Effects of Thyroid Hormone on Growth and Differentiation of L6 Muscle Cells

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Abstract

The effects of thyroid hormone (3,3',5-triiodothyronine, T_3) on growth and differentiation of L6 cells were investigated. Accordingly, thyroid hormone-depleted culture conditions were required, which were achieved by AG-1X-8 resin treatment of the serum. Growth-promoting properties of the serum either with or without addition of insulin, were not affected by resin treatment, but differentiation potency was enhanced as demonstrated by increased fusion of myoblasts to myotubes and increased creatine phosphokinase (CPK) activity. It was shown that addition of endogenous amounts of T_3 (0.04 nM) and 3,3'5,5'-tetraiodothyronine (T_4; 5 nM) to medium containing 2.5% resin-treated serum significantly inhibited the CPK activity stimulation (15%). This implies that part of the stimulation of CPK activity by resin treatment can be accounted for by depletion of the endogenous thyroid hormone content from serum. No effect of resin treatment was observed on the activities of lactate dehydrogenase (LDH), succinate dehydrogenase (SDH), or on the levels of Myosin Heavy Chain (MHC).

The effects of exogenous T_3 (5 nM) on growth and differentiation of L6 cells in the presence or absence of insulin were examined. T_3 did not affect the growth rate of these cells. The effect of T_3 on differentiation was evaluated using CPK activity and the fusion index as parameters. T_3 was shown to reduce CPK activity significantly by approximately 30%, which is in support of the results obtained with endogenous amounts of T_3 and T_4. No effect of T_3 was observed on fusion; the reduction of CPK activity by T_3 can therefore not be explained by inhibition of fusion. Since CPK activity was also inhibited by T_3 in maximally fused cultures it can be inferred that CPK expression in myotubes is controlled by T_3 independently of the fusion process. LDH and SDH activity were not influenced by T_3, nor did T_3 affect the level or isotype of MHC. Sarcoplasmic reticulum (SR) Ca^{2+}-ATPase levels increased by 100% in the presence of endogenous amounts of T_3 and T_4 and were maximally stimulated (300%) by 5 nM T_3. In conclusion: 1. T_3 does not influence myoblast growth and the fusion of myoblasts to myotubes in the presence or absence of insulin 2. CPK activity is an unsuitable biochemical marker for establishing the role of T_3 on myogenic differentiation 3. SR Ca^{2+}-ATPase content is already increased by addition of serum to the medium to a final concentration of 2.5%, due to the presence of endogenous thyroid hormones.

Key words: thyroid hormone; L6 muscle cell line; CPK activity; differentiation conditions.

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Thyroid hormone is a major regulator of growth and maturation of many tissues *in vivo* [23, 30]. Also *in vitro*, it has been shown that proliferation of several cell types is dependent on thyroid hormone, including pituitary tumor cells [8, 20], hepatoma cells [7] and osteoblast-like cells [11]. Its role in differentiation has been established in osteoblast-like cells [11].

Skeletal muscle growth and development *in vivo* is particularly sensitive to thyroid hormones [1, 23]. Hypothyroidism results in severe retardation of muscle growth, and in addition some specific aspects of muscle differentiation are impaired, including the development of the sarcoplasmic reticulum which is very strikingly affected [33-35]. Although *in vivo* studies have pointed out the necessity of thyroid hormone for muscle development, they are not

conclusive on the direct or indirect nature of thyroid hormone action. Unfortunately, only few data are available on the effects of thyroid hormone on growth and differentiation of muscle cells *in vitro*. To our knowledge, there is only one study that describes a stimulatory effect of thyroid hormone on muscle cell proliferation, using primary chicken myoblasts as an experimental model [21]. This study also reports a stimulation of differentiation by thyroid hormone, as inferred from an increase of the percentage fusion and induction of creatin phosphokinase (CPK) activity. The latter observation is in contrast with the finding of Shainberg *et al.* [32] who reported that thyroid hormone decreases CPK activity in primary cultures of rat myoblasts. This convinced us that more knowledge of the role of thyroid hormone in growth and differentiation of muscle cells *in vitro* was required, since this is essential for the interpretation of thyroid hormone actions on specific muscle proteins like SR Ca^{2+}-ATPase. Therefore, we investigated the effects of thyroid hormone on skeletal muscle cell growth and differentiation, employing the L6 muscle cell line which is being widely used in myogenesis studies. Differentiation is here defined as the process of fusion of mononucleated myoblasts to multinucleated myotubes followed by the expression of muscle specific proteins. To establish the role of thyroid hormone in the growth and differentiation process, serum was treated with AG-1X-8 anion resin, which has been successfully used to remove thyroid hormones from serum [28]. We took into account that his procedure may remove other essential factors in addition to thyroid hormones, possibly leading to altered growth- and differentiation -promoting characteristics of the serum. We employed the most commonly used indicators of myogenic differentiation i.e. fusion index (% nuclei in myotubes) and induction of CPK activity, complemented by estimations of the degree of transition of the embryonal BB- to the adult MM-isoform of CPK.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM) and fetal calf serum (FCS) were obtained from Flow Laboratories (Irvine, UK). Penicillin, streptomycin and trypsin were purchased from Gibco (Paisley, UK). Tissue culture flasks were from Nunc (Kalstrup, Denmark). T_3, T_4, 1-ß-D-arabinofuranosylcytosine (AraC), Bis-Tris electrophoresis buffer, CPK equilibration reagent, CPK Agaro-Stain I and II, myosin standard and calf thymus DNA type II were obtained from Sigma (St. Louis, MD). 3,5',3'-Triiodothyronine (rT_3) was a generous gift from Dr. Krenning (Erasmus University, Rotterdam, the Netherlands). Insulin (bovine; Zn-complex) was obtained from Serva (Heidelberg, FRG). Insulin-like growth factor-I (IGF-I; human recombinant) was obtained from Boehringer (Mannheim, FRG). AG-1X-8 (chloride form, 200-400 mesh) was purchased from BioRad (Richmond, CA). All other chemicals were of the highest reagent grade available.

Preparation of thyroid hormone-depleted serum (T_xS)

FCS was treated with AG-1X-8 resin essentially as described by Samuels *et al.* [28]. The resin was first washed three times in distilled water, and was then autoclaved twice for 15 min at 1 bar. Next, the serum was incubated with 50 mg resin/ml (dry weight) for 5 h at room temperature. The mixture was stirred to keep the resin in suspension. Next, the resin was removed by centrifugation at 1000x*g* for 10 min. Fresh resin (50 mg/ml serum) was added and the serum was incubated for an additional 19 h at room temperature. Finally, the resin was removed by centrifugation at 1000x*g* for 10 min and fine resin components were removed by subsequent centrifugation at 30,000x*g* for 20 min. Thyroid hormone-depleted serum (T_xS) was sterilized by filtration through a 0.22 µm filter.

Cell culture

The $L6_{AM}$ subclone of the L6 rat myogenic cell line, originally isolated by Yaffe [42] was used in this study. Cells were seeded at a density of 10^5 cells per 25 cm^2 tissue culture area in DMEM + 10% FCS or T_xS. After four days (day 0), when the cells had reached confluence, medium was replaced by DMEM + 2.5% FCS or T_xS. In most experiments, insulin (1 µg/ml) or IGF-I (25 or 100 ng/ml) was added at day 0, to obtain maximal differentiation of $L6_{AM}$ cells. T_3 and rT_3 were added at day 0 at a concentration of 5 nM. At day 2, AraC (4 g/ml) was added to prevent non-fusing myoblasts from overgrowing the cultures. Medium was changed at day 2, 6 and 8.

Biochemical analysis of differentiation

The activities of CPK (EC 2.7.3.2.) , SDH (EC 1.3.9.9.) and LDH (EC 1.1.1.2.7.) were determined as described in [15], [12] and [35] respectively.

CPK isoenzymes separation was achieved by agarose gel electrophoresis, using 1 M Bis-Tris-acetate buffer, pH 6.9. 1 µl samples of cell homogenate, equivalent to 0.3-0.5 mU CPK activity, were applied to the gel. Prior to application of the samples, the gel was incubated 1.5 h in 0.5 U/ml hexokinase and 0.4 U/ml glucose-6-phosphate dehydrogenase in Bis-Tris electrophoresis buffer (CPK equilibration reagent, Sigma). Electrophoresis was performed for 90 min at 300V, 40 mA in a LKB electrophoresis chamber. The gel was stained for CPK activity by incubation with Bis-Tris buffer containing 30 mM phosphocreatine, 20 mM glucose, 2 mM ADP, 10 mM Mg^{2+}, 5 mM AMP, 21.8 mM phenazine methosulfate, 2 U/ml hexokinase, 1.6 U/ml glucose-6-phosphate dehydrogenase, 2 mM NADP, 0.36 mM tetranitroblue tetrazolium, protected from light. The gels were scanned using a LKB 2202 Ultrascan, equipped with a laser densitometer.

SR Ca^{2+}-ATPase was quantified in muscle cell homogenates by competitive e.l.i.s.a., essentially as described in [10]. A specific monoclonal antibody, A52, against the fast type SR Ca^{2+}-ATPase from rabbit [44], was used in this

assay. Purified rat skeletal muscle SR that contained 4.25 nmol of Ca^{2+}-ATPase/mg of protein [33] was used for constructing a calibration curve.

MHC analysis proceeded as follows: MHC was extracted according to the procedure of Giulian *et al.* [16]. Extracted proteins were separated on 6% separating gels [22]. Gels were stained with Coomassie Brilliant Blue R250. Absorption profiles were obtained using an LKB Ultrascan laser densitometer, in combination with the LKB software program. Myosin standards were included in each run, which yielded a straight calibration line, with a detection limit of 1 μg protein.

Morphological analysis of differentiation

Cultures to be scored for cell fusion were fixed with methanol/acetic acid (3:1) and stained with haematoxylin. The percentage of nuclei was determined by counting at least 1000 nuclei per flask in 15-30 different fields at random (magnification: 400x).

Other determinations

The T_3 and T_4 levels in FCS before and after resin treatment were determined by RIA (by courtesy of the Department of Endocrinology (T_3 measurements) and the Department of Clinical Chemistry (T_4 measurements), Free University Academic Hospital, Amsterdam, The Netherlands). DNA was extracted from cells and assayed by the method of Burton [5] with DNA from calf thymus as a standard. Protein was determined by the method of Lowry [24] using crystalline bovine serum albumin as a standard.

Statistics

Data were evaluated by Student's t-test for paired observations. Differences were considered significant at $p < 0.05$.

Results

Effects of resin treatment

The average T_3 and T_4 levels in serum before resin treatment were 1.5 nM and 200 nM, respectively. Resin treatment reduced the T_3 and T_4 levels in serum to less than 0.3 nM and 3 nM, respectively, which represent the lower limit of sensitivity of the RIA's.

Growth. Fig. 1 shows the growth curves in DMEM + 10% FCS or 10% T_xS. It demonstrates that resin treatment did not alter the growth promoting properties of the serum. Addition of 5 nM T_3 to T_xS-medium did not change the growth rate either. Population doubling time of $L6_{AM}$ cells was 16.8 hr during exponential growth in all media.

Differentiation. In FCS-medium, CPK activity remained practically unchanged during the experiment, while in T_xS-medium, CPK activity increased threefold. Supplying T_xS with endogenous amounts in T_3 and T_4 resulted in a decrease of CPK activity, but CPK activity remained higher than in FCS-medium (Fig. 2a). Similar results were obtained in the presence of insulin (Fig. 2b).

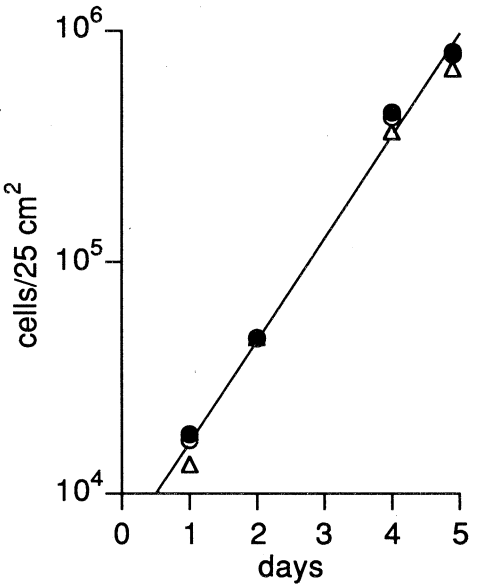

Figure 1. Effect of T_3 on growth rate. Cells were seeded at a density of 10^4 cells per 25cm^2 tissue culture area in DMEM + 10% FCS (open triangle); 10% T_xS (open circle); 10% T_xS + 5 nM T_3 (solid circle). Data represent means of triplicate values which showed less than 10% variation.

The increase in CPK activity in T_xS-medium coincided with a higher degree of fusion. In media without insulin, the percentage of nuclei found in myotubes at day 10 was 54.9 ± 2.7 (means ± S.E.M., n = 4) and 41.8 ± 2.1 (n = 3) in the presence of T_xS and FCS, respectively. The percentage of nuclei in myotubes cultured in media with insulin, at day 8, was 91.0 ± 1.9 (n = 4) and 70.3 ± 2.0 (n = 3) in T_xS and FCS-medium, respectively.

Effects of exogenous T3

DNA- and protein levels

We investigated the effect of T_3 (5 nM) on net DNA and protein synthesis which is a measure of the general condition of differentiating myoblasts. Figs. 3a and 3b show that there was no significant effect of T_3 on these parameters during differentiation, whether insulin was present in the medium or not. Protein levels increased more rapidly in the presence of insulin during the first four days on differentiation medium and then stabilized at a higher level. A transient stimulation of DNA net synthesis was observed at day two.

CPK activity

The effect of 5 nM T_3 on CPK activity in differentiating myoblasts is shown in Fig. 4a. A decrease of the CPK activity by T_3 was observed in the absence of insulin. Addition of 5 nM rT_3 had no effect on the CPK activity. Fig. 4b shows that T_3 also inhibited CPK activity (30%) in the presence of insulin. Again, no effect of rT_3 was seen.

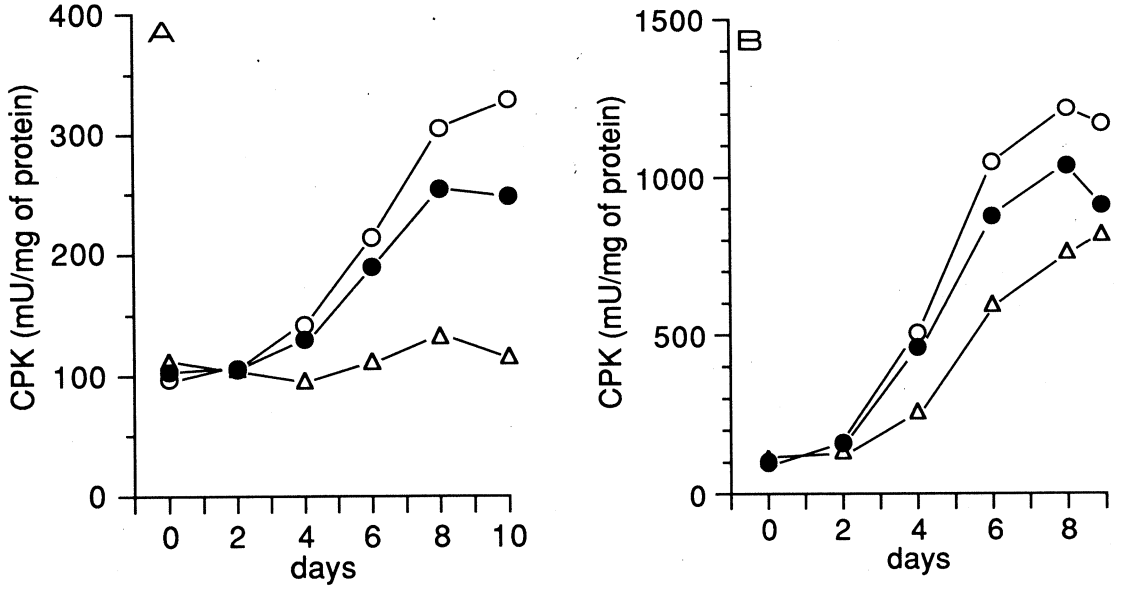

Figure 2. Effect of resin treatment on induction of CPK activity. Cells were seeded at a density of 10^5 cells per 25cm^2 tissue culture area in DMEM + 10% serum. At confluency (day 0), the medium was replaced by differentiation medium: DMEM + 2.5% serum (A) or DMEM + 2.5% serum + insulin (B). Three different sera were examined: FCS (open triangle); T_xS (open circle); T_xS supplied with endogenous amounts of T_3 and T_4 (solid circle), giving final concentrations of T_3 and T_4 of 0.15 and 19.8 nM, respectively, in DMEM + 10% serum and 0.0375 and 5 nM, respectively, in DMEM + 2.5% serum. Data represent means of duplicate values with less than 10% variation.

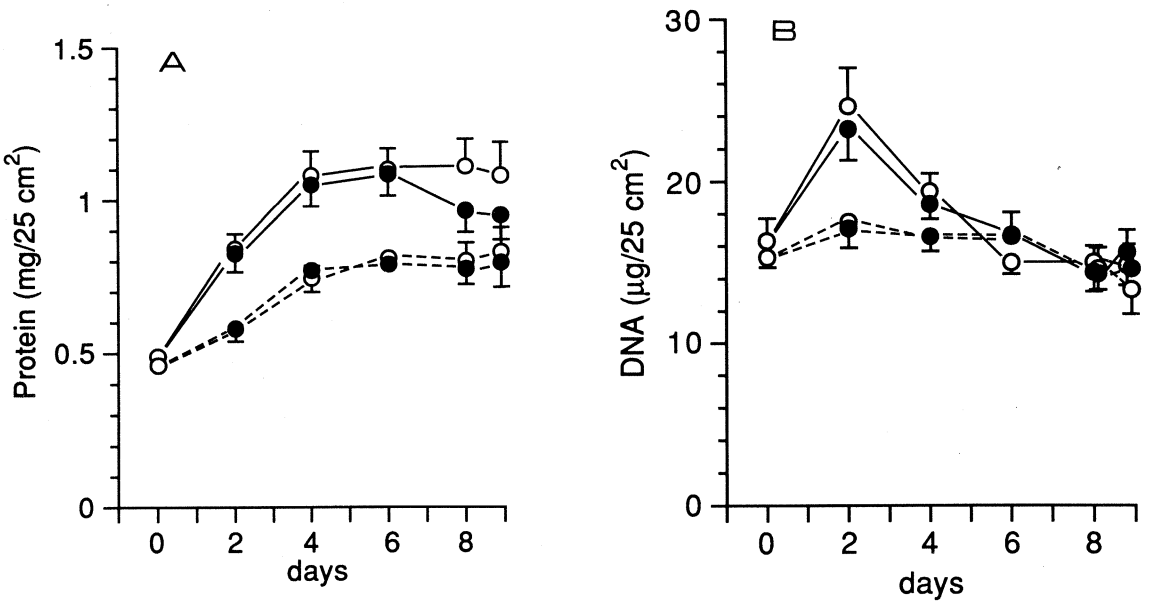

Figure 3. Effect of T_3 on the levels of protein and DNA in differentiating myoblasts. Cells were seeded at a density of 10^5 cells per 25cm^2 tissue culture area in DMEM + 10% T_xS. At confluency (day 0), the medium was replaced by or DMEM + 2.5% T_xS (discontinuous line) or DMEM + 2.5% T_xS + insulin (solid line). Control cells, no additions (open circle); 5 nM T_3 added at day 0 (solid circle). Protein levels (A) and DNA levels (B) were measured as described under Methods. Data represent means of six experiments ± S.E.M.

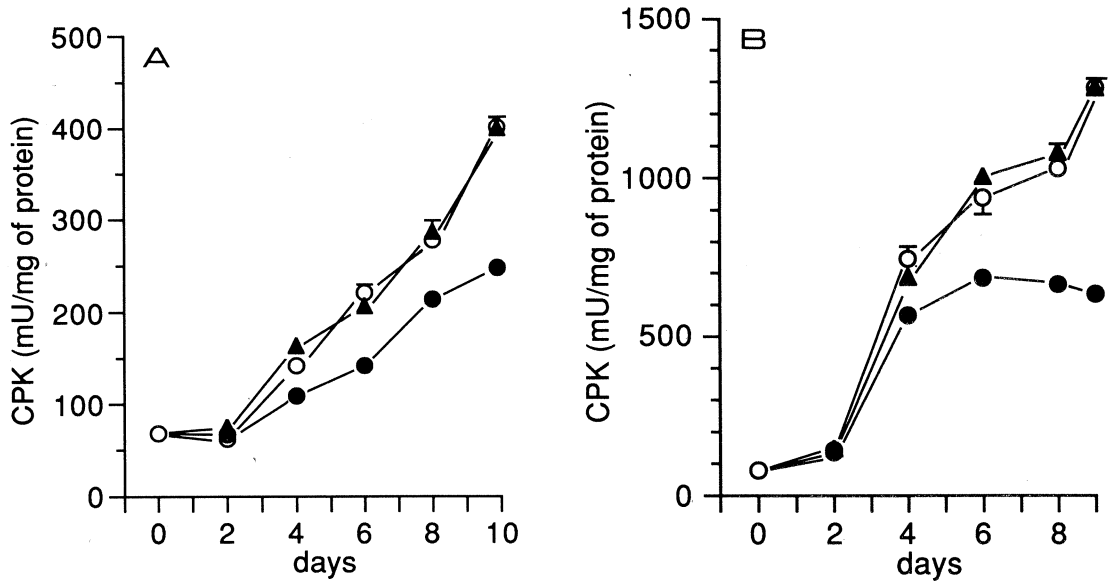

Figure 4. Effect of T_3 on CPK activity in differentiating myoblasts. Cells were seeded at a density of 10^5 cells per 25cm^2 tissue culture area in DMEM + 10% T_xS. At confluency (day 0), the medium was replaced by DMEM + 2.5% T_xS (A) or DMEM + 2.5% T_xS + insulin (B). Controls, no additions (open circle); 5 nM T_3, added at day 0 (closed circle); 5 nM rT_3, added at day 0 (solid triangle). Data represent means ± S.E.M. (n = 3).

Addition of T_3 to cultures that were already maximally fused (day 6), also resulted in a decrease of CPK activity (Fig. 5). When IGF-I (100 or 25 ng/ml) was added to the medium instead of insulin, essentially the same results were obtained: CPK activity was reduced by 20.4 ± 4.2% at day 6 and by 14.5 ± 2.3% at day 8 in the presence of T_3. [Values represent means ± S.E.M. (n = 6)].

During differentiation of $L6_{AM}$ cells, a CPK-isoenzyme transition takes place from the BB-form in undifferentiated myoblasts into the MM-form in myotubes. We studied T_3 effects on the ratio of the BB- and the MM-isoform after 8 days of culture in the presence of insulin. No effect of T_3 on the isoenzymes ratio was observed. The BB/MM ratio was 0.127 ± 0.022 (mean ± S.E.M., n = 3) in the absence of T_3, and 0.135 ± 0.035 (mean ± S.E.M., n = 3) in the presence of T_3.

Fusion

To investigate whether the lower CPK activity in the presence of T_3 resulted from a lower degree of fusion, the percentage of nuclei found in myotubes was determined in the presence and absence of 5 nM T_3. Table 1 shows that in the presence of T_3 the percentage of nuclei in myotubes remained unchanged, while at the same time the CPK activity was reduced by 35%. These results were obtained in the presence as well as in the absence of insulin.

SR Ca^{2+}-ATPase

We investigated the effect of exogenous T_3 as well as of endogenous thyroid hormones on SR Ca^{2+}-ATPase levels in differentiating myoblasts. The results, presented in Fig. 6, show that 5 nM T_3 caused a more than 4-fold

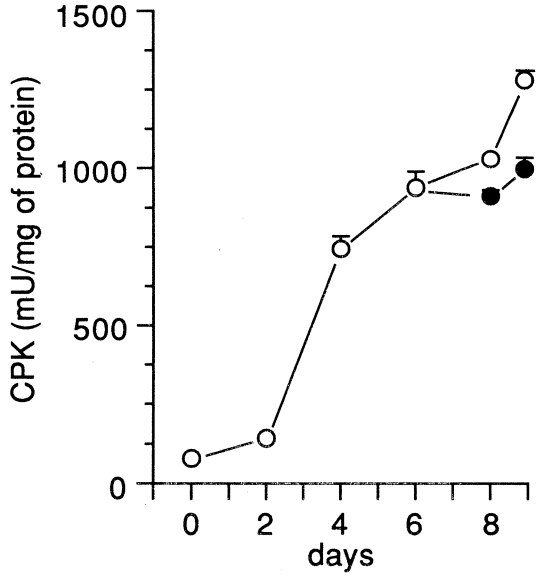

Figure 5. Effect of T_3 on CPK activity in myotubes. Cells were cultured as described in the legend to Fig. 4b. Control cells, no additions (open circle); 5 nM T_3 added at day 6 (solid circle). Data represent means ± S.E.M. (n = 3).

Table 1. Effects of T_3 on fusion and CPK activity. Cells were seeded at a density of 10^5 cells per 25cm^2 tissue culture area in DMEM + 10% T_xS. At confluency (day 0), the medium was replaced by DMEM + 2.5% T_xS, with or without insulin and T_3 (5 nM). The fusion index (% of nuclei in myotubes) and CPK activity were measured at day 8 (in presence of insulin) or day 10 (in the absence of insulin). Data represent means ± S.E.M. (n = 4).

Additions	% of nuclei in myotubes	CPK activity (mU/mg ofprotein)
insulin	91.0 ± 1.8	1013 ± 35
insulin + 5 nM T_3	90.5 ± 1.6	627 ± 77
none	54.9 ± 2.7	378 ± 29
5 nM T_3	56.2 ± 3.3	253 ± 6

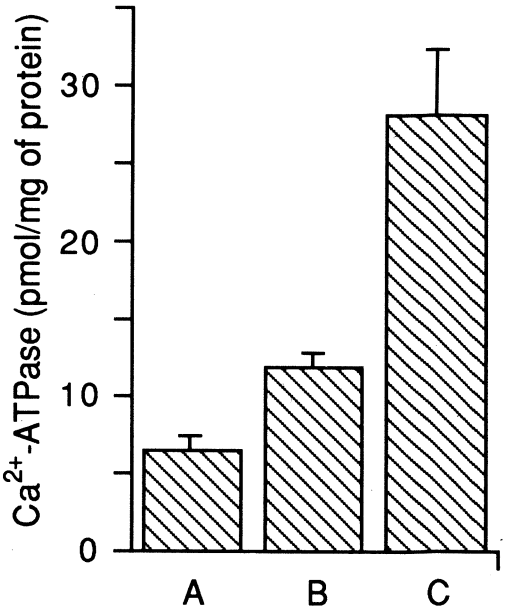

Figure 6. Effect of T_3 on SR Ca^{2+}-ATPase levels. Cells were cultured as described in the legend to Fig. 4b. At day 0 the different additions were made and the cells were harvested six days thereafter. Control cells, no additions (A); endogenous amounts of T_3 and T_4 present in T_xS, giving final concentrations of T_3 and T_4 of 0.0375 and 5 nM, respectively (B); 5 nM T_3 (C). Data represent means ± S.E.M. (n = 3).

increase of the SR Ca^{2+}-ATPase level. The addition of endogenous amounts of thyroid hormones (T_3 and T_4), present in untreated serum, induced already a nearly doubling of the SR Ca^{2+}-ATPase levels.

Other enzymes

The effects of T_3 on the activity of two other enzymes, SDH and LDH, as well as on MHC levels are shown in Fig. 7. SDH (a) and LDH (b) activity were not affected by T_3 and no change in isoform of MHC was observed either (results not shown). The levels of MHC were elevated in the presence of T_3 on day 7 and 9 (Fig. 7c), but the limited number of observations do not permit a conclusion regarding the significance of this effect. No effects of resin treatment on the LDH- and SDH activity or on the induction of MHC were observed (Figs. 7a,b,c).

Discussion

Resin treatment of serum

Serum, which is routinely added to culture medium contains quantities of endogenous thyroid hormones that may be sufficient to elicit biological effects in cultured cells. This was confirmed by our experiments since endogenous levels of T_3 and T_4, in our case 0.04 nM and 5 nM respectively, already influenced CPK and SR Ca^{2+}-ATPase levels. This necessitates the removal of thyroid hormones from serum by resin treatment, yet this procedure may remove other factors from serum that influence myogenesis. The latter possibility has also been confirmed by our experiments. Although $L6_{AM}$ cells proliferated at a normal rate in T_xS-medium, differentiation was enhanced in T_xS-medium compared to FCS-medium, as illustrated by a higher fusion-index and increased CPK activity. The fusion process is coupled to the induction of muscle specific proteins, including CPK, which is therefore often used as a biochemical marker indicating the degree of differentiation. Removal of thyroid hormones appeared to be partially responsible for the increase of CPK activity in T_xS-medium, since the supply of T_xS with endogenous amounts of T_3 and T_4 resulted in a significant decrease of CPK activity, yet not sufficient to return to the original level in FCS-medium. This was observed both in the presence and absence of insulin. Since the fusion index in T_xS-medium is 10-20% higher than in FCS-medium irrespective of the presence of insulin, the residual stimulation of CPK activity in T_xS-medium is most likely explained by the presence of relativily more myotubes. This indicates that apart from thyroid hormone, factors that inhibit the fusion process were also removed by resin treatment. Although resin treatment did not change the total protein content of serum, this does not exclude the possibility that minor serum components with a high potency of action were removed. TGF-ß might be such a factor, as it was shown to inhibit myogenic differentiation and the subsequent induction of CPK in L6 cells with very high potency [13]. It should be noted however that resin treatment did not affect LDH or SDH activity or induction of MHC during differentiation. A possible explanation is that changes in LDH and SDH activity are much less dramatic than the change in CPK activity during myogenesis. Comparisons between FCS- and T_xS-medium with respect to

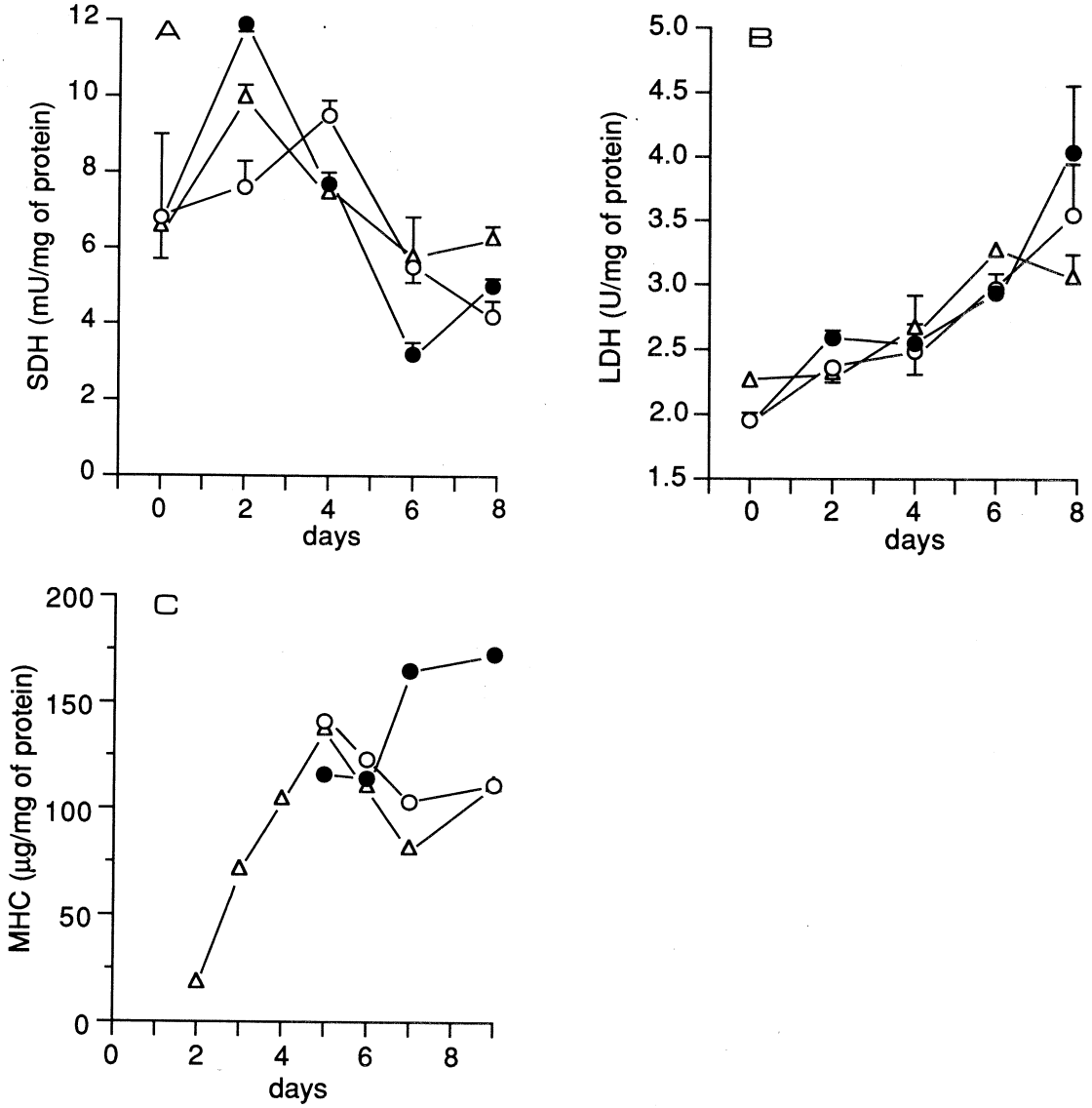

Figure 7. Effect of resin treatment and T_3 on other enzymes. Cells were seeded at a density of 10^5 cells per 25cm^2 tissue culture area in DMEM + 10% FCS (open triangle) or T_xS (open circle). At confluency (day 0), the medium was replaced by DMEM + 2.5% FCS + insulin (open triangle); DMEM + 2.5% T_xS + insulin (open circle); DMEM + 2.5% T_xS + insulin + 5 nM T_3 (solid circle). SDH (A) and LDH (B) activities and MHC (C) levels were determined as described under Methods.Data represent means ± S.E.M. of three (A) or four (B) values. Data in (C) represent means of duplicate determinations, which show 20-50% variation.

MHC were only made between day 5 and 8 after the change to differentiation medium. It can not be excluded that T_3 stimulates MHC synthesis. Assuming this, resin treatment might cause two counteracting effects on MHC synthesis, i.e., removal of T_3 and fusion stimulation.

In summary, resin treatment of serum had no effect on growth of $L6_{AM}$ cells, but differentiation, as defined by CPK activity and fusion-index, was lifted to a higher level in T_xS-medium. The finding that endogenous thyroid hormone levels in serum (2.5%) already influenced SR Ca^{2+}-ATPase levels and CPK activity in $L6_{AM}$ cells indicate that the use of thyroid hormone-depleted serum is required for studying T_3 effects in this cell line.

Effect of T_3 on growth

Proliferation has been shown to be dependent on thyroid hormone in pituitary [8, 20] and epithelial [4, 38] cell lines, as well as in cultured hepatoma [7] and osteoblast-like cells [11]. There is evidence that T_3 regulation of pituitary cell growth is mediated by the production of an autocrine factor [18, 25], possibly IGF-I [43]. Also in osteoblast-like cells, it was shown that T_3 stimulates IGF-I production [29]. To our knowledge, there has been only one report on the growth stimulating action of thyroid hormone on cultured

skeletal muscle cells [21]. In this study, a stimulatory action of T_4 on proliferation of chick myoblasts was found. In contrast we found no effect of T_3 on the proliferation of $L6_{AM}$ cells although the concentration (5 nM) we used lies within the range of concentrations found in rat muscle and other tissues [27]. One may speculate that this discrepancy may be caused by differences in autocrine production of growth factors in primary chick myoblasts and L6 cells. It was shown that primary rat myoblasts produce biologically significant amounts of IGF-I and IGF-II [17], while L6 cells secrete only IGF-II at a very low level [14]. However, no data are available on T_3 regulation of growth factor production in cultured skeletal muscle cells. The complete absence of T_3 effects on the proliferation of $L6_{AM}$ cells in our study is consistent with the findings of Brown *et al.*, who reported that T_3 does not play a direct role in regulating muscle DNA synthesis and formation of muscle satellite cells [3], in spite of the fact that T_3 has major effects on the development of muscle *in vivo*. This suggests that the stimulation of skeletal muscle growth by thyroid hormone *in vivo* is mediated by an thyroid hormone-induced factor, the most likely candidates being growth hormone and insulin-like growth factor-I.

Effect of T3 on differentiation and CPK activity

The observed increase of CPK activity upon resin treatment of the serum already indicated that thyroid hormones caused a reduction of the CPK activity in the $L6_{AM}$ cell line. This was confirmed by addition of 5 nM T_3 to the medium which resulted in a decrease of the CPK activity of 30%. Nonspecifity of this effect could be excluded, since rT_3, the biological inactive analogue of T_3, did not affect CPK activity. The DNA- and protein levels of the cultures were not significantly changed by T_3, indicating that the partial inhibition of CPK activity was not due to a general effect of T_3 on protein synthesis or on the condition of the cultures. CPK activity is generally used as a biochemical marker for the degree of fusion, therefore our CPK data suggested an inhibitory action of T_3 on the fusion process. However this was not supported by microscopical analysis of the fusion index. It appeared that fusion was not influenced by T_3, neither in presence or absence of insulin. The observation that T_3 also inhibited CPK activity in maximally fused cultures implies a specific T_3 effect on CPK activity in differentiated myotubes, analogous to the CPK activity stimulation by insulin in maximally fused cultures. One may conclude therefore that CPK activity is no suitable marker for establishing the role of T_3 in myogenic differentiation.

Literature data of T_3 effects on CPK activity in cultured muscle cells are not uniform. Shainberg *et al.* [32] reported a (not significant) reduction of CPK activity caused by T_3 and T_4 in primary cultures of rat myoblasts, which agrees with our observations, but Kumegawa *et al.* [21] found that T_4 stimulated CPK activity in primary cultures of chick myoblasts, by stimulating fusion. Possibly species differences underlie this inconsistency. The absence of a T_3 effect on the CPK iso-enzyme pattern in heart [19] is in agreement with our results.

The mechanism of the inhibitory action of T_3 on CPK activity remains a matter of speculation and lies beyond the scope of this study. A few remarks however can be made. Apparently the T_3 effect on CPK activity is independent of insulin and of resin treatment, because it can be observed under all conditions in the $L6_{AM}$ cell line. Furthermore it is not necessarily an inhibitory action of T_3 at the trancriptional level. For instance the specific stimulation of CPK activity by insulin has been ascribed to enhanced stabilization of mRNA of CPK [26]. Finally it should be noted that in developing muscle *in vivo* CPK activity is stimulated by T_3 [36; see also below].

Other enzymes

During neonatal development T_3 has been shown to stimulate the activities of LDH and SDH in fast skeletal muscle of the rat [1, 35] and also the transition of the neonatal to the adult fast isoform of myosin or MHC [6, 41]. We found no evidence for such stimulations in the $L6_{AM}$ cell line. The absence of a T_3-induced shift in MHC isoform could be ascribed to the fact that only the embryonic form of MHC is synthetized in L6 myotubes. This illustrates that L6 cells only partially complete the programme of muscle development, probably because essential factors are missing or are not present in the right proportions [36]. The absence of innervation and contractile activity in L6 cells are factors which may certainly contribute to this. In primary rat or chick myoblast cultures, manipulation of contractile activity by electrostimulation or tetrodotoxin was shown to affect the levels of various proteins that are synthesized in myotubes. Increased frequency of contraction, brought about by electrostimulation, has been reported to increase myosin synthesis [2] and to decrease synthesis of acetylcholine receptors and acetylcholinesterase [31, 39]. Inhibition of contractions by tetrodotoxin, on the other hand, was shown to increase MHC degradation [40] and to increase acetylcholine receptor and acetylcholinesterase synthesis [31, 39]. Furthermore, changes in the pattern of myosin light chain isotypes have been demonstrated upon chronic electrostimulation of chick myotubes [9, 37].

In spite of the limited maturation of our muscle cell line the more than fourfold stimulation of SR Ca^{2+}-ATPase net synthesis by T_3 proves the suitability of the $L6_{AM}$ cell line as a model for the study of the regulation of this enzyme by T_3 in concert with other factors.

In summary our experiments showed that resin treated serum does not interfere with myogenic differentiation but even slightly stimulates the fusion process. No effects of T_3 on fusion of $L6_{AM}$ cells were observed, which means that any effect of T_3 on muscle specific proteins will not be clouded by effects of T_3 on the fusion-coupled basal expression of muscle proteins.

List of abbreviations

AraC = 1-(ß-D-arabinofuranosyl)cytosine; Ca^{2+}-ATPase = Ca^{2+}-dependent Mg^{2+}-ATPase from sarcoplasmic reticulum (EC 3.6.1.38); CPK = creatin phosphokinase; E.l.i.s.a. = enzyme-lined immunosorbent assay; DMEM = Dulbecco's Modified Eagle's Medium; FCS = fetal calf serum; IGF = insulin-like growth factor; LDH = lactate dehydrogenase; MHC = myosin heavy chain; PBS = phosphate buffered saline; SDH = succinate dehydrogenase; SR = sarcoplasmic reticulum; T_3 = 3,3',5-triiodothyronine, thyroid, hormone; T_4 = 3,3',5,5'-tetraiodothyronine; rT_3 = 3,5',3'-triiodothyronine ('reverse T_3'); T_xS = thyroid hormone-depleted fetal calf serum.

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References

[1] Baldwin KM, Hooker AM, Campbell PJ, Lewis RE: Enzyme changes in neonatal skeletal muscle: effect of thyroid deficiency. *Am J Physiol* 1978; 235: C97-C102.

[2] Brevet A, Pinto E, Peacock J, Stockdale FE: Myosin synthesis increased by electrical stimulation of skeletal muscle cell cultures. *Science* 1976; 193: 1152-1154.

[3] Brown JG,Bates PC, Holliday MA, Millward DJ: Thyroid hormones and muscle protein turover. *Biochem J* 1981; 194: 771-782.

[4] Burke RE, McGuire WL: Nuclear thyroid hormone receptors in a human breast cancer cell line. *Canc Res* 1978; 38: 3769-3773.

[5] Burton K: A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem J* 1956; 62: 315-323.

[6] Butler-Browne GS, Herlicoviez D, Whalen RG: Effects of hypothyroidism on myosin isozyme transitions in developing rat muscle. *FEBS Lett* 1984; 166: 71-75.

[7] Chou CK, Ho LT, Ting LP, Hu CP, Su TS, Chang WC, Suen CS, Huang MY, Chang C: Selective suppression of insulin-induced proliferation of cultured human hepatoma cells by somatostatin. *J Clin Invest* 1987; 79: 175-178.

[8] DeFesi CR, Fels EC, Surks MI: Triiodothryonine stimulates growth of cultured GC cells by action early in the G1 period. *Endocrinology* 1984; 114: 293-295.

[9] Düsterhöft S, Pette D: Changes in myosin light chains by chronic stimulation of chick myotubes in culture. *J Physiol (London)* 1985; 361: 33P.

[10] Engvall E: Enzyme immunoassay ELISA and EMIT, in Van Vunakis H, Langone JJ (eds): *Methods in Enzymology (Vol.70)*. New York, Academic press, 1980; pp 419-438.

[11] Ernst M, Froesch ER: Triiodothyronine stimulates proliferation of osteoblastlike cells in serum-free culture. *FEBS Lett* 1987; 220: 163-166.

[12] Fischer JC, Ruitenbeek W, Berden JA, Trijbels JMF, Veerkamp JH, Stadhouders AM, Sengers RCA, Janssen AJM: Differential investigation of the capacity of succinate oxidation in human skeletal muscle. *Clin Chem Acta* 1985; 153: 23-36.

[13] Florini JR, Magri KA: Effects of growth factors on myogenic differentiation. *Am J Physiol* 1989; 256: C701-C711.

[14] Florini JR, Magri KA, Ewton DZ, James PL, Grindstaff K, Rotwein PS: "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *J Biol Chem* 1991; 266: 15917-15923.

[15] Forster G, Brent E, Bergmeyer HU: Creatin Kinase, in Bergmeyer HH (ed): *Methoden der enzymatischen analyse*. Weinheim, Verlag Chemie, 1986, pp 812-821.

[16] Guilian GG, Moss RL, Greaser ML: Improved methodology for analysis and quantification of proteins on one-dimensional silver stained slab gels. *Anal. Biochem.* 1983; 129: 277-287.

[17] Hill DJ, Crace CJ, Nissley SP, Morrell D, Holder AT, Milner RDG: Fetal rat myoblasts release both rat somatomedin-C (SM-C)/insulin-like growth factor I (IGF-I) and multiplication-stimulating activity *in vitro*: partial characterization and biological activity of myoblast-derived SM-C/IGF-I. *Endocrinology* 1985; 117: 2061-2072.

[18] Hinkle PM, Kinsella PA: Thyroid hormone induction of an autocrine growth factor secreted by pituitary tumor cells. *Science* 1986; 234: 1549-1552.

[19] Kessler-Icekson G: Effect of triiodothyronine on cultured neonatal rat heart cells: beating rate, myosin subunits and CK-isozymes. *J Mol Cell Cardiol* 1988; 20: 649-655.

[20] Kitagawa S, Obata T, Willingham MC, Cheng SY: Thyroid hormone action: induction of morphological changes and stimulation of cell growth in rat pituitary tumor GH_3 cells. *Endocrinology* 1987; 120: 2591-2596.

[21] Kumegawa M, Ikeda E, Hosoda S, Takuma T: *In vitro* effects of thyroxine and insulin on myoblasts from chick embryo skeletal muscle. *Dev Biol* 1980; 79: 493-499.

[22] Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227: 680-685.

[23] Legrand J: Thyroid hormone effects on growth and development, in Henneman G (ed): *Thyroid hormone metabolism.* New York, Marcel Dekker, 1986, pp 503-534.

[24] Lowry OH, Rosebrough NJ, Farr NJ, Randall RJ: Protein measurement with Folin phenol reagent. *J Biol Chem* 1951; 193: 1107-1111.

[25] Miller MJ, Fels EC, Shapiro LE, Surks MI: L-Triiodothyronine stimulates growth by means of an autocrine factor in a cultured growth hormone-producing cell line. *J Clin Invest* 1987; 79: 1773-1781.

[26] Pontecorvi A, Tata JR, Phyllaier M, Robbins J: Selective degradation of mRNA: the role of short-lived proteins in differential destabilization of insulin-induced creatin phosphokinase and myosin heavy chain mRNAs during rat skeletal muscle L_6 cell diffentiation. *EMBO* 1988; 7: 1489-1495.

[27] Samuels HH, Tsai JR, Cintron R: Thyroid hormone action: a cell-culture system responsive to physiological concentrations of thyroid hormones. *Science* 1973; 181: 1253-1256.

[28] Samuels HH, Stanley F, Casanova J: Depletion of L-3,5,3'-triiodothyronine and L-thyroxine in euthyroid calf serum for use in cell culture studies of the action of thyroid hormone. *Endocrinology* 1979; 105: 80-85.

[29] Schmid C, Schläpfer I, Waldvogel M, Schwander J, Zapf J, Froesch ER: Triiodothyronine (T_3) stimulates insulin-like growth factor (IGF)-1 and IGF binding protein (IGFBP)-2 production by rat osteoblasts in vitro. *Acta Endocrinol (Copenh)* 1992; 126: 467-473.

[30] Schwartz HL: Effect of thryoid hormone on growth and development, in Oppenheimer JH, Samuels HH (eds): *Molecular basis of thyroid hormone action.* New York, Academic Press, 1983; pp 413-440.

[31] Shainberg A, Burstein M: Decrease of acetylcholine receptor synthesis in muscle cultures by electrical stimulation. *Nature* 1976; 264: 368-369.

[32] Shainberg A, Brik H, Bar-Shavit R, Sampson SR: Inhibition of acetylcholine receptor synthesis by thyroid hormones. *J Endocrinol* 1984; 101: 141-147.

[33] Simonides WS, van Hardeveld C: The effect of hypothyroidism on sarcoplasmic reticulum in fast-twitch muscle of the rat. *Biochem Biophys Acta* 1985; 844: 129-141.

[34] Simonides WS, van Hardeveld C: Effects of the thyroid status on the sarcoplasmic reticulum in slow sketal muscle of the rat. *Cell Calcium* 1986; 7: 147-160.

[35] Simonides WS, van Hardeveld, C: The postnatal development of sarcoplasmic reticulum Ca^{2+} transport activity in skeletal muscle of the rat is critically dependent on thyroid hormone. *Endocrinology* 1989; 124: 1145-1153.

[36] Sporn MB, Roberts AB: Peptide growth factors are multifunctional. *Nature* 1988; 332: 217-219.

[37] Srihari T, Pette D: Myosin light chains in normal and electrostimulated cultures of embryonic chicken breast muscle. *FEBS Lett* 1981; 123: 312-314.

[38] Taub M, Chuman L, Saier MH, Sato G: Growth of Madin-Darby canine kidney epithelial cell (MDCK) line in hormone-supplemented, serum-free medium. *Proc Natl Acad Sci USA* 1979; 76: 3338-3342.

[39] Walker CR, Wilson BW: Regulation of acetylcholinesterase in cultured muscle by chemical agents and electrical stimulation. *Neuroscience* 1976; 1: 191-196.

[40] Walker CR, Strohman R: Myosin turnover in cultured muscle fibers relaxed by tetrodotoxin. *Exp Cell Res* 1978; 116: 341-349.

[41] Whalen RG, Schwartz K, Bouveret P, Sell SM, Gros F: Contractile protein isozymes in muscle development: identification of an embryonic form of myosin heavy chain. *Proc Natl Acad Sci USA* 1979; 76: 5197-5201.

[42] Yaffe D: Retention of differentiation potentialities during prolonged cultivation of myogenic cells. *Proc Natl Acad Sci USA* 1968; 61: 477-483.

[43] Zhou-Li F, Joly-Pharaboz MO, Bouillard B, Albaladejo V, Nicolas B, Andre J: Multihormonal control of cell proliferation: opposite effects of two stimulators (17ß-estradiol and L-triiodothyronine) and one inhibitor (dexamethasone) on F_4Z_2 pituitary tumor cells. *Endocrinology* 1991; 128: 2761-2768.

[44] Zubrzycka-Gaarn E, MacDonald G, Phillips L, Jorgensen AO, MacLennan DH: Monoclonal antibodies to the Ca^{2+}+Mg^{2+}-dependent ATPase of sarcoplasmic reticulum identify polymorphic forms of the enzyme and indicate the presence of a classical high-affinity Ca^{2+}-binding site. *J Bioenerg Biomembr* 1984; 16: 441-464.