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Characterization of propranolol-resistant (−)-[^{125}I]-cyanopindolol binding sites in rat soleus muscle

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1 The characteristics of a propranolol-resistant (−)-[^{125}I]-cyanopindolol (CYP) binding site in rat soleus muscle were determined.

2 Saturation studies performed on homogenates of rat soleus muscle showed two phases of (−)-[^{125}I]-CYP binding, a high affinity site (K_{D1} 30.5 ± 16.3 pM, B_{max} 9.4 ± 1.38 fmol mg^{-1} protein) and a lower affinity site (K_{D2} 522.5 ± 29.1 pM, B_{max} 62.19 ± 11.76 fmol mg^{-1} protein, $n = 4$).

3 In rat soleus muscle homogenates labelled with (−)-[^{125}I]-CYP (500 pM), (−)-propranolol competition curves were biphasic with pK_D values of 8.30 ± 0.19, and 5.33 ± 0.08, $n = 7$.

4 Competition between (−)-[^{125}I]-CYP (500 pM) and (±)-tertatolol, (±)-nadolol, (±)-alprenolol, (±)-CYP, and (−) and (+)-pindolol showed that these compounds competed for binding at the propranolol-resistant site with affinities lower than those displayed at typical β-adrenoceptors. The atypical β-adrenoceptor agonists BRL 37344, SR58611A and ICI D7114 and the partial agonist (±)-CGP 12177 also competed for (−)-[^{125}I]-CYP binding.

5 Stereoselectivity was demonstrated for the stereoisomers of alprenolol and tertatolol. The (−)-isomers of alprenolol and tertatolol had higher affinity than their corresponding (+)-isomers (3.1 and 2.6 fold respectively). These low stereoselectivity values are a characteristic of atypical β-adrenoceptors.

6 The β-adrenoceptor agonists, (−)-adrenaline, (−)-isoprenaline and (−)-noradrenaline, all showed lower affinity than the atypical β-adrenoceptor agonists and competition curves appeared biphasic in nature.

7 These results confirm the presence of a propranolol-resistant (−)-[^{125}I]-CYP binding site in rat soleus muscle. The affinities of the tested compounds at the propranolol-resistant (−)-[^{125}I]-CYP binding site show similarities to their affinities at 'atypical' β-adrenoceptors in adipocytes and gastrointestinal tissues and at the cloned β_3-adrenoceptor.

Keywords: Rat soleus muscle; (−)-[^{125}I]-cyanopindolol; receptor binding; atypical β-adrenoceptors; β_3-adrenoceptors

Introduction

The existence of β-adrenoceptors that differed from β_1- and β_2-subtypes was first indicated by functional studies on rat adipocytes. Isoprenaline-induced lipolysis was blocked by stereoisomers of β-adrenoceptor antagonists with lower stereoselectivity ratios than in tissues with predominantly β_1-(atrial) or β_2-(diaphragm) adrenoceptors (Harms *et al.*, 1977) and the affinity of β-adrenoceptor antagonists was lower at the adipocyte β-adrenoceptor than at typical β_1- and β_2-adrenoceptors (Bojanic *et al.*, 1985). This was further supported by the development of novel β-adrenoceptor agonists which selectively stimulated lipolysis in brown adipocytes (Arch *et al.*, 1984). These novel phenethanolamine analogues, BRL 37344, BRL 28410 and BRL 35113 were found to be more potent as stimulators of lipolysis than of either atrial rate (β_1-adrenoceptor mediated) or tracheal relaxation (β_2-adrenoceptor mediated).

Functional studies in gastrointestinal tissues such as guinea-pig gastric fundus (Coleman *et al.*, 1987) and ileum (Bond *et al.*, 1986; Bond & Clarke, 1988), rabbit stomach (Bristow *et al.*, 1970) and jejunum (Wikberg, 1977; Norman & Leathard, 1990), rat oesophageal muscle (Buckner & Christopherson, 1974), gastric fundus (Dettmar *et al.*, 1986; McLaughlin & McDonald, 1991), colon (Croci *et al.*, 1988; Bianchetti & Manara, 1990), distal colon (McLaughlin & McDonald, 1990), jejunum (Van der Vliet *et al.*, 1990), dog distal colon (Grivegnee *et al.*, 1984) and human colon (McLaughlin *et al.*, 1988) showed that adrenergic inhibition of tension development in these tissues was mediated by a β-adrenoceptor which was resistant to blockade by propranolol and other conventional β-adrenoceptor antagonists. In addition to brown and white fat cells there is evidence that other tissues such as heart (Kaumann, 1989), pancreas (Arch *et al.*, 1991) and liver (Emorine *et al.*, 1989) also contain atypical β-adrenoceptors, while functional studies in rat soleus muscle have identified a thermic response mediated by propranolol-resistant β-adrenoceptors (Challis *et al.*, 1988; see also Arch & Kaumann, 1993 for review).

The recent isolation and cloning of genes coding for β_3-adrenoceptors in man (Emorine *et al.*, 1989; Tate *et al.*, 1991), rat (Granneman *et al.*, 1991; Muzzin *et al.*, 1991) and mouse (Nahmias *et al.*, 1991) further supports the functional evidence that more than two subtypes exist. When expressed in cultured CHO cells the cloned β_3-adrenoceptors display a lower affinity for the radioligand (−)-[^{125}I]-cyanopindolol (CYP) than either β_1- and β_2-adrenoceptors. Identification of these receptors using receptor binding studies in tissues with atypical β-adrenoceptors has only recently been achieved in 3T3-F442A adipocytes with (−)-[^{125}I]-CYP and [^{3}H]-CGP 12177 (Feve *et al.*, 1991; 1992; Thomas *et al.*, 1992) and in rat brown adipose tissue membranes with [^{3}H]-CGP 12177 (Muzzin *et al.*, 1992).

Recent autoradiographic studies of β-adrenoceptors in rat skeletal muscle have revealed the presence of propranolol (1 μM)-resistant (−)-[^{125}I]-CYP binding (Molenaar *et al.*, 1991) distributed across the muscle bundle and most abundant in the soleus muscle. The present study was aimed at characterizing propranolol-resistant binding in rat soleus muscle with a view to the development of a receptor binding assay for atypical β-adrenoceptors.

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Methods

Membrane preparation

Sprague-Dawley rats of either sex (250–450 *g*) were stunned by a blow to the back of the head and exsanguinated. The soleus muscle was dissected and homogenized (4 muscles per homogenate) in 20 vol of ice cold Krebs buffer containing phenylmethylsulphonylfluoride (PMSF) (10 μM) pH 7.4 or in Tris buffer pH 7.4 containing $MgCl_2$ (5 mM) and PMSF (10 μM) for competition studies with some agonists. Homogenates were filtered through a nylon filter (210 μm) and centrifuged twice at 4°C for 15 min at 50,000 *g* in a Sorvall RC-5 superspeed centrifuge. The final pellet was resuspended in 15 volumes of buffer and stored on ice until use. Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as a standard.

Kinetic studies

The association of (−)-[125I]-CYP (500 pM) with specific binding sites in 50 μl aliquots of soleus muscle homogenates incubated with 5-hydroxytryptamine (5-HT) at 10 μM to block 5-HT-sensitive (−)-[125I]-CYP binding sites (Molenaar *et al.*, 1991) and (−)-propranolol (0.1 μM) to block β_1- and β_2-adrenoceptors was determined at various times up to 90 min. Non-specific binding was determined with (±)-alprenolol (500 μM). Preliminary studies showed that this concentration identified maximal 'specific binding' and as it is approximately 200 × K_D value (2.2 μM, present study) it will therefore block 99.6% of the propranolol-resistant (−)-[125I]-CYP binding sites. The dissociation of (−)-[125I]-CYP was determined after a 60 min incubation with (−)-[125I]-CYP (500 pM) at 37°C at intervals up to 90 min after the addition of (±)-alprenolol (500 μM).

Saturation binding

Aliquots of membrane suspension (50 μl) were incubated with (−)-[125I]-CYP (10–1000 pM) and 5-HT (10 μM) at 37°C for 60 min in a final volume of 100 μl. Non-specific binding was defined by (±)-alprenolol (500 μM).

Competition binding

Aliquots of membrane suspension (50 μl) were incubated with (−)-[125I]-CYP (500 pM), 5-HT (10 μM), with or without (−)-propranolol (0.1 μM) and a range of concentrations of competing agent. Non-specific binding was defined by (±)-alprenolol (500 μM). In competition experiments using (−)-isoprenaline, (−)-adrenaline and (−)-noradrenaline the incubation mixture also contained 0.1 mM ascorbic acid to prevent catecholamine oxidation. Solutions were incubated in duplicate or triplicate for 60 min at 37°C in a final volume of 100 μl and incubation was terminated by the addition of 4 ml 20°C buffer solution followed by rapid vacuum filtration. Membranes were washed onto Whatman GF/B filters presoaked in 2% polyethylenimine and washed with 3 × 4 ml buffer (Brandel M-30R Cell Harvester). Radioactivity retained on the filters was measured in a Packard gamma counter (Model B5424) with 79% efficiency.

Analysis of results

Results are expressed as means ± standard error (s.e.) of *n* experiments. Kinetic constants were obtained using KINFIT (Williams & Summers, 1990). Saturation data were analysed with EBDA (McPherson, 1983) to obtain Hill coefficient and LIGAND (Munson & Rodbard, 1980) to obtain dissociation constant (K_D) and receptor density (B_{max}) values. Competition data were analysed with EBDA to obtain preliminary K_D values and the non-linear curve fitting programme GraphPad (Intuitive Software for Science) to obtain final K_D and pseudo Hill coefficient values. All curves were analysed for a one site fit (propranolol-resistant site) and tested for two sites where competition for typical β-adrenoceptors was also expected ((−)-[125I]-CYP saturation, propranolol, ICI 118551 and CGP 20712A competition binding curves). Significant line shifts were determined with ANCOVA which is part of the REAP package (Gamma Research Systems, Knoxfield, Australia).

Radioiodination of (−)-CYP

(−)-[125I]-CYP was prepared from (−)-CYP and Na125I as described by Lew & Summers (1985).

Materials

(−)-Propranolol, ICI D7114 ((S)-4-[2-hydroxy-3-phenoxypropylamino ethoxy]-*N*-(2-methoxyethyl)phenoxyacetamide), (±)-ICI 118551 (erythro-DL-1(7-methylindian-4-yloxy)-3-isopropylaminobutan-2-ol) (Imperial Chemical Industries, Wilmslow, Cheshire, England); 5-HT, (±)-nadolol, prazosin, (±)-alprenolol hydrochloride, (−)-alprenolol, (−)-isoprenaline, (−)-adrenaline (epinephrine), (−)-noradrenaline (norepinephrine) (Sigma, St Louis, U.S.A.); (+)-alprenolol (Professor B. Jarrott, Monash University); atropine sulphate (BDH Chemicals Ltd, Poole, Dorset); (±)-CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl) 1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulphonate) (Ciba-Geigy AG Australia); (−)-CYP, (±)-CYP (Sandoz, Basel, Switzerland); Na [125I] (Amersham International, Buckinghamshire), BRL 37344 (sodium-4-[-2[-2-hydroxy-2-(-3-chloro-phenyl) ethylamino] propyl] phenoxyacetate) (Smith Kline Beecham, Great Burgh, Epsom, Surrey); (±)-tertatolol hydrochloride, (−)-tertatolol, (+)-tertatolol (Servier, Paris, France); SR58611A (**RS**-N-(7-carbethoxymethoxyl 1,2,3,4-tetrahydronaphth-2-yl)-2 hydroxy 2-(3-chlorophenyl)ethanamine) (SANOFI-MIDY S.p.A. Research Centre, Milan, Italy); (±)-CGP 12177 hydrochloride ((−)-4-)3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one) (Research Biochemicals Inc., Massachusetts, U.S.A.); (−)-pindolol, (+)-pindolol (Professor C. Raper, Victorian College of Pharmacy).

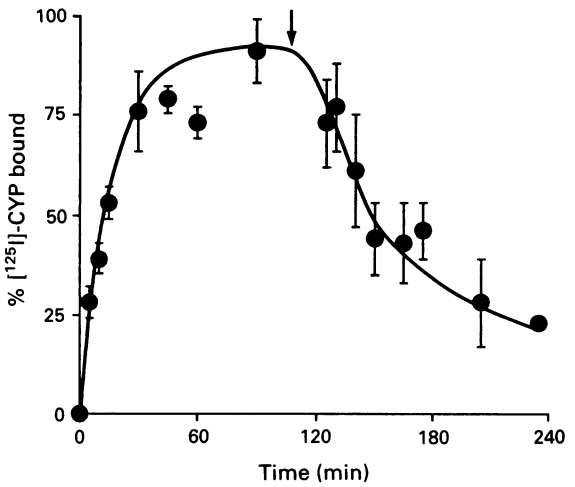


Figure 1 Association and dissociation of specific binding of (−)-[125I]-cyanopindolol ((−)-[125I]-CYP) to rat soleus muscle at 37°C. Dissociation curve represents specific binding at various times after the addition of (±)-alprenolol (arrow, 500 μM) to membranes incubated with (−)-[125I]-CYP for 60 min. Each point is expressed as a percentage of the binding occurring at equilibrium in association experiments. Points show mean ± s.e. ($n = 4$).

Results

Characterization of propranolol-resistant sites

Association kinetics of (−)-[^{125}I]-CYP binding to soleus muscle membranes Association kinetics of the site were determined at 37°C and showed that association of (−)-[^{125}I]-CYP to the propranolol-resistant site reached equilibrium in 60 min (Figure 1). The observed association rate constant, (K_{obs}) was 0.062/min. Dissociation kinetics determined at 37°C showed that binding was reversible on the addition of 500 μM (±)-alprenolol. Dissociation occurred in two phases, K_{-1} = 0.112/min (phase 1) and 0.008/min (phase 2) (Figure 1).

Saturation of (−)-[^{125}I]-CYP binding to soleus muscle membranes (−)-[^{125}I]-CYP bound to soleus muscle homogenates in a concentration-dependent manner. The specific binding defined by 500 μM (±)-alprenolol ranged from 45–80% at 10 pM, 76–86% at 160–180 pM and 71–78% at 530–580 pM. (−)-[^{125}I]-CYP bound with two affinities and analysis using LIGAND revealed a high affinity site with a K_D of 30.5 ± 16.3 pM (B_{max} = 9.4 ± 1.38 fmol mg^{-1} protein) (typical β-adrenoceptors) and a site of lower affinity with a K_D of 522.5 ± 29.1 pM (B_{max} = 62.19 ± 11.76 fmol mg^{-1} protein) (n = 4). A representative saturation isotherm is shown in Figure 2.

Competition by (−)-propranolol (−)-Propranolol competed for (−)-[^{125}I]-CYP binding in soleus muscle homogenates (Figure 3). The competition curve could be separated into high and low affinity components with pK_D values of 8.30 and 5.33 respectively. Using the mass action equation ([A]/[A] + K_D) we were able to establish that a concentration of 0.1 μM (−)-propranolol would block 95% of β_2-adrenoceptors but only 2% of the atypical site. Therefore subsequent competition studies were performed in the presence of (−)-propranolol (0.1 μM) to block the low density typical β-adrenoceptor sites so that competition for the more abundant atypical site could be examined.

In addition, competition between (−)-[^{125}I]-CYP and ICI 118551 (selective β_2-antagonist) in the absence of (−)-propranolol produced a biphasic curve with high and low affinity components (Table 1, Figure 4a) while competition with CGP 20712A (selective β_1-antagonist) showed a single low affinity binding site (Table 1, Figure 4b).

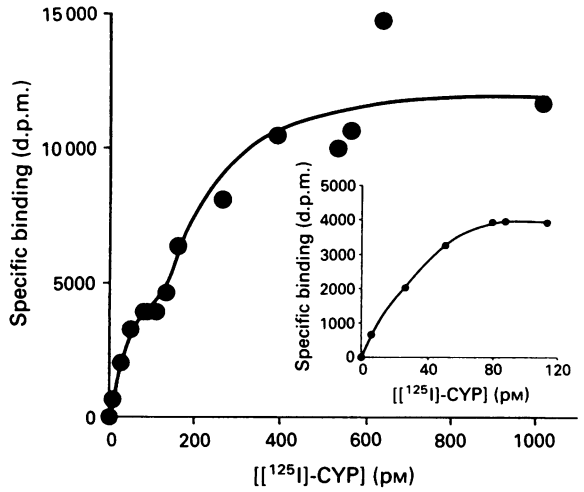


Figure 2 Representative saturation binding curve of specific (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) binding to rat soleus muscle membranes. Non-specific binding was defined by 500 μM (±)-alprenolol. Two phases of binding were observed, a high affinity site K_{D1} 30.5 ± 16.3 pM (B_{max} = 9.4 ± 1.38 fmol mg^{-1} protein) and low affinity site K_{D2} 522.5 ± 29.1 pM (B_{max} = 62.19 ± 11.76 fmol mg^{-1} protein) (n = 4). Inset shows saturation binding data between 0–120 pM.

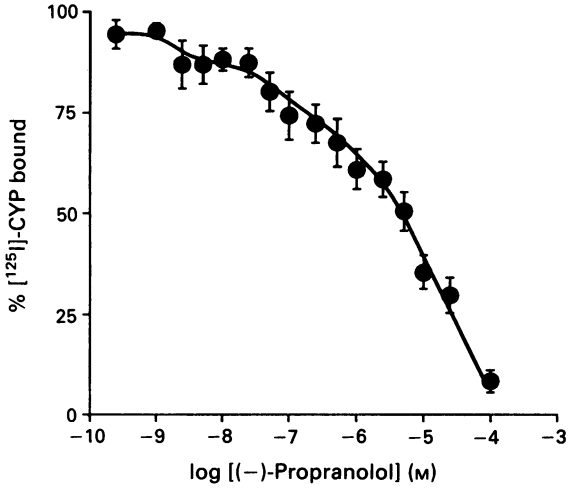


Figure 3 Competition between (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) and (−)-propranolol for binding sites in homogenates of rat soleus muscle. Incubations were for 1 h at 37°C and non-specific binding was defined by 500 μM (±)-alprenolol. The biphasic curve indicates two sites. Corresponding affinity and pseudo Hill coefficient values are given in Table 1.

Table 1 Affinity and pseudo Hill coefficient values obtained in competition binding experiments against (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) in rat soleus muscle

Competitor	n	*pK_D ± s.e.*	*nH ± s.e.*
(−)-Propranolol	7	8.30 ± 0.19[1] 5.33 ± 0.08	0.38 ± 0.03
(±)-ICI 118551	4	9.78 ± 0.33[1] 5.45 ± 0.09	0.40 ± 0.04
(±)-CGP 20712A	4	3.68 ± 0.06	1.44 ± 0.31
(±)-Cyanopindolol	6	7.63 ± 0.09	0.70 ± 0.11
(−)-[^{125}I]-CYP	4	10.52 ± 0.19[1] 9.28 ± 0.02	
(±)-Alprenolol	6	5.66 ± 0.05	0.62 ± 0.04
(±)-Tertatolol	6	6.70 ± 0.06	0.66 ± 0.06
(±)-Nadolol	6	5.09 ± 0.07	0.50 ± 0.04
(−)-Pindolol	7	4.61 ± 0.08	0.64 ± 0.07
(+)-Pindolol	7	4.48 ± 0.05	0.83 ± 0.07
(−)-Tertatolol	4	6.82 ± 0.04	0.70 ± 0.05
(+)-Tertatolol	4	6.40 ± 0.04	0.81 ± 0.06
(−)-Alprenolol	4	6.16 ± 0.03	0.88 ± 0.05
(+)-Alprenolol	4	5.67 ± 0.02	0.95 ± 0.03
Prazosin	3	3.71 ± 0.30	0.61 ± 0.22
Atropine	3	2.89 ± 0.20	0.62 ± 0.14
SR 58611A (Krebs)	4	5.50 ± 0.06	0.80 ± 0.08
SR 58611A (Tris)	8	5.34 ± 0.04	0.84 ± 0.05
ICI D7114 (Krebs)	5	5.12 ± 0.05	0.71 ± 0.05
ICI D7114 (Tris)	6	4.96 ± 0.07	0.64 ± 0.62
BRL 37344 (Krebs)	4	3.39 ± 0.10	0.56 ± 0.09
BRL 37344 (Tris)	6	3.95 ± 0.12	0.46 ± 0.06
(±)-CGP 12177A (Krebs)	4	5.11 ± 0.08	0.64 ± 0.69
(±)-CGP 12177A (Tris)	5	4.80 ± 0.04	0.68 ± 0.04
(−)-Isoprenaline	4	3.55 ± 0.10	0.68 ± 0.10
(−)-Adrenaline	4	3.57 ± 0.15	0.56 ± 0.10
(−)-Noradrenaline	4	3.20 ± 0.15	0.62 ± 0.14

[1]pK_D values obtained at classical β-adrenoceptors
Values are mean ± s.e. from n separate experiments.

Competition binding in the presence of (−)-propranolol

Antagonists Competition studies were performed to determine the affinities of the non-selective β-adrenoceptor antagonists (±)-alprenolol, (±)-nadolol and (±)-tertatolol

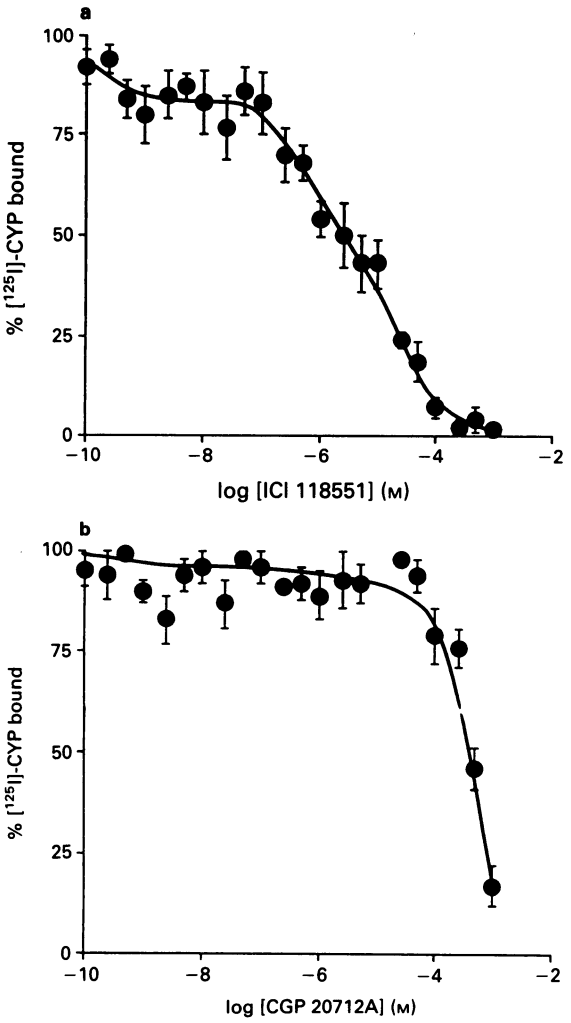


Figure 4 Competition between (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) and (a) the selective β_2-adrenoceptor antagonist (±)-ICI 118551 and (b) the selective β_1-adrenoceptor antagonist (±)-CGP 20712A in the absence of (−)-propranolol. Note the biphasic nature of competition by (±)-ICI 118551. Corresponding affinity and pseudo Hill coefficient values are given in Table 1.

for the propranolol-resistant (−)-[^{125}I]-CYP binding site (Table 1). (±)-Tertatolol was the most effective compound, and the order of potency (pK_D) for these antagonists was (±)-tertatolol (6.70) > (±)-alprenolol (5.66) > (±)-nadolol (5.09) (Figure 5a).

The structurally related compounds (±)-cyanopindolol (CYP) and the isomers of pindolol competed for the propranolol-resistant site (Table 1, Figure 5b). These competitors showed the following order of potency (pK_D): (±)-CYP (7.63) > (−)-pindolol (4.61) ≥ (+)-pindolol (4.49). The site failed to show significant stereoselectivity ($P > 0.05$) for the stereoisomers of pindolol.

Stereoselectivity was further examined by use of stereoisomers of compounds with higher affinity for the atypical site (Table 1, Figure 5c). Stereoselective competition for (−)-[^{125}I]-CYP binding was demonstrated for (−)-tertatolol (pK_D 6.82) > (+)-tertatolol (pK_D 6.40) and (−)-alprenolol (pK_D 6.16) > (+)-alprenolol (pK_D 5.67). These isomers produced curves that were significantly different in position ($P < 0.05$) with stereoselectivity indices (pK_D (−)-isomer minus pK_D (+)-isomer) of 0.42 ± 0.09 for tertatolol and 0.49 ± 0.04 for alprenolol. Table 1 gives the mean pK_D values together with pseudo Hill coefficient values.

Agonists The agonists SR58611A, ICI D7114 and BRL 37344, which are active at atypical β-adrenoceptors, competed at the propranolol-resistant (−)-[^{125}I]-CYP binding site with an order of potency SR 58611A > ICI D7114 > BRL 37344. Table 1 gives mean pK_D and pseudo Hill coefficient values. The partial agonist (±)-CGP 12177 also competed for (−)-[^{125}I]-CYP binding with an affinity similar to that of ICI D7114 (Table 1). These studies were performed in both Krebs phosphate and Tris buffer and curves were not significantly different ($P > 0.05$) with the change of assay buffer (Figure 6).

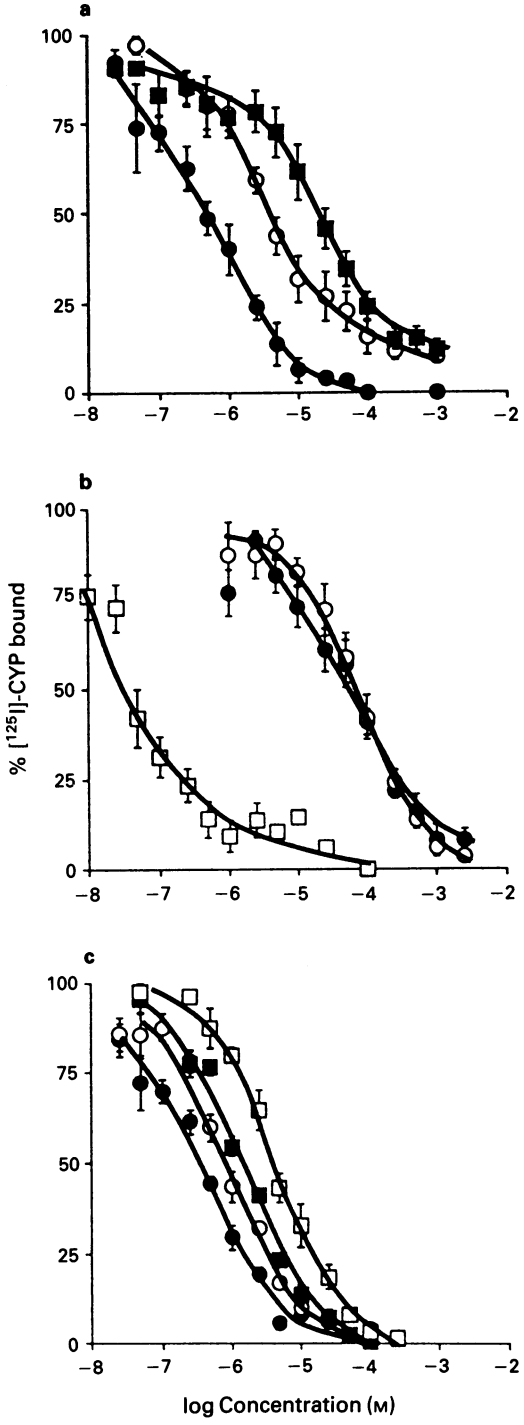
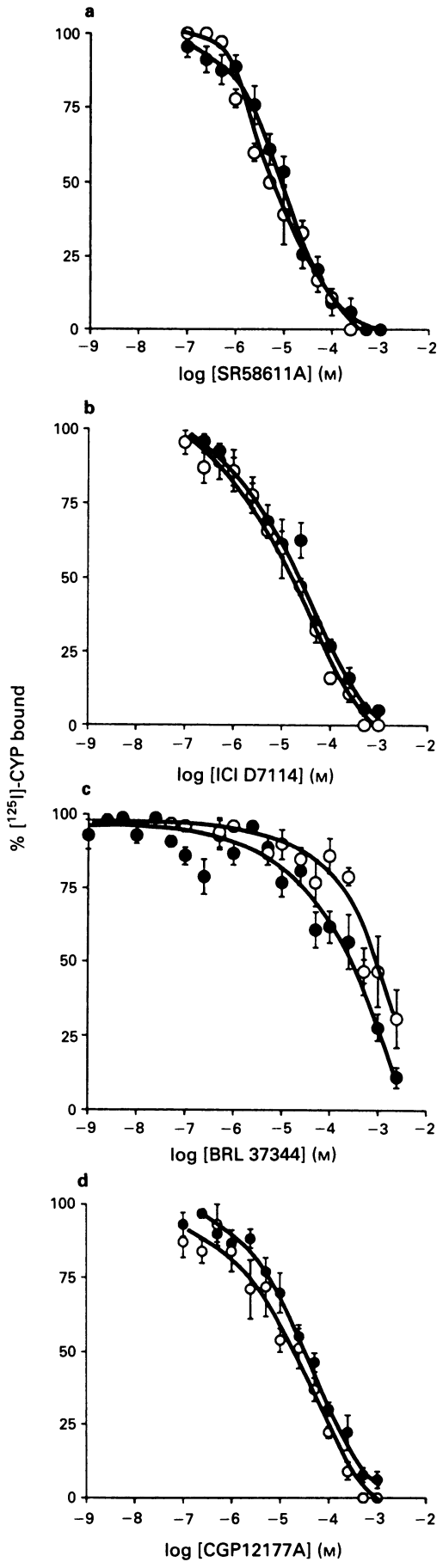


Figure 5 Mean curves for competition between (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) and (a) the antagonists (±)-tertatolol (●); (±)-nadolol (■); and (±)-alprenolol (○); (b) (±)-CYP (□); (−)-pindolol (●); and (+)-pindolol (○); (c) the stereoisomers of alprenolol (−) (■); (+) (□); and tertatolol (−) (●); (+) (○); in the presence of 0.1 μM (−)-propranolol. Points show mean ± s.e. Corresponding affinity and pseudo Hill coefficient values are given in Table 1.



Competition between (−)-[^{125}I]-CYP and the catecholamines, (−)-adrenaline, (−)-noradrenaline and (−)-isoprenaline, was also examined. Table 1 shows that these agonists all had low affinity for the propranolol-resistant site and that the curves were distinctly biphasic in nature (Figure 7). The addition of GTP to the incubation mixture was found to have no effect (results not shown). Competition between (−)-[^{125}I]-CYP binding and the α-adrenoceptor antagonist, prazosin, and the muscarinic antagonist, atropine, were examined. Competition by these compounds was of extremely low affinity (Table 1, Figure 8).

Discussion

The rat soleus muscle contains at least four distinct binding sites for (−)-[^{125}I]-CYP, β_1- and β_2-adrenoceptors (Elfellah & Reid, 1987; Kim *et al.*, 1991), a highly localized 5-HT-sensitive site (Molenaar *et al.*, 1991) and a propranolol-resistant site. The affinity of (−)-[^{125}I]-CYP for the propranolol resistant site (K_D = 522 pM) has close similarities with its affinity at the cloned human β_3-adrenoceptor (K_D = 500 pM, Emorine *et al.*, 1989) and the β_3-adrenoceptor expressed in 3T3-F442A cells ($K_D \sim$ 500 pM, Thomas *et al.*, 1992). Other reports of K_D values for (−)-[^{125}I]-CYP at β_3-adrenoceptors have ranged from 1.3 nM for rat β_3-adrenoceptors expressed in CHO cells (Muzzin *et al.*, 1991) to 1.9 nM in 3T3-F442A adipocytes (Feve *et al.*, 1992). This variation in reported K_D values is not uncommon for binding studies performed in different laboratories under slightly different conditions and all show clearly the lower affinity of (−)-[^{125}I]-CYP for the β_3-adrenoceptor compared to typical β_1- and β_2-adrenoceptors (Neve *et al.*, 1986).

The existence of a low affinity β-adrenoceptor site on the muscle is further supported by the two affinity values obtained for (−)-propranolol in competition experiments. The pK_D value (8.30) for the high affinity site corresponds to the affinity of propranolol in tissues containing typical β-adrenoceptors (Molenaar *et al.*, 1987) while the low affinity site with a pK_D of 5.33 (Table 1) is indicative of a propranolol-resistant site similar in affinity to those described in adipocytes (Arch, 1989) and gastrointestinal tissues such as ileum (Blue *et al.*, 1990) and oesophagus (Ford *et al.*, 1992). In radioligand binding experiments, biphasic competition by (−)-propranolol has also been observed in intestinal membranes (Van der Vliet *et al.*, 1990). In the present study a biphasic curve was also seen with the selective β_2-adrenoceptor antagonist (±)-ICI 118551. The lower affinity pK_D values obtained for both of these antagonists correspond well with values obtained for (−)-[^{125}I]-CYP competition in adipocytes (Muzzin *et al.*, 1991) (Table 2). The low affinity shown by the selective β_1-adrenoceptor antagonist (±)-CGP 20712A suggests that few (Kim *et al.*, 1991) if any β_1-adrenoceptors exist in the preparation and only competition for the more abundant propranolol-resistant site is observed. (±)-CGP 20712A is a weak antagonist of BRL 37344-induced adenylate cyclase stimulation in adipocytes, producing a clear depression of the response only at 1 mM (Hollenga *et al.*, 1991).

Many of the compounds that compete with (−)-[^{125}I]-CYP for the propranolol-resistant site also have pharmacological activity at atypical β-adrenoceptors in adipocytes and gastrointestinal tissues (Table 2). The pharmacological tools for

Figure 6 Mean curves for competition between (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) and various atypical β-adrenoceptor agonists: (a) SR 58611A; (b) ICI D7114; (c) BRL 37344, and (d) (±)-CGP 12177A in the presence of 0.1 μM (−)-propranolol. Curves are shown for competition in the two buffer systems Krebs (○) and Tris (●). Points show mean ± s.e. Corresponding affinity and pseudo Hill coefficient values are given in Table 1.

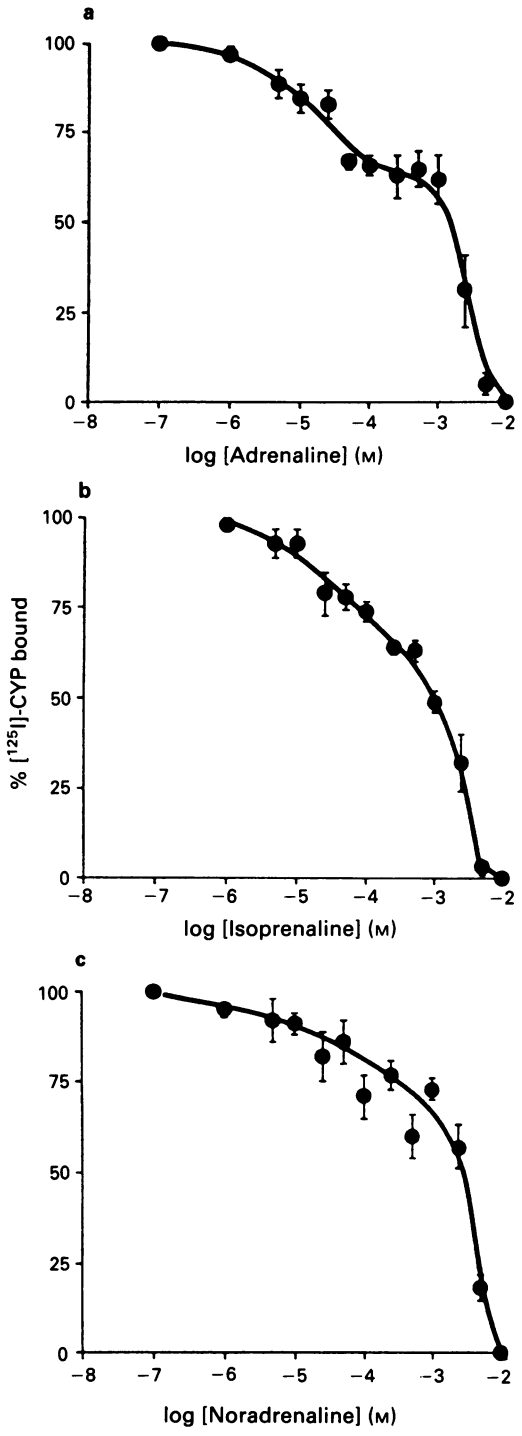


Figure 7 Mean curves for competition between (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) and the typical β-adrenoceptor catecholamine agonists: (a) (−)-adrenaline; (b) (−)-isoprenaline; and (c) (−)-noradrenaline in the presence of 0.1 μM (−)-propranolol. Competition by each of these agonists is clearly biphasic. Points show mean ± s.e. Corresponding affinity and pseudo Hill coefficient values are given in Table 1.

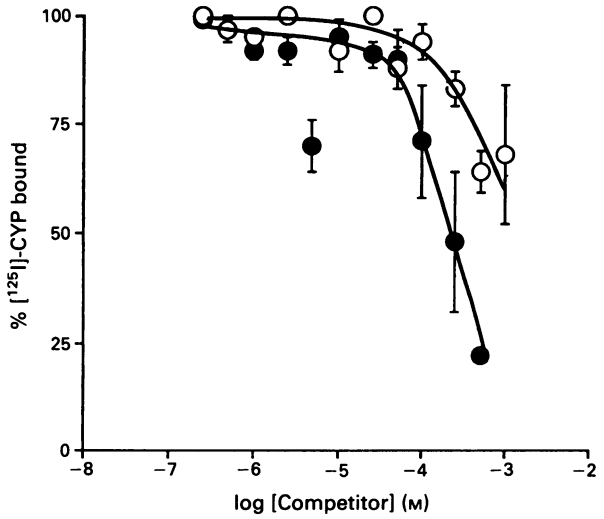


Figure 8 Mean competition binding curves between (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) and the α_1-adrenoceptor antagonist prazosin (●) and the muscarinic antagonist atropine (○) in the presence of 0.1 μM (−)-propranolol. Points show mean ± s.e. Corresponding affinity and pseudo Hill coefficient values are given in Table 1.

atypical β-adrenoceptor identification include the typical β-adrenoceptor antagonists, alprenolol, nadolol and pindolol. Studies on guinea-pig ileum found that nadolol was an inhibitor of BRL 37344-induced relaxation, exhibiting a low affinity for the site with a pA_2 of 4.31 (Bond & Clarke, 1988). This value was 3 orders of magnitude lower than the pA_2 of 7.7 at typical β-adrenoceptors (Lee *et al.*, 1975), but compared favourably with the pA_2 value of 5.1 in rat oesophagus (Ford *et al.*, 1992) and the pK_D of 5.09 found in the present study for soleus muscle. The action of a novel β-adrenoceptor agonist, SR58306A on rat colon preparations was most effectively antagonized by alprenolol (pA_2 7) (Croci *et al.*, 1988). Alprenolol also has a higher affinity than nadolol in blocking atypical responses in guinea-pig ileum (pA_2 6.47) and rat oesophagus (pA_2 6.6) (Ford *et al.*, 1992). These values are lower than its affinity (pA_2 8.2) at typical β-adrenoceptors (Blue *et al.*, 1990).

The action of tertatolol as an antagonist varies from the typical β-adrenoceptor blockers. It has been suggested that tertatolol binds competitively to typical β-adrenoceptors and also modifies the receptor so that it is no longer available for ligand binding (De Blasi *et al.*, 1988). At atypical β-adrenoceptors tertatolol has a pA_2 value of 6.8 in guinea-pig ileum (Bond & Vanhoutte, 1992) and 6.1 in rat oesophagus which correspond to the value of 6.70 in this study, these values being 100 fold lower than the affinity of tertatolol at typical β-adrenoceptors. The affinity of (±)-CYP (pK_D 7.63) in this study is lower than its affinity at typical β-adrenoceptors (pK_D 9.4, Meunier & Labrie, 1982) but compares with the pA_2 values for (±)-CYP antagonism of isoprenaline responses in guinea-pig ileum in the presence of propranolol with a pA_2 value of 7.63 (Blue *et al.*, 1989) and more recently in rat oesophagus pA_2 7.3 (Ford *et al.*, 1992). These values are also similar to those found for the (±)-CYP antagonism of BRL 37344 mediated responses in rat gastric fundus (McLaughlin & MacDonald, 1991) and distal colon (McLaughlin & MacDonald, 1990) (Table 2).

Some authors argue that describing a new binding site as a receptor requires that the site fits certain criteria including:- affinity, reversibility, saturability, distribution, a rank order of potency of compounds and stereospecificity (Laduron, 1984). The above criteria have been satisfied in this study, for although the site showed a lack of stereoselectivity for the isomers of pindolol it shows small but significant selectivity for the stereoisomers of both tertatolol and alprenolol. One of the earliest differences reported which distinguished adipocyte adrenoceptors from typical β_1- (atrial) and β_2- (diaphragm) adrenoceptors was the lower stereoselectivity ratios of antagonists such as the isomers of alprenolol which showed a 10 fold difference for total β-adrenoceptors in adipocytes compared to a 200 fold difference for total β-adrenoceptors in atria (Harms *et al.*, 1977). The suggestion was that β-adrenoceptors existed as isoreceptors in different

Table 2 Comparison of affinities of various compounds for atypical β-adrenoceptors

Compound	*Soleus*[a*]	*Adipocytes*	*Gut*	*Oesophagus*[p]	*Cloned β_3-AR*
(−)-Propranolol	5.33	6.8[b], 5.8[c*]	6.1[g], 6.1[h]	5.9	6.35[q]
(±)-Alprenolol	5.66	6.8[b], 6.4[c*]	6.47[g], 7[i]	–	–
(−)-Alprenolol	6.16	–	–	6.6	–
(±)-Nadolol	5.09	–	4.68[g], 4.31[j]	5.1	–
(±)-Tertatolol	6.70	–	6.8[k]	6.1	–
(−)-Pindolol	4.61	–	–	–	–
(±)-Pindolol	–	–	–	5.9	–
(±)-CYP	7.63	7[b]	6.56[l], 6.67[m], 7.63[n]	7.3	–
(−)-[^{125}I]-CYP	9.28	8.82[d]	–	–	9.31[r] 9.06[q]
(±)-ICI 118551	5.45	5.3[c*], 5.3[d*]	5.5[h]	–	6.6[r*]
(±)-CGP 20712A	3.68	4.6[e], 5.3[d*]	–	–	5.6[r*]
ICI D7114	5.12	–	–	–	–
SR 58611A	5.50	–	8.4[o]	–	–
(±)-CGP 12177A	5.11	6.6[f], 6.8[d*]	–	–	6.86[r*]
BRL 37344	3.39	6.6[c*], 6.9[d*]	5.3[l]	–	6.7[r*]
(−)-Isoprenaline	3.55	4.2[c*], 5.2[d*]	6.3[l]	–	6.2[r*]
(−)-Adrenaline	3.57	3.8[c*], 3.6[d*]	5.8[h]	–	4.7[r*]
(−)-Noradrenaline	3.20	3.3[c*], 3.9[d*]	6.7[l]	–	6.3[r*]

*Values obtained from (−)-[^{125}I]-cyanopindolol ((−)-[^{125}I]-CYP) competition data; other values are functionally obtained EC_{50} or pK_A values.

[a]Present study; [b]Arch (1989); [c]Muzzin *et al.* (1991); [d]Feve *et al.* (1991); [e]Hollenga *et al.* (1991); [f]Granneman & Whitty (1991); [g]Blue *et al.* (1990); [h]Van der Vliet *et al.* (1990); [i]Croci *et al.* (1988); [j]Bond & Clarke (1988); [k]Bond & Vanhoutte (1992); [l]McLaughlin & McDonald (1991); [m]McLaughlin & McDonald (1990); [n]Blue *et al.* (1989); [o]Bianchetti & Manara (1990); [p]Ford *et al.* (1992); [q]Nahmias *et al.* (1991); [r]Emorine *et al.* (1989).

species and tissues. A different degree of stereoselectivity in two tissues is a strong indication for a difference between the receptors involved. A lower steroselectivity for antagonists is a feature of atypical β-adrenoceptors (Bojanic *et al.*, 1985; Zaagsma & Nahorski, 1990) and distinguishes a site from typical β-adrenoceptors. Autoradiographic studies of propranolol resistant (−)-[^{125}I]-CYP binding in rat skeletal muscle sections have also shown stereoselectivity for the isomers of alprenolol (Molenaar *et al.*, 1991).

A lower affinity displayed by agonists in receptor binding assays compared to their potency in functional assays is not unusual, as agonist binding prefers the high affinity state acquired in functional studies. In binding studies the receptors tend to be predominantly in the low affinity state, regardless of the assay buffer used, although agonist binding to typical β-adrenoceptors is affected by the change in assay buffer (McPherson *et al.*, 1985). However, competition by atypical agonists in this study for the propranolol-resistant site was not influenced by the choice of assay buffer (Krebs phosphate buffer or Tris-Mg^{2+} buffer). The agonist with the highest affinity for the skeletal muscle site was a phenylethanolaminotetraline, SR58611A which has previously been shown to have selective agonist activity at atypical β-adrenoceptors in rat colon (Croci *et al.*, 1988; Bianchetti & Manara, 1990) and in mediating adipocyte lipolysis (Manara & Bianchetti, 1990). The action of SR58611A in these tissues was blocked by alprenolol. BRL 37344 is a phenylethylamine analogue which stimulates lipolysis via an atypical β-adrenoceptor in rat adipocytes (Arch *et al.*, 1984; Wilson *et al.*, 1984). The action of BRL 37344 tends to be variable in different tissues. In rat gastric fundus (McLaughlin & MacDonald, 1991) and rat distal colon (McLaughlin & MacDonald, 1990) it produced tachyphylaxis to itself and to isoprenaline and was only weakly antagonized by propranolol (1 μM). ICI D7114 shows selectivity for effects on brown fat and in stimulating whole body oxygen consumption at doses which show no chronotropic effects in the heart (Holloway *et al.*, 1991). CGP 12177 appears to act as a partial agonist in brown adipose tissue where it stimulates adenylate cyclase activity (Granneman & Whitty, 1991). The partial agonist activity of CGP 12177 has also been described at the cloned β_3-adrenoceptor in rat (Granneman *et al.*, 1991) and mouse (Nahmias *et al.*, 1991). A comparison of atypical β-adrenoceptor agonist affinities obtained in various studies is given in Table 2.

Biphasic competition by the catecholamines, (−)-adrenaline, (−)-noradrenaline and (−)-isoprenaline, may be due to the low affinity that they show for this site. Table 2 shows however that low affinities for these catacholamines have also been reported in other binding studies. It is possible that the very high concentrations used could displace (−)-[^{125}I]-CYP from other sites and for this reason it is wise to limit the emphasis placed on displacement by exceptionally high concentrations of competitor. This is illustrated by the displacement by atropine and prazosin at concentrations in the millimolar range. This phenomenon may also be relevant to the displacement seen by (±)-CGP 20712A and BRL 37344 at very high concentrations.

The functional role of such receptors and their physiological significance has yet to be established. A different order of potency for endogenous agonists such as seen between β_1- and β_2- subtypes allows tissues to have different sensitivities to components of the sympathetic nervous system (Arch, 1989). The cloned β_3-adrenoceptor shows a higher sensitivity to noradrenaline than to adrenaline which has led to the suggestion that the sympathetic innervation in response to stress, high energy intake or cold acclimatization may modulate β_3-adrenoceptor activity (Emorine *et al.*, 1989). Evidence for sympathetic innervation of atypical β-adrenoceptors has been shown in guinea-pig ileum preparations (Taneja & Clarke, 1992). In these preparations the responses to para-arterial sympathetic stimulation in the presence of ICI 118551 and CGP 20712A were blocked by compounds such as (−)-alprenolol and nadolol indicating that the relaxant response could partly be attributed to the involvement of innervated atypical β-adrenoceptors.

Based on the differences in molecular structure of the β-adrenoceptor subtypes it has been suggested that the importance of a third β-adrenoceptor may relate to differences in regulation. Both β_1- and β_2-adrenoceptors are readily desensitized upon exposure to β-adrenoceptor agonists (Lefkowitz *et al.*, 1990) due to phosphorylation of Ser/Thr residues in the carboxy terminus. In contrast, the β_3-adrenoceptor is predicted to be less susceptible to desensitiza-

tion due to the presence of fewer phosphorylation sites (Emorine *et al.*, 1991).

The role of an atypical β-adrenoceptor in skeletal muscle has yet to be determined. Due to its bulk, skeletal muscle may be an important site of adrenergically induced thermogenesis (Yang & McElligott, 1989). BRL 26830A has been observed to stimulate lactate formation and inhibit glycogen synthesis in soleus muscle (Challiss *et al.*, 1988). This thermogenic response is resistant to blockade by propranolol. Also of interest is the propranolol resistance of clenbuterol-induced protein anabolism in rat soleus and plantaris muscle (Maltin *et al.*, 1989).

Taken together these functional data tend to suggest a family of β-adrenoceptors in skeletal muscle that are distinct from the β_1- and β_2-subtypes. Whether or not the receptors described in binding assays in this study are the same receptors as those identified functionally has yet to be established. However the affinity of (−)-[125I]-CYP, the resistance of (−)-[125I]-CYP binding to (−)-propranolol, the low affinity of other β-adrenoceptor antagonists, the low stereoselectivity ratios displayed by antagonists, the affinity of atypical β-adrenoceptor agonists and the potent competition shown by (±)-CYP suggests that the site present on soleus muscle is similar to atypical β-adrenoceptors described in adipocytes and gastrointestinal tissues.

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