Electrical stimulation of C2C12 myotubes induces contractions and represses thyroid-hormone-dependent transcription of the fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase gene

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Chronic low-frequency contraction of skeletal muscle, either induced by a slow motor nerve or through direct electrical stimulation, generally induces expression of proteins associated with the slow phenotype, while repressing the corresponding fast isoforms. Contractions thereby counteract the primarily transcriptional effect of thyroid hormone (T_3), which results in the selective induction and stimulation of expression of fast isoforms. We studied the regulation of expression of the fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase (SERCA1), a characteristic component of the fast phenotype. Previous work suggested that reduction of SERCA1 expression by contractile activity might result from interference with the T_3-dependent transcriptional stimulation of the SERCA1 gene. The present study was set up to test this unexpected mode of action of contractile activity. We show that electrical stimulation of C2C12 mouse myotubes, which results in synchronous contractions at the imposed frequency, reduces basal but virtually abolishes T_3-dependent SERCA1 expression. T_3-dependent expression of a reporter gene driven by the SERCA1 promoter was similarly affected by electrical stimulation. This is the first demonstration that the counteracting effects on muscle gene expression of electrically induced contractions and T_3 may interact at the transcriptional level.

INTRODUCTION

Most skeletal muscles have a mixed composition of slow and fast fibres. The phenotype of these fibres is characterized by their metabolic rate and the isoforms of the muscle genes they express. The phenotype of skeletal-muscle fibres in the rat is primarily determined by the type of innervation and the thyroid status. Prolonged low-frequency contractile activity imposed by a slow motor nerve stimulates expression of muscle proteins associated with the slow phenotype and represses expression of proteins associated with the fast phenotype [1–4]. It is now known that it is not the nerve itself but the activity induced by it that triggers this shift in phenotype [1,5,6]. Thyroid hormone (T_3), on the other hand, stimulates expression of proteins associated with the fast phenotype at the expense of their slow counterparts [7,8]. We studied the expression of the fast-type skeletal-muscle sarcoplasmic-reticulum Ca^{2+}-ATPase (SERCA1), the induction of which is dependent on T_3 [9–11], and we have shown that, in rat soleus muscle, T_3 co-ordinately replaces slow isoforms of myosin heavy chain and sarcoplasmic reticulum Ca^{2+}-ATPase with fast isoforms [12,13]. The stimulation of SERCA1 expression by T_3 is brought about by an increase in the transcription frequency of the SERCA1 gene [14], mediated by thyroid-hormone-response elements in the promoter of the gene [15], as has also been shown for other genes up-regulated by T_3 (reviewed in [16,17]).

How contractile activity exerts its effect on muscle phenotype is unknown, but a mediating role for the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$), which increases with contraction, has been suggested [18]. We found in rat L6 myotubes that T_3-dependent SERCA1 expression in particular was greatly reduced when $[Ca^{2+}]_i$ was increased above the resting level of 120 nM [19]. However, increased steady-state $[Ca^{2+}]_i$ in these non-contracting cells is not the same as an increase in the average $[Ca^{2+}]_i$ as a result of continuous repetitive contraction. Therefore we have developed an *in vitro* system that allows direct determination of the effects of contractions and T_3 on muscle gene expression, without the disturbing influence of secondary effects of altered innervation inherent *in vivo*. We used mouse C2C12 muscle cells, which, unlike L6 cells, can be induced to contract by direct electrical stimulation, and determined the effect of T_3 and contractile activity on SERCA1 expression and SERCA1 promoter activity. We demonstrate specific interaction between electrically induced contractile activity and transcriptional stimulation by T_3.

MATERIALS AND METHODS

Materials

For electrical stimulation of C2C12 myotubes we used custom-built electrodes which were made of 1 mm platinum wire integrated into lids of six-well dishes (well diameter 36 mm). For each well two half-circle electrodes were aligned to the side of the well in order to obtain a maximal area of stimulation.

Fetal calf serum (FCS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Gibco–BRL (Paisley, Scotland, U.K.) and insulin (bovine) was from Serva (Heidelberg, Germany). 1-(β-D-Arabinofuranosyl)cytosine (AraC) and T_3 were obtained from Sigma (St. Louis, MO, U.S.A.). Culture

Abbreviations used: AraC, 1-(β-D-arabinofuranosyl)cytosine; DMEM, Dulbecco's modified Eagle's medium; SERCA1, fast-type sarcoplasmic-reticulum Ca^{2+}-ATPase; T_3, L-tri-iodothyronine (thyroid hormone); FCS, fetal calf serum; TxFCS, thyroid-hormone-depleted FCS; CAT, chloramphenicol acetyltransferase; $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration.

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Part of the 5′ flanking sequence of the rat SERCA1 gene has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U34282.

plastics were from Nunc (Roskilde, Denmark). AG-1-X8 anion-exchange resin was from Bio-Rad (Richmond, CA, U.S.A.). [^{3}H]Chloramphenicol was from Du Pont–New England Nuclear (Wilmington, DE, U.S.A.) and all DNA-modification enzymes were obtained from Boehringer-Mannheim (Mannheim, Germany). The β-galactosidase determination kit was from Promega (Madison, WI, U.S.A.).

Cell culture and electrical stimulation

C2C12 cells were grown to confluence in 0.1 % gelatin-coated six-well multidishes in DMEM containing 10 % (v/v) FCS depleted of thyroid hormones (TxFCS) by AG-1-X8 resin treatment [20]. At day 0 differentiation was induced by lowering the serum concentration to 2.5 % (v/v) as described by Carnac et al. [21]. To stimulate the fusion and differentiation process this 'differentiation medium' was supplemented with insulin (1 μg/ml) [22]. Cultures were divided into three groups: one group received 100 nM T_3 from day 0, one group received T_3 from day 3, and one group received no T_3. At 2 days after the switch to differentiation medium, AraC (4 μg/ml) was added to the medium to eliminate any proliferating cells (myoblasts) [11]. The medium was then changed every 24 h. Starting from day 3, when fusion was complete, cells were stimulated to contract using 6 ms pulses of 3 V/cm², at 2 Hz. Cells were harvested at day 3 (when electrical stimulations were started) and at day 5.

SERCA1 protein analysis

For the determination of total protein and SERCA1 protein levels, cultures were washed twice with PBS and then covered with 300 μl of PBS and frozen at −20 °C. After thawing, the cells were harvested by scraping with a rubber 'policeman' and stored at −20 °C. SERCA1 protein levels were determined by ELISA as described by Muller et al. [11] using the SERCA1-specific antibody A52, which was a gift from Dr. MacLennan (Banting and Best Institute, Toronto, Ontario, Canada). SERCA1 levels were expressed relative to total protein as determined by the method of Lowry et al. [23].

Transient transfections and expression assay

Expression constructs of the rat SERCA1 promoter driving the chloramphenicol acetyltransferase (CAT) gene were made by subcloning deletion constructs of the previously described [15] SERCA1 gene 5′ flanking sequence with 5′ ends at −2658 or −141 and a common 3′ end at +91 (+1 is the transcription-initiation site) into the pOCAT2 chloramphenicol [24] expression vector. Part of the 5′ flanking sequence of the rat SERCA1 gene has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U34282.

C2C12 myoblasts were seeded at 22×10^3 cells/cm² on 0.1 % gelatin-coated six-well multidishes. The next day, when cultures were 70–80 % confluent, cells were transiently transfected using the calcium phosphate precipitation method [25]. Together with the SERCA1/pOCAT construct we co-transfected the construct pCDM13, which expresses the thyroid hormone receptor α1 [kindly provided by Dr. G. A. Brent (Brigham and Women's Hospital, Boston, MA, U.S.A.)] as described by Prost et al. [26]. Cells were also co-transfected with a construct in which the β-galactosidase gene was driven by the cytomegalovirus promoter (pCMV-β-Gal) to allow correction for differences in transfection efficiency between wells. Transfection mixes were prepared for 12 wells (two six-well dishes) and contained per well: 2 μg of SERCA1/CAT construct, 0.3 μg of pCDM13 and 1 μg of pCMV-β-Gal. The next day (day 0) cells were washed twice with PBS and once with DMEM containing 5 % TxFCS after which cells were switched to differentiation medium [DMEM with 2.5 % TxFCS, insulin (1 μg/ml)]. At day 2, AraC (4 μg/ml) was added to the medium, which was changed every 24 h from then on. At day 3, T_3 treatment (50 nM) and electrical stimulations were started. Of the sets of two dishes that received the same transfection mixture, one dish was stimulated and the other served as a control. At day 5, cultures were harvested and analysed for CAT activity by the phase-extraction method (overnight incubations at 37 °C) [27] and normalized to β-galactosidase activity, which was assayed according to the manufacturer's protocol. Transfections with the promoterless pOCAT2 vector served as a control for non-specific effects of T_3 or electrical stimulation on CAT activity. Parallel cultures were used for the determination of the transfection efficiency, which was at least 10 % as determined by *in situ* β-galactosidase activity assay [28].

Statistics

Data are displayed as means ± S.E.M. for n experiments and were evaluated by two-sided Student's t test. Data for individual experiments were obtained from duplicate (SERCA1 protein level) or triplicate (transfections) cell cultures. Differences were considered significant at $P < 0.05$.

RESULTS

Effect of T_3 on SERCA1 expression in C2C12 cells

To determine the optimal time points for the assessment of the effect of contractions on basal and T_3-induced SERCA1 expression in C2C12 cells, we performed an experiment to establish the time course of the expression of SERCA1 and its stimulation by T_3 in these cells. Fusion, which was complete at day 3, and myotube morphology were not affected by T_3 treatment. Figure 1 shows that basal as well as T_3-induced expression increased gradually to reach a maximal level at day 5. Beyond this time point the quality of the cultures started to deteriorate.

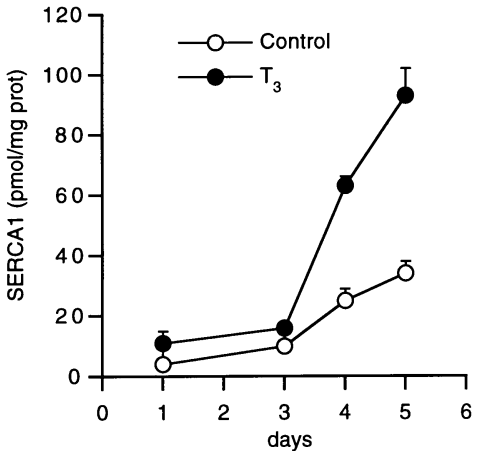

Figure 1 Effect of T_3 on SERCA1 expression in C2C12 myotubes

Cells were grown as described in the Materials and methods section in the absence (○) or presence (●) of T_3. Data points represent means ± S.D. of a representative experiment in duplicate.

Table 1 Effect of contractile activity on the expression of SERCA1 protein

Cells were grown and SERCA1 protein expression was determined as described in the Materials and methods section. Differentiation was induced at day 0 and electrical stimulations were started at day 3. T_3 treatment was started at either day 0 or day 3. Data for individual experiments were normalized to the value found in quiescent controls at day 5 and are presented as percentage of the SERCA1 protein level at day 3. Data represent means ± S.E.M. for three experiments. *$P < 0.05$ for T_3 versus control; †$P < 0.05$ for contractions versus no contractions.

	Day 3	Day 5	
		No contractions	Contractions
No T_3	100 ± 31	197	97 ± 8.7†
T_3 from day 3	–	278 ± 18.3*	102 ± 9.7†
T_3 from day 0	139 ± 33.1*	500 ± 93.7*	147 ± 8.8*†

Electrical stimulation of C2C12 myotubes

Electrical stimulation of C2C12 myotubes with 6 ms pulses of 3 V/cm² resulted in synchronous contractions at the frequency of pacing (2 Hz), and this was maintained for 48 h in at least 70% of the myotubes. Variation of this voltage or pulse duration either compromised participation or the period over which contractions followed the stimulation frequency. In cultures that were not electrically stimulated, no contractions were observed. Contractions, in either the presence or absence of T_3, did not affect myotube morphology and no signs of cell death were observed in any group. Coating of the culture surface with a 0.1% gelatin solution was found to be necessary to prevent detachment of contracting cells. In non-stimulated cultures grown in the presence of T_3, the protein content decreased slightly between day 3 and day 5 resulting in a small but significant difference from the T_3-treated contracting cultures ($-16 \pm 3\%$, $n = 3$, $P < 0.05$).

Effect of electrical stimulation on SERCA1 expression

Starting from day 3, when fusion was complete, C2C12 myotubes were stimulated to contract at 2 Hz and were given T_3. Table 1 shows that contractions completely abolished T_3 stimulation of SERCA1 expression, which at day 5 amounted to 1.4 ± 0.1-fold ($n = 3$, $P < 0.05$) in quiescent cells. The basal level of expression of SERCA1 in contracting cells was reduced compared with quiescent cells by 50 ± 5% ($n = 3$, $P < 0.05$) at day 5.

Because of the general stimulatory effect of T_3 on muscle cell differentiation [29], we also studied the effect of contractions in cultures in which T_3 treatment was started, together with the induction of differentiation (day 0), 2 days before the onset of electrical stimulation. This protocol allowed undisturbed development of factors possibly involved in T_3 stimulation that might otherwise be inhibited by contractions between day 3 and day 5. At day 3, when contractions were started, the T_3 stimulation of SERCA1 expression was 1.4 ± 0.1-fold ($n = 3$, $P < 0.05$). Over the period between day 3 and day 5, the fold T_3 stimulation of SERCA1 expression in quiescent cells increased to 2.5 ± 0.2 ($n = 3$, $P < 0.05$). In contracting cells, however, the T_3 stimulation of SERCA1 expression did not increase further, remaining at 1.5 ± 0.1 at day 5 ($n = 3$, $P < 0.05$).

Effect of electrical stimulation on SERCA 1 promoter activity

Whereas contractions reduced basal SERCA1 expression, they abolished its stimulation by T_3. The fact that T_3 mediates its effect on SERCA1 expression at the transcriptional level [14,15] suggests that contractile activity interferes with the effect of T_3 at that level. To study the effect of contractions on SERCA1 promoter activity and its stimulation by T_3 we have developed a system that allows electrical stimulation in transiently transfected cells. C2C12 myoblasts were transfected with pOCAT2 constructs containing the CAT reporter gene driven by SERCA1 promoter fragments. After transfection, these myoblasts were allowed to fuse and differentiate into myotubes in which contractions were induced by electrical stimulation. It was found that cultures needed to be thoroughly washed after transfection, as described in the Materials and methods section, to allow normal fusion and contractile activity in response to electrical stimulation. As in control cultures, contractions could be maintained for 48 h in transfected cells and despite the relatively long period between transfection and harvesting of the cells, the expression of the pOCAT2/SERCA1 constructs did not deteriorate (not shown). Furthermore contractions had no effect on β-galactosidase expression from the co-transfected CMV-Gal plasmid used for normalization.

Table 2 summarizes the results of the transfection study. Cells transfected with pOCAT2/−2658, containing 2658 nucleotides of the SERCA1 5′ flanking sequence, showed a 7.0 ± 0.6-fold ($n = 4$, $P < 0.05$) stimulation of normalized CAT activity by T_3 in quiescent cells. Contractions significantly reduced this stimulation to 2.5 ± 1.0-fold ($n = 4$, $P < 0.05$). The basal activity of this construct was not significantly altered by contractile activity. When the SERCA1 5′ sequence was truncated to −141, leaving a minimal functional promoter, T_3 and contractions no longer affected transcription. Also in transfections with the promoterless pOCAT2 expression vector, CAT activity was independent of T_3 or contractile activity. This implies that the observed reduction

Table 2 Effect of contractile activity on T_3 induction of SERCA1 promoter activity

C2C12 myoblasts were transfected with pOCAT constructs containing 5′ flanking sequence of the SERCA1 gene. The SERCA1 inserts had a common 3′ end at +91. The 5′ end of the construct is indicated by the name of the construct. Cell culture and transfections were performed as described in the Materials and methods section. CAT activities were normalized to β-galatosidase activity which was unaffected by contractile activity. Data are presented as percentage of the normalized CAT activity found in quiescent cultures without addition of T_3 and represent the means ± S.E.M. for four (three for the promoterless pOCAT2) experiments. *$P < 0.05$ for T_3 versus control; †$P < 0.05$ for contractions versus no contractions. Promoter activity of the −141 and −2658 fragment resulted in a stimulation of basal CAT expression of 10-fold and 40-fold relative to the pOCAT2 vector respectively.

	$-T_3$		$+T_3$		T_3 induction ratio	
Contractions...	−	+	−	+	−	+
pOCAT2/−2658	100	118 ± 31	700 ± 57*	296 ± 119	7.0 ± 0.6 *	2.5 ± 1.0†
pOCAT2/−141	100	94 ± 7	104 ± 4	97 ± 18	1.0 ± 0.1	1.0 ± 0.1
pOCAT2	100	86 ± 17	101 ± 18	86 ± 5	1.0 ± 0.2	1.0 ± 0.1

in T_3 stimulation of CAT activity in experiments with pOCAT/−2658 is not due to a general non-specific effect of contractions, but results from an almost complete inhibition of T_3 stimulation of SERCA1 promoter activity.

DISCUSSION

In agreement with results for skeletal muscle *in vivo* and the skeletal-muscle cell line L6, T_3 markedly increased SERCA1 expression in C2C12 cells. Electrical stimulation of C2C12 myotubes, resulting in synchronous contractions at the imposed frequency (2 Hz), repressed basal but abolished T_3-dependent SERCA1 expression. Since this effect also occurred in cells that were allowed to grow in the presence of T_3 before contractions were started, we conclude that contractions inhibit the T_3 stimulation of SERCA1 expression rather than processes responsible for the development of this effect, such as the production of thyroid hormone receptor or cofactors that participate in the transcriptional activation by thyroid hormone receptor.

We have previously shown that T_3 stimulates the expression of SERCA1 by increasing the activity of the SERCA1 promoter [14,15]. Analysis of SERCA1-promoter activity in C2C12 cells revealed a reduction in T_3 stimulation from 7-fold in non-contracting cells to a no longer significant 2.5-fold stimulation in contracting cells. This effect of contractions was promoter-specific, and depended on sequences upstream of position −141 relative to the transcription-initiation site. This strongly suggests that the repression of the T_3-dependent SERCA1 expression by contractions is at least in part a result of decreased transcription frequency of the SERCA1 gene, rather than altered stability of SERCA1 protein or the mRNA coding for it. The repression of basal SERCA1 protein expression by contractions did not appear to result from an effect on transcription driven by the first 2658 bp of the SERCA1 5′ flanking sequence. However, it cannot be excluded that further upstream sequences are involved in such regulation. Alternatively post-transcriptional events may be involved.

Although effects of electrical stimulation not related to contraction cannot be excluded, the findings of the present study provide a mechanism of interaction by which the opposing stimuli of T_3 and (chronic) contractile activity determine the expression of SERCA1, a typical fast isoform, in skeletal muscle. Previous work had already indicated that the proportion of fast isoforms in skeletal-muscle fibres is determined by the balance between the stimulating effect of T_3 and the inhibiting effect of the so-called slow-type contractile activity [1–4,7,8,12,13,30]. The co-ordinated regulation of several phenotype-specific proteins, such as SERCA and myosin heavy and light chain isoforms [1,4,12,13,30], suggests that the mechanism of interaction described may apply to other muscle proteins as well.

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