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No functional β3-adrenergic receptors expressed in rat skeletal muscle cells[1]

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KEY WORDS beta adrenergic receptors; cyclic AMP; skeletal muscle; isoproterenol; CGP12177A; SR59230A

AIM: To determine the functional role of β_3-adrenoceptors (β_3-AR) in rat skeletal muscle cells. **METHODS:** Nonselective β-AR agonist isoprenaline (isoproterenol, Iso), β_3-AR agonist CGP12177A which is a β_1-/β_2-AR antagonist and selective β_3-AR antagonist SR59230A on cAMP accumulation was studied in primary cultured rat skeletal muscle cells. **RESULTS:** Iso stimulated cAMP accumulation in a concentration-dependent manner with EC_{50} of 1.51 nmol · L^{-1} and propranolol inhibited cAMP accumulation stimulated by Iso with K_B of 3.47 nmol · L^{-1}. CGP12177A had no effect on cAMP accumulation but inhibited cAMP production induced by Iso. SR59230A 10 nmol · L^{-1} did not inhibit cAMP production induced by Iso. **CONCLUSION:** The functional β_3-AR are not present or at least not coupled to adenylyl cyclase activity in skeletal muscle cells.

Epinephrine exerts several physiologic effects on skeletal muscle through both α- and β-adrenoceptors (AR). β-AR in skeletal muscle are mainly composed of β_2-AR and a small proportion of β_1-AR[1]. There were also some evidences that putative β_3-AR might exit in skeletal muscle. Early functional studies indicated that atypical β-AR might be involved in the control of glycogen synthesis in rat soleus muscle[2]. Autoradiographic studies of β-AR in rat skeletal muscle have revealed the presence of propranolol-resistant [^{125}I] cyanopindolol binding sites distributed in the soleus muscle[3]. Subsequently, these sites were found to be identical to putative β-AR binding sites found in rat brown adipose tissue, and were strongly resemble to the cloned human β_3-AR expressed in Chinese hamster ovary cells[4,5].

Molecular evidence for the presence of β_3-AR in skeletal muscle is equivocal. Northern blot analysis and nuclease protection assays of RNA from tissue indicate that the β_3-AR is not expressed in skeletal muscle[6], However, β_3-AR mRNA was detected in soleus muscle by reverse transcriptional polymerase chain reaction[7].

The β_3-AR is a member of the superfamily of G protein-coupled receptors. The mechanism by which the activated β_3-AR transmit the signals across the plasma membrane involves the stimulation of G_s, which in turn activates adenyl cyclase, yielding the second messenger cAMP, although β_3-AR can also be coupled to G_i protein.

Establishing whether or not skeletal muscle contains functional β_3-AR will help to improve our understanding of the mechanism of skeletal muscle growth control and to apply β_3-AR selective drugs in therapeutics. Therefore, in present study, the effect of β_3-AR and their post-receptor pathways, β_3-AR agonist CGP12177A which is a β_1-/β_2-AR antagonist in the meantime and selective β_3-AR antagonist SR59230A on cAMP accumulation was investigated in cultured rat skeletal muscle cells.

MATERIALS AND METHODS

Chemicals SR59230A {(3-(2-ethylphenoxy)-1-[(1S)-1,2,3,4-tetra-hydronaphth-1-ylaminol]-(2S)-2-propanol oxalate} was synthesized in the Chemistry Section of the Sanofi Midy SpA Research Centre (Milan, Italy). CGP 12177A [*dl*-4-(3-t-butyl-amino-2-hydroxy-propoxy) benzimidazol-2-one hydro-chlo-ride] was purchased from RBI (Natick MA, USA). 3-Iso-butyl-1-methylxanthine, *dl*-isoprenaline (Iso) HCl, *l*-propranolol, and cAMP were purchased from Sigma. [^{3}H] adenine (710.4

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$TBq \cdot mol^{-1}$) was purchased from NEN Products (Boston MA, USA).

Rats Neonatal Wistar rats (age 2 – 3 d), which were Grade Ⅱ and Certificate № 013056, were obtained from Experimental Animal Center of Beijing Medical University and used on the same day.

Skeletal muscle cell culture Muscles were dissected from hind limb under sterile conditions and placed in Dulbecco's modified Eagle's medium (DMEM). After trimming off the excess connective tissue, the muscle tissue was minced and placed in the Kreb's solution containing 0.15 % trypsin, and stirred in 75-mL flask at 37 ℃ for 8 min. The supernatant liquid was centrifuged (1500 × g for 5 min) and washed in 5 mL DMEM. The digesting procedure was repeated 4 – 5 times. The washed pellet was resuspended in DMEM supplemented with 10 % newborn calf serum, benzylpenicillin 50 U, streptomycin 50 μg and filtered through a nylon screen. The suspension was incubated at 37 ℃ in a humidified, 5 % CO_2 atmosphere for 50 min. The cell suspension was then plated at 1.5×10^5 cells per well in 6-well plates and incubated. The medium was completely exchanged with fresh prewarmed medium on day 1, 3, 5, and 7.

Determination of cAMP Cells were grown for 3 – 4 d (preconfluented) or 7 – 8 d (confluented). cAMP was assessed by the [^{3}H]adenine prelabeling method[8]. Briefly, the cultured cells were prelabeled with [^{3}H]adenine 37 $MBq \cdot L^{-1}$ for 2 h and washed twice with Krebs-Ringer bicarbonate buffer 2 mL at 37 ℃. The buffer was aspirated and 1 mL of warm Krebs-Ringer bicarbonate buffer containing 3-isobutyl-1-methylxanthine 0.2 $mmol \cdot L^{-1}$ was added. Cell cultures were incubated at 37 ℃ for 15 min with agonists. Antagonists were added 30 min before agonists. The reactions were terminated by the addition of trichloroacetic acids 100 μL, 50 μL of unlabeled cAMP were added as a carrier, and the plates were scraped. [^{3}H]cAMP was isolated by sequential Dowex, alumina chromatography and the radioactivity was determined (Beckman LS6500). Data were calculated as a percentage of the conversion of [^{3}H]ATP to [^{3}H]cAMP.

Statistics Each incubation with a particular drug concentration and combination was replicated 4 – 5 times, and the results was presented as $\bar{x} \pm s$. The EC_{50} was obtained by using the four-parameter logistic equation in nonlinear regression analysis in Graphpad Prism software. The negative logarithms of antagonist dissociation constant K_B was calculated from the Schild equation: $\lg(d_r - 1) = \lg[B] - \lg K_B$, in which $[B]$ was the concentration of antagonist. The statistical significance of difference between control and experimental groups was assessed by two-side paired t-test.

RESULTS

cAMP accumulation induced by Iso and effect of propranolol on the response Iso stimulated cAMP accumulation in a concentration-dependent manner with EC_{50} value of 1.51 $nmol \cdot L^{-1}$ (95 % confidence limits 0.18 – 12.50 $nmol \cdot L^{-1}$). The K_B value of propranolol 0.1 $\mu mol \cdot L^{-1}$ was 3.47 $nmol \cdot L^{-1}$ (Fig 1).

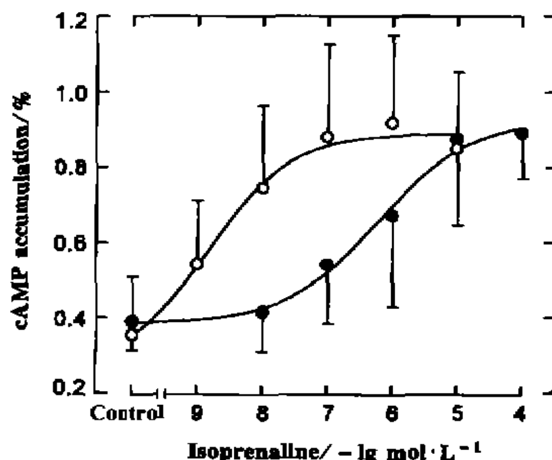

Fig 1. The Iso-induced (○) cAMP accumulation and the antagonistic effect of propranolol 0.1 $\mu mol \cdot L^{-1}$ on it (●) in cultured skeletal muscle cells. n = 4 samples from 4 rats. $\bar{x} \pm s$.

Effect of CGP12177A on cAMP accumulation CGP12177A 10 $nmol \cdot L^{-1}$ to 100 $\mu mol \cdot L^{-1}$ were used in SR59230A 10 $nmol \cdot L^{-1}$-treated or untreated cells, and no response was observed.

Effects of CGP12177A and SR59230A on Iso-induced cAMP accumulation in immature and mature skeletal muscle cells Iso 0.1

μmol·L^{-1} induced an increase in cAMP levels in cultured skeletal muscle cells. CGP12177A 0.1 μmol·L^{-1} produced inhibition, but SR59230A (10 nmol·L^{-1}) did not affect the increase of cAMP accumulation induced by Iso. There was no significant difference between the immature cells (3 – 4 d) and the mature cells (7 – 8 d) (Tab 1).

Tab 1. Effects of CGP12177A (CGP) and SR59230A (SR) (10 nmol·L^{-1}) on basal and isoprenaline (Iso, 0.1 μmol·L^{-1})-induced cAMP accumulation in immature (n = 4 samples from 4 rats) and mature (n = 5 samples from 5 rats) skeletal muscle cells.
$^bP < 0.05$, $^cP < 0.01$ *vs* Basal. $^eP < 0.05$ *vs* Iso.

Group	cAMP accumulation/%	
	Immature	Mature
Basal	0.30±0.09	0.27±0.07
Iso	0.82±0.21^c	0.63±0.14^b
Iso + CGP 0.1 μmol·L^{-1}	0.36±0.15^e	0.34±0.08^e
Iso + SR	0.75±0.19	0.56±0.15
CGP 1 μmol·L^{-1}	0.28±0.10	0.28±0.02
CGP 1 μmol·L^{-1} + SR	0.27±0.11	0.26±0.04

DISCUSSION

When developing the method for measuring cAMP accumulation in cultured skeletal muscle cells, an Iso concentration-response curve for the stimulation of cAMP accumulation were performed in control and propranolol (0.1 μmol·L^{-1})-treated confluent skeletal muscle cells. Iso stimulated cAMP accumulation in a concentration-dependent manner and propranolol shifted the concentration-response curve for Iso to the right with calculated K_B value of 3.47 nmol·L^{-1}. The mean K_B value is in accordance with reported K_B value for β_1- or β_2-AR (0.75 nmol·L^{-1})[9]. The result suggests that Iso stimulated cAMP accumulation via β_1-or β_2-AR in skeletal muscle cells.

CGP12177A possessed a partial agonistic activity with high potency in stimulating adenylyl cyclase in CHO-rat β_3 cells and rat brown adipocytes. CGP12177A 10 nmol·L^{-1} to 100 μmol·L^{-1} were used in our experiments and had no effect on cAMP accumulation. However CGP12177A inhibited cAMP production induced by Iso. SR59230A is a selective and potent β_3-AR antagonist and therefore a suitable and useful tool to study the functional role of the β_3-AR in various tissue[10,11]. Theoretically SR59230A 10 nmol·L^{-1} used in our experiment should be able to antagonize β_3-AR but not β_1-and β_2-AR. The addition of SR59230A, however, did not inhibit cAMP production induced by Iso. These data together with Iso-induced cAMP production which were not resistant to propranolol indicate that functional β_3-AR are not present or at least not coupled to adenylyl cyclase activity in skeletal muscle cells.

In brown fat cells, a switch occurs in β-AR subtypes which is coupled to cAMP production, from being β_1-AR in immature cells to being β_3-AR in mature cells[10,12]. To further confirm whether or not a similar switch occurs in skeletal muscle cells, studies were performed on proliferating (immature) and confluent (mature) skeletal muscle cells in culture. There were no evidence of functional β_3-AR exiting in the proliferating or the confluent cells and no significant difference between the proliferating cells and the confluent cells.

Our finding is similar to the results of Sillence *et al*[13]. Their results of second-messenger studies did not support the hypothesis that bovine skeletal muscle tissue membranes contained functional β_3-AR: based on the failure of a β_3-AR agonist of BRL37344 to stimulate cAMP production and that cAMP production induced by Iso was not resistant to blockade by either ICI118551 or CGP20712. Our results supported that low concentration of BRL37344 stimulated glucose utilization via an atypical β-AR and the stimulation is negated by the activation of β_2-AR that occurred at higher concentration of BRL37344[14]. The opposing effects of β_2-AR and atypical β-AR activation suggested that in skeletal muscle the atypical β-AR seemed unlikely the β_3-AR and they linked to different post-receptor pathways.

In conclusion, functional β_3-AR are not present or at least not coupled to adenylyl cyclase activity in skeletal muscle cells and the possibility of another β-AR subtype in skeletal muscle could not be excluded.

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大鼠骨骼肌细胞不存在功能性 β_3-肾上腺素受体[1]

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关键词 β-肾上腺素受体；环腺苷一磷酸；骨骼肌；异丙肾上腺素；CGP12177A；SR59230A

目的：研究原代培养的大鼠骨骼肌细胞中是否存在功能性 β_3-肾上腺素受体(β_3-AR)．**方法**：利用柱层析方法测定异丙肾上腺素(Iso)，β_3-AR 激动剂 CGP12177A 和 β_3-AR 拮抗剂 SR59230A 对培养骨骼肌细胞环磷腺苷(cAMP)生成作用．**结果**：Iso 剂量依赖性刺激骨骼肌细胞 cAMP 的生成，EC_{50} 为 1.51 nmol·L^{-1}．普萘洛尔 0.1 μmol·L^{-1} 抑制 Iso 刺激的 cAMP 的生成，K_B 值为 3.47 nmol·L^{-1}．CGP12177A 无刺激 cAMP 生成作用，但可抑制 Iso 的作用．SR59230A 10 nmol·L^{-1} 不能抑制 Iso 刺激 cAMP 的产生．**结论**：大鼠骨骼肌细胞中不存在功能性 β_3-AR 或至少不与腺苷酸环化酶耦联．

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