The elevation of sarcoplasmic reticulum Ca^{2+} -ATPase levels by thyroid hormone in the L6 muscle cell line is potentiated by insulin-like growth factor-I

Alice MULLER,*† Cornelis VAN HARDEVELD,* Warner S. SIMONIDES* and Johan VAN RIJN†

*Laboratory for Physiology and †Department of Radiation Oncology, Free University, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

Net synthesis of the fast-type sarcoplasmic reticulum (SR) Ca^{2+} -ATPase was studied in the muscle cell line L6_{AM} using an immunochemical assay (e.l.i.s.a.). In addition, Ca^{2+} uptake by SR was monitored in muscle cell homogenates by a method employing the fluorescent Ca^{2+} indicator fura-2. Measurements were done both in differentiating myoblasts and in myotubes. Ca^{2+} -ATPase levels were low (1 pmol/mg of protein) in undifferentiated myoblasts (controls) and only doubled over a period of 8 days in the absence of thyroid hormone (L-tri-iodothyronine; T_3). This corresponded to a similar increase in Ca^{2+} uptake activity. Only half of the myoblasts fused under these conditions. Fusion was not increased in the presence of T_3 (5 nM), but Ca^{2+} -ATPase levels increased 4-fold and the Ca^{2+} uptake activity doubled compared with controls. In contrast, insulin-like growth factor-I (IGF-I) induced almost complete myotube formation (> 90% fusion), but only slightly stimulated (50%) net Ca^{2+} -ATPase synthesis above control levels. However, the doubling of the Ca^{2+} uptake stimulation by IGF-I was comparable with that caused by T_3 . The effects of T_3 plus IGF-I on Ca^{2+} -ATPase levels and Ca^{2+} uptake activity were more than additive. Furthermore, the temporal relationship between the induction of Ca^{2+} -ATPase net synthesis and Ca^{2+} uptake activity was identical with the two hormones. Qualitatively similar results were obtained when T_3 and IGF-I were added to maximally fused cell cultures. The enhanced effect of T_3 on Ca^{2+} -ATPase net synthesis and Ca^{2+} uptake activity in the presence of IGF-I cannot therefore be explained by an increased myotube formation stimulated by the latter. In both differentiating myoblasts and myotubes the effect of T_3 was more prominent on Ca^{2+} -ATPase net synthesis than on Ca^{2+} uptake activity, whereas in myotubes the opposite was observed for IGF-I. This could imply complementary actions of the two agents in the development of a functional SR.

INTRODUCTION

Thyroid hormone (L-tri-iodothyronine; T_3) has major effects on Ca^{2+} homeostasis in heart and skeletal muscle [1,2]. One of the most striking effects is the increased speed of muscle relaxation in hyperthyroidism and the decreased relaxation rate in hypothyroidism [3–6]. In the last decade it has become clear through work by ourselves and others that T_3 -dependent changes in the sarcoplasmic reticulum (SR) underlie these phenomena. T_3 administration *in vivo* causes an increase of the amounts of Ca^{2+} pumps (Ca^{2+} -ATPase) and SR, thereby increasing the rate of Ca^{2+} removal from the sarcoplasm as well as the capacity for Ca^{2+} storage [7–11]. As a consequence of the increased Ca^{2+} release and re-uptake (Ca^{2+} cycling) the metabolic rate is stimulated in skeletal muscle by consuming more ATP, which contributes to the well known thermogenic effect of thyroid hormone [12]. We recently became interested in the mechanism by which T_3 regulates SR synthesis, in particular its major constituent the Ca^{2+} pump, since it was shown by us that the postnatal development of Ca^{2+} uptake activity in rat skeletal muscle is virtually completely dependent on T_3 [13]. The results of that study gave rise to the question of how the synthesis of SR Ca^{2+} -ATPase is coupled to its function, i.e. Ca^{2+} uptake by the SR. To exert this function the SR membrane system has to develop properly. This brings us to the more specific questions of whether Ca^{2+} -ATPase synthesis and Ca^{2+} uptake activity, i.e. functional SR membrane, are equally well stimulated by T_3 , and

whether there are more factors involved that are possibly under T_3 control. A possible candidate is insulin-like growth factor-I (IGF-I), which promotes growth and differentiation in skeletal muscle cells [14]. IGF-I production is controlled by growth hormone (GH), which in turn is dependent on T_3 for its production [15]. Thus part of the influence of the thyroid status on the SR might be derived from IGF-I action. To examine the possible inter-relationship between the effects of T_3 and IGF-I on SR, we chose an *in vitro* cell culture model. The rat myogenic L6 cell line [16], which is not contaminated with other cell types and has been shown to possess T_3 nuclear receptors [17], was considered to be suitable.

The results of this study show that, in L6_{AM} cells (subcloned from the L6 myogenic cell line) IGF-I is required for maximal stimulation of Ca^{2+} -ATPase net synthesis and Ca^{2+} uptake activity by T_3 .

MATERIALS AND METHODS

Materials

Fetal calf serum and Dulbecco's modified Eagle's medium (DMEM) were purchased from Flow Laboratories (Irvine, Scotland, U.K.). IGF-I (human recombinant) was obtained from Boehringer (Mannheim, Germany). 1-(β -D-Arabinofuranosyl)-cytosine (AraC) and T_3 were from Sigma (St. Louis, MO, U.S.A.). 3,5',3'-Tri-iodothyronine ('reverse T_3 ') was a gift

Abbreviations used: ANOVA, analysis of variance; AraC, 1-(β -D-arabinofuranosyl)cytosine; Ca^{2+} -ATPase, Ca^{2+} -dependent Mg^{2+} -ATPase from sarcoplasmic reticulum (EC 3.6.1.38); DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; GH, growth hormone; IGF-I, insulin-like growth factor-I; MAb, monoclonal antibody; PBS, phosphate-buffered saline (2.68 mM-KCl, 1.47 mM- KH_2PO_4 , 1.37 mM-NaCl and 8.06 mM- Na_2HPO_4); SR, sarcoplasmic reticulum; T_3 , L-tri-iodothyronine (thyroid hormone); T_3S , thyroid-hormone-depleted fetal calf serum.

† To whom correspondence should be addressed.

Fig. 1. Immunoblot analysis of MAb A52 against fast-type Ca^{2+} -ATPase

Portions of purified SR from rat gastrocnemius muscle (4.25 nmol/mg of SR protein) and L6_{AM} myotube homogenate were electrophoresed in a 6% polyacrylamide gel and then transferred to nitrocellulose sheets. One half of the blot was immunostained with MAb A52, as described in the Materials and methods section. The other half of the blot was stained for protein with Indian Ink. L6_{AM} myotube samples contained 15 μg of protein; SR samples contained 50 ng of protein (immunostain) or 1 μg of protein (protein stain).

Fig. 2. Ca^{2+} uptake activity in mixtures of myoblast and myotube homogenates

Homogenates from myoblasts, containing low Ca^{2+} uptake activity, were mixed with homogenates of myotubes, containing high Ca^{2+} uptake activity, in different ratios. The total amount of protein in the assay was kept constant at 300 μg in all measurements. On the abscissa, the percentage of myotube protein in the mixture is plotted. For further experimental details, see the Materials and methods section. Values represent means of duplicate experiments.

from Dr. E. Krenning (Erasmus University, Rotterdam, The Netherlands). AG-1-X8 anion-exchange resin was obtained from Bio-Rad (Utrecht, The Netherlands).

Cell culture and sample preparation

A subclone, L6_{AM} , of the rat myogenic cell line L6 [16] was used in this study. Cells were plated and grown in DMEM containing 10% (v/v) fetal calf serum depleted of thyroid hormones (T_3S). After 4 days (day 0) the medium was replaced by 2.5% (v/v) T_3S in DMEM either with or without added T_3 or IGF-I, as indicated in the legends to the Figures. From day 2,

AraC (4 $\mu\text{g}/\text{ml}$) was present in the medium. The % fusion (% nuclei in myotubes) was determined by counting at least 1000 nuclei per flask in 15–30 different fields at random. Thyroid hormones were removed from serum by AG-1-X8 resin treatment [18].

For quantification of Ca^{2+} -ATPase by e.l.i.s.a., cell cultures were washed twice with phosphate-buffered saline (PBS), and then covered with 300 μl of PBS and frozen at -20°C . After thawing, the cells were harvested and stored at -20°C until analysis. For measuring the Ca^{2+} uptake activity, the washed cell material was centrifuged at 1000 g at 4°C for 10 min. The supernatant was aspirated and the pellet was homogenized in 150 μl of buffer containing 40 mM-histidine and 120 mM-KCl (pH 7.0) by repeatedly pipetting with a syringe equipped with a 19 gauge needle. The samples were frozen in liquid N_2 and were stored at -80°C until analysis.

The protein content was determined by the method of Lowry *et al.* [19], using BSA as a standard.

E.l.i.s.a.

A specific monoclonal antibody (MAb), A52, raised against the fast-type SR Ca^{2+} -ATPase from rabbit [20], was obtained from Dr. D. H. MacLennan (Toronto, Canada) for use in an e.l.i.s.a. The specificity of the antibody for fast-type rat Ca^{2+} -ATPase was further examined by immunoblot analysis. For immunoblotting, protein samples from rat skeletal muscle SR and L6_{AM} homogenates were separated on 7% separating gels [21] and electrophoretically transferred to nitrocellulose paper (0.45 μm pore size), as described in [22]. Immunoblotting and colour development of the blots were performed as described in [22]. Primary incubation of the blots was carried out for 1 h at room temperature with MAb A52 and secondary incubation was performed with alkaline phosphatase-conjugated anti-mouse IgG for 1 h at room temperature. The result is shown in Fig. 1.

Ca^{2+} -ATPase was determined in muscle cell homogenates by competitive e.l.i.s.a., essentially as described in [23,24]. Quantification of Ca^{2+} -ATPase occurred by constructing a standard curve in each assay from samples with known amounts of purified rat skeletal muscle SR (0.04–3.00 μg of protein/ml). This purified SR contained 4.25 nmol of Ca^{2+} -ATPase/mg of protein [7].

Ca^{2+} uptake measurements

The Ca^{2+} uptake activity of muscle cell homogenates was determined by a Ca^{2+} uptake assay derived from the earlier-employed Ca^{2+} -stat method [3]. The method is based on the idea that there is a simple relationship between the time required to take up a fixed small amount of Ca^{2+} from the medium and the SR Ca^{2+} -ATPase activity of a muscle cell homogenate. The assay was performed at 25°C in 2 ml of buffer containing 40 mM-histidine, 120 mM-KCl, 3 mM- MgCl_2 , 5 mM- NaN_3 , 5 mM-sodium oxalate and 0.5 μM -fura-2 to monitor the changes in free Ca^{2+} concentration ($[\text{Ca}^{2+}]$). Fluorescence was measured in an Aminco Bowman spectrofluorimeter equipped with a thermostatted cuvette holder. The excitation and emission wavelength settings were 336 and 492 nm respectively. A 300 μg sample of cell homogenate protein was added to the reaction medium, which resulted in a $[\text{Ca}^{2+}]$ of $4.7 \pm 0.1 \mu\text{M}$ (mean $\pm$ S.E.M., $n = 39$). The assay was started by the addition of 1 mM-ATP. Ca^{2+} uptake was recorded by monitoring the decrease in the fura-2 fluorescence signal in the incubation mixture to at least 60% of the maximal fluorescence (F_{max}). Ca^{2+} concentrations were calculated according to [3]. The time required to return Ca^{2+} levels in the cuvette from 3.15 μM to 0.81 μM was taken as a measure of Ca^{2+} uptake activity of muscle cell homogenates. A Ca^{2+} uptake activity of 1.0 indicates that 4.66 nmol of Ca^{2+} is taken up by

1 mg of cell homogenate protein in 1 min. It was demonstrated that, at fixed protein concentrations (routinely 150 $\mu\text{g}/\text{ml}$), there was a linear relationship between the Ca^{2+} uptake activities in muscle cell homogenates and the reciprocal value of the Ca^{2+} uptake time. This was shown by mixing homogenates from myoblasts containing low Ca^{2+} uptake activity with those from myotubes exhibiting high Ca^{2+} uptake activity in different proportions (see Fig. 2). The observed linear relationship between Ca^{2+} uptake activity and different proportions of myoblasts and myotubes in the homogenate was taken as evidence that there were no specific factors in either myoblast or myotube material influencing Ca^{2+} uptake under the conditions of the assay.

Statistics

All data were evaluated by analysis of variance (ANOVA) for repeated experiments [25], and when stated in the text by Student's paired t test. Differences were considered significant at $P < 0.05$.

RESULTS

Differentiating myoblasts

Fig. 3 shows that Ca^{2+} -ATPase levels were doubled in controls (no addition) after 8 days of culture in 2.5% (v/v) T_3 S. The addition of T_3 , however, elevated the Ca^{2+} -ATPase level 4-fold above the control level. Under these circumstances myotube formation, as indicated by the percentage fusion, was $54.9 \pm 1.3\%$ in controls and $56.2 \pm 1.6\%$ in T_3 treated cultures (values represent means $\pm$ S.E.M., $n = 4$). No effect was observed with reverse- T_3 (5–50 nM) (results not shown). Although IGF-I (3.3 nM) increased Ca^{2+} -ATPase levels above control levels, this was significantly less than that found in presence of T_3 . IGF-I, however, increased the percentage fusion to $91.0 \pm 1.8\%$ ($n = 4$). The combined effects of IGF-I and T_3 on Ca^{2+} -ATPase levels were more than additive.

In order to examine how the observed induction of Ca^{2+} -ATPase protein levels by T_3 and IGF-I was expressed by its function, i.e. Ca^{2+} sequestration into the SR, the Ca^{2+} uptake activity of the SR was measured. Fig. 4 shows representative traces of the Ca^{2+} uptake activity under various conditions. Myoblast homogenates hardly showed any Ca^{2+} uptake activity. The Ca^{2+} uptake activity of the myotube homogenate was much greater, implying that SR had developed in the myotubes. Myotubes grown in the presence of T_3 showed a still higher Ca^{2+} uptake activity of the SR. T_3 and IGF-I stimulated Ca^{2+} uptake activity significantly (Fig. 5). Once more the combined effects of T_3 and IGF-I relative to the controls were more than additive.

Fig. 6 shows the time course of Ca^{2+} -ATPase levels and Ca^{2+} uptake activity in cultures grown in the presence of T_3 and IGF-I. No significant difference was observed in the time course of the relative rates of increase of these two parameters.

Myotubes

The separate and combined effects of T_3 and IGF-I were studied under conditions where myotube formation was virtually complete (from day 5). These experiments were undertaken to eliminate the variable of percentage fusion, which differed between cultures grown in the presence or absence of IGF-I. Fig. 7 shows that in the absence of IGF-I the Ca^{2+} -ATPase content gradually decreased, by approx. 22% from day 5 to day 8. T_3 and IGF-I, when added separately, increased the Ca^{2+} -ATPase content significantly compared with control levels. The effect of T_3 was significantly larger than that of IGF-I at days 7 and 8 (Student's paired t test). The simultaneous addition of T_3 and IGF-I showed a more than additive increase in net synthesis of Ca^{2+} -ATPase compared with the effects of T_3 or IGF-I alone.

Fig. 3. Effects of T_3 and IGF-I on Ca^{2+} -ATPase accumulation in differentiating myoblasts

Cells were grown to confluency on DMEM containing 10% (v/v) T_3 S. Medium was then replaced by 2.5% (v/v) T_3 S (day 0) and additions were made as indicated in the Figure. ○, Without additions; ●, 5 nM- T_3 ; △, 3.3 nM-IGF-I; ▲, T_3 +IGF-I. Ca^{2+} -ATPase was determined in homogenates by competitive e.l.i.s.a. Data represent the means of four experiments $\pm$ S.E.M. Individual values within each experiment were determined in duplicate. Data were analysed by ANOVA for repeated experiments. IGF-I, T_3 or IGF-I+ T_3 versus control, $P < 0.02$ or less; T_3 versus IGF-I, $P < 0.001$; (IGF-I+ T_3) minus IGF-I versus T_3 minus control, $P < 0.003$.

Fig. 4. Fluorescence traces of the Ca^{2+} uptake activity assay

The fluorescence values (in arbitrary units) are given on the left axis. The corresponding $[\text{Ca}^{2+}]$ values in the medium are given on the right axis. The assay was started by the addition of ATP to the reaction medium as indicated in the Figure. The upper trace shows the fluorescence trace measured in the presence of a cell homogenate of myoblasts. The myoblasts were harvested 4 days after seeding the cells at a density of 10^5 cells/ 25 cm^2 in DMEM+10% (v/v) T_3 S. The middle trace is a fluorescence trace measured in the presence of a cell homogenate of myotubes, grown without T_3 . The myotubes were harvested 8 days after switching confluent cultures of myoblasts to differentiation medium containing 2.5% T_3 S and 3.3 nM-IGF-I. The lower trace shows the fluorescence measured in presence of a cell homogenate of myotubes, grown in the presence of T_3 . The myotubes were harvested 8 days after switching confluent cultures of myoblasts to differentiation medium containing 2.5% T_3 S, 3.3 nM-IGF-I and 5 nM- T_3 . The protein concentration in the cuvette was 150 $\mu\text{g}/\text{ml}$ under all conditions.

Fig. 5. Effects of T_3 and IGF-I on Ca^{2+} uptake activity in differentiating myoblasts

Cell cultures were grown as indicated in the legend to Fig. 2. Ca^{2+} uptake activity was measured as described in the legend to Fig. 4. $\circ$, Control; $\bullet$, T_3 ; $\triangle$, IGF-I; $\blacktriangle$, T_3 + IGF-I. Data represent the means of four experiments $\pm$ S.E.M. Data were analysed by ANOVA for repeated experiments. T_3 , IGF-I or IGF-I + T_3 versus control, $P < 0.001$; (IGF-I + T_3) minus IGF-I versus T_3 minus control, $P < 0.03$.

Fig. 6. Time course of the induction of Ca^{2+} -ATPase net synthesis ($\circ$) and Ca^{2+} uptake activity ($\bullet$)

Cells were grown under conditions as described in the legend to Fig. 3. After the cells had grown to confluency, the medium was replaced by 2.5% (v/v) T_x S (day 0) supplemented with IGF-I (3.3 nM) and T_3 (5 nM). The increases in Ca^{2+} -ATPase net synthesis and Ca^{2+} -uptake activity are expressed as percentages of the maximal values reached in the time period of the experiment. Data represent means $\pm$ S.E.M. ($n = 4$).

Fig. 8 shows that in the absence of IGF-I a decrease in the Ca^{2+} uptake activity of approx. 27% from day 5 to day 8 was observed. It was striking to observe that Ca^{2+} uptake activity was stimulated significantly more by IGF-I than by T_3 , which was

Fig. 7. Effects of T_3 and IGF-I on Ca^{2+} -ATPase accumulation in myotubes

Cells were grown to confluency on DMEM containing 10% (v/v) T_x S. Medium was then replaced by 2.5% (v/v) T_x S containing 3.3 nM-IGF-I (day 0). At day 4, medium was changed to 2.5% (v/v) T_x S from which IGF-I was omitted. At day 5 additions were made: $\circ$, none (control); $\bullet$, 5 nM- T_3 ; $\triangle$, 3.3 nM-IGF-I; $\blacktriangle$, T_3 + IGF-I. Ca^{2+} -ATPase was determined by competitive e.l.i.s.a. Data represent the means of nine experiments $\pm$ S.E.M. Data were analysed by ANOVA for repeated experiments. IGF-I, T_3 or IGF-I + T_3 versus control, $P < 0.003$; day 7 and 8, T_3 versus IGF-I, $P < 0.05$ (Student's paired t test); (IGF-I + T_3) minus IGF-I versus T_3 minus control, $P < 0.006$.

Fig. 8. Effects of T_3 and IGF-I on Ca^{2+} uptake activity in myotubes

Cell cultures were grown as indicated in the legend to Fig. 7. Ca^{2+} uptake activity was measured as described in the legend to Fig. 4. $\circ$, Control; $\bullet$, 5 nM- T_3 ; $\triangle$, 3.3 nM-IGF-I; $\blacktriangle$, T_3 + IGF-I. Data represent the means of six experiments $\pm$ S.E.M. Data were analysed by ANOVA for repeated experiments. IGF-I or IGF-I + T_3 versus control, $P < 0.001$; T_3 versus control, $P < 0.10$; (IGF-I + T_3) minus IGF-I versus T_3 minus control, $P < 0.001$.

Table 1. Relative stimulation of SR Ca²⁺-ATPase synthesis and Ca²⁺ uptake activity

The data represent the relative stimulation of Ca²⁺-ATPase net synthesis and Ca²⁺ uptake activity, measured in the same batch of cells, induced by the indicated additions (T₃, 5 nM; IGF-I, 3.3 nM) relative to the controls (= 1, no additions). The values were calculated from individual results of the experiments presented in Figs. 3 and 5 (exposure time 5 days) and Figs. 7 and 8 (exposure time 3 days). For further experimental details, see the legends of the cited Figures. Values represent means ± S.E.M. (n = 4). *P < 0.05 versus IGF-I; †P < 0.05 versus T₃ (Student's paired *t* test).

Condition	Additions	Exposure time (days)	Ca ²⁺ -ATPase (a)	Ca ²⁺ uptake (b)	Ratio (a/b)
Differentiating myoblasts (4)	IGF-I	5	2.1 ± 0.4	2.0 ± 0.3	1.0 ± 0.1
	T ₃	5	4.6 ± 0.6*	1.9 ± 0.1	2.4 ± 0.3*
	T ₃ + IGF-I	5	8.5 ± 1.6*†	3.3 ± 0.4*†	2.5 ± 0.5*
Myotubes (4)	IGF-I	3	1.8 ± 0.1	1.4 ± 0.1	1.2 ± 0.1
	T ₃	3	2.7 ± 0.2*	1.2 ± 0.1*	2.2 ± 0.2*
	T ₃ + IGF-I	3	5.3 ± 0.2*†	1.9 ± 0.1*†	3.2 ± 0.3*†

contrary to the effects of these compounds on the Ca²⁺-ATPase levels (Fig. 7). Stimulation of Ca²⁺ uptake activity in cultures exposed to both T₃ and IGF-I was significantly greater than the added effects of T₃ or IGF-I alone.

We examined whether Ca²⁺-ATPase levels and Ca²⁺ uptake activities in differentiating myoblasts and myotubes were increased proportionally by addition of T₃, IGF-I or IGF-I plus T₃. Table 1 shows that the relative stimulations of net Ca²⁺-ATPase synthesis and Ca²⁺ uptake activity by IGF-I were similar. In contrast, the relative stimulation of Ca²⁺-ATPase net synthesis by T₃ was more than twice as high as the stimulation of the Ca²⁺ uptake activity. The latter also holds for the effects of the combined addition of T₃ and IGF-I.

DISCUSSION

Factors regulating Ca²⁺-ATPase synthesis *in vitro*

There are still many gaps in the knowledge of the regulation of Ca²⁺-ATPase synthesis in cultured skeletal muscle cells. It is, however, well established that the rate of Ca²⁺-ATPase synthesis sharply increases after fusion of myoblasts into multinucleated myotubes [26–28]. Innervation, which determines the activity pattern of muscle, and T₃ are major determinants of Ca²⁺-ATPase activity *in vivo* [1,29]. It was shown earlier that Ca²⁺-ATPase synthesis *in vitro* is stimulated after spontaneous contractions in myotubes [30]. The present study shows that T₃ is also able to stimulate Ca²⁺-ATPase synthesis *in vitro*. Thus the effects of T₃ and muscle contraction on Ca²⁺-ATPase synthesis *in vivo* can be reproduced *in vitro*, at least qualitatively. How these actions are related to the genomic expression of Ca²⁺-ATPase is still an open question. Martonosi and co-workers found evidence that changes in intracellular free Ca²⁺ could play a pivotal role in the regulation of Ca²⁺-ATPase synthesis [31,32]. Thyroid status and muscle contraction both strongly influence Ca²⁺ homeostasis. Thus it cannot be excluded that the effects of T₃ and muscle contraction are at least partially mediated by changes in intracellular free Ca²⁺.

In recent years it has become clear that growth factors play an essential role in muscle differentiation, and the significance of insulin-like growth factors among others for differentiation in the L6 muscle cell line was convincingly demonstrated [14]. The stimulation of Ca²⁺-ATPase by IGF-I and its potentiating effect on T₃-mediated stimulation as described here is a novel aspect of the regulation of Ca²⁺-ATPase synthesis, at least in the L6 muscle cell line.

It should be noted that T₃ and IGF-I, when both present, stimulate Ca²⁺-ATPase synthesis to levels (approx. 15 pmol/mg

of protein) which are of the order of magnitude of the concentrations found in rabbit soleus muscle (35 pmol/mg of protein) [24] and in rat soleus muscle (40–60 pmol/mg of protein) ([33]; A. Muller, unpublished work). This is, however, still more than 10-fold lower than the Ca²⁺-ATPase concentrations found in fast extensor digitorum longus muscle of both species ([24,33]; A. Muller, unpublished work).

Effects of T₃ and IGF-I on Ca²⁺-ATPase synthesis and Ca²⁺ uptake activity

In spite of the lower degree of myotube formation in T₃-treated cultures (55 % fusion), the stimulatory effect of T₃ on net Ca²⁺-ATPase synthesis was twice as large as the effect seen in the presence of IGF-I (91 % fusion). The fusion process of myoblasts and the synthesis of muscle-specific proteins, including Ca²⁺-ATPase, coincide in many myogenic systems. According to the results of Pinset & Whalen [34], these events are coupled in L6 cells. This is claimed not to be the case for Ca²⁺-ATPase and myofibrillar proteins in primary myoblasts [35]. Although the synthesis of Ca²⁺-ATPase was greatly accelerated upon fusion of myoblasts and differentiation into myotubes, fusion was not essential. In Ca²⁺-deprived medium myoblast fusion was inhibited but Ca²⁺-ATPase synthesis, although delayed for 50 h, still took place. To date it is not known whether T₃ induces Ca²⁺-ATPase synthesis in the L6_{AM} cell line in both myoblasts and myotubes or only in myotubes. In the latter case this would mean that the T₃-stimulated Ca²⁺-ATPase accumulation is 4-fold higher than the IGF-I effect, since, as we mentioned previously, the percentage fusion was approximately doubled in IGF-I treated cultures compared with T₃-treated ones. On the other hand, it would also mean that the potentiating effect of IGF-I on T₃ stimulation of Ca²⁺-ATPase synthesis and Ca²⁺ uptake activity could be largely ascribed to the IGF-induced increase in fusion (from 55 % to 91 %). However, the experiments with cultures consisting of more than 90 % myotubes (Figs. 7 and 8) show once more that the combined effects of IGF-I and T₃ on Ca²⁺-ATPase synthesis and Ca²⁺ uptake activity are greater when they are added separately. From this it can be concluded that increased fusion cannot be the only mechanism by which IGF-I increases the effects of T₃.

Time course of Ca²⁺-ATPase synthesis and Ca²⁺ uptake activity

We showed that the induction of Ca²⁺-ATPase synthesis and Ca²⁺ uptake activity by T₃ proceeded synchronously. Everts [36] found that the Ca²⁺-ATPase content in soleus muscle from developing rats increased significantly after 1 day of T₃ treatment and Ca²⁺ uptake activity increased only after 2 days. It was, however, noted that this could be caused by differences in the

sensitivity of the methods used. Two models of SR biogenesis have been proposed: (1) the random incorporation model [27] and (2) the growing point model [28]. The first model assumes Ca^{2+} -ATPase incorporation into multifunctional endoplasmic reticulum (ER), which would gradually be transformed into a monofunctional SR. The growing-point model assumes lateral displacement of Ca^{2+} -ATPase from the rough ER to fully differentiated SR, with a growing point of SR in between the ER and SR. In both models the possibility of a lag time between the new synthesis of Ca^{2+} -ATPase and its operation in SR, i.e. Ca^{2+} uptake, is not excluded. Our results do not support an appreciable difference (at least 1 day) in the time sequence of Ca^{2+} -ATPase accumulation and Ca^{2+} uptake activity in L6_{AM} cells.

Concluding remarks

For the present, the inter-relationship of the actions of T_3 and IGF-I in the L6_{AM} muscle cell line can only be a matter of speculation. Very recently we have found that fast Ca^{2+} -ATPase mRNA levels were elevated 8-fold by T_3 , relative to controls, in differentiating myoblasts (A. Muller, unpublished work), which is in agreement with a similar study in heart, where T_3 increased mRNA levels for slow Ca^{2+} -ATPase [37]. These results suggest a role for T_3 at a level higher than protein turnover. This is also possible for IGF-I, since it has been reported that insulin, acting as an IGF-I analogue, enhanced stabilization of mRNA for creatine kinase in the L6A_1 muscle cell line [38]. On the other hand, the relatively more prominent effect of IGF-I on Ca^{2+} uptake activity could point to an essential role for IGF-I in the maturation of a functional SR membrane. Finally, since IGF-I production is indirectly controlled by T_3 (see the Introduction section), the results of this study could be relevant for the thyroid hormone control of SR function *in vivo*. Additional evidence for this will, however, have to be provided from studies in primary muscle cell cultures of neonatal rats.

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