Cross-talk between transcriptional regulation by thyroid hormone and myogenin: new aspects of the Ca^{2+}-dependent expression of the fast-type sarcoplasmic reticulum Ca^{2+}-ATPase

Marc H. M. THELEN[1], Warner S. SIMONIDES, Alice MULLER and Cornelis VAN HARDEVELD

Laboratory for Physiology, Institute for Cardiovascular Research (ICaR-VU), Vrije Universiteit Amsterdam, van der Boechorststraat 7, 1081 BT Amsterdam, The Netherlands

We have previously demonstrated an interaction between the major determinants of skeletal muscle phenotype by showing that continuous contractile activity represses the thyroid hormone (3,3′,5-tri-iodothyronine; T_3)-dependent transcriptional activity of fast-type sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA1), a characteristic of the fast phenotype. Both the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and the myogenic determination factors MyoD and myogenin have been implicated as mediators of the effect of contractile activity on skeletal muscle phenotype. Using L6 cells we have shown that an increase in the steady-state $[Ca^{2+}]_i$ above the resting level of 120 nM indeed can mimic the effect of contractile activity on T_3-dependent SERCA1 expression. We now show that the repressing effect of increased $[Ca^{2+}]_i$ on T_3-dependent SERCA1 expression in L6 cells is exerted at a pre-translational level and is accompanied by increased myogenin mRNA expression. Myogenin overexpression in these cells revealed that increased expression of myogenin alone strongly decreases the T_3-dependent stimulation of SERCA1 promoter activity. These results suggest a pathway for the regulation of skeletal muscle phenotype in which $[Ca^{2+}]_i$ mediates the effect of contractile activity by regulating the expression of myogenin, which in turn interferes with transcriptional regulation by T_3.

INTRODUCTION

The phenotype of skeletal muscle fibres is determined by an interplay of thyroid hormone (3,3′,5-tri-iodothyronine; T_3) and contractile activity. T_3 favours the expression of proteins associated with the fast phenotype [1,2], whereas chronic low-frequency contraction results in the repression of these proteins and their replacement with isoforms characteristic of the slow phenotype (reviewed in [3]). We study the fast-type sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase (SERCA1), which is a characteristic component of the fast phenotype. The expression of this Ca^{2+} pump and other components of the fast phenotype is induced by T_3 [4,5] and repressed by continuous contractile activity [6,7]. The stimulation of SERCA1 expression by T_3 is brought about by an increase in the transcription frequency of the SERCA1 gene [8], mediated by T_3 response elements in the promoter of the gene [9], as has also been shown for other genes up-regulated by T_3 (reviewed in [10,11]).

How contractile activity exerts its effect on skeletal muscle phenotype is as yet unknown. Using rat primary myotubes we showed that spontaneous contractions strongly repress SERCA1 expression, indicating that the effect of contractile activity on the fast phenotype is direct, rather than being the result of innervation or electrical stimulation [12,13]. In C2C12 myotubes contractile activity particularly represses T_3-dependent transcription of the SERCA1 gene, suggesting direct interference between both determinants of muscle phenotype [14]. In rat L6 myotubes, which do not contract, T_3-dependent SERCA1 protein expression was strongly decreased when the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) was increased above the resting level of 120 nM [12]. These results suggest that the effect of contractile activity on SERCA1 gene expression could be mediated by an increase in the average $[Ca^{2+}]_i$, which varies in proportion to the frequency of contraction.

The myogenic determination factors (MDFs) have also been suggested to be involved in the effect of contractile activity on muscle phenotype and could provide a link between $[Ca^{2+}]_i$ and gene expression. The MDFs are a family of transcription factors essential for development and differentiation of skeletal muscle *in vivo* and of muscle cells *in vitro* (reviewed in [15–17]). The consensus binding nucleotide sequence for the MDFs is CANNTG, referred to as an E-box, which can be found in the promoter region of virtually every muscle gene [18], including that of SERCA1 [9]. When differentiation is complete, MDF expression decreases, but low levels of expression persist in adult muscle. Several observations have led to the hypothesis that these levels of MDFs, and particularly those of MyoD and myogenin, have a role in co-ordinating the expression of phenotype-specific genes, with myogenin being associated with the slow phenotype and MyoD with the fast phenotype [19,20]. It should be noted that no causal relation between different skeletal muscle phenotypes and the expression of either MyoD or myogenin has yet been shown. However, in line with the hypothesis, transformation of adult fast muscle to a slow phenotype, induced by a slow-type contraction pattern, was recently shown to be accompanied by increased expression of myogenin and down-regulation of the expression of MyoD [21]. Although SERCA1 expression is usually correlated with high levels of MyoD, it is not dependent on this myofactor [13]. Suppression of the SERCA1 isoform in the contraction-induced transformation to a slow phenotype must therefore be assumed to be mediated by myogenin. However, studies of E-box-containing promoters *in*

Abbreviations used: $[Ca^{2+}]_i$, free cytosolic Ca^{2+} concentration; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; MDF, myogenic determination factor; SERCA1, fast-type sarcoplasmic/endoplasmic-reticulum Ca^{2+}-ATPase; SSPE, 180 mM NaCl/0.1 mM EDTA/10 mM sodium phosphate (pH 7.4); T_3, 3,3′,5-tri-iodothyronine (thyroid hormone); TR, T_3 receptor; TRE, thyroid response element; T_xFCS, thyroid-hormone-depleted fetal calf serum.

[1] To whom correspondence should be addressed.

vitro have so far shown different degrees of stimulation of transcription by all MDFs, but not repression.

The aim of the present study was, first, to analyse in more detail the repressive effect of increased $[Ca^{2+}]_i$ on the expression of SERCA1 and, secondly, to test the possible mediatory role of myogenin in the active repression of this fast isoform. Because $[Ca^{2+}]_i$ can be manipulated in L6 muscle cells and myogenin is the primary MDF in these cells [22,23], we used this model system to investigate this possible mechanism of phenotypic regulation in skeletal muscle.

MATERIALS AND METHODS

Variation in $[Ca^{2+}]_i$ in L6 myotubes

Variation in $[Ca^{2+}]_i$) was achieved by exposing the cultures to ionomycin (Sigma), leading to supranormal $[Ca^{2+}]_i$. As described by Muller et al. [12], growing L6 myotubes in the presence of 0.1 or 0.25 μM ionomycin resulted in an increase of the average $[Ca^{2+}]_i$ from approx. 120 nM (without ionomycin) to approx. 150 nM or approx. 185 nM respectively. In the present study, when discussing the effect of ionomycin, we refer to the corresponding $[Ca^{2+}]_i$ of the study of Muller et al.

L6 myoblasts of the previously described $L6_{AM}$ subclone of the rat myogenic cell line L6 [24] were grown to confluence in Dulbecco's modified Eagle's medium (DMEM; Gibco) containing 10 % (v/v) T_xFCS [fetal calf serum (Gibco) depleted of thyroid hormones by treatment with AG-1-X8 resin (Bio-Rad)] [25]. Cells were allowed to differentiate by replacing the medium with DMEM containing 2.5 % (v/v) T_xFCS and insulin (Serva; 1 μg/ml). After 2 days (assigned as day 0), the medium was replaced by DMEM containing 2.5 % T_xFCS, 1 μg/ml insulin and 4 μg/ml 1-(β-D-arabinofuranosyl)cytosine (Sigma), supplemented with 0.1 or 0.25 μM ionomycin [control cultures received 0.004 % (v/v) DMSO, which was used as solvent], with or without T_3 (Sigma; 5 nM). All media were changed every 24 h and cells were harvested for analysis of proteins and RNA on days 0, 1, 2, and 3. After this time point the quality of the cultures began to deteriorate.

Protein analysis

For the determination of SERCA1 protein levels, cultures were washed twice with PBS and then covered with 300 μl of PBS and frozen at −20 °C. After being thawed, the cells were harvested by scraping with a rubber 'policeman' and stored at −20 °C in separate samples for analysis of total protein and SERCA1. SERCA1 protein levels were determined by ELISA as described by Muller et al. [24] by using the SERCA1-specific antibody A52, which was a gift from Professor MacLennan (Banting and Best Institute, Toronto, Canada). SERCA1 levels were normalized to total protein as determined by the method of Lowry [26].

Northern blot analysis

RNA was isolated, subjected to electrophoresis, blotted to Hybond-N nylon membranes (Amersham) and hybridized with random-prime ^{32}P-labelled cDNA probes as described previously [8]. Hybridizations were performed in 50 % (v/v) formamide/5 × SSPE/5 × Denhardt's solution/1 % (w/v) SDS/60 μg/ml sheared denatured herring sperm [SSPE is 180 mM NaCl/0.1 mM EDTA/10 mM sodium phosphate (pH 7.4); 100 × Denhardt's solution is 2 % (w/v) Ficoll/2 % (w/v) polyvinylpyrrolidone/2 % (w/v) BSA] at 42 °C for 16 h. The following cDNA probes were used: SERCA1, a +586 bp cDNA fragment that we have previously shown to be isoform-specific [8]; myogenin, a 380 bp cDNA fragment consisting of the last 45 bp of exon 1, all 82 bp of exon 2 and the first 253 bp of exon 3 of the mouse myogenin gene; MyoD, a 376 bp cDNA fragment containing the last 42 bp of exon 2 and the first 334 bp of exon 3 of the rat MyoD gene; myf5, a 292 bp fragment consisting of nt 500–791 of the mouse cDNA-coding fragment. All MDF probes were kindly made available and checked on a Northern blot for specificity by G. Molnar and N. A. Schroedl (Alfred E. duPont Institute, Wilmington, DE, U.S.A.). Blots were washed to a stringency of 0.1 × SSPE/0.1 % SDS at 52 °C. After hybridization to the cDNA probes the blots were hybridized to an end-labelled 18 S rRNA oligonucleotide probe in 5 × SSPE/5 × Denhardt's solution/60 μg/ml sheared, denatured herring sperm at 52 °C and subsequently washed to a stringency of 2 × SSPE/0.1 % SDS at 52 °C. Between hybridizations with different probes the probe was stripped from the membranes in accordance with the protocol provided by the manufacturer of the membranes (Amersham). Blots were subjected to autoradiography for up to 15 days and signals were quantified by laser-densitometric scanning. Signals of hybridizations with the cDNA probes were normalized with the 18 S rRNA signal as an independent standard, as described by Samarel et al. [27].

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 [9] 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 CAT [28] expression vector. Part of the 5′ flanking sequence of the rat SERCA1 gene has been submitted to Genbank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number U34282.

L6 myoblasts were seeded at 25×10^3 cells/cm^2 on six-well multidishes (Nunc) in DMEM containing 10 % (v/v) T_xFCS. The next day, when cultures were 50–70 % confluent, cells were transfected with the calcium phosphate precipitation method [29]. Together with the SERCA1/pOCAT construct we co-transfected the thyroid hormone α1 receptor-expressing construct pCDM13 as described by Prost et al. [30]. To permit correction for differences in transfection efficiency between wells, we also co-transfected the β-galactosidase-expressing construct pCMV-βGAL. Myogenin overexpression was achieved by co-transfection with the pEMSV-myo8 myogenin expression construct, which was a gift from Dr. E. N. Olson (MD Anderson Cancer Center, University of Texas, Houston, TX, U.S.A.) [31]. Transfection mixes were prepared for two wells and contained, per well: 3 μg of SERCA1/pOCAT construct, 0.6 μg of pCDM13, 1 μg pCMV-βGAL and 1 μg of pEMSV-myo8. The next day (assigned as day 0) cells were washed twice with PBS and once with DMEM containing 5 % (v/v) T_xFCS, after which the medium was replaced by DMEM containing 10 % (v/v) T_xFCS with or without 50 nM T_3. At day 1, 40 h after transfection, cells were harvested and analysed for CAT activity with the organic extraction method [32] and normalized to βGAL activity, which was assayed in accordance with the manufacturer's protocol (Promega). Parallel cultures were used for the determination of the transfection efficiency, which was at least 5 % as determined by β-galactosidase activity assay *in situ* [33].

Statistics

Data were evaluated by Student's paired or unpaired t test. Differences as function of time of treatment were tested by analysis of variance for repeated experiments. Differences were considered significant at $P < 0.05$.

RESULTS

$[Ca^{2+}]_i$ and levels of SERCA1 protein and mRNA

Previous work has shown that the addition of 0.1 or 0.25 μM ionomycin to L6 myotube cultures increases the $[Ca^{2+}]_i$ from 120 nm to 150 nm or 185 nM respectively [12]. Figure 1 shows that in the absence of T_3 such an increase in the $[Ca^{2+}]_i$ slightly decreased the basal expression of SERCA1 protein. This $[Ca^{2+}]_i$ dependence was much more pronounced in cultures grown in the presence of T_3, confirming previous results [12]. In cells with a $[Ca^{2+}]_i$ of 120 nM, T_3 increased SERCA1 expression to reach a 3.8-fold stimulation compared with controls at day 3 (3.8 ± 0.5, mean $\pm$ S.E.M., $n = 4$; $P < 0.05$). However, elevation of the $[Ca^{2+}]_i$ to 185 nM significantly decreased this effect of T_3 to a 1.5-fold stimulation (1.5 ± 0.1, mean $\pm$ S.E.M., $n = 4$; $P < 0.01$) (see also Figure 3).

To investigate whether $[Ca^{2+}]_i$ interferes with the T_3-stimulated expression of SERCA1 at a pre-translational or a post-translational level, we quantified SERCA1 mRNA by Northern blot analysis with RNA isolated from parallel cultures of the experiments presented above. The expression of SERCA1 mRNA gradually increased to reach its maximal level at day 3, in line with the protein results presented in Figure 1. Also the stimulation of SERCA1 mRNA expression by T_3 was maximal at day 3. This pre-translational effect of T_3 proved to be dependent on $[Ca^{2+}]_i$ (Table 1). This is illustrated in Figure 2, which depicts a typical Northern blot of RNA isolated from cultures maintained at 120, 150 or 185 nM $[Ca^{2+}]_i$ in the absence or presence of T_3, and

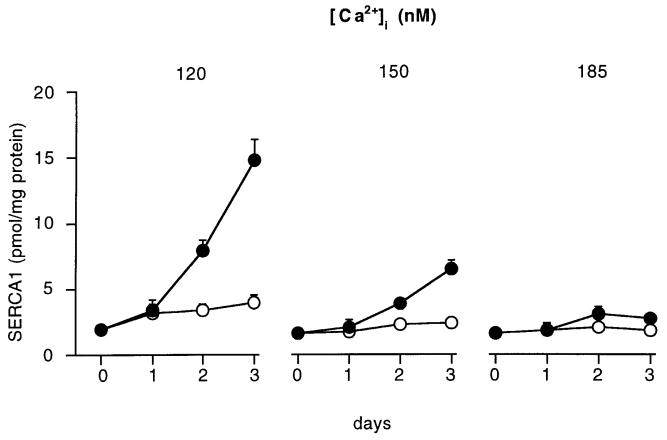

Figure 1 Effect of variation of $[Ca^{2+}]_i$ on SERCA1 expression in L6 myotubes

T_3 treatment and variation of $[Ca^{2+}]_i$ was started at day 0 as described in the Materials and methods section. SERCA1 content was determined in homogenates by competitive ELISA and expressed relative to the total protein content, which was not affected by T_3 treatment or variation of $[Ca^{2+}]_i$. Results are means $\pm$ S.E.M. for four independent experiments. Symbols: ○, control, no additions; ●, 5 nM T_3 added at day 0. Analysis of variance revealed significant effects of T_3 at all $[Ca^{2+}]_i$. An increase in $[Ca^{2+}]_i$ above 120 nM significantly decreased SERCA1 expression in the presence or absence of T_3.

Table 1 Effect of variation of $[Ca^{2+}]_i$ on the stimulation of SERCA1 mRNA expression by T_3

Data represent the combined results of four independent experiments in which $[Ca^{2+}]_i$ was varied between 120 and 185 nM. T_3 stimulations of SERCA1 mRNA expression, as determined by Northern blot analysis (see Figure 2), are shown as means $\pm$ S.E.M. for four individual experiments. T_3 stimulation of SERCA1 mRNA at day 0 and day 1 could not be quantified in all experiments owing to low signals. At both days 2 and 3, T_3 stimulation of SERCA1 mRNA was statistically significant at all $[Ca^{2+}]_i$ ($P < 0.05$). Asterisks denote significant differences ($P < 0.05$) of T_3 stimulations of SERCA1 mRNA between 120 nM and higher $[Ca^{2+}]_i$ as determined by Student's paired t test.

		T_3 induction ratio		
Day	$[Ca^{2+}]_i$ (nM) ...	120	150	185
2		6.7 ± 0.8	3.2 ± 0.8	2.3 ± 0.6*
3		9.7 ± 2.2	6.1 ± 1.2*	4.3 ± 1.6*

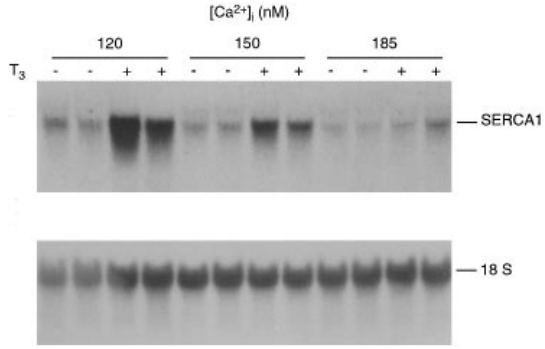

Figure 2 Autoradiogram of a Northern blot of total RNA from L6 myotubes with different $[Ca^{2+}]_i$ values

Two days after the induction of differentiation, $[Ca^{2+}]_i$ was varied by the addition of ionomycin to the culture medium and T_3 treatment was started (day 0). Total RNA isolated at day 3 (40 μg) was subjected to electrophoresis, blotted and probed with a rat SERCA1 cDNA probe as described in the Materials and methods section. The blot was stripped and reprobed with an 18 S rRNA hybridizing oligonucleotide.

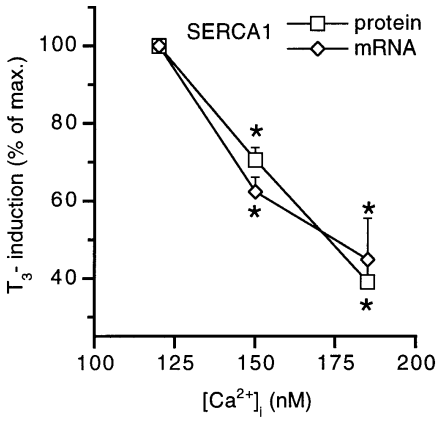

Figure 3 Effect of variation of the $[Ca^{2+}]_i$ on the T_3 stimulation of SERCA1 protein and mRNA expression at day 3 after onset of T_3 treatment and establishment of $[Ca^{2+}]_i$

Data represent the combined results of four independent experiments in which $[Ca^{2+}]_i$ was varied between 120 and 185 nM. T_3 stimulation of SERCA1 protein expression was calculated as the mean T_3 stimulation in four individual experiments (see Figure 1). T_3 stimulation of SERCA1 mRNA expression, as determined by Northern blot analysis (see Figure 2), was calculated as the mean T_3 stimulation of SERCA1 mRNA expression in four individual experiments. Stimulations are plotted as percentages of the maximal stimulation reached at a $[Ca^{2+}]_i$ of 120 nM for both SERCA1 protein and mRNA. T_3 stimulation of SERCA1 protein and mRNA was statistically significant at all $[Ca^{2+}]_i$ values ($P < 0.05$). Asterisks denote significant differences ($P < 0.05$) of T_3 stimulations of either protein or mRNA between 120 nM and higher $[Ca^{2+}]_i$ as determined by Student's paired t test.

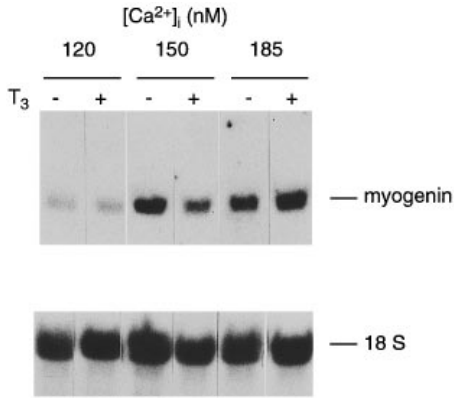

Figure 4 Autoradiogram of a Northern blot of total RNA from L6 myotubes with different $[Ca^{2+}]_i$ values

Two days after the induction of differentiation, $[Ca^{2+}]_i$ was varied by addition of ionomycin to the culture medium and T_3-treatment was started (day 0). Total RNA isolated at day 3 (30 μg) was subjected to electrophoresis, blotted and probed with a mouse myogenin cDNA probe as described in the Materials and methods section. The blot was stripped and reprobed with an 18 S rRNA hybridizing oligonucleotide.

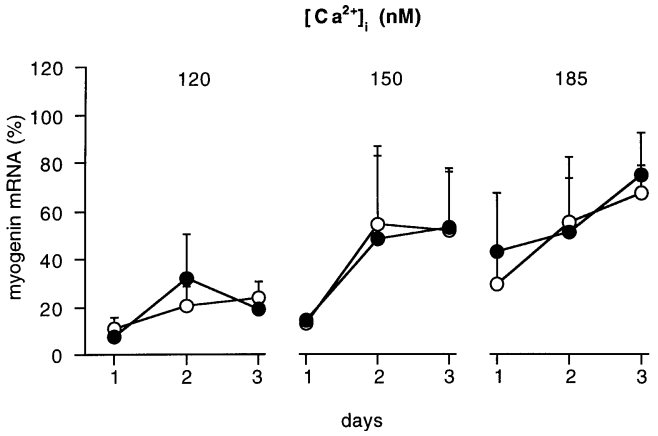

Figure 5 Effect of $[Ca^{2+}]_i$ on the expression of myogenin mRNA in L6 myotubes

T_3 treatment and variation of $[Ca^{2+}]_i$, as described in the Materials and methods section, were started at day 0. The expression of myogenin mRNA was determined by Northern blot analysis (see also Figure 4) and normalized to the amount of 18 S rRNA in each sample. Total RNA was not significantly affected by variation of $[Ca^{2+}]_i$. Results are means $\pm$ S.E.M. for three experiments in duplicate, and are shown as percentages of the level at day 0 (100%). Symbols: ●, 5 nM T_3 added at day 0; ○, no T_3 added. Data were subjected to analysis of variance, which revealed a significant effect of $[Ca^{2+}]_i$ on myogenin mRNA expression both in the presence and in the absence of T_3. T_3 did not affect myogenin mRNA expression at any $[Ca^{2+}]_i$.

probed for SERCA1 mRNA and 18 S rRNA. The combined results of four independent experiments are presented in Figure 3. In this graph the stimulation of both SERCA1 protein and mRNA by T_3 is shown as a function of $[Ca^{2+}]_i$ and normalized to the maximal stimulation at 120 nM. The correlation between the $[Ca^{2+}]_i$ dependence of stimulation of protein and mRNA by T_3 implies that $[Ca^{2+}]_i$ interferes with the action of T_3 at a pre-translational level.

There was no significant effect of treatment with T_3 or ionomycin on total protein content, on total RNA content, on myotube formation or on myotube morphology.

$[Ca^{2+}]_i$ and MDF mRNA expression

Next we investigated the possible $[Ca^{2+}]_i$ dependence of the expression of myogenin, MyoD and myf5 in L6 cells. Although L6 cells do not normally express MyoD [22,23], we checked whether it became detectable when cells were grown at a higher $[Ca^{2+}]_i$. myf5 has not been implicated in phenotype-specific gene expression but it can substitute for MyoD [34].

The blots used for the determination of SERCA1 mRNA expression were stripped and hybridized with mouse cDNA probes for myogenin, MyoD and myf5. MyoD expression was not detectable in any culture. A single product of the expected length was identified with myogenin and myf5. However, the expression level of myf5 was just above the detection level and insufficient for quantitative analysis by laser-densitometric scanning of autoradiograms. Nevertheless an increase in the $[Ca^{2+}]_i$ did not seem to alter the low level of expression. Myogenin mRNA, in contrast, was readily detectable and its expression depended on $[Ca^{2+}]_i$. Figure 4 depicts an autoradiogram of a Northern blot showing that 3 days after adjustment of the $[Ca^{2+}]_i$ to 150 or 185 nM the expression of myogenin mRNA was increased significantly compared with that in cells maintained at a $[Ca^{2+}]_i$ of 120 nM. Figure 5 shows the time course of the increase in the myogenin mRNA level. In line with results of Wright et al. [22], myogenin expression quickly returns to a low level after the transient increase associated with differentation. However, in cells with $[Ca^{2+}]_i$ above the resting value of 120 nM, the level of myogenin mRNA did not fully return to a low level and subsequently showed a gradual increase, reaching its maximal value at day 3. At this time the expression of myogenin mRNA in cells with a $[Ca^{2+}]_i$ of 185 nM was 3.8 ± 0.7-fold higher than in cells with a $[Ca^{2+}]_i$ of 120 nM (mean $\pm$ S.E.M for three duplicate experiments; $P < 0.05$). T_3 did not affect the expression of myogenin mRNA (Figure 5).

Effect of myogenin on the stimulation of SERCA1 promoter activity by T_3

Finally, we employed myogenin overexpression in transient transfections to investigate whether this MDF can interfere with T_3-dependent SERCA1 expression, thus accounting for the effect of increased $[Ca^{2+}]_i$ on T_3-dependent SERCA1 expression.

L6 myoblasts were seeded at low density and transfected with SERCA1/pOCAT2 constructs in which SERCA1 promoter fragments drive the CAT reporter gene. Transfections did not affect myoblast morphology. Myogenin levels were increased in transfected L6 cells by co-transfection of the pEMSV–myo8 myogenin expression construct. Low-density cultures were used because endogenous myogenin levels are low under these conditions, whereas confluent, fusing cultures show a transient increase in myogenin expression [22].

Table 2 shows that T_3 induced an almost 4-fold increase in promoter activity, with the full-length SERCA1 5′ flanking region driving the CAT gene (induction ratio 3.7 ± 0.2, mean $\pm$ S.E.M., $n = 11$; $P < 0.001$). Co-transfection with the myogenin expression construct decreased the absolute T_3-dependent increase by more than 50% (induction ratio 2.2 ± 0.1, mean $\pm$ S.E.M., $n = 9$; $P < 0.001$ compared with the ratio in the absence of myogenin).

When the 5′ flanking region was truncated to -141, thereby deleting its thyroid response elements (TREs) and all except one E-box, a marginal stimulation of promoter activity by T_3 remained, which was also slightly decreased by myogenin overexpression. The functional significance of this effect on non-

Table 2 Effect of myogenin overexpression on T_3 induction of SERCA1 promoter activity

L6 myoblasts were transfected and analysed for CAT activity as described in the Materials and methods section. CAT reporter gene expression was driven by either the full-length promoter sequence of the rat SERCA1 gene (pOCAT2/−2658) or the minimal functional promoter (pOCAT2/−141).The SERCA1 fragments had a common 3′ end at +91. In pUTKAT3, CAT expression is driven by the viral thymidine kinase promoter. Results are shown as T_3 induction ratios and are means ± S.E.M. for the number of experiments shown in parentheses. *$P < 0.05$ for myogenin compared with no myogenin, as determined by Student's t test.

	T_3 induction ratio	
SERCA1 fragment	−Myogenin	+Myogenin
pOCAT2/−2658	3.7 ± 0.2 (11)	2.2 ± 0.1 (9)*
pOCAT2/−141	1.3 ± 0.1 (12)	1.1 ± 0.1 (8)*
pUTKAT3	2.5 ± 0.2 (5)	2.2 ± 0.3 (5)

specific T_3 stimulation (TRE-independent) is questionable. However, to exclude the possibility of a general effect of myogenin on T_3-mediated effects, we examined the effect of myogenin overexpression on the induction of the pUTKAT3 vector by T_3 [28]. This plasmid is fully identical with pOCAT2, except that the CAT gene is driven by the viral thymidine kinase promoter, which is T_3-responsive [35]. The 2.5-fold induction with T_3 (2.5 ± 0.2, mean ± S.E.M., $n = 5$; $P < 0.001$) that we observed agrees with previous reports [36,37]; Table 2 shows that this stimulation was not significantly affected by myogenin overexpression ($P = 0.25$; $n = 5$).

Myogenin overexpression had no specific effect on basal SERCA1 promoter activity (results not shown).

DISCUSSION

An increase in $[Ca^{2+}]_i$, as well as increased expression of myogenin, have been suggested to mediate the repressing effect of continuous contractile activity on fast-muscle gene expression [12,19,38–41]. Expression of the fast phenotype is to a large extent dependent on T_3; the results of the present study show, first, that an increase in the $[Ca^{2+}]_i$ above the resting value of 120 nM represses the T_3-dependent expression of a typical fast isoform (SERCA1) at a pre-translational level. Secondly, myogenin was shown to be capable of actively repressing the expression of SERCA1 by interfering with T_3-dependent transcriptional activity. Finally, the observation that an increase in $[Ca^{2+}]_i$ stimulates expression of myogenin mRNA suggests a mechanism for at least part of the contraction-related effects on gene expression in muscle. In this mechanism $[Ca^{2+}]_i$ and MDFs are part of the same regulatory pathway, i.e. an altered level of contractile activity leads to altered $[Ca^{2+}]_i$, leading to altered MDF expression, which leads to altered gene expression. Although these results obtained *in vitro* with a non-contracting muscle cell line preclude firm conclusions, several earlier observations lend support to the proposed scheme.

Recently we have shown that the major part of the repressing effect of continuous contractile activity on SERCA1 expression in C2C12 myotubes relies on interference with T_3-dependent transcription of the SERCA1 gene [14]. The pre-translational modulation of T_3-dependent SERCA1 expression by $[Ca^{2+}]_i$ reported here for non-contracting L6 myotubes suggests that the mechanical component of the excitation–contraction cycle is not necessary for this effect. Earlier, Walke et al. [39] and Huang and Schmidt [40] reported that Ca^{2+} influx across the plasma membrane can mimic the repressing effect of contractile activity on the expression of all nicotine acetylcholine receptor (nAChR) subunit genes in non-contracting myotubes in rat and chicken respectively. The requirement of protein synthesis *de novo* for this effect of $[Ca^{2+}]_i$ implies the involvement of a mediatory factor [42]. An increasing number of studies suggest that this mediatory role is played by one or more members of the MDF family of muscle transcription factors. The stimulation of nAChR expression associated with denervation is preceded by altered expression of myogenin and MyoD [41,43–45] and is dependent on an E-box containing promoter fragment [45–47]. The direct relation between altered $[Ca^{2+}]_i$ and myogenin expression, suggested by our results, is in line with two studies of Adamo and co-workers which showed that arginine-vasopressin markedly increased both $[Ca^{2+}]_i$ and the expression of myogenin [48,49].

Analysis of the expression of MDFs in various types of skeletal muscle led Hughes et al. [19] and Voytic et al. [20] to propose that the expression of myogenin and MyoD determines the slow and fast muscle phenotypes respectively. The effect of myogenin on the expression of the SERCA1 gene observed by us is a first demonstration that a 'slow-specific' MDF can repress the expression of a 'fast' gene. Because L6 cells do not express MyoD ([22,23] and the present study), our results support only one aspect of the proposed role of MDFs in phenotypic regulation, and conclusions about a differential effect of $[Ca^{2+}]_i$ on MyoD and myogenin cannot be drawn However, the only other MDF expressed in L6 cells, myf5 [22,23,50], which can substitute for MyoD, was not affected by an increase in $[Ca^{2+}]_i$: up-regulation of myogenin is therefore not part of a general effect of $[Ca^{2+}]_i$ on MDF expression.

It is noteworthy that we did not observe any effect of T_3 on myogenin expression, because Downes et al. [51] have characterized a fragment with multiple TREs in the promoter region of the mouse myogenin gene. However, a functional role for this element in muscle cells has not yet been shown, which is perhaps not surprising given the proposed antagonistic effects of T_3 and myogenin on muscle gene expression.

Although a detailed analysis of the mechanism by which myogenin affects transcriptional regulation by T_3 was beyond the scope of this study, the absence of an effect of myogenin on the T_3-induction of the viral thymidine kinase promoter rules out the possibility of interference with T_3 receptor (TR) expression or with TR's binding to its TRE. The promoter specificity of the effect implies that sequences within the SERCA1 promoter, e.g. E-boxes, are required for the action of myogenin. It is tempting to speculate that the binding of myogenin to an appropriately situated E-box might interfere with the binding of a recently discovered class of co-activators and co-repressors involved in the interaction between the TR and the transcription–initiation complex [52,53]. In addition, one of the 14 putative E-boxes present in the first 2658 nt of the 5′ flanking region of the SERCA1 gene ([9], and M. H. M. Thelen, W. S. Simonides and C. van Hardeveld, unpublished work) splits the potent TRE at −550 nt in two [9]. This might allow the interference of myogenin with T_3 receptor binding and trans-activation.

In conclusion it seems fitting that the stimulator of the slow phenotype, contractile activity, should repress the fast phenotype by direct interference with the mechanism of action of T_3, the stimulator of the fast phenotype. The co-ordinated regulation by T_3 and contractile activity of the expression of SERCA1 and the 'fast' isoforms of other muscle proteins, such as the myosin heavy chain [4,5], suggests that the mechanism presented here might also be involved in the regulation of other 'fast' muscle genes. This implies that the repression of the fast phenotype is at least partly dependent on the modulation of the transcriptional

effect of T_3. The present study suggests that $[Ca^{2+}]_i$-dependent regulation of the expression of myogenin might play a key role in such regulation of muscle phenotype.

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