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EFFECTS OF A BETA-ADRENERGIC AGONIST ON PROTEIN TURNOVER IN MUSCLE CELLS IN CULTURE

MARY ANN MCELLIGOTT,* LEE-YUH CHAUNG and ALBERT BARRETO, JR.
Merck Sharp & Dohme Research Laboratories, Department of Animal Drug Discovery, Rahway, NJ 07065, U.S.A.

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Abstract—β-Adrenergic agents powerfully stimulate muscle growth in animals. Whether the mechanism of action involves a direct effect on muscle cell β-receptors or is secondarily due to a β-induced alteration in the hormonal environment is not known. To assess whether direct β-receptor activation results in muscle protein accretion, we examined the effect of the β-agonist zinterol on several anabolic processes in L8 muscle cells in culture. *In vivo* feeding of zinterol (26.5 ppm) to rats significantly increased muscle weight by 15%. *In vitro*, zinterol stimulated lactate release from L8 cells whereas propranolol inhibited this process, demonstrating that these cells have functional β-receptors both before and after fusion. We measured several anabolic processes, in both serum-stimulated and quiescent cells, over a wide range of zinterol concentrations. Zinterol had no effect on protein or DNA synthesis, protein degradation, or rates of amino acid uptake. These data suggest that the *in vivo* muscle growth stimulation is either indirect or some *in vivo* requirements (e.g. tension and nerve interactions) are necessary for expression of the effect.

Certain β-adrenergic agonists increase muscle mass in chronically treated rats [1–3]. It is not established whether the increased protein accretion in muscle is a primary effect, due to a direct stimulation of muscle β-adrenergic receptors, or due to a secondary β-adrenergic effect, mediated by alterations in the levels of circulating hormones or other factors.

Muscle size is controlled by a balance between rates of protein synthesis and protein degradation. There are several reports in the literature suggesting that β-adrenergic agonists alter protein turnover in muscle in an anabolic direction. Isoproterenol inhibits protein degradation in perfused rat hemicorpus [4], in incubated rat epitrochlaris muscle [5] and in incubated rat diaphragm [6]. Epinephrine reportedly stimulates protein synthesis in incubated diaphragm from hypophysectomized rats but not in normal rats [7]. Isoproterenol has no effect on protein synthesis in perfused rat hemicorpus [4]. However, *in vivo*, the fractional rate of protein synthesis increases in muscle after treatment with the β-adrenergic agonist clenbuterol [2] and isoproterenol [1]. Net protein synthesis (rate of total incorporation of radiolabeled amino acid into muscle protein − rate of protein degradation) and protein degradation increased and decreased, respectively, in the EDL muscle from rats fed clenbuterol for 2 days (protein turnover measurements in the EDL were made *in vitro*). Incubated soleus muscles from these same rats showed higher rates of protein degradation but no alteration in synthesis (Yang YT, Firman LS and Boeninghaus JE, unpublished results†). These protein turnover measurements were only made after 2 days of treatment and thus only reflect the physiological state at this time. β-Agonists stimulate growth of both the EDL and soleus muscles in rats fed β-agonists and thus the effect is not fiber type specific [3]. The above data from *in vitro* muscle preparations suggest the possibility that β-adrenergic agonists may have a direct action on rates of protein turnover in muscle under certain conditions.

We used a muscle cell culture model to clarify the relationship between the β-adrenergic receptor and regulation of rates of protein turnover in muscle. Several physiological functions of cells in culture are reported to be affected by β-adrenergic agonists. Beta-adrenergic agents promote precocious fusion in primary chick myoblasts [8], by stimulating cAMP which is critical for initiating fusion, although the fusion process is not dependent on activation of the β-adrenergic receptors. Isoproterenol is shown to increase the phosphorylation of desmin and vimentin, intermediate filament proteins, in 8-day myotube cultures; however, the biological function is not established [9]. Differentiated muscle cells in culture are reported to possess β-adrenergic receptors capable of transducing a stimulus to a biological response [8, 10–12], but a direct effect of β-adrenergic agonists on protein turnover has not been demonstrated. Use of a muscle cell culture system in these experiments allows us to circumvent *in vivo* problems, such as the complexity of the hormonal environment *in vivo* and the non-homogeneous cellular composition of whole muscle preparations, and to determine directly whether stimulation of β-receptors in L8 muscle cultures regulates rates of protein turnover.

We have examined the *in vivo* and *in vitro* effects of zinterol, a β-adrenergic agonist. Zinterol was fed to rats for 1 week to determine the effect on muscle growth, and the *in vitro* effects on rates of amino acid

* Send correspondence and reprint requests to: Mary Ann McElligott, Ph.D., Roberts Pharmaceutical Corp., Meridian Center III, 6 Industrial Way West, Eatontown, NJ 07724.

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uptake, DNA and protein syntheses, and protein degradation in cultures of L8 muscle cells were measured. These metabolic processes were assessed in both quiescent and serum-stimulated cells to determine whether zinterol could directly stimulate anabolic processes or potentiate an already stimulated system. Zinterol was used in these experiments because this β-adrenergic agonist has been demonstrated to have full intrinsic activity and a high affinity for the β-agonist receptors in L6 cells [11]. Furthermore, zinterol is very stable at 37°. Isoproterenol which is easily oxidized was not used since long incubation times were required for some experiments. We found that zinterol stimulated muscle growth in rats and, although L8 muscle cells have functional β-adrenergic receptors, we could not demonstrate a direct anabolic effect of zinterol on these cells in culture.

MATERIALS AND METHODS

Treatment of animals. Male Charles River CD (average weight 145 g) were fed zinterol (26.5 ppm) mixed in the feed (Ralston Purina powdered rat chow) for 1 week. Rats were weighed at the end of the experiment, killed by decapitation, and the gastrocnemius muscle removed and weighed.

Tissue culture. L8 muscle cells (a gift from Dr F. J. Roisen) were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 1% penicillin streptomycin and 1% nystatin (all from Grand Island Biological Co., Grand Island, NY) in a humidified atmosphere of 5% CO_2/95% air at 37°. Cells were plated in multiwell plates at a density of 3×10^4 cells/cm^2. L8 myotube cultures were used for experiment 1 week or more after plating at which time maximum fusion (60–90%) was attained [13]. Most experiments were repeated two to four times, and a representative experiment is presented here. The effect of zinterol on several anabolic processes was also assessed in primary chick muscle cells. These data were similar to results presented for L8 cells; therefore, we have not shown the results here.

Lactate determination. The growth medium was removed and the cultures were rinsed with Hanks' balanced salt solution (Hanks) and incubated for 3 hr at 37° in DMEM containing 1 μg/ml ascorbic acid and various concentrations of zinterol (Mead Johnson & Co.). Lactate released into the medium was determined enzymatically using lactate dehydrogenase as a catalyst, and the formation of reduced nicotinamide adenine dinucleotide was measured fluorometrically by the method of Gutmann and Wahlefeld [14].

Protein synthesis. Cells were rinsed with Hanks and incubated overnight in DMEM to bring the cells into a quiescent state (basal rates of protein synthesis). DMEM or 1% FBS in DMEM, both containing 2 mM unlabeled tyrosine and various concentrations of zinterol, was added to cells and incubated for the indicated time periods. A 1-hr pulse of [^{3}H]tyrosine (3 μCi/ml final concentration, New England Nuclear, Boston, MA) was added at the indicated times. The cells were rinsed three times with ice-cold Hanks and scraped from the plates in 20% trichloroacetic acid (TCA). Cells were pelleted by centrifugation and dissolved in 0.5 ml of 1 N sodium hydroxide (NaOH) and 1% deoxycholate (DOC). Protein was measured in a small sample using a fluorometric assay [15]. Glacial acetic acid (100 μl) and 10 ml of Aquasol (New England Nuclear) were added to the remainder of the sample, and the radioactivity was determined. The protein synthesis rate is expressed as the dpm/μg protein/hr.

Amino acid uptake. Cells were rinsed with Hanks and incubated overnight in DMEM containing 0.045 mM unlabeled aminoisobutyric acid (AIB). Various concentrations of zinterol and either 1% bovine serum albumin or 1% FBS were then added to the cultures which were incubated for the indicated times. [^{14}C]AIB (0.5 μCi/ml final concentration, New England Nuclear) was added to each well for the last 40 min. The cultures were rinsed three times in ice-cold Hanks, and the cells were collected in 1 ml of 0.1 N NaOH and 0.1% DOC. Protein was measured and the sample counted for radioactivity as described above. AIB uptake is expressed as the dpm/mg protein/40 min.

Protein degradation. Cellular protein was labeled with [^{3}H]tyrosine (0.2 μCi/ml final concentration, New England Nuclear) for 18 hr. The cultures were rinsed with Hanks to remove excess label and incubated in DMEM or DMEM plus 4% FBS. Degradation medium contained 2 mM unlabeled tyrosine to prevent reutilization of the radiolabeled tyrosine [16] and various concentrations of zinterol. A zero-time point sample of medium was taken, followed by a 17.5-hr sample. The percentage of protein degraded was determined as described previously [17].

Thymidine uptake. Sparsely plated muscle cells (3×10^3 cells/cm^2) were used for thymidine uptake studies. Two days after plating, he medium was replaced with either DMEM or DMEM containing 2% fresh FBS and various concentrations of zinterol. A 1-hr pulse of [^{3}H]thymidine (0.5 μCi/ml final concentration, Amersham, Arlington Heights, IL) was given at the indicated time points during a 24-hr period. Cells were rinsed three times with ice-cold Hanks to remove the label. The cells were scraped from the plates in 1.5 ml of 20% TCA and centrifuged to pellet the sample. The pellet was dissolved in 0.5 ml of 1 N NaOH and 1% DOC. An aliquot of the sample was used for protein determination and the remainder counted for radioactivity as described above. The rate of thymidine uptake is expressed as dpm/μg protein/hr.

RESULTS

Zinterol dramatically increased the weight of the gastrocnemius muscle 15% in just 1 week (Table 1). The average daily gain of these rats increased 11%.

Zinterol stimulated lactate release from L8 muscle cells both before (Fig. 1A) and after (Fig. 1B) fusion. The stimulation of lactate release was dose dependent. Zinterol, at the highest concentration used, 10^{-3} M, increased lactate release 115% in the nonfused muscle cells and 240% in fused muscle cells. Propranolol (10^{-4} M) inhibited the zinterol-stimulated lactate release.

Table 1. Zinterol stimulation of muscle growth and body weight gain

Treatment	Initial weight (g)	Average daily gain (g)	Gastrocnemius muscle (g)
Control	144.6 ± 1.8	8.23 ± 0.26	0.9700 ± 0.0135
Zinterol (26.5 ppm)	144.7 ± 2.8	9.12 ± 0.36*	1.1189 ± 0.0186†

Values are means ± SE; N = 19 for control and N = 10 for zinterol-treated.
*† Significantly different from control: * $P < 0.01$, and † $P < 0.001$ (Student's t-test).

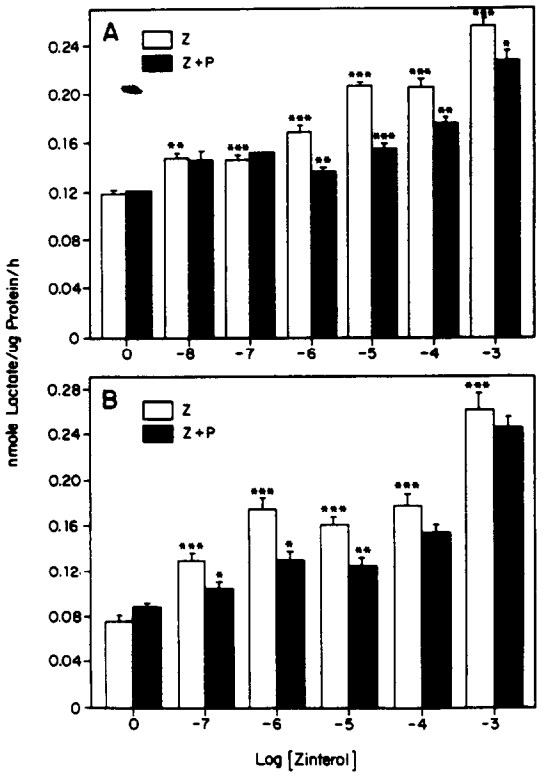

Fig. 1. Effect of zinterol on lactate release from L8 muscle cells before and after fusion. (A) Muscle cells before fusion (myoblasts). (B) Muscle cells after fusion (myotubes). Key: (Z): zinterol included at the indicated concentration. Significance was determined using Student's t-test: ** $P < 0.01$ and *** $P < 0.001$ vs control without zinterol. (Z + P): zinterol + 10^{-4} M propranolol added at the same time as zinterol. Significance: * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ vs cultures with zinterol alone. Values are means ± SE (N = 6); no error bar indicates that the error is included within the bar.

FBS stimulated rates of protein synthesis in muscle cells within hours after the addition of serum (Fig. 2A). The rate of protein synthesis returned to the basal levels observed in DMEM by 48 hr after serum addition. Zinterol (10^{-9}–10^{-4} M) did not affect significantly basal rates of protein synthesis measured in DMEM at any time during the 48-hr period (Fig. 2B). Similarly, zinterol did not augment rates of protein synthesis in muscle cells stimulated by the addition of serum (Fig. 2C).

The serum stimulation of AIB uptake by muscle cells was rapid and reached maximum by 4 hr (Fig. 3A). Various concentrations of zinterol (10^{-8}–10^{-4} M) did not consistently alter basal rates of AIB uptake in L8 cells incubated in DMEM for a 24-hr period (Fig. 3B). Zinterol (10^{-9}–10^{-4} M) also had no effect on the serum-stimulated rates of AIB uptake (Fig. 3C).

Muscle tissue contains undifferentiated satellite cells which are recruited under certain conditions such as compensatory muscle hypertrophy [18]. Satellite cells and myofibers derive from the same somitic origin [19] and behave in a manner similar to myoblasts, with an anabolic response to serum [20]. Therefore, we explored the possibility that β-agonists might recruit undifferentiated muscle cells prior to protein accretion, and examined the response of undifferentiated myoblasts to zinterol treatment. Peak stimulation of DNA synthesis in sparsely plated muscle cells was observed 24 hr after the addition of 1% FBS (Fig. 4A). Basal rates of thymidine uptake in muscle cells incubated in DMEM (Fig. 4B) were not affected by the addition of various concentrations of zinterol (10^{-9}–10^{-5} M). The serum stimulation of thymidine incorporation (Fig. 4C) was not altered by the addition of zinterol (10^{-8}–10^{-5} M). Serum stimulated protein accumulation in cultures treated for 48 hr (Fig. 5A). Zinterol (10^{-9}–10^{-4} M) treatment for a 48-hr period did not affect the protein concentration per well either in non-fused (myoblast) cells incubated in DMEM (Fig. 5B), or in myoblasts which were stimulated to divide by addition of FBS (Fig. 5C).

Rates of proteolysis were accelerated in fused muscle cells incubated in DMEM without serum. Zinterol (10^{-12}–10^{-7} M) did not affect the percentage of protein degraded under these conditions. Zinterol also did not affect basal proteolysis in cells incubated in 4% FBS (Fig. 6).

DISCUSSION

Lactate release from L8 muscle cells was stimulated by zinterol both before and after fusion. Concentrations of zinterol as low as 10^{-8} M stimulated lactate release from L8 cells in the myoblast stage in our experiments. The lactate production was inhibited by propranolol, a β-adrenergic receptor antagonist (Fig. 1). These data indicate that both L8 myoblasts and differentiated L8 myotubes possess β-receptors capable of a biological response when stimulated directly in culture with a β-adrenergic agent. These data also indicate that β-agonists act *in vitro* through β-receptors. It has not been fully

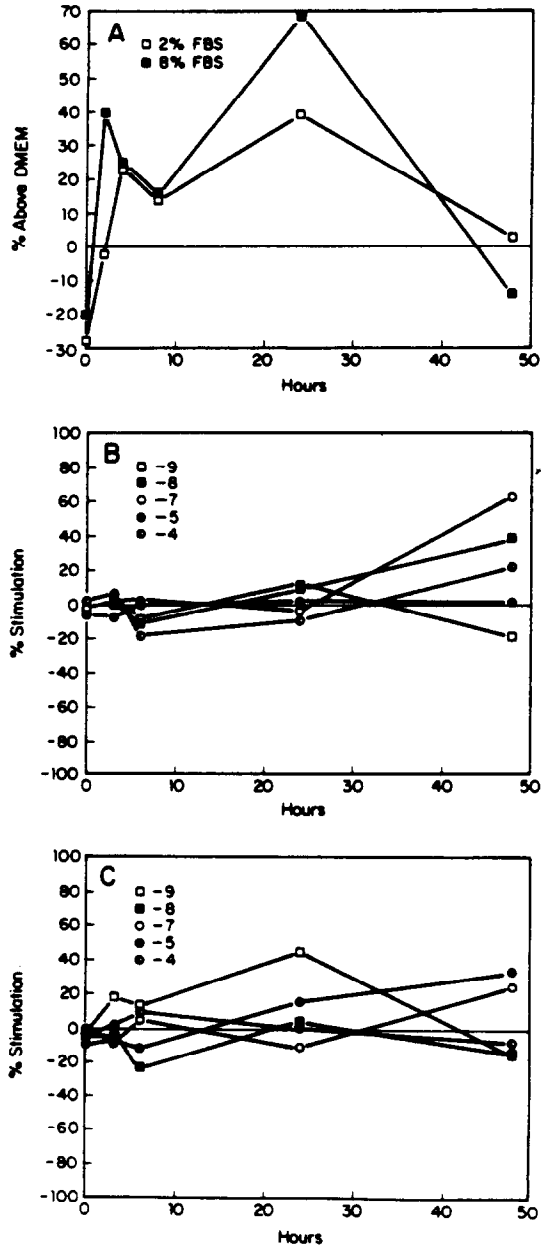

Fig. 2. Effect of zinterol and fetal bovine serum on protein synthesis in L8 muscle cells. (A) Cultures were incubated in 2% or 8% FBS for the indicated time period, and the rate of protein synthesis was determined in myotube cultures. The percentage increase in protein synthesis in the presence of serum compared to DMEM alone was plotted (N = 4). (B) Cells were incubated in DMEM (basal protein synthetic rate) with or without zinterol at the following concentrations: (−9) 10^{-9} M, (−8) 10^{-8} M, (−7) 10^{-7} M, (−5) 10^{-5} M and (−4) 10^{-4} M. Protein synthesis was measured at the indicated time points. The percentage difference between the rate of protein synthesis in the presence and absence of zinterol was calculated (N = 3). (C) Cells were incubated in DMEM + 1% FBS (stimulated rates of protein synthesis) with or without the following concentrations of zinterol: (−9) 10^{-9} M, (−8) 10^{-8} M, (−7) 10^{-7} M, (−5) 10^{-5} M and (−4) 10^{-4} M. Protein synthesis was measured at the indicated time points. The percentage difference between the rate of protein synthesis in the presence and absence of zinterol was plotted (N = 3).

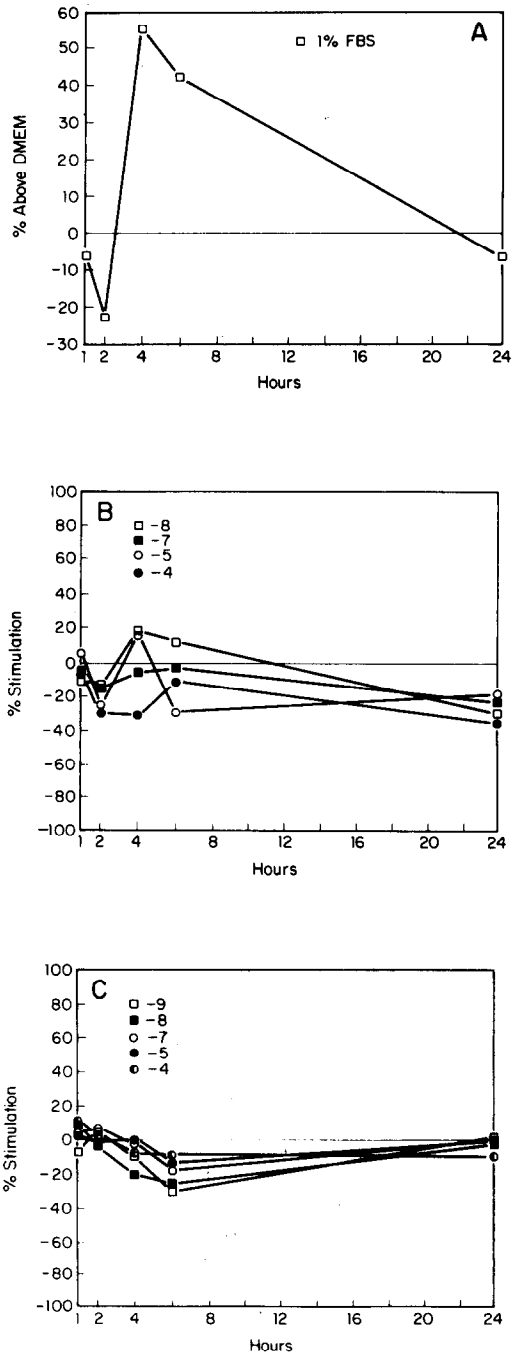

Fig. 3. Effect of zinterol and FBS on AIB uptake in L8 muscle cells. (A) Myotube cultures were incubated in 1% FBS for 24 hr. AIB uptake was determined at the indicated time points. The percentage difference between AIB uptake in the presence and absence of FBS was plotted (N = 3). (B) AIB uptake was measured in myotube cultures incubated in DMEM (basal rates) containing various concentrations of zinterol: (−8) 10^{-8} M, (−7) 10^{-7} M, (−5) 10^{-5} M and (−4) 10^{-4} M. The percentage difference in AIB uptake in the presence and absence of zinterol is plotted (N = 3). (C) AIB uptake was measured in myotube cultures incubated in DMEM containing 1% FBS (stimulated rates) and the following concentrations of zinterol: (−9) 10^{-9} M, (−8) 10^{-8} M, (−7) 10^{-7} M, (−5) 10^{-5} M and (−4) 10^{-4} M. The percentage difference in AIB uptake in the presence and absence of zinterol was plotted (N = 3).

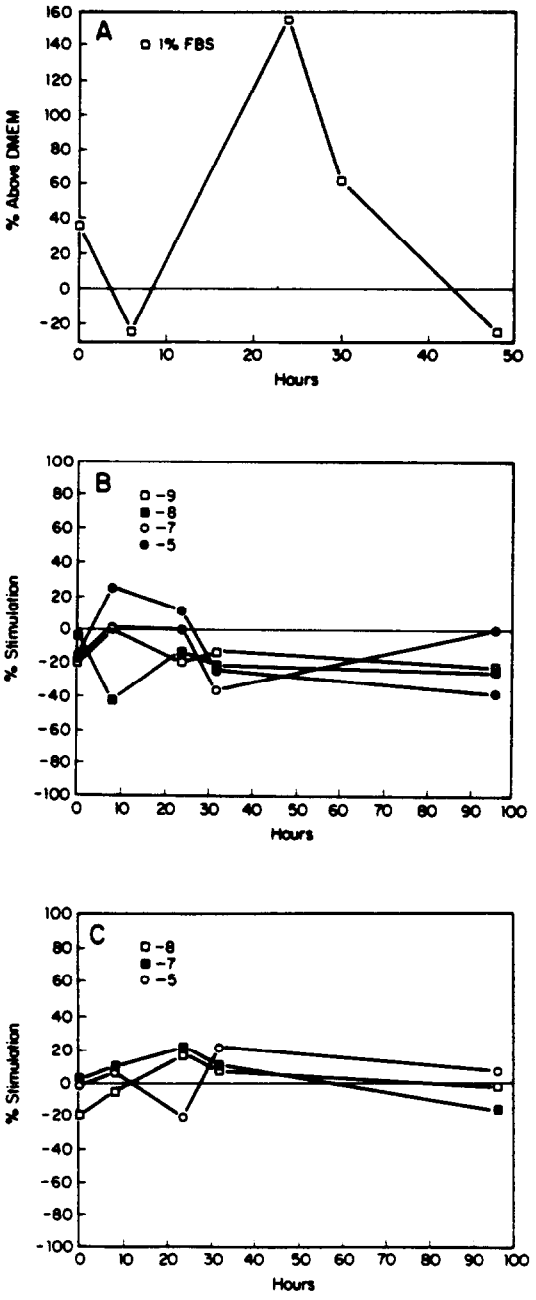

Fig. 4. Effect of zinterol and FBS on rates of thymidine uptake in L8 myoblasts. (A) The percentage stimulation of thymidine uptake in the presence of serum (1% FBS) compared to the absence of serum was measured at the indicated time points, in myoblast cultures (N = 3). (B) Myoblast cultures were incubated in DMEM (basal thymidine uptake) containing zinterol at the following concentrations: (−9) 10^{-9} M, (−8) 10^{-8} M, (−7) 10^{-7} M and (−5) 10^{-5} M. Thymidine uptake was determined at the indicated time points, and the percentage difference between the rate of uptake in the presence and absence of zinterol was plotted (N = 3). (C) Myoblast cultures were incubated in DMEM containing 2% FBS (stimulated thymidine uptake) and the following concentrations of zinterol: (−8) 10^{-8} M, (−7) 10^{-7} M and (−5) 10^{-5} M. Thymidine uptake was determined at the indicated time points. The percentage difference between the rate of uptake in the presence and absence of zinterol was plotted (N = 3).

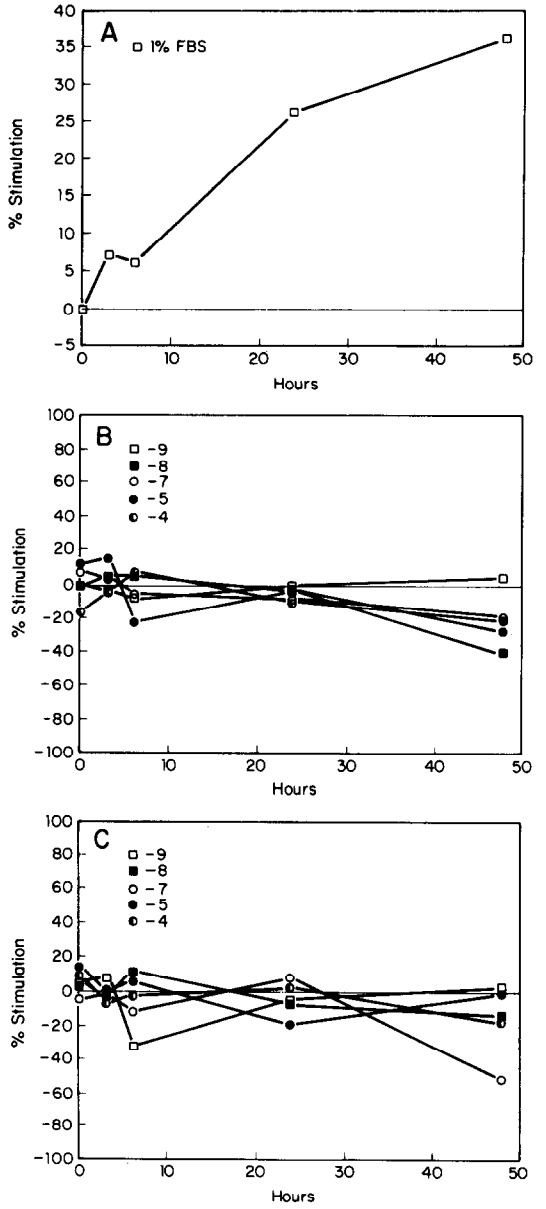

Fig. 5. Effect of zinterol and FBS on protein accumulation in muscle cultures. (A) Myoblast cultures were incubated in DMEM or DMEM + 1% FBS for the indicated times. The protein content per well was determined, and the percentage increase in the presence of FBS was plotted. (B) Myoblast cultures were incubated in DMEM containing zinterol at the following concentrations: (−9) 10^{-9} M, (−8) 10^{-8} M, (−7) 10^{-7} M, (−5) 10^{-5} M and (−4) 10^{-4} M. (C) Same as panel B but myoblast cultures were incubated in DMEM containing 1% FBS (N = 3).

established whether all myoblasts have functional β-adrenergic receptors. Primary quail [10] and primary human [21] myoblast cultures are reported not to have functional β-adrenergic receptors. There is evidence that β-receptors are expressed in L8 myoblasts, and receptor number, as determined by binding, increases with differentiation of the cells [12] but the functionality of the receptors was not

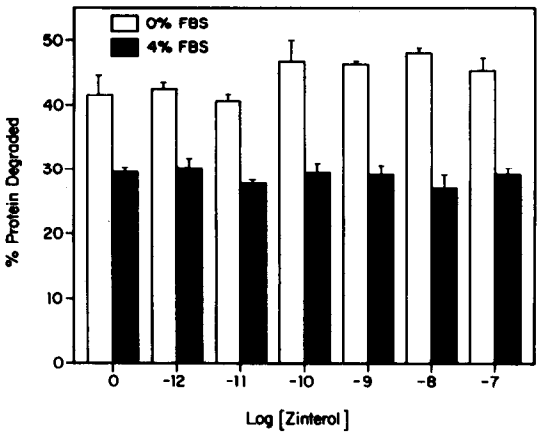

Fig. 6. Effect of zinterol on protein degradation in muscle cells. The percentage protein degraded in a 17.5-hr period in the presence of 4% FBS and in DMEM alone (0% FBS) was plotted versus the log concentration of zinterol in the incubation medium. Values are means ± SE (N = 4).

investigated. Whether fully functional β-receptors can be demonstrated in myoblasts derived from other sources remains to be determined.

Zinterol is an effective rat muscle growth enhancer *in vivo* (Table 1) with results comparable to clenbuterol [2, 3]. However, *in vitro*, although β-receptors were functional as discussed above, there was no activation of cellular anabolic processes [protein and deoxyribonucleic acid (DNA) syntheses, protein degradation, amino acid transport] by zinterol and thus no increase in muscle protein mass *in vitro* in muscle cells in culture (Fig. 5). Results from *in vitro* studies, in the literature, concerning the measurable effects of β-agonists on muscle protein turnover which we discussed at the beginning of the paper suggested that β-agonists could directly affect rates of protein turnover. However, the ability of β-adrenergic inhibitors to block the measured effects of these β-adrenergic agonists on protein turnover was not established in any of the reports with the exception of Garber *et al.* [5] who showed that the inhibition of protein degradation by isoproterenol in incubated rat epitrochlaris muscle was blocked by propranolol. This β-antagonist experiment is crucial in determining a direct relationship between stimulation of the β-adrenergic receptor and alterations in protein turnover for any model system. Therefore, it is premature to definitively conclude that stimulation of the β-adrenergic receptor of muscle incubated *in vitro* directly leads to modulation of the rates of either protein synthesis or protein degradation. We have demonstrated that zinterol stimulated lactate release in L8 muscle cells in culture and that stimulation of these receptors did not result in an alteration of protein turnover. Thus, we conclude that the β-adrenergic receptors in these cells are functional but are not coupled to the processes which regulate protein turnover.

It is possible that we did not observe any direct effects of zinterol on protein turnover in muscle cultures because certain *in vivo* requirements are necessary for expression of the anabolic effect of β-adrenergic agonists. Isolated muscles given subtetanic electrical stimulation show a dose-dependent alteration in the force of contraction due to isoprenaline which is blocked by propranolol [22, 23]. Time-to-peak tension, the half-relaxation time of the twitch, and the membrane resting potential are also altered by isoprenaline treatment and blocked by the addition of propranolol. These changes in contractility due to β-agonist stimulation of muscle may be the primary response which secondarily results in muscle growth. This contractile component is not present in studies measuring protein turnover in incubated muscle. Although myotubes in culture spontaneously contract, the level of activity is most likely not comparable to an *in vivo* situation. Nerve–muscle interactions, also not present *in vitro*, could be the component necessary for expression of the stimulatory mechanism *in vivo*. Further *in vivo* studies are required before this issue can be resolved.

In summary, our studies demonstrated that zinterol stimulates muscle growth in rats and, although this β-adrenergic agonist affected certain aspects of cellular metabolism (lactate release), activation of the β-adrenergic receptors did not modulate anabolic processes in cultured muscle cells.

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