (b) were 100 nM (■) and 37 μM (▼). Concentrations of ICI 118551 used in skeletal muscle (c) were 1 μM (△), 2.7 μM (▲), 10 μM (▽) and 1 mM (◆); concentrations used in adipose tissue (d) were 38 nM (□) and 48 μM (◇).

produced by low concentrations of antagonist (36 and 100 nM) were similar in both tissues (Table 2), and were consistent with the activation of β_1-adrenoceptors by (−)-isoprenaline. However, when higher concentrations of the β_1-antagonist were used (in the range 10 to 37 μM), the shift in the concentration-response curve to isoprenaline was less than that expected from the activation of β_1-adrenoceptors alone. The shift was apparently greater in skeletal muscle than in adipose tissue, being reflected by pseudo pA_2 values of 7 and 6.5 respectively (determined from a single experiment in each tissue). Cyclic AMP production in skeletal muscle membranes was abolished by 1 mM CGP 20712A.

Next, pseudo pA_2 values were calculated from the shift in concentration-response curves caused by ICI 118551 (Table 2). The pseudo pA_2 value determined at 38 nM ICI 118551 in adipose tissue was higher than that determined using 1 μM in skeletal muscle. However, both values were closer to that expected for blockade of β_2-adrenoceptors, than for blockade of β_1- or β_3-adrenoceptors. Concentrations of ICI 118551 above 1 μM caused a non-parallel shift in the concentration-response curve to isoprenaline, and at anatagonist concentrations above 10 μM, cyclic AMP production was abolished.

Discussion

The results of this study show that bovine skeletal muscle has a low-affinity, 'atypical' [^{125}I]-ICYP binding site, which has binding characteristics almost identical to those of the 'atypical' site described in rat soleus muscle (Roberts *et al.*, 1993; Sillence *et al.*, 1993) and brown adipose tissue (Sillence *et al.*, 1993). The 'atypical' binding site is similarly abundant in bovine and rat skeletal muscle: representing 89% and 81%

respectively, of the total population of [^{125}I]-ICYP binding sites in muscle from these two species.

Having identified the 'atypical' binding site, our next aim was to test the hypothesis that this site represents a functional β_3-adrenoceptor coupled to adenylyl cyclase. The most extensive studies of β_3-adrenoceptor-mediated cyclic AMP responses are those which have been conducted on rat adipose tissue (Granneman, 1990; 1992; Hollenga *et al.*, 1991a,b). This tissue can be stimulated by BRL 37344 and in the presence of (−)-isoprenaline, gives a bi-phasic response which is resistant to blockade by either CGP 20712A or ICI 118551, yielding pA_2 values of 4.53 (Hollenga *et al.*, 1991b) and 5.49 (Hollenga & Zaagsma, 1989) respectively. None of these features were observed in the present study, suggesting that β_3-adrenoceptors are not expressed at a functional level in either bovine skeletal muscle or adipose tissue membranes.

This finding contrasts with the results of studies in rat soleus muscle (Challis *et al.*, 1988) and white adipose tissues (Hollenga & Zaagsma, 1989; Granneman, 1992), but is similar to the results of functional studies in human tissue. More specifically, human subcutaneous adipose tissue shows little or no response to BRL 37344 (Hollenga *et al.*, 1991a; Langin *et al.*, 1991), whereas functional β_3-adrenoceptors can be detected in human omental adipose tissue, with a lipolysis assay of appropriate sensitivity (Lonnqvist *et al.*, 1993). The second-messenger assay employed in the present study is not particularly sensitive, and bovine β_3-adrenoceptors may be expressed to a greater degree in other adipose tissue depots. Nevertheless, the results of the present study do not support the hypothesis that the abundant 'atypical' [^{125}I]-ICYP binding sites described above, represent functional bovine β_3-adrenoceptors.

The possibility that the 'atypical' [^{125}I]-ICYP binding sites represent β_3-adrenoceptors which are not coupled to cyclic AMP, is suggested from ligand binding data using adrenoceptor antagonists. The pK value for [^{125}I]-ICYP at the 'atypical' bovine site (8.84) is not only similar to that seen in our earlier experiments (Sillence *et al.*, 1993) using rat brown adipose tissue (8.77), but is also in close agreement with the value reported for a putative β_3-adrenoceptor in cultured murine adipocytes (8.82) (Fève *et al.*, 1991), and with those pK values for cloned human (8.72) and mouse (9.06) β_3-adrenoceptors expressed in Chinese hamster ovary (CHO) cells (Féve *et al.*, 1991; Nahmias *et al.*, 1991). In previous papers, we and others discussed further similarities between the 'atypical' [^{125}I]-ICYP binding site, and the putative rat β_3-adrenoceptor (Sillence *et al.*, 1993; Roberts *et al.*, 1993). However, despite these similarities, the pK values for the binding of agonists to the 'atypical' site are disturbingly low. Because β-agonists are generally less lipophilic than antagonists, it might be argued that the 'atypical' [^{125}I]-ICYP binding site is simply a nonspecific, lipophilic binding site. Rademaker *et al.* (1985) identified such a site in Cheng liver cells, by observing a strong correlation between binding affinity and hydrophobicity for 10 ligands, and a high affinity for the lipophilic α-adrenoceptor antagonist, phentolamine (pK 5.5), relative to that of the hydrophilic β-agonist isoprenaline. Our own analysis of log P and pK values for 11 ligands tested in rat skeletal muscle, shows a correlation which is not stronger for the 'atypical' binding site than for binding to classical β_2-adrenoceptors (unpublished data). Furthermore, the pK value for phentolamine observed in the present study (3.64) is not consistent with that reported in Cheng liver cells, so we conclude that the 'atypical' rat and bovine [^{125}I]-ICYP binding sites are not the same as the non-β-adrenoceptor site described by Rademaker *et al.* (1985). As well as binding to lipophilic sites, [^{125}I]-ICYP has been shown to label 5-HT receptors (Hoyer *et al.*, 1985) and to be susceptible to tissue peroxidases, which cause the nonspecific incorporation of iodide free radicles into tyrosine residues of membrane proteins (Rigby *et al.*, 1988). However, in contrast to these non-β-adrenoceptor binding sites, the site described in this and in our previous study, is not sensitive either to 5-HT (Sillence *et al.*, 1993) or to ascorbic acid concentration (unpublished observation).

Despite having shown that our 'atypical' [^{125}I]-ICYP site does not correspond to a number of previously-identified non-β-adrenoceptor binding sites, the low affinity observed for the binding of β-adrenoceptor agonists and the lack of a functional β_3-adrenoceptor-mediated response in tissues which express this binding site, are of concern. Further studies will need to be performed, therefore, before the nature of this 'atypical' site can be fully understood.

As well as identifying 'atypical' [^{125}I]-ICYP binding sites by using high concentrations of radioligand, we showed in the present study that under more conventional binding conditions, both skeletal muscle and white adipose tissue appear to have homogeneous populations of β_2-adrenoceptors. The present results for muscle agree with those of earlier studies in cattle (Ijzerman *et al.*, 1984; Sillence *et al.*, 1991), guinea-pigs (Elfellah & Reid, 1989), humans (Liggett *et al.*, 1988), and rat plantaris muscle (Kim *et al.*, 1991); whereas in rat soleus muscle, a minor population of β_1-adrenoceptors was identified with the highly sensitive technique of autoradiography (Kim *et al.*, 1991). In contrast to skeletal muscle, white adipose tissue from a number of species including pigs (Coutinho *et al.*, 1992), hamsters (Bjorgell & Belfrage, 1982) and man (Mauriege *et al.*, 1988), is well-recognised as being a source of both β_1- and β_2-adrenoceptors. The β_1-adrenoceptor is thought to govern lipolysis, whereas the contribution of β_2-adrenoceptors is thought to vary according to the species of animal (Arner, 1992). Thus, the present finding that no β_1-adrenoceptors could be detected in bovine adipose tissue with ligand binding techniques was surprising, and to our knowledge is in accord with only one previous study which examined sheep adipose tissue (R.G. Vernon, personal communication).

Our finding that no β_1-adrenoceptors could be detected in bovine muscle or adipose tissue using ligand binding techniques, is clearly at variance with the results of our adenylyl cyclase experiments. Although we have not attempted to quantify the relative contribution of β_1- and β_2-adrenoceptors to the production of cyclic AMP, our results show clearly that both adrenoceptor sub-types are involved, because the selective antagonists of CGP 20712A and ICI 118551 were both effective at low concentrations in causing a rightward shift in the concentration-response curve to (−)-isoprenaline. In addition, it was apparent that the generation of second-messenger by the two adrenoceptor sub-types was not additive. For this reason we have not attempted to generate Schild plots from our data, nor to define precisely pA_2 values for the antagonists used. The pseudo pA_2 values we report are estimates made with the lower concentrations of antagonists tested. Under these conditions, the occupation of either receptor sub-type attenuates the response of the tissue to (−)-isoprenaline markedly. However, when one or other receptor subtype is blocked to an extent greater than 99% by using high concentrations of antagonist e.g. 10 to 37 μM CGP 20712A, then maximum cyclic AMP production is still achievable, presumably through the agonist interacting with the unbound receptor sub-type. Our results suggest that the generation of second-messenger in these tissues involves a system which is more complex than can be described using standard pharmacological equations and we propose that this system warrants further investigation.

At the outset of this study, our primary aim was to determine which β-adrenoceptor subtypes are functional in bovine skeletal muscle. A secondary aim was to characterize bovine adipose tissue. The role of β_2-adrenoceptors in skeletal muscle is well-established for many species, and the results of the present work confirm the presence and coupling to second-messenger of this sub-type in cattle. We have also demonstrated that bovine skeletal muscle contains a small population of β_1-adrenoceptors, which are undetectable by ligand binding methods, but which nevertheless are strongly

coupled to the production of cyclic AMP. It remains to be determined whether β_1-adrenoceptors are present in skeletal muscle from other species, and whether these receptors have a physiological role, or represent a potential therapeutic target. As in skeletal muscle, both β_1- and β_2-adrenoceptors contribute to the production of second-messenger in bovine adipose tissue. Finally, although we have identified a binding site for [^{125}I]-ICYP which shares certain characteristics with the putative β_3-adrenoceptor, our second-messenger data do not support the hypothesis that functional β_3-adrenoceptors are present in either bovine skeletal muscle or adipose tissue.

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References

ARCH, J.R.S., AINSWORTH, A.T., CAWTHORNE, M.A., PIERCY, V., SENNITT, M.V., THODY, V.E., WILSON, C. & WILSON, S. (1984). Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature*, **309**, 163–165.

ARNER, P. (1992). Adrenergic receptor function in fat cells. *Am. J. Clin. Nutr.*, **55**, 228S–236S.

BJORGELL, P. & BELFRAGE, P. (1982). Characteristics of the lipolytic beta-adrenergic receptors in hamster adipocytes. *Biochim. Biophys. Acta*, **713**, 80–85.

CHALLISS, R.A.J., LEIGHTON, B., WILSON, S., THURLBY, P.L. & ARCH, J.R.S. (1988). An investigation of the β-adrenoceptor that mediates metabolic responses to the novel agonist BRL 28410 in rat soleus muscle. *Biochem. Pharmacol.*, **37**, 947–950.

COUTINHO, L.L., BERGEN, W.G., MERKEL, R.A. & SMITH, C.K. (1992). Quantitative characterization of beta-adrenergic receptor subtypes in porcine adipocytes. *Comp. Biochem. Physiol.*, **101C**, 481–485.

DISATNIK, M.H., SAMPSON, S.R. & SHAINBERG, A. (1990). Characterization of β-adrenoceptors on rat skeletal muscle cells grown *in vitro*. *Biochem. Pharmacol.*, **40**, 1043–1048.

ELFELLAH, M.S. & REID, J.L. (1989). Regulation of β_1- and β_2-adrenoceptors following chronic treatment with β-adrenoceptor antagonists. *Eur. J. Pharmacol.*, **173**, 85–92.

FEVE, B., EMORINE, L.J., LASNIER, F., BLIN, N., BAUDE, B., NAHMIAS, C., STROSBERG, A.R. & PAIRAULT, J. (1991). Atypical β-adrenergic receptor in 3T3-F442A adipocytes: pharmacological and molecular relationship with the human β_3-adrenergic receptor. *J. Biol. Chem.*, **266**, 20329–20336.

GRANNEMAN, J.G. (1990). Norepinephrine and BRL 37344 stimulate adenylate cyclase by different receptors in rat brown adipose tissue. *J. Pharmacol. Exp. Ther.*, **254**, 508–513.

GRANNEMAN, J.G. (1992). Effects of agonist exposure on the coupling of $beta_1$ and $beta_3$ adrenergic receptors to adenylyl cyclase in isolated adipocytes. *J. Pharmacol. Exp. Ther.*, **261**, 638–642.

HOLLENGA, C., BROUWER, F. & ZAAGSMA, J. (1991a). Differences in functional cyclic AMP compartments mediating lipolysis by isoprenaline and BRL 37344 in four adipocyte types. *Eur. J. Pharmacol.*, **200**, 325–330.

HOLLENGA, Ch., BROUWER, F. & ZAAGSMA, J. (1991b). Relationship between lipolysis and cyclic AMP generation mediated by atypical β-adrenoceptors in rat adipocytes. *Br. J. Pharmacol.*, **102**, 577–580.

HOLLENGA, Ch. & ZAAGSMA, J. (1989). Direct evidence for the atypical nature of functional β-adrenoceptors in rat adipocytes. *Br. J. Pharmacol.*, **98**, 1420–1424.

HOYER, D., ENGLE, G. & KALKMAN, H.O. (1985). Characterization of the 5-HT_{1B} recognition site in rat brain; binding studies with (−)[^{125}I]iodocyanopindolol. *Eur. J. Pharmacol.*, **118**, 1–12.

IJZERMAN, A.P., BULTSMA, T., TIMMERMAN, H. & ZAAGSMA, J. (1984). The relationship between ionization and affinity of β-adrenoceptor ligands. *Naunyn-Schmied. Arch. Pharmacol.*, **327**, 293–298.

KIM, Y.S., SAINZ, R.D., MOLENAAR, P. & SUMMERS, R.J. (1991). Characterization of β_1- and β_2-adrenoceptors in rat skeletal muscles. *Biochem. Pharmacol.*, **42**, 1783–1789.

LANGIN, D., PORTILLO, M.P., SAULINER-BLACHE, J.S. & LAFONTAN, M. (1991). Coexistence of three β-adrenoceptor subtypes in white fat cells of various mammalian species. *Eur. J. Pharmacol.*, **199**, 291–301.

LIGGETT, S.B., SHAH, S.D. & CRYER, P.E. (1988). Characterization of β-adrenergic receptors of human skeletal muscle obtained by needle biopsy. *Am. J. Physiol.*, **254**, (Endocrinol. Metab. 17), E795–798.

LONNQVIST, F., KRIEF, S., STROSBERG, A.D., NYBERG, B., EMORINE, L.J. & ARNER, P. (1993). Evidence for a functional β_3-adrenergic receptor in man. *Br. J. Pharmacol.* (in press).

MACKAY. D. (1978). How should values of pA_2 and affinity constants for pharmacological competitive antagonists be estimated? *J. Pharm. Pharmacol.*, **30**, 312–313.

MAURIEGE, P., DEPERGOLA, G., BERLAN, M. & LAFONTAN, M. (1988). Human fat cell beta-adrenergic receptors: beta-agonist-dependent lipolytic responses and characterization of beta-adrenergic binding sites on human fat cell membranes with highly selective beta-antagonists. *J. Lipid Res.*, **29**, 587–601.

MOLENAAR, P., ROBERTS, S.J., KIM, Y.S., PAK, H.S., SAINZ, R.D. & SUMMERS, R.J. (1991). Localization and characterizaton of two propranolol resistant (−)-[^{125}I] cyanopindolol binding sites in rat skeletal muscle. *Eur. J. Pharmacol.*, **209**, 257–262.

MUNSON, P.J. & RODBARD, D. (1980). Ligand: a versatile computerized approach for characterization of ligand-binding studies. *Anal. Biochem.*, **107**, 220–239.

NAHMIAS, C., BLIN, N., ELALOUF, J.M., MATTEI, M.G., STROSBERG, A.D. & EMORINE, L.J. (1991). Molecular characterization of the mouse β_3-adrenoceptor: relationship with the atypical receptor of adipocytes. *EMBO J.*, **10**, 3721–3727.

RADEMAKER, B., KRAMER, K., STROES, J.W., VLUG, J., KRIELAART, M. & ZAAGSMA, J. (1985). High affinity non-β-adrenoceptor binding of β-adrenergic ligands. *Eur. J. Pharmacol.*, **111**, 31–36.

RIGBY, P.J., PASSARELLI, M.C., SELF, G.J., PREUSS, J.M.H. & GOLDIE, R.G. (1988). Ascorbic acid-induced binding of [^{125}I]I-iodocyanopindolol to non-beta-adrenoceptor sites in guinea-pig trachea. *Biochem. Pharmacol.*, **37**, 1421–1424.

ROBERTS, S.J., MOLENAAR, P. & SUMMERS, R.J. (1993). Characterization of propranolol-resistant (−)-[^{125}I]-cyanopindolol binding sites in rat soleus muscle. *Br. J. Pharmacol.*, **109**, 344–352.

SILLENCE, M.N., MOORE, N.G., PEGG, G.G. & LINDSAY, D.B. (1993). Ligand binding properties of putative β_3-adrenoceptors compared in brown adipose tissue and in skeletal muscle membranes. *Br. J. Pharmacol.*, **109**, 1157–1163.

SILLENCE, M.N., PEGG, G.G. & LINDSAY, D.B. (1991). Affinity of clenbuterol analogues for β_2-adrenoceptors in bovine skeletal muscle and the effect of these compounds on urinary nitrogen excretion in female rats. *Naunyn-Schmied. Arch. Pharmacol.*, **344**, 442–448.

SMITH, S.A., SENNITT, M.V. & CAWTHORNE, M.A. (1990). BRL 35135: an orally active antihyperglycaemic agent with weight reducing effects. In *New Antidiabetic Drugs*. ed. Bailey, C.J. & Flatt, P.R. pp. 177–189. London: Smith-Gordon.

WILSON, C., WILSON, S., PIERCY, V., SENNITT, M.W. & ARCH, J.R.S. (1984). The rat lipolytic β-adrenoceptor studies using novel β-adrenoceptor agonists. *Eur. J. Pharmacol.*, **100**, 309–319.

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