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β-ADRENOCEPTORS AND THE EFFECT OF β-AGONISTS ON PROTEIN METABOLISM IN OVINE PRIMARY MUSCLE CULTURES

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Abstract—The β-adrenergic receptors of differentiated ovine muscle cultures derived from either fetal or pre-pubertal lambs were characterized by binding of (±)-[^{3}H]CGP-12177, directly to intact cells in monolayer. Fetal muscle cells contained a single class of specific and saturable binding sites which had a dissociation constant (K_d) of 0.38×10^{-9} M and a binding capacity of 55.2 fmol/μg protein. β-Adrenergic agonists competed for the specific binding sites with a typical β_2-adrenergic specificity. Satellite muscle cells derived from pre-pubertal lambs contained two classes of binding site. The high affinity site had a K_d of 1.02×10^{-9} M and a binding capacity of 28.4 fmol/μg protein and the low affinity site a K_d of 12.1×10^{-9} M and a binding capacity of 389 fmol/μg protein. β-Adrenergic agonists competed for the specific binding sites with a typical β_1-adrenergic specificity. The β-agonist cimaterol had no effect on either protein synthesis or degradation in fetal muscle cells. In cultures derived from satellite cells cimaterol significantly stimulated protein synthesis at concentrations of 10^{-8}–10^{-7} M and at 10^{-8}–10^{-6} M in the presence of serum. These effects were maintained if 10^{-5} M propranolol was added to the incubation media, but were blocked by 10^{-6} M isoproterenol. Propranolol and isoproterenol had no stimulatory effects on protein synthesis. Cimaterol also had no detectable effects on protein degradation or the transport of amino acids or glucose. It is concluded that although β-adrenergic receptors are present in ovine muscle cultures they may not play a role in the anabolic effect of β-agonists observed in cultured muscle cells.

The addition of β-agonists to the diet of commercially important livestock species has been clearly shown to have positive effects on both protein deposition and lipolysis whilst lipogenesis is reduced [1]. The β-agonists clenbuterol and cimaterol, which have been most widely used, have a greater affinity for the β_2-receptor and elicit marked repartitioning effects in most species studied. There are several studies in young rats which have demonstrated that the stimulatory effect of clenbuterol on either muscle weight or total carcass protein is unaffected by either thyroidectomy [2], adrenalectomy or castration [3]. These results indicate that the anabolic effect of β-agonists on skeletal muscle could be mediated directly via the β_2-receptor. However, when young rats were fed clenbuterol plus the β-antagonist propranolol for a 7 day period a significant stimulation of protein accretion still occurred, although the increase in muscle fibre size was reduced [4]. It is possible, therefore, that although some of the changes in muscle metabolism caused by β-agonists may be mediated via the β-receptor this does not include its stimulatory effect on muscle growth.

Differentiated muscle cells in culture of both rodent [5] and avian origin [6] have been shown to possess predominantly β_2-receptors. These receptors are functional in L8 muscle cells, but do not appear to be involved in the regulation of protein turnover [7]. It is not known if a similar system is present in sheep muscle in which the anabolic effect of clenbuterol on muscle deposition *in vivo* appears to be mediated largely via a reduction in protein breakdown, although the response of individual muscles is not uniform [8]. The aim of this study was therefore to determine if ovine primary muscle cultures derived from fetal [9] or pre-pubertal lambs [10] possess β-receptors and if these play a role in the anabolic effect of the β-agonist cimaterol.

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MATERIALS AND METHODS

Reagents. Dulbecco's modification of Eagle's medium (DMEM) lacking glutamine (Imperial Laboratories, Andover, U.K.), in powder form, was dissolved in distilled deionized water and buffered with 0.37% (w/v) $NaHCO_3$ and gaseous CO_2. Hepes-buffered DMEM was prepared using powdered DMEM as above, to which was added 0.477% (w/v) Hepes buffer (Imperial Laboratories) and 0.085% (w/v) $NaHCO_3$. The pH was adjusted to 7.5–7.6 at 15° which gives a pH of 7.2–7.35 at 37°. Earle's balanced salts (EBS; Imperial Laboratories) in powder form, was dissolved in distilled deionized water and buffered with 0.22% (w/v) $NaHCO_3$ and the pH adjusted to 7.2–7.4 using HCl. Glucose-free DMEM (Sigma Chemical Co., Poole, U.K.) in powder form was dissolved in distilled deionized water with 0.011% (w/v) sodium pyruvate (Sigma) and 0.0015% (w/v) phenol red were added. The

media was buffered with 0.37% (w/v) $NaHCO_3$ and gaseous CO_2. All solutions were sterilized by passage through a 0.22 μm membrane filter and penicillin (1×10^5 I.U./L) and streptomycin (100 mg/L; Sigma) were routinely added to all culture media. Glutamine (4 mM; Imperial Laboratories) was also added to all culture media with the exception of EBS.

Crystalline grade bovine serum albumin (BSA), (±)-propranolol, (−)-isoproterenol bitartrate, (−)-epinephrine and (−)-norepinephrine were all obtained from Sigma and (±)-cimaterol was supplied by Boehringer Ingelhiem (F.R.G.). Stock solutions of epinephrine (0.1 M), norepinephrine (0.1 M) and cimaterol (10 mM) were prepared in 0.1 M acetic acid and isoproterenol (10 mM), propranolol (10 mM) and 5.0% (w/v) BSA were prepared in minimal phosphate-buffered saline (PBS-M; Imperial Laboratories). Stock solutions of bovine insulin (Sigma; 26.2×10^3 I.U./g) were dissolved in 0.9% (w/v) NaCl containing HCl (10 mM). These were all stored in aliquots at −40°. All other chemicals were analytical grade (Fisons plc, Scientific Equipment Division, Loughborough, U.K.) unless otherwise stated.

Cell culture. Fetal ovine primary muscle cultures of myogenic cells from pooled hind-limb muscle of 12-week-old fetuses were prepared as described by Harper *et al.* [9]. The cells were seeded in 24-well "Primaria" tissue culture trays at 5×10^3 cell/cm^2 in 8% fetal calf serum (FCS; Imperial Laboratories) growth medium which was renewed 2 days later. Then after a further 2 days, when the cells had achieved confluence, this medium was replaced with 2% FCS-maintenance medium. This caused a slowing of cell proliferation and differentiation into an array of small myotubes. Experiments were carried out between 8 and 10 days after initial seeding. Each fetal preparation was also subjected to clonal analysis [9] and shown to contain a myogenic population of 80 ± 4% (±SD) (N = 3).

Satellite cells were also used to grow ovine primary muscle cultures, which were prepared from the semitendinosus muscle of 3–4-month-old pre-pubertal Suffolk cross lambs as described by Roe *et al.* [10]. The cells were seeded in 24-well "Primaria" tissue culture trays at 10^4 cells/cm^2 in 8% FCS growth medium which was renewed 2 days later. Then after 4–5 days when the cells had achieved confluence the growth medium was replaced with 2% FCS-maintenance medium. Experiments were carried out 7–8 days after initial seeding. Each satellite cell preparation was also subjected to clonal analysis [9] and shown to contain a myogenic population of 85 ± 6% (±SD) (N = 4).

Radioisotope techniques. (±)-[^{3}H]CGP-12177 (4-(3-tertiarybutylamino-2-hydroxypropoxy)-benzimidazole-2-on hydrochloride; 1.61 TBq/mmol) was the isotope used for the measurement of β-receptors, [3,5-^{3}H]Tyrosine (2.0 TBq/mmol) was used in measurements of protein metabolism and 2-deoxy-D-[1-^{3}H]glucose (2-DOG; 629 GBq/mmol) plus 2-amino-[1-^{14}C]isobutyric acid (AIB; 2.18 GBq/mmol) were used in the transport studies. All radiochemicals were obtained from Amersham International plc (Aylesbury, U.K.).

Receptor-binding assay. Binding assays for intact cells in monolayer culture were performed using a modification of the methods described by Pittman and Molinoff [5]. The cells were initially washed with Hepes-buffered DMEM containing 2% FCS and were then incubated in 500 μL of this medium at 37° in a non-gassed incubator for 2 hr. The binding reaction was then initiated by adding [^{3}H]CGP in 20-μL aliquots to give final concentrations of 4, 2, 1, 0.5, 0.25 and 0.125×10^{-9} M and the incubation continued for a further 2 hr after which the assays were terminated by the aspiration of the reaction mixture and the washing of each monolayer three times with 1.0 mL of ice-cold 10 mM Tris-isosaline pH 7.5. Non-specific binding was determined in the presence of 10^{-5} M *dl*-propranolol. The cells in each well were then dissolved in 0.5 mL 0.5 M NaOH at 37° for 2 hr. The specific radioactivity in each well was then determined by measuring the protein content [11] and the radioactivity in 0.2 mL. Each sample was counted in "Optiphase" scintillation fluid (Fisons) with the addition of 0.1 mL formic acid to aid emulsification of the alkaline samples.

The competition studies were performed using a similar method to that described above with the modification that the wells were initially incubated in 450 μL of Hepes-buffered DMEM containing 2% FCS. The binding reaction was then initiated by adding 50 μL of the competitor plus 10 μL of [^{3}H]CGP. These experiments were initially performed using 1×10^{-9} M [^{3}H]CGP but the coefficient of variation for each displacement point was between 20 and 30% due to the low number of counts. This problem was removed using 2×10^{-9} M [^{3}H]CGP in which the coefficient of variation decreased to 10% or less and gave similar results with respect to the ability of each agonist to displace the ligand.

Receptor concentration and affinity were calculated using computer Scatchard analysis of the binding displacement curves (EBDA Programme; G. A. McPherson, Elsevier-Biosoft, Cambridge, U.K.). The receptor-binding activity of isoproterenol, epinephrine, norepinephrine and cimaterol was assessed by the ability of increasing concentrations of these peptides to displace [^{3}H]CGP in comparison with that measured using 10^{-5} M propranolol. The dissociation constant (K'_d) was calculated from the concentration of unlabelled agonist yielding 50% displacement (IC_{50}) using the equation of Cheng and Prusoff [12].

Measurement of protein synthesis and degradation. Protein synthesis was measured as the incorporation of [^{3}H]tyrosine (0.37 MBq/mL) into 5% trichloroacetic acid (TCA)-insoluble material over 6 hr, as described by Harper *et al.* [9]. Protein degradation was measured after the cell monolayers had been labelled for 2 days with [^{3}H]tyrosine (0.37 MBq/mL). DMEM containing 0.1% BSA was then added to the cells and degradation was measured as the release of this label into a 5% (w/v) TCA-soluble form during a 24 hr experimental period as described by Harper *et al.* [9]. Before the experimental period fetal cells were incubated for 22 hr in DMEM containing 0.1% BSA. This period was decreased to 6 hr for satellite cells as it was found that these cells were

more sensitive to serum starvation than fetal muscle cells [10]. Unlabelled tyrosine in excess of 2 mM was also added to DMEM to minimize reincorporation of the released [^{3}H]tyrosine. In all studies treatment with 10^{-6} M insulin was included as a positive control.

Transport studies. The uptake of AIB by confluent satellite cell monolayers was measured using a modification of the method described by Hollenberg and Cuatrecasas [13]. The cells were initially washed with EBS and were then incubated in 200 μL of EBS containing 0.1% BSA at 37° in a 6% CO_2 atmosphere for 1 hr. The media was then removed and replaced with the same volume of EBS containing 0.1% BSA plus the test substrate (cimaterol or insulin) and the incubation continued for a further 1.5 hr, AIB was then added in 20-μL aliquots (0.19 MBq/mL) and its uptake measured over a 15 min incubation period. The test media was then aspirated and the monolayers washed three times with 1.0 mL of ice-cold EBS. The cells were then solubilized and the specific radioactivity measured as described above for the receptor-binding assay. The uptake of 2-DOG was also measured over a 10 min period using an identical method with the modification that glucose-free DMEM was used as the incubation and washing media and the amount of 2-DOG added to each well was 0.81 MBq/mL.

Statistical analysis. Experiments were set up with treatments randomly assigned within each tray. Each experiment was conducted using two separate trays with six wells used per treatment and all studies were performed on a minimum of two separate cell preparations. Analysis of variance was carried out, allowing tray-to-tray differences as a source of variation. Variance ratios were used to test for the overall significance of treatment-specific differences. Treatments were compared with the control in each experiment using a Student's *t*-test with the pooled standard error of the difference (SED).

RESULTS

The binding of [^{3}H]CGP to cellular receptors in fetal muscle cells was saturable (Fig. 1a) and the specific binding of [^{3}H]CGP ranged from 95 to 30% of total binding at low and high concentrations of ligand respectively. Scatchard analysis of these binding data revealed a single class of binding sites with a dissociation constant (K_d) of $0.38 \pm 0.12 \times 10^{-9}$ M and a maximum number of binding sites of 55.2 ± 0.67 fmol/μg protein. The Hill coefficient for binding was 1.07 ($r = 0.97$).

In satellite cell preparations the specific binding of [^{3}H]CGP ranged from 45 to 15% of total binding at low and high concentrations of ligand, respectively. Scatchard analysis of these binding data revealed two classes of binding sites (Fig. 1b) which were shown to be of low and high affinities using iterative analysis. The high affinity site had a K_d of $1.02 \pm 0.10 \times 10^{-9}$ M and a maximum number of binding sites of 28.4 pmol/μg protein, while the low affinity site with a K_d of $12.1 \pm 0.6 \times 10^{-9}$ M and a maximum number of binding sites of 389 ± 14 fmol/μg protein. The Hill coefficients for binding were 0.91 ($r = 0.91$) and 0.99 ($r = 0.99$) for the high and low affinity sites, respectively.

In both the fetal and satellite muscle cell preparations β-adrenergic agonists competed for [^{3}H]CGP binding sites with affinities significantly lower than the antagonist propranolol (Table 1). In fetal muscle cells the order of potency was isoproterenol > epinephrine > norepinephrine which is characteristic for the β_2-subtype of adrenergic receptors [14]. In satellite cells, however, this order of potency was isoproterenol ≫ norepinephrine = epinephrine which is characteristic for the β_1-subtype of adrenergic receptors. In both fetal and satellite cells cimaterol displaced [^{3}H]CGP with an affinity similar to that recorded for epinephrine. It is however, acknowledged that in the present study it was difficult to determine the β-receptor subtype because of the inability to measure accurately the displacement by β-agonists at low concentrations of [^{3}H]CGP.

The β-agonist cimaterol significantly stimulated protein synthesis by between 8 and 12% when incubated at concentrations of 10^{-8}–10^{-7} M, respectively (Fig. 2), in differentiated muscle cells derived from satellite cells. When these studies were repeated in the presence of 2% FCS in the incubation media protein synthesis was 7% higher than that observed in controls and this significant effect was observed at cimaterol concentrations of 10^{-8}–10^{-6} M. All these positive effects by cimaterol on protein synthesis were maintained if the β-antagonist propranolol was also added to the incubation media at a concentration of 10^{-5} M, but were removed by the addition of 10^{-6} M isoproterenol to the incubation media. Propranolol alone had no significant effects on protein synthesis at concentrations of between 10^{-9} and 10^{-5} M, but caused a 22% ($P < 0.05$) decrease in protein synthesis at concentration of 10^{-4} M. Isoproterenol had no significant effects on protein synthesis at concentrations ranging from 10^{-9} to 10^{-5} M either in the presence or absence of 2% FCS. In all experiments the addition of insulin at a concentration of 10^{-6} M stimulated protein synthesis by between 30 and 35% ($P < 0.001$), except when performed in the presence of 2% FCS when this stimulatory effect was reduced to 11% ($P < 0.01$). Cimaterol did not have any significant effects on the rate of uptake of either AIB or 2-DOG or on protein degradation in satellite cells. Insulin (10^{-6} M), however, significantly stimulated the rate of uptake of both AIB and 2-DOG by between 55 and 60% ($P < 0.001$) and inhibited protein degradation by 35% ($P < 0.001$). In fetal muscle cells cimaterol had no effect on either protein synthesis or degradation whilst the addition of insulin (10^{-6} M) increased protein synthesis by 18% ($P < 0.001$) and reduced protein degradation by 9% ($P < 0.05$).

DISCUSSION

The present study is the first to demonstrate the presence of β-adrenergic receptors in cultured muscle cells of ruminant origin. In the two ovine muscle cell culture preparations studied the number and dominant β-receptor sub-type differed. In fetal muscle cultures a single high affinity β-receptor that

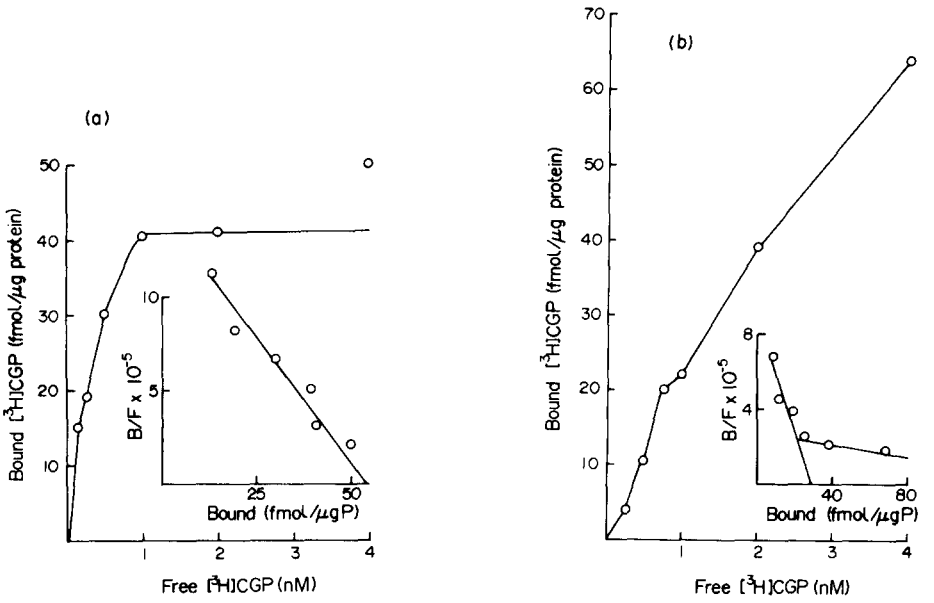

Fig. 1. [^{3}H]CGP binding to ovine primary muscle cultures derived from muscle of (a) fetal or (b) pre-pubertal (satellite cells) lambs, with the corresponding Scatchard plot (inset) of a typical experiment (B/F units = fmol/μg protein/nM).

Table 1. Comparison of the ability of β-agonists to displace [^{3}H]CGP-12177 in relation to that of 10 μmol/L propranolol (IC_{50}) and apparent equilibrium dissociation constants (K'_d) for the interaction of the antagonists and agonists with [^{3}H]CGP-12177 binding sites in ovine primary muscle cultures derived from either fetal or pre-pubertal (satellite cells) lambs

Competitor	Fetal			Pre-pubertal		
	Relative binding affinity*	IC_{50} (μmol/L)	K'_d (μmol/L)	Relative binding affinity*	IC_{50} (μmol/L)	K'_d (μmol/L)
(±)Propranolol	1.0	0.04 ± 0.01	0.002 ± 0.001	1.0	0.009 ± 0.01	0.016 ± 0.009
(−)Isoproterenol	0.4	0.11 ± 0.07	0.01 ± 0.007	0.02	0.46 ± 0.09	0.41 ± 0.01
(−)Epinephrine	0.002	20.5 ± 4.0	1.2 ± 0.3	0.0001	82.9 ± 38.2	78.5 ± 35.4
(−)Norepinephrine	0.0004	101.0 ± 19.9	13.5 ± 1.7	0.0002	43.7 ± 15.6	40.5 ± 14.5
(±)Cimaterol	0.002	16.8 ± 7.0	1.0 ± 0.6	0.0003	35.6 ± 11.2	32.9 ± 10.3

Values are means ± SE.

* The relative binding affinity was calculated as the increase of the ratio of the IC_{50} of the agonist relative to the IC_{50} of propranolol.

was predominantly β_2 was found. In cultures derived from satellite cells a dual class of receptor was present of which the majority were of the β_1 sub-type. The high affinity β-receptor present in satellite cells exhibited a similar K_d and number of binding sites to that found in fetal muscle cells. This contrasts with the low affinity class of β-receptor which had 14 times more binding sites than the high affinity β-receptor in the same satellite cell, but with a 10-fold higher K_d. These differences in receptor population between fetal and satellite muscle cell culture preparations were obtained despite both preparations being grown and differentiated under identical experimental conditions. This effect cannot be explained by differences in the number of myotubes between differentiated fetal and satellite muscle cells because when each preparation was observed under phase-contrast microscope they were judged to contain very similar numbers of myotubes. This was to be expected given that the initial seeding density of both the fetal and satellite cells was selected in order to give a maximal number of myotubes [9, 10]. Furthermore, the myogenic populations of the fetal and satellite cells were also observed to be identical when subjected to clonal analysis.

The difference between fetal and satellite cell preparations was not only confined to receptor populations but was also apparent in the effects of insulin and cimaterol on protein metabolism. Insulin caused a larger stimulation of protein synthesis and inhibition of protein degradation in satellite cells compared with fetal cells, and cimaterol only increased

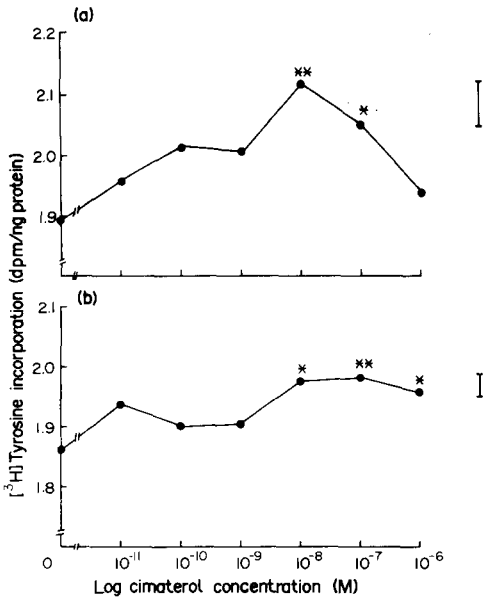

Fig. 2. Dose–response of protein synthesis in ovine primary muscle cultures (satellite cells) derived from pre-pubertal lambs to cimaterol in the presence of (a) 0.1% bovine serum albumin and (b) 2% fetal calf serum. Values are plotted as means and the pooled standard error of the difference (SED) is given; N = 6. *P < 0.05, **P < 0.01 compared with control values (Student's *t*-test).

protein synthesis in satellite cells. These results accord with our earlier findings that major quantitative differences exist between cultured fetal and satellite cells, in their response to anabolic hormones and growth factors [10], which may be related to significant changes in receptor population.

It has been suggested that the differentiation of myogenic cells in tissue culture is similar to that observed *in vivo* [15]. The above differences in β-receptor populations measured *in vitro* may therefore be explained by developmental adaptations in muscle metabolism between fetal and pre-pubertal ruminants. This proposal is supported by previous studies on the effects of anabolic hormones in fetal [9] and satellite cells [10] which are in agreement with studies using whole animal or organ systems. The predominance of β_2-receptors in fetal myoblasts could therefore be related to the immaturity of the sympathetic nervous system in the fetus, as shown by studies on the heart [16]. Consequently oxidative metabolism of fetal myoblasts may be under a greater influence from epinephrine than norepinephrine. This proposal that adrenergic rather than sympathetic stimulation has a dominant role in the control of fetal metabolism is supported by the finding that fetal sensitivity to the hyperglycaemic effect of epinephrine is much greater than that observed for norepinephrine [17].

In the adult rat long-term adaptations to exercise include a rise in β-adrenergic receptor density which is positively correlated to the oxidative capacity of skeletal muscle [18]. A similar situation may pertain in ruminants with the developmental changes in receptor population, affinity and number of binding sites found between muscle cells isolated from 12-week-old fetuses and 3–4-month-old lambs, occurring as a result of changes in oxidative metabolism by muscle. This effect is likely to be influenced by changes in nutrient supply to skeletal muscle which occur between the fetus and pre-pubertal animal *in vivo*. Glucose is the main substrate taken up by the hind-limb in the late gestation fetus and its oxidation can account for 70% of oxygen utilization [19]. This contrasts with a figure of 35% in the adult sheep, in which acetate metabolism can account for a further 35% of oxygen utilization [20]. The predominance of the β_1-receptor sub-type in satellite cells contrasts with other studies on skeletal muscle which have demonstrated that β_2-receptors dominate [21]. This difference can be explained by the fact that satellite cells were investigated as opposed to hind-limb preparations [18] or continuous cell lines [22] in earlier studies.

The β-agonist cimaterol significantly affected protein metabolism in primary ovine muscle cells to the extent that protein synthesis was stimulated in satellite cells, but there was no effect on protein degradation. These results contrast with the anabolic effect of β-agonists observed in ruminant muscle *in vivo*, which is thought to be primarily mediated via an inhibition of protein degradation [8]. However, a stimulation of whole-body protein synthetic rate has been recorded after 11 days of clenbuterol treatment in adult wether sheep [23]. Cimaterol has also been shown to demonstrate an anabolic effect on the continuous rodent muscle cell lines G8-1 (mouse) and L6 (rat) via a stimulation of protein synthesis, with this effect being blocked by propranolol [24]. This contrasts with results from the present study using ovine satellite cells in which propranolol was unable to block the stimulation of protein synthesis induced by cimaterol, which accords with *in vivo* studies in rats [4]. The β-receptor may not always be involved in the anabolic effect of β-agonists, a proposal which is supported by the absence of any significant effects on protein metabolism by cimaterol on fetal muscle cells in this study, plus experiments using zinterol on rat L8 muscle cells [7] which both contain predominantly β_2-receptors.

Cimaterol did not significantly affect the rate of uptake of AIB or 2-DOG, thereby demonstrating that neither amino acids nor glucose are the rate limiting substrate on the stimulatory effect cimaterol has on protein synthesis. This result is not due to errors in the measurement of AIB or 2-DOG uptake for which the mean coefficient of variation for each point was between 4 and 5%. It is therefore not possible to state what the rate limiting substrates are for the anabolic effect cimaterol causes on protein synthesis in satellite cells. Protein synthesis in satellite cells was stimulated using a concentration of cimaterol of 10^{-8}–10^{-7} M in the absence of serum, but a significant effect was observed using 10^{-6} M cimaterol if 2% FCS was present. In these experiments the measurement of protein synthesis in controls was identical under both conditions. A possible explanation for this result is that β-agonists have both stimulatory and inhibitory effects on protein synthesis in muscle cells, with this latter response

being blocked in the presence of serum. This proposal is supported by the finding that isoproterenol had no significant effects on protein synthesis, but was able to block the stimulatory response by cimaterol on protein synthesis. Therefore, given the contrasting effects of adding β-antagonists (propranolol) or β-agonists (isoproterenol) to the incubation medium at the same time as cimaterol it has yet to be fully established whether the anabolic effect of cimaterol is mediated via the β-receptor.

It is concluded that β-adrenergic receptors are present in both ovine fetal and satellite muscle cells, but these do not appear to play any role in the anabolic effect of β-agonists in ruminant muscle. Cimaterol is therefore able to elicit a direct stimulation of protein synthesis in satellite cells although this response may not be related to its ability to bind to the β-receptor.

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