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# Cyclic AMP accumulation in rat soleus muscle: stimulation by $\beta_2$- but not $\beta_3$-adrenoceptors

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**Abstract**

The $\beta$-adrenoceptor subtypes involved in cyclic AMP accumulation in rat soleus muscle were studied using $\beta_1$- $\beta_2$- and $\beta_3$-adrenoceptor agonists and antagonists. Responses to (−)-isoprenaline were antagonised by (−)-propranolol ($pK_B = 8.32$ at 0.1 $\mu$M) and by erythro-DL-1(7-methylindian-4-yloxy)-3-isopropylaminobutan-2-ol ((±)-ICI 118551) ($pK_B = 9.38$ at 10 nM and 9.65 at 100 nM) but not by 2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1H-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulfonate ((±)-CGP 20712A at 10 nM or 100 nM). The $\beta_3$-adrenoceptor agonist sodium-4-[-2[-2-hydroxy-2-(-3-chlorophenyl)ethylamino]propyl]phenoxyacetate (BRL 37344 at 10 pM or 10 $\mu$M) caused no significant change in basal cyclic AMP levels and had no effect on the level of cyclic AMP accumulation stimulated by (−)-isoprenaline, zinterol or forskolin. (−)-Isoprenaline pretreatment (400 $\mu$g kg$^{-1}$ h$^{-1}$, 14 days) abolished responses to (−)-isoprenaline (10 $\mu$M) and zinterol (1 $\mu$M) while BRL 37344 had no effect in either isoprenaline or vehicle-treated groups. These results show that $\beta_3$-adrenoceptor agonists do not stimulate cyclic AMP accumulation in rat soleus muscle and that (−)-isoprenaline induced increases in cyclic AMP levels are mediated predominantly by $\beta_2$-adrenoceptors. This suggests that the previously reported increase in glucose uptake by $\beta_3$-adrenoceptor agonists in skeletal muscle does not involve direct stimulation of adenylate cyclase. © 1998 Elsevier Science B.V.

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## 1. Introduction

Activation of $\beta$-adrenoceptors influences a number of metabolic and physiological processes in skeletal muscle including glucose uptake and metabolism, growth promotion, increased heat production (thermogenesis) and contractility (for review, see Yang and McElligott, 1989). The biological effects of catecholamines on skeletal muscle are believed to be mediated by elevation of tissue cyclic AMP levels through the activation of adenylate cyclase. Proteins phosphorylated as a result of cyclic AMP elevation and protein kinase A activation in skeletal muscle include: glycolytic enzymes, sarcolemmal $Na^+/K^+$ pumps, phospholamban and voltage-sensitive and sarcolemmal $Ca^{2+}$ channels (Yang and McElligott, 1989). Most functional and receptor binding studies indicate that the $\beta_2$-adrenoceptor is the predominant $\beta$-adrenoceptor subtype in skeletal muscle (Elfellah and Reid, 1987; Liggett et al., 1988); however, there is increasing evidence for the presence of a $\beta$-adrenoceptor in skeletal muscle that is distinct from $\beta_1$- and $\beta_2$-adrenoceptors.

Skeletal muscle plays a major role in the thermogenic action of $\beta$-adrenoceptor agonists and a range of selective $\beta_3$-adrenoceptor compounds have been found to be potent and selective stimulants of non-shivering thermogenesis (Astrup, 1986; Thurlby and Ellis, 1986). Challiss et al. (1988) identified a propranolol-resistant component of glycogen synthesis in rat soleus muscle that was stimulated by the $\beta_3$-adrenoceptor agonist BRL 28410. Skeletal muscle is also the major site of insulin-stimulated glucose disposal in humans and rats; hence, it is regarded as a target tissue of selective $\beta_3$-adrenoceptor compounds that produce anti-diabetic and anti-hyperglycaemic effects (Arch et al., 1991). Stimulation of the sympathetic nervous system via the rat ventromedial hypothalamus increased glucose uptake in brown adipose tissue, heart and skeletal muscle (Sudo et al., 1991). Similarly, the $\beta_3$-adrenoceptor agonists BRL 37344 and BRL 35135 increase the rate of

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glucose uptake and tissue glucose utilisation in brown and white adipose tissue, heart, skeletal muscle and diaphragm (Abe et al., 1993; Liu and Stock, 1995). Additionally, the propranolol-resistance of clenbuterol and cimaterol stimulated protein accretion in skeletal muscle has led some authors to describe the predominant skeletal muscle $\beta$-adrenoceptor as an 'atypical $\beta_2$-adrenoceptor' (Arch et al., 1991). Collectively, these functional studies support a role for skeletal muscle as a target tissue for the $\beta_3$-adrenoceptor anti-obesity/thermogenic agents.

Autoradiographic studies have identified propranolol-resistant [$^{125}$I](−)-cyanopindolol binding sites distributed across the rat hindlimb muscle bundle and most abundant in the soleus muscle (Molenaar et al., 1991). Further characterisation of this site showed the presence of both $\beta_2$-adrenoceptor and atypical $\beta$-adrenoceptor binding sites in rat soleus muscle (Roberts et al., 1993; Sillence et al., 1993). This atypical binding site shares several features in common with the adipocyte $\beta_3$-adrenoceptor including the low affinity of catecholamines, propranolol and other $\beta$-adrenoceptor antagonists compared to their affinities/potencies at $\beta_1$- and $\beta_2$-adrenoceptors (for review, see Arch and Kaumann, 1993). Early indications that $\beta_3$-adrenoceptor mRNA was present in skeletal muscle (Emorine et al., 1989) have not been confirmed by more specific molecular techniques that have conclusively shown the presence of a strong $\beta_3$-adrenoceptor mRNA signal in white and brown fat, ileum, colon and brain (Granneman et al., 1991; Krief et al., 1993; Evans et al., 1996; Summers et al., 1995a).

The present study was designed to characterise the $\beta$-adrenoceptor subtypes involved in the stimulation of cyclic AMP accumulation in rat soleus muscle, and to determine whether $\beta_3$-adrenoceptors contribute to this response. The regulation of the $\beta$-adrenoceptor subtypes involved were also examined using soleus muscle from animals chronically pretreated with (−)-isoprenaline.

## 2. Materials and methods

### 2.1. Soleus muscle tissue preparation

Male Sprague–Dawley rats (250–350 g) were anaesthetised with 80% $C0_2$/20% $O_2$ and exsanguinated (two animals were required to provide adequate tissue for each experiment). Hindleg muscle bundles were dissected from both hindlimbs and placed into ice-cold Krebs bicarbonate solution (composition mM: NaCl 118.4, KCl 4.7, $CaCl_2$ 1.9, $NaHCO_3$ 25, $MgSO_4$ 1.2, D-glucose 11.7, $NaH_2PO_4$ 1.2.) previously gassed with carbogen (95% $O_2$/5% $CO_2$, pH 7.4) containing 0.1 mM ascorbic acid and 0.1 mM ethylenediaminetetraacetic acid (EDTA). The soleus muscles were removed, divided into two equal parts along the length of the muscle and chopped into smaller slices (0.5 $mm^2$) on a McIlwain tissue chopper.

Soleus muscle slices (10–20 mg) were transferred into individually carbogenated 5 ml tubes and incubated at 37°C for 25 min in the absence or presence of antagonists. Agonists were added in 20 $\mu$l volume with 100 $\mu$l 3-isobutyl-1-methyl-xanthine (IBMX) (0.1 mM), the tubes were recarbogenated and incubated for a further 5 min. To determine basal cyclic AMP production one set of tubes had no drug added. All incubations were performed in duplicate. After the stimulation period, the incubation medium was removed to prevent interference by residual salt (Albano and Barnes, 1974) and replaced with 1 ml ice-cold 6% trichloracetic acid for cellular extraction of cyclic AMP.

### 2.2. Extraction and determination of cyclic AMP

The trichloracetic acid was removed from each tube and treated with 3 ml of 0.5 M tri-*n*-octylamine dissolved in 1,1,2-trichloro-trifluorethane. The cyclic AMP-containing neutralised aqueous phase was removed from each tube and diluted 1 in 4 with a 50-mM sodium acetate assay buffer (containing 0.1% bovine serum albumin (BSA) and 1 mM theophylline) (pH 5.0) to eliminate residual trichloracetic acid interference (Albano and Barnes, 1974). Each sample (600 $\mu$l) was acetylated, and duplicate 100 $\mu$l aliquots of the acetylated samples and unknowns were assayed for cyclic AMP by radioimmunoassay (RIA) as described by Marley et al. (1991). The soleus muscle pellet remaining after trichloracetic acid removal was dissolved in 2 M NaOH, aliquots of each sample (50 $\mu$l) were diluted with 150 $\mu$l $dH_2O$, and proteins were measured by the method of Lowry et al. (1951).

### 2.3. Implantation of osmotic mini pumps

Male Sprague–Dawley rats (280–320 g) were anaesthetised with pentobarbitone sodium (Nembutal) 25 mg/kg i.p., the back of the neck shaved and a local subcutaneous injection of lignocaine (2%) made. Osmotic mini pumps (Alzet, model 2002) were implanted subcutaneously to deliver 0.5 $\mu$l/h of either (−)-isoprenaline (400 $\mu$g/kg per h) or vehicle (1 mM HCl). After 14 days, the animals were anaesthetised with 80% $C0_2$/20% $O_2$ and exsanguinated. Soleus muscles were removed and prepared as described above.

### 2.4. Analysis of cyclic AMP studies

Standard curves were generated of the % $^{125}$I-cyclic AMP bound as a function of the log cyclic AMP concentration using PRISM (Intuitive Software for Science) and interpolation of unknowns was performed with an internal standard curve measured in each experiment. The amount of cyclic AMP produced was normalised to the protein content of the muscle slices (fmol/$\mu$g protein) and cyclic AMP accumulation was expressed as a % of the maximum

response to (−)-isoprenaline. Nonlinear regression analysis (PRISM2) was used to fit curves to the grouped concentration response data and $pEC_{50}$ values were calculated. An estimate of the antagonist affinity was obtained from the shift to the right of the mean concentration–response curve produced by a single concentration of antagonist giving an apparent $pA_2$ ($pK_B$ value),

$$pK_B = \log(\text{agonist ratio} - 1) - \log[\text{antagonist}],$$

according to Furchgott (1972). Significance was established using a Student's paired *t*-test with $P < 0.05$ considered significant. All results were presented as means ± S.E.M. for the stated number of observations (*n*).

### 2.5. Drugs and reagents

The drugs and reagents used were as follows: (−)-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); (−)-isoprenaline bitartrate, (−)-isoprenaline hydrochloride, (−)-epinephrine, (−)-norepinephrine, IBMX (3-isobutyl-1-methylxanthine), forskolin, adenosine-3′:5′-cyclic monophosphate sodium salt (cyclic AMP) (Sigma, St. Louis, MO, USA); (±)-CGP 20712A (2-hydroxy-5(2-((2-hydroxy-3-(4-((1-methyl-4-trifluoromethyl)1*H*-imidazole-2-yl)-phenoxy)propyl)amino)ethoxy)-benzamide monomethane sulfonate), BRL 37344 (sodium-4-[-2[-2-hydroxy-2-(-3-chloro-phenyl) ethylamino] propyl] phenoxyacetate) (Smith Kline Beecham, Great Burgh, Epsom, UK); SR 58611A (RS-*N*-(7-carbethoxymethoxyl 1,2,3,4-tetrahydronaphth-2-yl)-2 hydroxy 2-(3-chlorophenyl)ethanamine) (Sanofi-Midy Research Centre, Milan, Italy); (±)-CGP 12177 hydrochloride ((−)-4-)3-*t*-butylamino-2-hydroxypropoxy)benzimidazol-2-one) (Research Biochemicals, Massachusetts, USA); zinterol HCl (Bristol-Myer Squibb, Victoria, Australia); $^{125}$I-2′-*O*-monosuccinyladenosine-3′:5′-cyclic monophosphate tyrosylmethylester, rabbit polyclonal antiserum Ab 5102 (Dr. P. Marley, University of Melbourne, Australia).

## 3. Results

### 3.1. Stimulation of cyclic AMP accumulation by catecholamines

A concentration-dependent increase in cyclic AMP accumulation in rat soleus muscle was produced by the three catecholamines with a rank order of potency as follows: (−)-isoprenaline > (−)-adrenaline ≥ (−)-noradrenaline (Table 1, Fig. 1a). Forskolin also produced a concentration dependent increase in cyclic AMP to levels 7–8-fold higher than those caused by (−)-isoprenaline (Fig. 1b).

Table 1
Effects of agonists on cyclic AMP accumulation in rat soleus muscle

| Agonist | (*n*) | $pEC_{50}$ | ($EC_{50}$) |
|---|---|---|---|
| (−)-Isoprenaline | 4 | 7.21 ± 0.10 | ($6.24\times10^{-8}$) |
| (−)-Adrenaline | 4 | 5.66 ± 0.22 | ($2.21\times10^{-6}$) |
| (−)-Noradrenaline | 4 | 5.40 ± 0.11 | ($3.98\times10^{-6}$) |
| BRL 37344 | 5 | No response | |
| SR 58611A | 3 | No response | |
| ICI D7114 | 3 | No response | |
| CGP 12177A | 3 | No response | |

Values are mean ± S.E.M. from *n* separate experiments.

### 3.2. Effects of antagonists on cyclic AMP accumulation

Concentration–response curves to (−)-isoprenaline were performed in the presence of (−)-propranolol, CGP 20712A and ICI 118551 to determine the β-adrenoceptor subtypes involved in the stimulation of cyclic AMP production in rat soleus muscle. (−)-Propranolol (0.1 μM) produced a rightward shift in the concentration–response curve with a $pK_B$ value of 8.32 suggesting involvement of $\beta_1$- and/or $\beta_2$-adrenoceptors (Fig. 2a). The selective $\beta_2$-

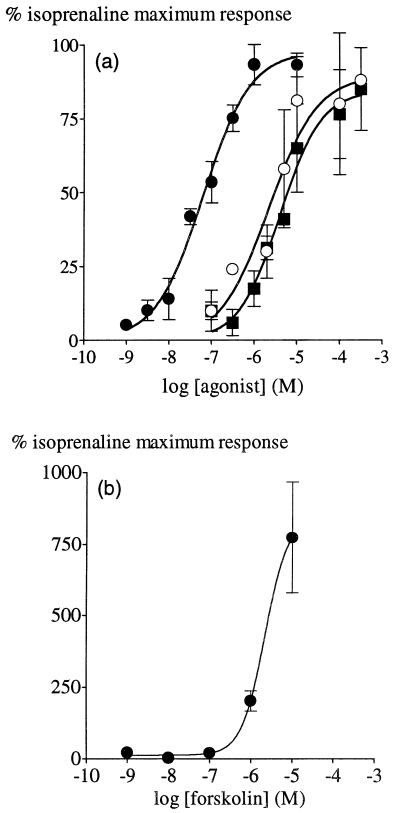


Fig. 1. Concentration–response curves of cyclic AMP accumulation in rat soleus muscle slices after stimulation with (a) the catecholamines (−)-isoprenaline (•), (−)-adrenaline (O) and (−)-noradrenaline (■) and (b) forskolin. Values are expressed as % of the maximum (−)-isoprenaline response in each tissue. Points show mean ± S.E.M. ($n = 4$). $EC_{50}$ values are shown in Table 1.

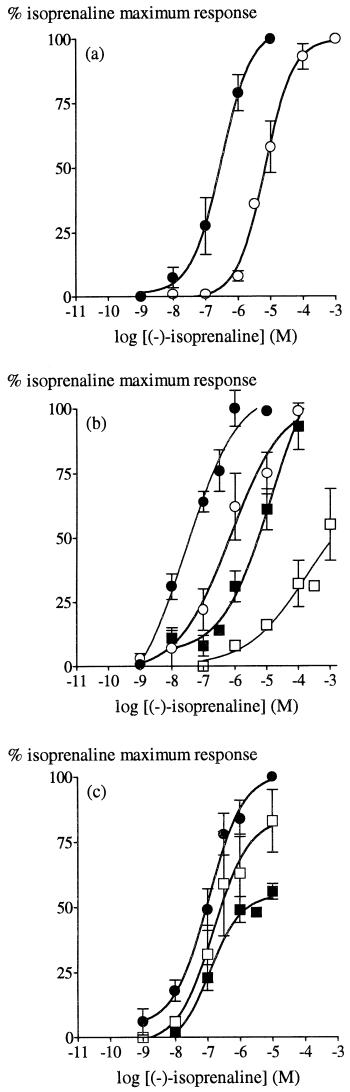


Fig. 2. Mean cumulative concentration–response curves showing the effect of various $\beta$-adrenoceptor antagonists on (−)-isoprenaline induced (•) cyclic AMP accumulation in rat soleus muscle. Graph (a) 0.1 $\mu$M (−)-propranolol (O), graph (b) the selective $\beta_2$-adrenoceptor antagonist ICI 118551 10 nM (O), 100 nM (■) and 1 $\mu$M (□) and graph (c) the selective $\beta_1$-adrenoceptor antagonist CGP 20712A 10 nM (■) or 100 nM (□). The results show a significant rightward shift of the (−)-isoprenaline concentration–response curve with propranolol and ICI 118551, while CGP 20712A did not significantly shift the (−)-isoprenaline concentration–response curve ($P > 0.05$), but a reduction of the maximum (−)-isoprenaline response was observed. Apparent $pA_2$ values are given in Table 2. Points are expressed as % of the maximum (−)-isoprenaline response and shown as mean ± S.E.M. ($n = 4–6$).

adrenoceptor antagonist ICI 118551 (10, 100, 1000 nM) was tested and found to produce concentration-dependent rightward shifts in the (−)-isoprenaline concentration–response curve (Fig. 2b). Concentrations of 10 nM and 100 nM ICI 118551 had $pK_B$ values of 9.38 and 9.65, respectively (Table 2). A $pK_B$ value could not be calculated for 1 $\mu$M ICI 118551 as the (−)-isoprenaline concentration–response curve was shifted too far to the right to obtain a maximum (−)-isoprenaline response.

Table 2
Apparent $pA_2$ ($pK_B$) values of compounds inhibiting (−)-isoprenaline stimulated cyclic AMP accumulation in rat soleus muscle

| Antagonist | $n$ | $pK_B$ value | $pA_2$ $\beta_1$–AR[a] | $pA_2$ $\beta_2$–AR[a] |
|---|---|---|---|---|
| (−)-propranolol 0.1 $\mu$M | 4 | 8.32 | 8.1–8.6 | 7.9–8.4 |
| ICI 118551 10 nM | 4 | 9.38 | 7.2 | 9.3 |
| ICI 118551 100 nM | 6 | 9.65 | | |
| ICI 118551 1 $\mu$M | 4 | – | | |
| CGP 20712A 10 nM | 4 | No shift | 9.6 | 5.4 |
| CGP 20712A 100 nM | 4 | No shift | | |

[a]Values from Arch and Kaumann, 1993.
Values are mean ± S.E.M. from $n$ separate experiments.

The selective $\beta_1$-adrenoceptor antagonist CGP 20712A (10 nM) caused a significant 40–50% reduction in the maximum response to (−)-isoprenaline ($P < 0.05$) (Fig. 2c). However, CGP 20712A (10 nM) caused no difference in the $pEC_{50}$ values of control (−)-isoprenaline curves

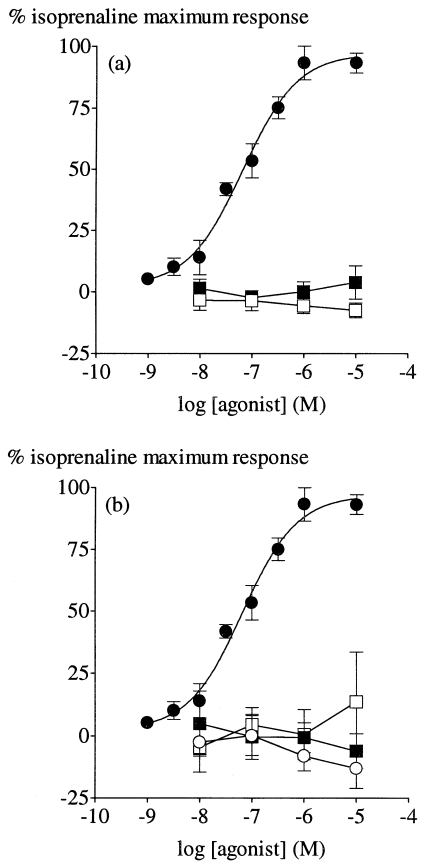


Fig. 3. Concentration–response curves of cyclic AMP accumulation in rat soleus muscle slices after addition of (−)-isoprenaline (•) or (a) the selective $\beta_3$-adrenoceptor agonist BRL 37344 in the absence (□) and presence (■) of the selective $\beta_2$-adrenoceptor antagonist ICI 118551 (100 nM) ($n = 5–6$). Also shown (b) are concentration–response curves for the selective $\beta_3$-adrenoceptor agonists ICI D7114 (O), SR 58611A (■) and CGP 12177A (□) ($n = 3$). Values were expressed as a % of the maximum (−)-isoprenaline (10 $\mu$M) response in each experiment and points show mean ± S.E.M.

($pEC_{50}$ 6.98 ± 0.14, $n = 8$) compared to curves in the presence of 10 nM CGP 20712A ($pEC_{50}$ 6.93 ± 0.28, $n = 4$). CGP 20712A (100 nM) also reduced the maximum (−)-isoprenaline response slightly, but caused no shift in the (−)-isoprenaline concentration–response curve ($pEC_{50}$ 6.82 ± 0.18, $n = 4$). The $pK_B$ values for (−)-propranolol, ICI 118551 and CGP 20712A are shown in Table 2, and these values are compared to the reported affinity of these compounds at $\beta_1$- and $\beta_2$-adrenoceptors.

### 3.3. Cyclic AMP accumulation by $\beta_3$-adrenoceptor agonists

The $\beta_3$-adrenoceptor agonist BRL 37344 failed to increase basal cyclic AMP levels even at 10 μM. It should be noted that BRL 37344 caused a slight, but not significant reduction in the basal levels of cyclic AMP at high concentrations, and this effect was not significantly altered in the presence of ICI 118551 (0.1 μM) (Fig. 3a). The $\beta_3$-adrenoceptor agonists CGP 12177A, SR 58611A and ICI D7114 (10 nM–10 μM) also failed to significantly increase basal cyclic AMP levels ($P > 0.05$) (Fig. 3b).

Any possible inhibitory effects of BRL 37344 (10 μM) were also examined against a higher background level of cyclic AMP production using maximum concentrations of (−)-isoprenaline (10 μM), forskolin (10 μM) and the selective $\beta_2$-adrenoceptor agonist zinterol (1 μM). Fig. 4 shows that BRL 37344 (10 μM) had no significant effect on the basal level of cyclic AMP ($P = 0.828$, $n = 6$), or on the level of cyclic AMP produced by either (−)-isoprenaline, forskolin or zinterol ($P > 0.05$, $n = 4$–6). Fig. 4 also shows the $\beta_2$-adrenoceptor antagonist ICI 118551 (100 nM) significantly blocks zinterol stimulated cyclic AMP accumulation ($P < 0.05$, $n = 4$).

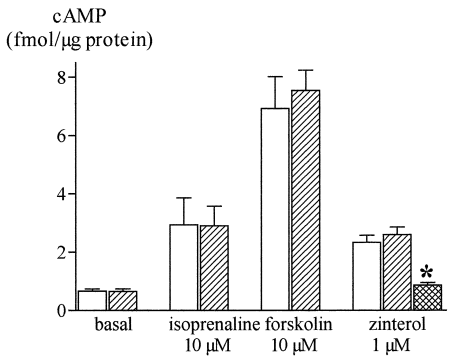


Fig. 4. The effect of BRL 37344 on basal and stimulated levels of cyclic AMP accumulation in rat soleus muscle. The bar graph shows basal levels of cyclic AMP accumulation ($n = 7$) and levels stimulated by (−)-isoprenaline (10 μM) ($n = 7$), forskolin (10 μM) ($n = 4$) and zinterol (1 μM) ($n = 4$) in the absence (open bars) and presence (hatched bars) of BRL 37344 (10 μM). BRL 37344 had no significant effect ($P > 0.05$) on basal or stimulated levels of cyclic AMP. Also shown is the significant ($* P < 0.05$) inhibition of the zinterol response by the $\beta_2$-adrenoceptor antagonist ICI 118551 (100 nM) (cross hatched bar) ($n = 4$). Bars show mean ± S.E.M. of $n$ experiments.

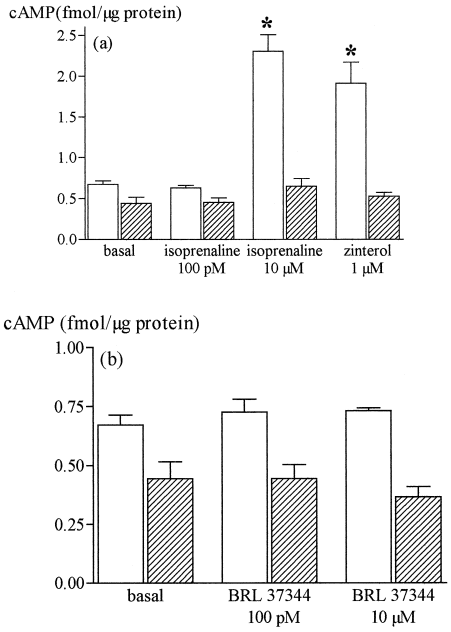


Fig. 5. The effect of chronic (−)-isoprenaline treatment (400 μg/kg per h, 14 days) on cyclic AMP accumulation. Responses in vehicle treated animals are shown (open bars) compared to (−)-isoprenaline treated animals (hatched bars) to the following agonists: (a) (−)-isoprenaline (100 pM, 10 μM) and zinterol (1 μM) and (b) to the $\beta_3$-adrenoceptor agonist BRL 37344 (100 pM, 10 μM). * Indicates responses that were significantly ($P < 0.05$) changed compared to the basal levels in the same treatment group of animals. Bars show mean ± S.E.M. of 4–5 experiments.

### 3.4. Regulation of β-ARS in rat soleus muscle with chronic (−)-isoprenaline infusion

The basal level of cyclic AMP was significantly lower ($P < 0.05$, $n = 5$) in animals pretreated with (−)-isoprenaline (400 μg/kg per h) for 14 days (0.44 ± 0.07 fmol/μg protein) compared with the vehicle-treated controls (0.67 ± 0.04 fmol/μg protein). (−)-Isoprenaline (100 pM) did not significantly change the cyclic AMP levels above basal in either vehicle- or isoprenaline-treated animals (Fig. 5). (−)-Isoprenaline (10 μM) and zinterol (1 μM) both caused an approximately 3-fold increase in cyclic AMP in vehicle-treated animals, and this response was abolished in (−)-isoprenaline-treated animals (Fig. 5a). In comparison, the $\beta_3$-adrenoceptor agonist BRL 37344 (at 100 pM or 10 μM) failed to cause a significant change in cyclic AMP from basal levels ($P > 0.05$) in either the vehicle or (−)-isoprenaline-treated animals (Fig. 5b). Forskolin (10 μM) increased cyclic AMP levels in both vehicle (16.5 ± 5.1 fmol/μg protein) and (−)-isoprenaline-treated animals (10.6 ± 2.0 fmol/μg protein), and although levels tended to decrease after (−)-isoprenaline treatment, this was not significantly different due to the large variation in forskolin responses in the control animals ($P = 0.29$, $n = 3$–4, data not shown).

## 4. Discussion

The present cyclic AMP accumulation studies in rat soleus muscle show that (−)-isoprenaline, (−)-adrenaline and (−)-noradrenaline produce concentration-dependent increases in cyclic AMP with a rank order of potency corresponding to their rank order of affinity in binding studies in human skeletal muscle (Liggett et al., 1988). The $pK_B$ values of (−)-propranolol and ICI 118551 against (−)-isoprenaline responses support the presence of $\beta_2$-adrenoceptors in mediating the increase of cyclic AMP production. In addition, we have shown that a supramaximal concentration of the $\beta_2$-adrenoceptor agonist zinterol (Zhong et al., 1996) increased cyclic AMP accumulation, and that this response was inhibited by the $\beta_2$-adrenoceptor antagonist ICI 118551. The lack of a rightward shift in the isoprenaline concentration–response curve with the $\beta_1$-adrenoceptor antagonist CGP 20712A correlates well with our previous studies that showed biphasic competition of [$^{125}$I](−)-cyanopindolol binding with ICI 118551 ($pK_D$ values 9.78, 5.45), while CGP 20712A showed competition at a low affinity (atypical $\beta$-adrenoceptor) site only ($pK_D$ 3.68) (Roberts et al., 1993). Other studies in rat soleus muscle, using conditions designed to identify only $\beta_1$- and $\beta_2$-adrenoceptors, have revealed a small amount of total $\beta$-adrenoceptor binding was to an ICI 118551-resistant receptor (possibly $\beta_1$-adrenoceptor) (16%) compared to $\beta_2$-adrenoceptors (84%) (Kim et al., 1991). The observed reduction in the isoprenaline maximum response in the presence of CGP 20712A (10 nM) was unexpected, as was the reversal of this reduction by a higher concentration of CGP 20712A (100 nM). Whether this reduction was due to an interaction with a small population of $\beta_1$-adrenoceptors or due to a non-specific depressant effect of CGP 20712A was not determined. This observation appears to be unique, as a literature search failed to find any similar observations, and at present we are unable to provide an explanation for this result.

Liu et al. (1996) showed that glucose uptake by soleus muscle was significantly enhanced by low concentrations of BRL 37344 (1 pM–1 nM) but inhibited to below control levels by higher concentrations of 1 $\mu$M and 10 $\mu$M. Our experiments with selective $\beta_3$-adrenoceptor agonists showed no increases in cyclic AMP levels over a concentration range of 10 nM–10 $\mu$M. Lower concentrations of BRL 37344 and (−)-isoprenaline (100 pM) were also tested to compare cyclic AMP responses to the increase in glucose utilisation response observed by Liu et al. (1996). However, there was no significant change from basal levels of cyclic AMP accumulation with either a low or high concentration of these agonists. The failure of BRL 37344 to stimulate cyclic AMP production has also been observed in bovine skeletal muscle (Sillence and Matthews, 1994), although it is interesting to note that the same study also failed to show $\beta_3$-adrenoceptor cyclic AMP production in bovine adipose tissue.

Studies in adipocytes indicate that $\beta_3$-adrenoceptors can couple to adenylate cyclase via the inhibitory G protein ($G_i$) to decrease cyclic AMP (Chaudry et al., 1994). In addition, the involvement of $G_i$ proteins in the $\beta_3$-adrenoceptor signalling pathways in human heart (Gauthier et al., 1996) questions the previous assumption that $\beta$-adrenoceptor stimulation will always result in increased cyclic AMP production. We therefore postulated that the $\beta_3$-adrenoceptors in soleus muscle may couple to adenylate cyclase through $G_i$, but the basal level of cyclic AMP in the soleus muscle preparations was not high enough to observe this effect. However, a high concentration of BRL 37344 (10 $\mu$M) caused no statistically significant change in the level of cyclic AMP stimulated by the non-selective $\beta$-adrenoceptor agonist (−)-isoprenaline, the selective $\beta_2$-adrenoceptor agonist zinterol, or by direct activation of adenylate cyclase by forskolin.

Based on the differences in molecular structure of the three $\beta$-adrenoceptors, the importance of the third subtype may relate to differences in the regulation of the receptor by both circulating and neuronally released catecholamines. $\beta_1$- and $\beta_2$-adrenoceptors are desensitised and down-regulated after exposure to $\beta$-adrenoceptor agonists due to phosphorylation of serine and threonine residues in the carboxy terminus by $\beta$-adrenergic receptor kinase and protein kinase A (for review, see Hein and Kobilka, 1995). In contrast, the $\beta_3$-adrenoceptor is predicted to be less susceptible to desensitisation due to the presence of fewer phosphorylation sites and a lack of sequence homology with $\beta_2$-adrenoceptors in regions that are important for agonist-mediated sequestration and down-regulation (for review, see Strosberg and Pietri-Rouxel, 1996). There is evidence that a physiological desensitisation of the $\beta_3$-adrenoceptor response occurs in the absence of receptor density changes due to the down-regulation of the $G_s$ protein $\alpha$-subunit (Chambers et al., 1994). The $\beta_3$-adrenoceptor is also regulated at the level of the $\beta_3$-adrenoceptor mRNA by compounds such as glucocorticoids, insulin, butyrate and triiodothyronine, which regulate $\beta_3$-mRNA transcription and alter $\beta_3$-adrenoceptor density (for review, see Strosberg and Pietri-Rouxel, 1996). Our previous binding studies in soleus muscle homogenates showed that 14 days of treatment in vivo with a high dose of (−)-isoprenaline selectively and significantly down-regulates $\beta_2$-adrenoceptor density, while the low affinity [$^{125}$I](−)-cyanopindolol binding site remained unchanged (Summers et al., 1995b). Chronic pretreatment with adrenaline, but not noradrenaline, also caused a significant decrease in the number of $\beta_2$-adrenoceptor binding sites in rabbit gastrocnemius muscle (Elfellah et al., 1988). In the present study, we observed a significant decrease in basal cyclic AMP levels after chronic (−)-isoprenaline treatment. In agreement with our binding studies (Summers et al., 1995b), we found that the cyclic AMP stimulation by (−)-isoprenaline and zinterol in vehicle-treated control animals was abolished in the (−)-isoprenaline-treated animals. These re-

sults support the presence of a $\beta_2$-adrenoceptor that is susceptible to desensitisation and down-regulation after long-term agonist stimulation.

These studies indicate that functional $\beta_3$-adrenoceptors coupled to adenylate cyclase are not present in rat soleus muscle, and the role of the propranolol resistant [$^{125}$I](−)-cyanopindolol binding site (Roberts et al., 1993) remains unknown. It is possible that [$^{125}$I](−)-cyanopindolol may not be binding to $\beta_3$-adrenoceptors on soleus muscle fibres, but to $\beta_3$-adrenoceptors on the numerous blood vessels that supply this tissue (Martin et al., 1989). Alternatively, soleus muscle may contain infiltrating adipocytes (Nagase et al., 1996), which would explain the close correlation of the [$^{125}$I](−)-cyanopindolol binding sites in skeletal muscle with a similar site in adipocyte membranes (Sillence et al., 1993). This possibility seems unlikely however, as a $\beta_3$-adrenoceptor-mediated cyclase response from the infiltrating adipocytes would be expected. It is also possible that the [$^{125}$I](−)-cyanopindolol binding site is not a $\beta_3$-adrenoceptor, and the in vivo functional effects of $\beta_3$-adrenoceptor selective compounds such as BRL 37344 that are described in the literature result from activation of a non-muscle $\beta_3$-adrenoceptor, which leads to the production of hormones or other factors that act on the muscle to produce these effects (e.g., increased glucose tolerance).

It can be concluded that $\beta_2$-adrenoceptors play a dominant role in the cyclic AMP stimulatory response to $\beta$-adrenoceptor agonists in rat soleus muscle. The propranolol-resistant [$^{125}$I](−)-cyanopindolol binding site (Roberts et al., 1993) does not appear to represent a $\beta$-adrenoceptor in rat soleus muscle that is coupled to adenylate cyclase via a stimulatory G protein to produce an increase in cyclic AMP production, or via an inhibitory G protein to reduce cyclic AMP production. Further studies are necessary to establish the functional significance of the atypical [$^{125}$I](−)-cyanopindolol binding site in skeletal muscle.

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