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CHARACTERIZATION OF β_1- AND β_2-ADRENOCEPTORS IN RAT SKELETAL MUSCLES

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Abstract—Binding studies with (−)-[^{125}I]cyanopindolol (ICYP) were conducted to characterize β-adrenoceptors in plantaris and soleus muscles of rats (male, 250–300 g). The distribution of β_1- and β_2-adrenoceptors in different muscle fiber types, identified in serial sections by succinic dehydrogenase (SDH) staining, was studied by autoradiography. The densities of binding sites (B_{max}, fmol/mg protein) were 5.4 ± 0.9 (mean ± SEM) in plantaris and 11.5 ± 2.0 in soleus muscle. In plantaris muscle, monophasic competition curves were observed when binding experiments were performed using CGP 20712A (50 pM to 0.5 mM), a β_1-adrenoceptor selective antagonist, or ICI 118,551 (50 pM to 20 μM), a β_2-adrenoceptor selective antagonist, to compete for ICYP binding. Analysis with LIGAND revealed a single binding site with a K_D value of 2.41 ± 0.56 nM (mean ± SEM) for ICI 118,551 and 8.93 ± 3.00 μM for CGP 20712A, indicating the presence of a homogeneous population of β_2-adrenoceptors. In soleus muscle, competition curves were biphasic with 16–21% β_1-adrenoceptors. Autoradiographic studies supported the findings from binding studies with membrane homogenates. The ICYP binding pattern was associated closely with the muscle fiber types identified by SDH staining. Propranolol-resistant binding sites were observed, and these sites were associated with muscle fibers positive to SDH staining.

Chronic treatment with β-adrenoceptor agonists increases skeletal muscle growth in many species of animals [1–5]. The precise mode of action responsible for the increased hypertrophy of skeletal muscle produced by β-adrenoceptor agonists is not well understood. Since most growth-promoting β-adrenoceptor agonists such as clenbuterol and fenoterol are highly selective for β_2-adrenoceptors [6], it is possible that the growth-promoting action of β-adrenoceptor agonists in skeletal muscle occurs through activation of this receptor subtype.

In skeletal muscles, slow-twitch, oxidative fibers have a higher density of β-adrenoceptors than fast-twitch, glycolytic fibers [7–10]. In rats treated with the β-adrenoceptor agonist cimaterol, muscles composed mainly of fast-twitch, glycolytic fibers, (e.g. *m. gastrocnemius*, *m. plantaris* and *m. extensor digitorum longus*) exhibit a greater hypertrophy than those composed mainly of slow-twitch, oxidative fibers (e.g. *m. soleus*) [5, 11]. Thus the hypertrophic response to β-adrenoceptor agonists is not proportional to the β-adrenoceptor density in different skeletal muscles. Detailed information about the distribution of β-adrenoceptor subtypes in different muscle fiber types should improve our understanding of the mechanism(s) by which β-adrenoceptor agonists increase skeletal muscle growth.

In the present study, we characterized the β-adrenoceptor subtypes in membrane preparations from two types of skeletal muscles in rats using the high-affinity radioligand (−)-[^{125}I]cyanopindolol, and highly selective β_1-(CGP 20712A) and β_2-(ICI 118,551) adrenoceptor antagonists. We also investigated the distribution of β-adrenoceptors among different types of skeletal muscles using autoradiography.

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MATERIALS AND METHODS

Animals. Eight male Sprague–Dawley rats (250–300 g body weight) which were inbred and reared at the Department of Zoology, Melbourne University, were used. Four animals were used in each of the autoradiographic and homogenate membrane binding experiments.

Drugs. The compounds used were: (−)-propranolol and ICI 118,551 [erythro-DL-1(7-methylindian-4-yloxy)-3-isopropylaminobutan-2-ol] (Imperial Chemical Industries, Wilmslow, Cheshire, U.K.); 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} (Ciba–Geigy AG, Basel, Switzerland); Na^{125}I (Amersham International Ltd., Buckinghamshire, U.K.); (−)-cyanopindolol (Sandoz, Basel, Switzerland); and GTP (Boehringer Mannheim Corp., New York, NY).

Radioiodination of (−)-cyanopindolol. (−)-[^{125}I]-Cyanopindolol (ICYP) was prepared from (−)-cyanopindolol and Na^{125}I with a specific activity of 2200 Ci/mmol as previously described [12].

Preparation of skeletal muscle membranes. Four rats were killed by cervical dislocation. Plantaris and soleus muscles, composed mostly of fast-twitch, glycolytic/oxidative and slow-twitch, oxidative fibers, respectively, were dissected and frozen in liquid nitrogen for future analysis. The muscle membrane

suspension was prepared following the procedure described previously for guinea pig heart [13] with minor modifications. Muscles were homogenized in 10 vol. of ice-cold Krebs-phosphate buffer (composition, mM: NaCl, 119; KCl, 4.8; $MgSO_4$, 1.2; $CaCl_2$, 1.9; glucose, 11.7; NaH_2PO_4, 1.3; Na_2HPO_4, 8.7; pH 7.4) for 20 sec at high speed using a Polytron homogenizer. The homogenates were filtered through nylon mesh to remove connective tissue, and then centrifuged twice at 50,000 *g* for 10 min. The resulting pellets were resuspended in 100 vol. of Krebs-phosphate buffer to obtain the final stocks of membrane suspension. Protein was determined by the method of Lowry *et al.* [14].

Radioligand binding assay. For both saturation and competition binding experiments, tissue membrane aliquots of 150 μL each were combined with Krebs-phosphate buffer containing 0.1 mM GTP, 1 mM ascorbic acid and 0.1 mM EDTA in a final volume of 250 μL as described previously [13]. The tubes were then incubated at 37° for 70 min, and incubations were terminated by the addition of ice-cold buffer followed by rapid vacuum filtration. After two additional washings, radioactivity retained on the filters was measured using a gamma counter with an efficiency of 79%. Non-specific binding was estimated as the difference between total binding and binding in the presence of (−)-propranolol (1 μM). For saturation experiments, eight different concentrations of radioligand were used: 5, 10, 20, 40, 60, 80, 100 and 125 pM.

For the measurement of β-adrenoceptor subtypes, competition binding experiments were performed with CGP 20712A and ICI 118,551, β_1- and β_2-selective antagonists, respectively. Twenty-one concentrations of CGP 20712A (50 pM to 0.5 mM) and seventeen concentrations of ICI 118,551 (50 pM to 20 μM) were used together with 50 pM of the radioligand ICYP. Non-specific binding was estimated as the difference between total binding and binding in the presence of (−)-propranolol (1 μM). Data derived from saturation and competition binding experiments were analyzed using two computer programs: EBDA [15] which performed preliminary Scatchard, Hill and Hofstee analyses and created a file for LIGAND [16], which was used to obtain final parameter estimates.

Autoradiographic preparation. Four rats were killed by cervical dislocation. Bundles of soleus, gastrocnemius and plantaris muscles were frozen in dry-ice/acetone (−78.5°). Cross-sections (10 μm) of soleus, gastrocnemius and plantaris were cut on a cryostat (−20°) and mounted onto gelatin chrome alum coated microscope slides [17].

Slide mounted sections were preincubated in Krebs buffer containing 0.1 mM ascorbic acid, 10 μM phenylmethylsulfonylfluoride (PMSF) and 0.1 mM GTP for 30 min to prevent the masking of β-adrenoceptors by "tight" binding of endogenous catecholamines [18]. Sections were then transferred to Krebs buffer containing 0.1 mM ascorbic acid and 10 μM PMSF with ICYP (50 pM) in the absence or presence of ICI 118,551 (70 nM), CGP 20712A (100 nM) or (−)-propranolol (1 μM) for 150 min at 25° [19]. (−)-Propranolol (1 μM) was used to define non-specific binding. Labeled sections were rinsed quickly in buffer followed by 2 × 15 min washes at 37° in the same medium and finally rinsed in distilled water (22–25°). Sections were dried in a stream of dehumidified air and stored at 4° in sealed boxes containing silica gel.

Distribution and quantitation of photographic film images. Dried labeled sections were exposed to hyperfilm (Amersham) in light-tight boxes for 21 days. The film was developed with Kodak D19, briefly rinsed in water, and fixed with Kodak Rapid Fix.

Relative bindings of ICYP from film images were quantitated using the AVID system [20]. This system uses the Chromapro 45 Dumas (Circle S Inc.) light source, a Mintron 1801CB CCD video camera to view images which are digitized using the video Van Gogh (Tecmar) board and displayed using the Graphic Master (Tecmar). This system is installed in an IBM AT computer and the graphic input device is a Microsoft™ mouse.

Histology. To characterize muscle fiber types with regard to oxidative capacity, serial sections were stained for succinic dehydrogenase (SDH) as previously described [21] and with haematoxylin and eosin.

RESULTS

Characterization of skeletal muscle β-adrenoceptors. Figure 1 shows a typical binding isotherm and Scatchard plot of ICYP binding to membrane suspensions of rat plantaris and soleus muscles. In both preparations, the specific binding was saturable and ranged from 68–78% (5 pM) to 37–56% (100 pM) in plantaris muscle, and from 51–74% (5 pM) to 34–41% (100 pM) in soleus muscle. Scatchard plots were linear, indicating binding to a single class of sites. Table 1 shows mean dissociation constants (K_D), maximal densities of binding sites (B_{max}) and Hill coefficients (n_H) in plantaris and soleus muscles. The K_D values (11.7 ± 2.8 and 15.5 ± 7.2 pM) were not significantly different from each other, and the Hill coefficients were not significantly different from unity. The major difference between the muscles in binding characteristics was that the B_{max} in soleus muscle (11.5 ± 2.0 fmol/mg protein) was considerably higher ($P < 0.01$) than that in plantaris muscle (5.4 ± 0.9 fmol/mg protein).

Figure 2 shows mean competition binding curves for ICYP and CGP 20712A (50 pM–0.5 mM) or ICI 118,551 (50 pM–20 μM) in plantaris and soleus muscles. In plantaris muscle, the competition curves for each of the antagonists were monophasic (Fig. 2). The pseudo n_H value was close to unity for ICI 118,551 but was high (1.75) for CGP 20712A (Table 2). However, the value of the pseudo n_H obtained from CGP 20712A competition binding experiments is difficult to interpret since inhibition points were not evenly distributed across the curve; points were clustered mostly around the top portion of the curve (Fig. 2). Further analysis of the binding data by LIGAND [16] clearly revealed one binding site in plantaris muscle with K_D values of 8.93 ± 3.00 μM for CGP 20712A and 2.41 ± 0.56 nM for ICI 118,551 (Table 2). These are close to those previously

Table 1. Dissociation constants (K_D), maximal density of binding sites (B_{max}) and Hill coefficients (n_H) for (−)-[^{125}I]cyanopindolol binding to rat plantaris and soleus muscle membranes

Membranes	K_D (pM)	B_{max} (fmol/mg protein)	n_H
Plantaris	11.7 ± 2.8	5.4 ± 0.9	0.97 ± 0.08
Soleus	15.5 ± 7.2	11.5 ± 2.0*	0.94 ± 0.06

Values are means ± SEM from four animals.
* $P < 0.01$.

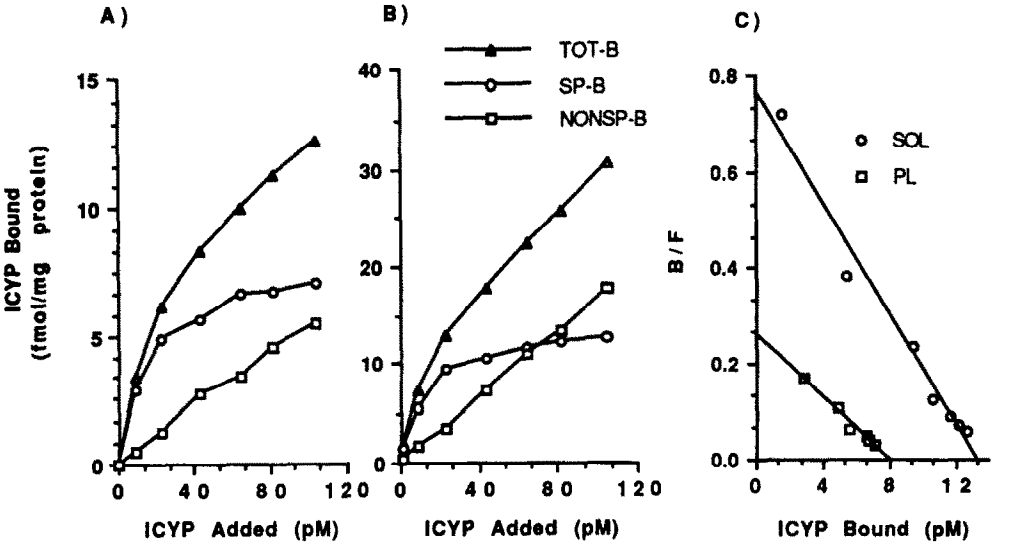

Fig. 1. Representative saturation binding experiment of (−)-[^{125}I]cyanopindolol (ICYP) to rat plantaris (A) and soleus (B) muscles, showing total (TOT-B), specific (SP-B) and non-specific binding (NONSP-B). Also shown are the Scatchard plots (C) for these two experiments.

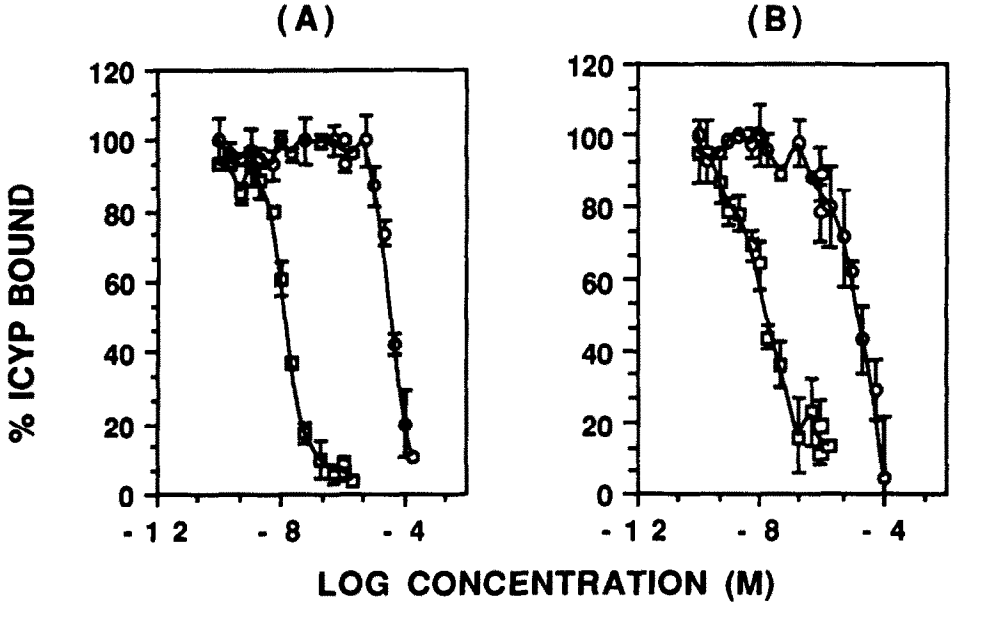

Fig. 2. Mean competition binding curves between (−)-[^{125}]cyanopindolol (ICYP) and the β_2-selective antagonist ICI 118,551 (□) and the β_1-selective antagonist CGP 20712A (○) in rat plantaris (A) and soleus (B). % ICYP bindings are specific bindings. Non-specific binding was estimated as the difference between total binding and binding in the presence of (−)-propranolol. Values shown are mean ± SEM of four measurements from four animals.

Table 2. Pseudo n_H values for competition binding curves between (−)-[^{125}I]cyanopindolol and CGP 20712A and ICI 118,551, dissociation constants (K_D) for CGP 20712A and ICI 118,551 and proportion of β_1- and β_2-adrenoceptor binding sites in rat plantaris and soleus muscles

	n_H	K_D	β_2
Plantaris			
CGP 20712A	1.75 ± 0.10	8.93 ± 3.00 μM	100%
ICI 118,551	1.10 ± 0.09	2.41 ± 0.56 nM	100%

	n_H	$K_{D,H}$	$K_{D,L}$	β_1	β_2
Soleus					
CGP 20712A	0.96 ± 0.25	0.29 ± 0.06 nM	7.38 ± 4.15 μM	15.7%	84.3%
ICI 118,551	0.55 ± 0.08	1.52 ± 0.74 nM	0.13 ± 0.05 μM	21.1%	78.9%

Values are means ± SEM from four animals.

reported at β_2-adrenoceptors in guinea pig atrial membranes [22].

In contrast, the mean competition curves for each of the antagonists were biphasic in soleus muscle (Fig. 2) indicating two binding sites with K_D values of 0.29 ± 0.06 nM and 7.38 ± 4.15 μM for CGP 20712A. These values are similar to the K_D values reported previously for CGP 20712A at β_1- and β_2-adrenoceptors [22, 23]. The proportion of β_1-: β_2-adrenoceptors was 15.7 : 84.3. Analysis of ICI 118,551 competition experiments in soleus muscle with a two binding site model failed to provide a significant improvement in fit compared to a one binding site model. Values determined with CGP 20712A are considered to be more reliable because of its greater selectivity (6763-fold) compared to ICI 118,551 (182-fold) [19]. Without further improvement, LIGAND [16] generated a two binding site model having values of 1.52 nM at high affinity and 0.13 μM at low affinity binding sites which were close to values ($K_{D,H}$ 2.32 nM, $K_{D,L}$ 0.2 μM) reported previously at β_2- and β_1-adrenoceptors in guinea pig atrial membranes [22]. In the two-site model, the populations of low and high affinity binding sites in soleus muscle for ICI 118,551 were 21.1 and 78.9%, respectively.

Autoradiographic distribution of β-adrenoceptors. Figure 3a is a diagram demonstrating the location of the gastrocnemius, plantaris and soleus muscles. Figure 3b is a serial section stained for SDH activity. Soleus muscle had SDH-positive muscle fibers evenly distributed throughout the muscle bundle. On the other hand, gastrocnemius and plantaris muscles had some SDH-positive muscle fibers which were aggregated near soleus muscle. Panels c–f of Fig. 3 show autoradiographic film images of serial sections labeled with ICYP in the absence or presence of ICI 118,551 (70 nM), CGP 20712A (100 nM) or (−)-propranolol (1 μM) to define non-specific binding. Table 3 summarizes the relative quantity of ICYP binding in each muscle, determined by quantitative autoradiography. ICYP binding in the presence of (−)-propranolol, ICI 118,551 or CGP 20712A in each muscle is expressed as the percentage of the total binding in each muscle. The plantaris and gastrocnemius muscles had a lower density of evenly distributed ICYP binding sites than the soleus muscle (Fig. 3c). When quantitated, the percentages of total binding in plantaris and gastrocnemius muscle relative to the total binding in soleus muscle were 72.4 ± 5.8% (SEM) and 58.3 ± 3.4% (SEM), respectively. In the plantaris and gastrocnemius muscles, the areas stained positively by SDH appeared to have more total ICYP binding sites than areas stained negatively by SDH (Fig. 3c). (−)-Propranolol (1 μM) did not inhibit ICYP binding completely (Fig. 3d); the percentages of propranolol-resistant ICYP binding relative to total binding were 41.5 ± 3.2, 34.3 ± 6.3 and 29.4 ± 4.5% in soleus, plantaris and gastrocnemius muscles, respectively (Table 3). These sites were associated with the SDH-positive muscle areas (Fig. 3d). ICI 118,551 inhibited 47.4 ± 5.9, 54.6 ± 6.2 and 56.7 ± 7.3% of total ICYP binding in soleus, plantaris and gastrocnemius muscles. The sites resistant to ICI 118,551 also appeared to be associated with the SDH-positive muscle areas (Fig. 3e). In contrast, CGP 20712A did not inhibit the ICYP binding in all three muscles (Fig. 3f); the percentage of ICYP binding relative to total binding were 106.9 ± 8.5, 105.2 ± 5.1 and 100.5 ± 7.1% in soleus, plantaris and gastrocnemius muscles.

DISCUSSION

This study demonstrates that soleus muscle, composed mostly of slow-twitch, oxidative fibers, has a 2-fold greater density of β-adrenoceptors than the plantaris muscle, which is composed mostly of fast-twitch, oxidative/glycolytic fibers. This result is in general agreement with previous findings [7–12], which reported a higher receptor density in slow-twitch, oxidative muscles such as soleus than that of fast-twitch, glycolytic muscles such as gastrocnemius. On the other hand, Reddy *et al.* [24] reported fewer β-adrenoceptors in soleus muscle than in extensor digitorum longus muscle (fast-twitch, glycolytic).

The β-adrenoceptors in plantaris muscles were almost exclusively of the β_2-subtype confirming previous reports [25, 26]. The K_D values obtained

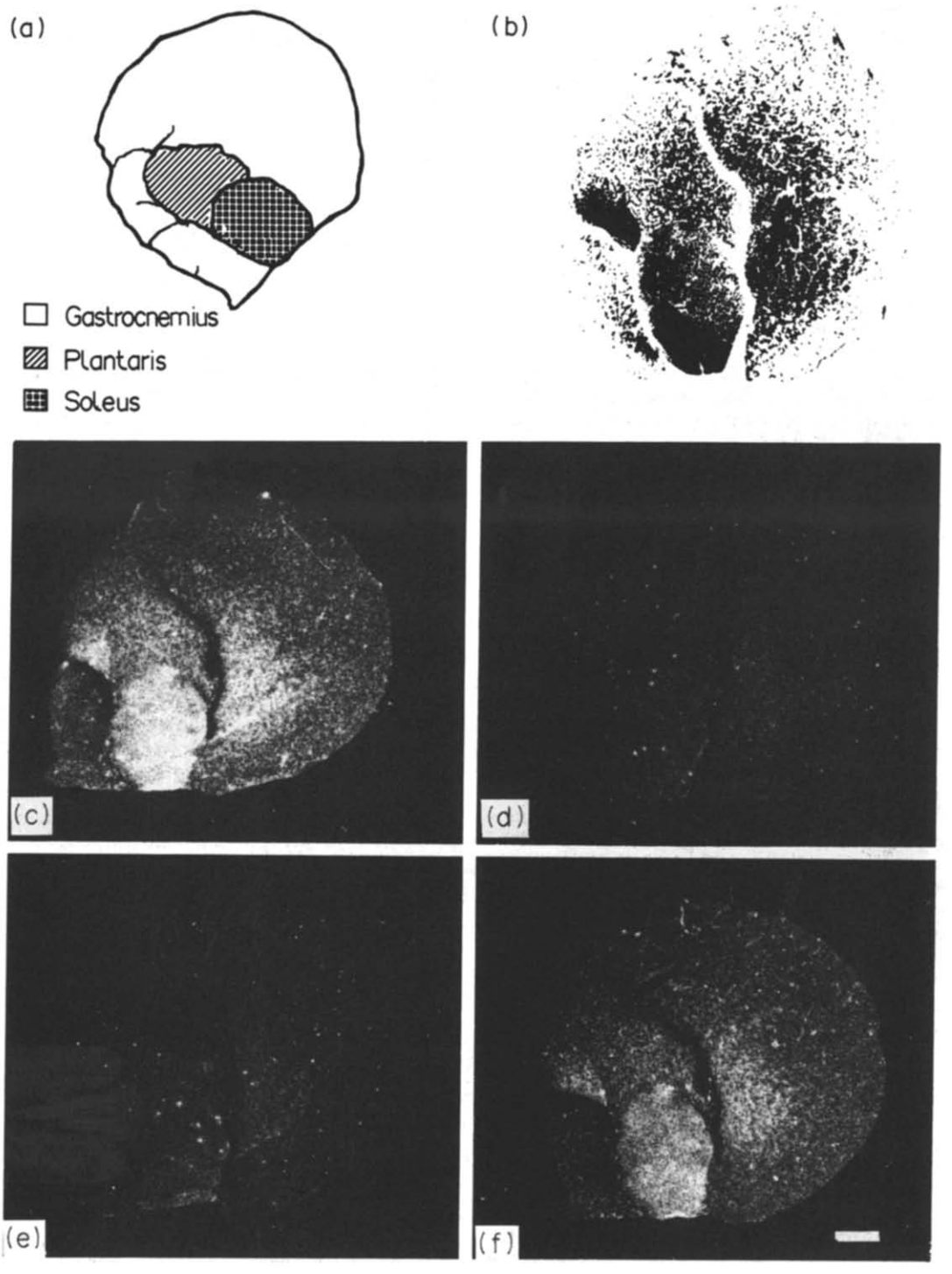

Fig. 3. Autoradiographic localization of (−)-[^{125}I]cyanopindolol (ICYP) binding to rat skeletal muscles. (a) Diagram illustrating the location of gastrocnemius, plantaris and soleus muscle. (b) Photograph of an SDH-stained section. (c)–(f) Photographs of hyperfilm images showing ICYP binding sites in serially cut sections in (c) the absence or (d) the presence of propranolol (1 μM), (e) ICI 118,551 (70 nM) or (f) CGP 20712A (100 nM). High binding is represented by the accumulation of white grains since the photographic prints from autoradiographic images were made directly from hyperfilm. The scale bar indicates 1 mm.

Table 3. Relative binding of (−)-[^{125}I]cyanopindolol in the presence of propranolol, ICI 118,551 or CGP 20712A in soleus, plantaris and gastrocnemius muscles

	Relative binding to total binding (%)		
Muscle	Propranolol	ICI 118,551	CGP 20712A
Soleus	41.5 ± 3.2	52.6 ± 5.9	106.9 ± 8.5
Plantaris	34.3 ± 6.3	45.4 ± 6.2	105.2 ± 5.1
Gastrocnemius	29.4 ± 4.5	43.3 ± 7.3	100.5 ± 7.1

Relative binding of ICYP was measured from autoradiographic film images using the AVID image processing system as described in the text. The binding of ICYP in the presence of each of the compounds at each muscle was expressed as a percentage of total binding in each muscle. Values are means ± SEM from four animals.

here for CGP 20712A and ICI 118,511 are close to those reported previously in guinea pig atrial membranes [22]. Results from autoradiography showed that β_2-adrenoceptors were evenly distributed over the plantaris, soleus and gastrocnemius muscles.

In contrast to the plantaris muscle, there was a minor population of β_1-adrenoceptors in soleus muscles. The physiological significance of this observation is not clear except that there may be a link between the variation in subtype population and the differences in metabolic and mechanical properties among different muscles. For example, the difference in the utilization of intramuscular triglycerides during exercise was noted between red and white muscles [27, 28]. Fundamental differences also exist between slow-twitch and fast-twitch muscles in mechanical responses following β-adrenergic stimulation [29]. Currently, it is not possible to know whether the β_1-adrenoceptors observed in soleus muscles are from selective skeletal muscle fiber types or from some other tissue compartments since this result was assessed in crude membrane fractions from muscle homogenate. The autoradiographic methods employed in this study do not allow resolution of the exact location of β-adrenoceptor subtypes within the soleus. Therefore, further studies investigating the location of β_1-adrenoceptors in skeletal muscle are warranted to understand the physiological significance of this receptor in skeletal muscles.

Propranolol is known to block 99.7% of β_1- and 99.9% of β_2-adrenoceptors [13] at the concentration used in our study. It is interesting therefore to note that the present autoradiographic study demonstrated a presence of propranolol- or ICI-resistant binding sites associated with muscle fibers stained positively to SDH. Currently, the nature of the propranolol- or ICI 118,551-resistant ICYP binding sites is not known. However, there are several lines of evidence suggesting that the propranolol- or ICI 118,551-resistant ICYP binding sites observed in SDH-positive muscle fibers may be of the "β_3-adrenoceptor" subtype [30]. The affinity of propranolol for atypical β-adrenoceptors present in some tissues including soleus muscle is 10–100 times lower than that for the typical β_1- or β_2-adrenoceptors observed in tracheal or atrial tissues [31–34]. The K_i value of ICI 118,551 for the β_3-adrenoceptors has been reported to be more than 100 times higher than for β_2-adrenoceptors [30]. In addition, we recently observed in a separate study that BRL 37344, an atypical β-adrenoceptor agonist [35], inhibited the binding of ICYP to the propranolol- or ICI 118,551-resistant binding sites (unpublished observations). At this stage, however, it is premature to classify propranolol- or ICI 118,551-resistant binding sites as β_3-adrenoceptors, and further characterization is projected.

In conclusion, in the present study we demonstrated that soleus muscle composed mostly of the slow-twitch, oxidative muscle fibers has a higher density of β-adrenoceptors than the plantaris muscle which is predominatly composed of fast-twitch glycolytic/oxidative muscle fibers. While the β-adrenoceptors in plantaris muscle appear to be exclusively of the β_2-subtype, the soleus muscle appears to have a minor population of β_1-adrenoceptors. The oxidative muscle fibers probably have, in addition, a significant proportion of atypical β-adrenoceptors. Further studies are being conducted to confirm and extend these observations.

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