Ca²⁺ homeostasis and fast-type sarcoplasmic reticulum Ca²⁺-ATPase expression in L6 muscle cells

Role of thyroid hormone

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

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

The effect of thyroid hormone (L-tri-iodothyronine; T₃) on the cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) in L6 myotubes was studied at rest and during activation to explore the possible mediating role of [Ca²⁺]_i in the T₃-induced net synthesis of fast-type sarcoplasmic reticulum (SR) Ca²⁺-ATPase. The mean [Ca²⁺]_i at rest was approx. 115 nM in myoblasts, control myotubes and T₃-treated myotubes. Therefore it is unlikely that the T₃-induced elevation of Ca²⁺-ATPase levels is mediated by [Ca²⁺]_i changes. To investigate the influence of the 4-fold higher Ca²⁺-ATPase levels in T₃-treated myotubes (compared with controls) on [Ca²⁺]_i, interventions with caffeine (10 mM) and a high extracellular K⁺ concentration ([K⁺]_o) (30 mM) were applied which initially mobilize Ca²⁺ predominantly from the SR. The results showed a lower (caffeine) or not significantly different (high [K⁺]_o) increase in [Ca²⁺]_i in T₃-treated myotubes compared with controls. No rise in [Ca²⁺]_i was found in myoblasts with caffeine or high [K⁺]_o. The role of [Ca²⁺]_i in the regulation of Ca²⁺-ATPase levels was investigated by varying [Ca²⁺]_i through exposure of cells to different concentrations of extracellular Ca²⁺ (0.2–1.8 mM) and ionomycin (0.1–0.25 μM). At subnormal [Ca²⁺]_i (55 nM) the T₃-induced net synthesis of Ca²⁺-ATPase was virtually abolished, and at supranormal [Ca²⁺]_i (195 nM) it was greatly depressed. Intermediate stimulation of net Ca²⁺-ATPase synthesis was found at [Ca²⁺]_i of 95 and 165 nM, with an optimum at approx. 125 nM. Similar but less pronounced effects were found for the basal Ca²⁺-ATPase levels. In contracting primary rat myotubes, Ca²⁺-ATPase levels were significantly lower than in tetrodotoxin-arrested myotubes. The same results were obtained in the presence of T₃. Since the mean [Ca²⁺]_i in contracting cells is higher than in resting cells, these data agree with those obtained in the L6 cells with ionomycin. A major conclusion of this study is the existence of a [Ca²⁺]_i optimum, near resting levels, for the expression of the fast-type Ca²⁺-ATPase in the L6 muscle cell line.

INTRODUCTION

Sarcoplasmic reticulum (SR) Ca²⁺-ATPase is one of the principal regulators of Ca²⁺ homeostasis in skeletal muscle cells. It is responsible for the removal of Ca²⁺ from the cytosol during a contraction–relaxation cycle, thereby returning the cytosolic Ca²⁺ to its original resting levels. Changes in the level of Ca²⁺-ATPase will therefore profoundly affect Ca²⁺ homeostasis, and consequently contractility and energy turnover [1]. It has been well documented that thyroid status and neural activity are major determinants of Ca²⁺-ATPase levels *in vivo* [1,2]. Changes in Ca²⁺-ATPase levels underlie the well-known alterations in relaxation rate of the muscle in different thyroid states [3–7]. Recently we have shown that thyroid hormone (L-tri-iodothyronine, T₃) increases the level of fast-type SR Ca²⁺-ATPase and the Ca²⁺-uptake activity of the SR in the L6 muscle cell line, and that these effects were much greater in the presence of insulin-like growth factor-I [8]. In view of these findings, we addressed the question of how these T₃-related changes in Ca²⁺-ATPase levels are reflected in the cytosolic free Ca²⁺ concentration ([Ca²⁺]_i) during rest and activation.

Directly related to this question is the putative role of [Ca²⁺]_i in the regulation of Ca²⁺-ATPase expression [9,10]. In chicken primary myotubes that were fusion-arrested by culturing in medium with low [Ca²⁺] (< 0.3 mM), the increased synthesis of

Ca²⁺-ATPase, which occurs during normal development, was inhibited [11]. However, in a study using rat primary myoblasts which were fusion-arrested by a low Ca²⁺-medium, Ca²⁺-ATPase synthesis was shown to increase normally [12]. Evidence also exists that interventions which increase Ca²⁺ influx (ionomycin, ionophore A23187) also elevate the level of a 100 kDa protein, the putative Ca²⁺-ATPase in chicken primary myotubes [13].

In view of these interesting but by no means unambiguous data, we searched for a role for [Ca²⁺]_i in the regulation of the net synthesis of Ca²⁺-ATPase (fast isoform) in the presence and absence of T₃, firstly by manipulating [Ca²⁺]_i in L6 cells with low-Ca²⁺ media and the Ca²⁺ ionophore ionomycin, and secondly by comparing contracting and tetrodotoxin (TTX)-arrested primary rat myotubes. [Ca²⁺]_i was monitored by single cell epifluorescence microscopy. These experiments were carried out in maximally fused cell cultures, thereby eliminating the effects of Ca²⁺ on fusion-related expression of proteins, including possibly Ca²⁺-ATPase. Insulin, serving as an insulin-like growth factor-I analogue [14], was added to the culture medium to maximize the T₃ stimulation of net Ca²⁺-ATPase synthesis.

This study provides strong evidence for the existence of a [Ca²⁺]_i optimum (around 125 nM) for the expression of the fast isoform of SR Ca²⁺-ATPase in the L6 muscle cell line, but does not point to a mediatory role for [Ca²⁺]_i in the induction of net synthesis of Ca²⁺-ATPase by T₃ in resting L6 muscle cells.

Abbreviations used: ANOVA, analysis of variance; AraC, 1-(β-D-arabinofuranosyl)cytosine; Ca²⁺-ATPase, Ca²⁺-dependent Mg²⁺-ATPase from sarcoplasmic reticulum (EC 3.6.1.38); [Ca²⁺]_i, cytosolic free Ca²⁺ concentration; [Ca²⁺]_o, extracellular Ca²⁺ concentration (similarly for K⁺); DMEM, Dulbecco's modified Eagle's medium; HS, horse serum; FCS, fetal calf serum; SR, sarcoplasmic reticulum; TTX, tetrodotoxin; T₃, L-tri-iodothyronine (thyroid hormone); T₃S, T₃-depleted FCS.

† To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Fetal calf serum (FCS), horse serum (HS) and Dulbecco's modified Eagle's medium (DMEM) were obtained from Flow Laboratories (Irvine, Scotland, U.K.). Insulin was purchased from Serva (Heidelberg, Germany). 1-(β -D-Arabinofuranosyl)-cytosine (AraC) and T_3 were obtained from Sigma (St. Louis, MO, U.S.A.), Fura-2/AM and pluronic F-127 were from Molecular Probes (Eugene, OR, U.S.A.); caffeine was from Brocacef (Maarsse, The Netherlands); ionomycin was from Calbiochem (San Diego, CA, U.S.A.), TTX was from Janssen (Beerse, Belgium); and AG-1-X8 anion-exchange resin was from Bio-Rad (Utrecht, The Netherlands).

Media

For the preparation of media with different extracellular Ca^{2+} concentrations ($[Ca^{2+}]_o$), DMEM was reconstituted from ingredients present in commercially available DMEM except for Ca^{2+} . $[Ca^{2+}]_o$ was brought to the desired concentration by adding EGTA (50 μ M) and different amounts of $CaCl_2$ from a stock solution. $[Ca^{2+}]_o$ was checked using a calibrated F2112 Ca^{2+} -selective electrode. T_3 -depleted FCS (T_3S) was obtained by AG-1-X8 resin treatment of FCS [15], and Ca^{2+} was removed from T_3S by exhaustive dialysis against 8.4 mM- Na_2HPO_4 /2.2 mM- NaH_2PO_4 /145 mM-NaCl, pH 7.35. Ca^{2+} -deficient DMEM (referred to as $DMEM_{CD}$) and Ca^{2+} -deficient T_3S (referred to as T_3S_{CD}) were sterilized by filtration through a 0.45 μ m pore size Millipore filter.

Cell culture

A subclone, L6_{AM}, of the rat myogenic cell line L6 was used in this study [16]. Cultures consigned to be used for Ca^{2+} -ATPase measurements by e.l.i.s.a. were plated in 25 cm² tissue culture flasks, whereas cultures used for $[Ca^{2+}]_i$ measurements were seeded on 25 mm coverslips, which were placed in 6-well tissue culture plates. The experiments with high $[K^+]_o$ and caffeine were designed as follows. The cells were grown to confluency on DMEM containing 10% (v/v) T_3S . Next, the medium was changed to DMEM containing 2.5% (v/v) T_3S and 1 μ g of insulin/ml, with or without T_3 (5 nM), and 6 or 7 days later, when the difference in Ca^{2+} -ATPase levels between controls and T_3 -treated cultures was maximal, the cells were used for $[Ca^{2+}]_i$ measurements. Cultures consisting of undifferentiated myoblasts were obtained by growing cells to confluency on DMEM containing 10% (v/v) T_3S .

In the experiments with variable $[Ca^{2+}]_i$, the following protocol was used. The cells were grown to confluency on DMEM containing 10% (v/v) T_3S . Next, the cultures were switched to $DMEM_{CD}$ containing 2.5% (v/v) T_3S_{CD} , 50 μ M-EGTA and 1.8 mM- $[Ca^{2+}]_o$, supplemented with 1 μ g of insulin/ml. After 4 days, when fusion was complete, the cultures were changed to $DMEM_{CD}$ containing 2.5% (v/v) T_3S_{CD} , 50 μ M-EGTA, 1 μ g of insulin/ml and 0.2, 0.4 or 1.8 mM- $[Ca^{2+}]_o$, with or without T_3 (5 nM). In the experiments using ionomycin, cells were grown to confluency on DMEM containing 10% (v/v) T_3S . Next, the cultures were switched to DMEM containing 2.5% (v/v) T_3S and 1 μ g of insulin/ml, and when fusion was maximal (after 4 days) ionomycin was added to a final concentration of 0.1 or 0.25 μ M (control cultures received no additions), and at the same time treatment with T_3 (5 nM) was started. The medium was changed every day to minimize possible changes in the ionomycin concentration during culturing. In all the experiments,

AraC (4 μ g/ml) was added to the medium from 2 days after switching to DMEM containing 2.5% (v/v) T_3S .

Primary myoblasts were prepared from 2-day-old neonatal rats essentially as described by Koningsberg [17]. Cells were seeded at a density of 2×10^3 cells/cm² on collagen-coated flasks, and were grown to confluency on DMEM containing 10% (v/v) HS and 5% (v/v) FCS. After 3 days, cells were switched to DMEM containing 10% (v/v) HS, with or without added T_3 (5 nM) and TTX (10 μ M).

E.l.i.s.a.

For the determination of Ca^{2+} -ATPase levels by e.l.i.s.a., cell cultures were washed twice with 8.4 mM- Na_2HPO_4 /2.2 mM- NaH_2PO_4 /145 mM-NaCl, pH 7.35, then covered with 300 μ l of this solution and frozen at -20°C . After thawing, the cells were harvested and stored at -20°C until analysis. A specific monoclonal antibody, A52, raised against the fast isoform of SR Ca^{2+} -ATPase from rabbit [18] but also specific for the rat fast isoform [8], was used to quantify Ca^{2+} -ATPase in muscle cell homogenates by competitive e.l.i.s.a. [8]. The protein content was determined by the method of Lowry *et al.* [19], using BSA as a standard.

Fluorescence measurements

Cells were loaded with Fura-2 for 15 min at 37°C by incubation with 2 μ M-Fura-2AM from a 1 mM stock in dimethyl sulphoxide containing 20% (w/v) pluronic F-127 in the same medium in which they had been cultured. The cells were washed three times prior to measurement. Coverslips with loaded cells were transferred to a temperature-controlled chamber [20] mounted on a microscope stage and superfused with measuring solution at a rate of 3 μ l/s at 37°C . Fluorescence measurements were carried out with a modified Zeiss IM inverted microscope suitable for dual wavelength excitation (340, 380 nm) and measurement of fluorescence emission (510 nm) as described earlier [21].

The following measuring solutions were used: DMEM containing 2.5% (v/v) FCS; Hepes buffer containing 20 mM-Hepes, 118 mM-NaCl, 12 mM- $NaHCO_3$, 2.6 mM-KCl, 1.2 mM- KH_2PO_4 , 1.2 mM- $MgSO_4$, 1 mM- $CaCl_2$, 10 mM-glucose, pH 7.4, supplemented with 2.5% (v/v) FCS; and hepes buffer without $CaCl_2$, supplemented with 50 μ M-EGTA, 2.5% (v/v) T_3S_{CD} and Ca^{2+} titrated to final concentrations of 0.2, 0.4 and 1.8 mM. In experiments with ionomycin, the medium in which the cells had been cultured was used as measuring solution.

Calibration

Intracellular calibration was performed essentially as described in [21]. In order to measure the maximal Fura-2 fluorescence at 340 and 380 nm excitation wavelengths, $[Ca^{2+}]_i$ was raised by exposing the cells to Hepes buffer (without glucose) containing 3 mM- $CaCl_2$, 10 μ M-ionomycin, 2 μ M of the mitochondrial uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazine (FCCP) and 5 mM of the glycolysis blocker iodoacetic acid. The presence of the latter two components was required to deplete the cell of energy-rich compounds to such an extent that a stable R_{max} was reached. For measurement of the minimal Fura-2 fluorescence, $[Ca^{2+}]_i$ was depleted by exposing the cells to Hepes buffer containing 10 μ M-ionomycin and 10 mM-EGTA. $[Ca^{2+}]_i$ was calculated from the ratio (R) of the fluorescence at the two wavelengths of excitation (340 and 380 nm) using the equation:

$$[Ca^{2+}] = K_d (R^{340}_{380} - R^{340}_{380min} / R^{340}_{380max} - R^{340}_{380}) \times (F^{380}_{min} / F^{380}_{max})$$

R_{max} and R_{min} are the values of R obtained under conditions of

Fig. 1. Effect of T₃ on Ca²⁺-ATPase levels in differentiating myoblasts

Cells were grown to confluency on DMEM containing 10% (v/v) T₃S. Medium was then replaced by 2.5% (v/v) T₃S containing 1 μg of insulin/ml, with or without T₃ (5 nM) (day 0). ○, Control; ●, T₃. Ca²⁺-ATPase was determined in homogenates by competitive c.l.i.s.a. Data represent the means ± S.E.M. of four experiments.

Fig. 2. Effect of caffeine on [Ca²⁺]_i in myoblasts, control myotubes and T₃-treated myotubes

Cultures were grown to confluency on DMEM containing 10% (v/v) T₃S (myoblasts), after which the medium was replaced by DMEM containing 2.5% (v/v) T₃S and 1 μg of insulin/ml (control myotubes) or DMEM containing 2.5% (v/v) T₃S, 1 μg of insulin/ml and 5 nM-T₃ (T₃-treated myotubes) and the cells were grown for 6 or 7 more days. The cells were loaded with Fura-2 and transferred to the fluorescence microscope for [Ca²⁺]_i measurements (see the Materials and methods section). After equilibration for 10 min there was a rapid switch to medium containing caffeine (10 mM) (*t* = 0) and the changes in the 340 and 380 nm fluorescence signals were recorded at 1 s time intervals. [Ca²⁺]_i values were normalized to [Ca²⁺]_i at *t* = 0 (100%). △, Myoblasts; ○, control myotubes; ●, T₃-treated myotubes. Data represent the means ± S.E.M. of five experiments and were analysed by ANOVA for repeated experiments. Significance of differences: myoblasts versus control myotubes, *P* < 0.005; myoblasts versus T₃-treated myotubes, *P* < 0.04; control myotubes versus T₃-treated myotubes, *P* < 0.04.

saturating Ca²⁺ and without Ca²⁺ respectively. $F_{380\text{ min.}}^{380}/F_{380\text{ max.}}^{380}$ is the ratio of the 380 nm fluorescence signals of Fura-2 without Ca²⁺ and with saturating Ca²⁺. The *K_d* (220 nM) was ex-

Fig. 3. Effect of 30 mM-[K⁺]_o on [Ca²⁺]_i in myoblasts, control myotubes and T₃-treated myotubes

Myoblasts and myotubes with or without T₃ were cultured and loaded with Fura-2 for [Ca²⁺]_i measurements as described in the legend to Fig. 2. After equilibration for 10 min there was a rapid switch to medium containing high [K⁺]_o (30 mM) (*t* = 0), and the changes in 340 and 380 nm fluorescence signals were recorded at 1 s time intervals. [Ca²⁺]_i values were normalized to [Ca²⁺]_i at *t* = 0 (100%). △, Myoblasts; ○, control myotubes; ●, T₃-treated myotubes. Data represent the means ± S.E.M. of ten experiments, and were analysed by ANOVA for repeated experiments. Significance of differences: myoblasts versus control myotubes, *P* < 0.005; myoblasts versus T₃-treated myotubes, *P* < 0.005; control myotubes versus T₃-treated myotubes, *P* < 0.29 (not significant).

perimentally determined according to Grynkiewicz [22]. The obtained values were (means ± S.D.): $R_{\text{min.}}$, 0.16 ± 0.02 (*n* = 7); $R_{\text{max.}}$, 2.76 ± 0.25 (*n* = 6); $F_{380\text{ min.}}^{380}/F_{380\text{ max.}}^{380}$, 7.

Statistics

Data were evaluated by analysis of variance (ANOVA) [23] or Student's paired *t* test. Differences were considered significant at *P* < 0.05.

RESULTS

[Ca²⁺]_i at rest

Unless stated otherwise, [Ca²⁺]_i measurements were carried out in HEPES-buffered solution instead of DMEM (in which the cells were grown), since the HEPES buffer showed a 4-fold lower autofluorescence. Accordingly, the effect of medium composition on resting [Ca²⁺]_i was first studied. In DMEM, resting [Ca²⁺]_i was 105 ± 6 nM (mean ± S.E.M., *n* = 5) in myoblasts and 118 ± 8 nM (*n* = 5) in myotubes. Similar values were also found in HEPES buffer, i.e. 104 ± 7 nM (*n* = 20) and 103 ± 6 nM (*n* = 20) in myoblasts and myotubes respectively. From this it was concluded that HEPES buffer could be used in further [Ca²⁺]_i measurements, and also that [Ca²⁺]_i levels did not differ between myoblasts and myotubes. In addition, it was shown that there was no effect of T₃ treatment on resting [Ca²⁺]_i levels in myotubes (103 ± 9 nM, *n* = 15).

[Ca²⁺]_i during activation

We addressed the question how the T₃-induced increase in Ca²⁺-ATPase levels (Fig. 1) influenced [Ca²⁺]_i during activation. For this purpose, two interventions were used to mobilize Ca²⁺ from the SR: caffeine (10 mM) and high [K⁺]_o (30 mM). Caffeine caused an increase in [Ca²⁺]_i in untreated myotubes (controls) and in T₃-treated myotubes, which persisted for at least 30 s (Fig.

Table 1. Effect of $[Ca^{2+}]_o$ and ionomycin on $[Ca^{2+}]_i$

In expt. A, cells were grown as described in the legend to Fig. 4. $[Ca^{2+}]_i$ was measured 3 days after switching to medium with various $[Ca^{2+}]_o$. Measurements were carried out in Hepes buffer without $CaCl_2$, supplemented with 50 μM -EGTA, 2.5% T_xS_{CD} and Ca^{2+} titrated to final concentrations of 0.2, 0.4 and 1.8 mM. $[Ca^{2+}]_i$ after 3 days of exposure to media with different $[Ca^{2+}]_o$ was not different from the $[Ca^{2+}]_i$ immediately following the change of medium. Data represent means $\pm$ S.E.M. of four experiments. Data were analysed by Student's paired *t* test. * $P < 0.05$ and ** $P < 0.005$ versus 1.8 mM- $[Ca^{2+}]_o$. In expt. B, cells were grown under conditions as described in the legend to Fig. 5. $[Ca^{2+}]_i$ was measured 24 h after adding fresh ionomycin, at days 1, 2 and 3. Measurements were carried out in the medium on which the cells had been cultured. $[Ca^{2+}]_i$ values were $34 \pm 9\%$ (mean $\pm$ S.E.M., $n = 21$) (0.1 μM) and $47 \pm 14\%$ ($n = 21$) (0.25 μM) higher when measured immediately after adding ionomycin. The initial and final values were averaged. Data represent means $\pm$ S.E.M. of four experiments, and were analysed by Student's paired *t* test: * $P < 0.05$ and ** $P < 0.01$ versus control (no ionomycin).

Experimental series	$[Ca^{2+}]_o$ (mM)	Ionomycin (μM)	$[Ca^{2+}]_i$ (nM)	
			Control	T_3
A	1.8	—	138 ± 8	133 ± 4
	0.4	—	$101 \pm 5^*$	$105 \pm 6^*$
	0.2	—	$55 \pm 3^{**}$	$60 \pm 6^{**}$
B	1.8	—	117 ± 10	116 ± 12
	1.8	0.1	$157 \pm 16^{**}$	$148 \pm 14^*$
	1.8	0.25	$187 \pm 14^{**}$	$181 \pm 12^*$

Fig. 4. Effect of $[Ca^{2+}]_o$ on Ca^{2+} -ATPase levels in myotubes

Cells were grown to confluency on DMEM containing 10% (v/v) T_xS . The medium was then changed to DMEM_{CD} containing 2.5% (v/v) T_xS_{CD} , 1 μg of insulin/ml, 50 μM -EGTA and 1.8 mM- $[Ca^{2+}]_o$. After fusion was complete, cells were changed to DMEM_{CD} containing 2.5% (v/v) T_xS_{CD} , 1 μg of insulin/ml and 50 μM EGTA, containing (a) 1.8, (b) 0.4 or (c) 0.2 mM- $[Ca^{2+}]_o$, with or without T_3 (5 nM) (day 0). $\circ$, Control; $\bullet$, T_3 . Ca^{2+} -ATPase was determined in homogenates by competitive e.l.i.s.a. Data represent means $\pm$ S.E.M. of four experiments. Data were analysed by Student's paired *t* test. Control versus T_3 : 1.8 mM, $P < 0.02$ (days 2 and 3); 0.4 mM, $P < 0.05$ (days 1 and 3). Control (1.8 mM) versus control (0.2 mM), $P < 0.02$ (day 2); T_3 (1.8 mM) versus T_3 (0.2 mM), $P < 0.02$ (days 1, 2 and 3).

2). However, the increase in $[Ca^{2+}]_i$ was significantly smaller in T_3 -treated myotubes than in the controls. Caffeine induced a significant decrease in $[Ca^{2+}]_i$ in myoblasts.

Exposure of cultures to high $[K^+]_o$ (30 mM) evoked a transient increase in $[Ca^{2+}]_i$ in T_3 -treated myotubes and in controls (Fig.

Fig. 5. Effect of ionomycin on Ca^{2+} -ATPase levels in myotubes

Cells were grown to confluency on DMEM containing 10% (v/v) T_xS . The medium was then changed to DMEM containing 2.5% (v/v) T_xS and 1 μg of insulin/ml. When fusion was complete, cells were changed to DMEM containing 2.5% (v/v) T_xS and 1 μg of insulin/ml, with or without T_3 (5 nM) and ionomycin [no ionomycin (a), 0.1 (b) or 0.25 μM (c)] (day 0). $\circ$, Control; $\bullet$, T_3 . Ca^{2+} -ATPase was determined in homogenates by competitive e.l.i.s.a. Data represent means $\pm$ S.E.M. of four experiments. Data were analysed by Student's paired *t* test. Control versus T_3 : without ionomycin, $P < 0.01$ (days 2 and 3); 0.1 μM , $P < 0.025$ (days 2 and 3); 0.25 μM , $P < 0.05$ (days 2 and 3). Control without ionomycin versus control 0.25 μM -ionomycin, $P < 0.01$ (day 3); T_3 without ionomycin versus T_3 0.25 μM -ionomycin, $P < 0.025$ (days 2 and 3).

3). In general, the response reached a peak within 8 s. Again the mean response to high $[K^+]_o$ was greater in untreated myotubes compared with T_3 -treated cultures, although the difference was not significant ($P = 0.29$) due to a large scatter of the results. Addition of 30 mM- $[K^+]_o$ had no effect on $[Ca^{2+}]_i$ in myoblasts.

Effect of subnormal $[Ca^{2+}]_i$ on Ca^{2+} -ATPase levels

Cells were exposed to various $[Ca^{2+}]_o$ from the time-point that fusion was complete. Lowering $[Ca^{2+}]_o$ from 1.8 to 0.4 or 0.2 mM resulted in a decrease in $[Ca^{2+}]_i$ immediately after switching the medium, which persisted during the period of the experiment (Table 1, expt. A). No significant effect of T_3 on $[Ca^{2+}]_i$ was observed. Fig. 4 shows that at $[Ca^{2+}]_o$ of 1.8 mM, Ca^{2+} -ATPase levels were increased by T_3 by 125% at day 3; the increase was 90% in myotubes grown at 0.4 mM- $[Ca^{2+}]_o$. In myotubes grown at 0.2 mM- $[Ca^{2+}]_o$, no significant effect of T_3 on Ca^{2+} -ATPase levels was observed. The Ca^{2+} -ATPase levels in control myotubes (without T_3) grown at 0.2 mM- $[Ca^{2+}]_o$ were significantly lower than the levels in the control myotubes cultured at 0.4 or 1.8 mM- $[Ca^{2+}]_o$ at day 2.

Total protein levels in myotubes cultured at 0.2 mM- $[Ca^{2+}]_o$ were 30% lower than in myotubes grown at 1.8 or 0.4 mM- $[Ca^{2+}]_o$. Protein levels in control myotubes and T_3 -treated myotubes were the same at all $[Ca^{2+}]_o$ values.

Effect of supranormal $[Ca^{2+}]_i$ on Ca^{2+} -ATPase levels

In order to elevate $[Ca^{2+}]_i$, cells were exposed to various concentrations of ionomycin. $[Ca^{2+}]_i$ significantly increased in the presence of ionomycin (0.1 and 0.25 μM) at 1.8 mM- $[Ca^{2+}]_o$ (Table 1, expt. B). No significant effect of T_3 on $[Ca^{2+}]_i$ was observed. Fig. 5 shows that, in the absence of ionomycin, Ca^{2+} -ATPase levels were increased by T_3 by 165% at day 3. This increase was less in the presence of 0.1 μM -ionomycin. In the presence of 0.25 μM -ionomycin, T_3 still increased Ca^{2+} -ATPase levels, but to much lower levels than in the absence of ionomycin. In the control myotubes (without T_3) Ca^{2+} -ATPase levels were

Fig. 6. Ca²⁺-ATPase levels at subnormal and supranormal [Ca²⁺]_i

Ca²⁺-ATPase levels at subnormal and supranormal [Ca²⁺]_i at day 3 (derived from the averaged data presented in Figs. 4 and 5) as a function of [Ca²⁺]_i (derived from the averaged data presented in Table 1). ○, Control; ●, T₃.

Fig. 7. Effect of muscle cell contractility on Ca²⁺-ATPase levels

Primary rat myoblasts were isolated as described in the Materials and methods section. Cells were grown to confluency in DMEM containing 10% (v/v) HS and 5% (v/v) FCS. Cells were then switched to DMEM containing 10% (v/v) HS, with or without T₃ (5 nM), and TTX (10 μM). ○, Control; ●, TTX; △, T₃; ▲, T₃ + TTX. Ca²⁺-ATPase was determined in homogenates by competitive e.l.i.s.a. An example is given of one of two qualitatively similar experiments. Each point was determined in duplicate.

only decreased significantly when grown in medium with 0.25 μM-ionomycin. Total protein levels were affected neither by 0.1 μM-ionomycin nor by 0.25 μM-ionomycin. No effect of T₃ on total protein levels was observed.

The effects of sub- and supra-normal [Ca²⁺]_i on Ca²⁺-ATPase levels are summarized in Fig. 6, which shows that there is an optimum [Ca²⁺]_i at approx. 125 nM for the basal and T₃-regulated Ca²⁺-ATPase levels.

Effect of muscle cell contractility on Ca²⁺-ATPase levels

Primary rat myotubes, which in contrast with L6 myotubes show spontaneous contractions, were used as a model to test the finding that an elevation of [Ca²⁺]_i above the resting concentration diminishes Ca²⁺-ATPase levels. Fig. 7 shows that when contractions were arrested by TTX, Ca²⁺-ATPase levels after 7

days of culture were twice as high compared with the controls. This was also observed in the presence of T₃. Ca²⁺-ATPase levels remained higher in the presence of T₃ in both contracting and TTX-arrested cells.

DISCUSSION

Effect of T₃ on [Ca²⁺]_i at rest and during activation

[Ca²⁺]_i at rest is ultimately determined by the influx-efflux balance across the plasma membrane. In spite of considerable changes in the amounts of Ca²⁺ channels in the plasma membrane during differentiation of myoblasts to myotubes [24], we found no difference in [Ca²⁺]_i in resting myotubes or myoblasts. This indicates that during muscle cell differentiation the development of Ca²⁺-influx and -efflux pathways are in balance, at least in L6 cells.

Our [Ca²⁺]_i values of approx. 115 nM in resting myoblasts and myotubes agree well with those found in primary rat myotubes [25] and primary chick myotubes [26]. In earlier studies with adult myocytes [21] and liver cells [27], we observed that resting [Ca²⁺]_i was dependent on thyroid status, generally increasing in the direction hypothyroid → hyperthyroid. In the L6 muscle cell line we found no effect of T₃ on resting [Ca²⁺]_i, implying that effects of T₃ on resting [Ca²⁺]_i are cell-type-specific. From this it also follows that the possibility can be excluded that the induction by T₃ of net Ca²⁺-ATPase synthesis at a [Ca²⁺]_o of 1.8 mM in L6 cells is (partly) mediated by changes in [Ca²⁺]_i.

The T₃-induced proliferation of the SR in rat skeletal muscle has been put forward as a possible explanation for the increasing rise in metabolic activity upon caffeine and high [K⁺]_o depolarization [28] that is observed in the direction hypothyroid → hyperthyroid. It was suggested that the increased capacity for Ca²⁺ storage would enhance the magnitude of Ca²⁺ release, thereby possibly leading to a higher [Ca²⁺]_i during activation. The results obtained in the present study do not support this explanation. The increase in [Ca²⁺]_i in myotubes upon exposure to caffeine or high [K⁺]_o indicates that an intact SR organelle has developed, serving as an intracellular Ca²⁺ pool, whereas no [Ca²⁺]_i rise was found in myoblasts. However, rather than enhancing the [Ca²⁺]_i rise, T₃ actually diminished or did not influence the [Ca²⁺]_i rise induced by caffeine or high [K⁺]_o, respectively. This implies that although the SR Ca²⁺-storage capacity (and likewise the magnitude of Ca²⁺ release) may be increased by T₃, it is more than compensated for by the increase in Ca²⁺ uptake activity through an increase in Ca²⁺ pump units. Furthermore, we have no information about possible T₃ effects on the ryanodine-sensitive Ca²⁺-channel.

Effect of [Ca²⁺]_i on Ca²⁺-ATPase levels

The most striking result of this study is the finding of an optimal [Ca²⁺]_i for the basal and T₃-regulated fast-type SR Ca²⁺-ATPase levels in L6 cells. This regulation takes place within relative small margins (55–195 nM), with an optimum around 125 nM which represents the resting [Ca²⁺]_i. Similar results were obtained by Berlin *et al.* [29], who found evidence for a 'bell-shaped dependence' of acetylcholine receptor synthesis on [Ca²⁺]_i, with both low and high [Ca²⁺]_i being inhibitory. The observation that at a [Ca²⁺]_o of 0.2 mM the net synthesis of Ca²⁺-ATPase is strongly decreased supports the idea that a minimal [Ca²⁺]_i is required for normal stimulation of the expression of fast-type Ca²⁺-ATPase.

The question arises as to how relevant the regulation of the fast-type Ca²⁺-ATPase level by [Ca²⁺]_i is for muscle cell function, i.e. during contraction. Since L6 cells do not display spontaneous contractions, primary muscle cells were used to study the effect of muscle contractility on net Ca²⁺-ATPase synthesis. The lower

fast-type Ca^{2+} -ATPase levels in contracting versus silent primary myotubes in the absence and presence of T_3 supports the idea that a mean $[\text{Ca}^{2+}]_i$ above resting levels has a suppressive action on the net expression of this Ca^{2+} -ATPase isotype. These results are not in accordance with earlier data obtained from chicken myotubes [30]. In that study, Ca^{2+} -uptake activity was reported to be higher in contracting myotubes than in TTX-treated myotubes. Apart from species differences we have no explanation for this discrepancy, although Ca^{2+} -uptake activity measurements are less specific since both the slow and fast isoforms of Ca^{2+} -ATPase may contribute to Ca^{2+} -uptake activity. Furthermore, no data on the corresponding Ca^{2+} -ATPase levels were provided. Martonosi and co-workers [11,13] reported that addition of ionomycin or A23187 (1–10 μM) stimulated synthesis of a 100 kDa protein, supposed to be identical with Ca^{2+} -ATPase, in primary chick myotubes. These findings were positively correlated with higher $[\text{Ca}^{2+}]_i$ in a similar later study [10]. However, $[\text{Ca}^{2+}]_i$ measurements were indirect in that study, based on the decay rate of aequorin [10], and no distinction could be made between the slow and the fast isoform. The divergence of the results in chicken myotubes from the above-cited studies and those from our study in the rat L6 muscle cell line could also suggest the possibility of different $[\text{Ca}^{2+}]_i$ optima for Ca^{2+} -ATPase expression in different species.

It is interesting to speculate on the significance of our findings in the light of existing models which try to explain muscle plasticity. Nowadays it is well accepted that the pattern of neuromuscular activity and T_3 are major determinants of muscle phenotype. A number of investigators have suggested that Ca^{2+} could be a mediator of changes in protein isoforms accompanying transformation of fast to slow muscle, or vice versa [30–32]. For instance, long-term low-frequency stimulation, as occurs in slow muscle, favours the expression of slow isoforms of myosin heavy chain [33] and Ca^{2+} -ATPase [34,35]. Since the mean $[\text{Ca}^{2+}]_i$ will differ depending on the type of neuromuscular activity, i.e. lower in fast than in slow muscle, it cannot be excluded that different $[\text{Ca}^{2+}]_i$ optima exist for slow- and fast-type Ca^{2+} -ATPases. A rough estimation of the rise in the mean $[\text{Ca}^{2+}]_i$ prevailing in fast muscle exposed to a stimulation protocol (10 Hz, 12 h/day during 52 days) which causes a complete exchange of the fast with the slow Ca^{2+} -ATPase isoform mRNA [34], may illustrate this. Assuming a twitch duration of 40 ms at 37 °C and a peak $[\text{Ca}^{2+}]_i$ of 1000 nM [36], the mean $[\text{Ca}^{2+}]_i$ elevation during 24 h would be approx. $500 \times (10 \times 40/1000) \times 0.5$, i.e. 100 nM. In spite of considerable uncertainties in this calculation, it demonstrates that the order of magnitude of the mean $[\text{Ca}^{2+}]_i$ changes accompanying relative changes in fast Ca^{2+} -ATPase *in vivo* is the same as that found *in vitro*. Nevertheless, it is difficult to understand why contracting myotubes which are repetitively exposed to a high Ca^{2+} load react by decreasing their Ca^{2+} -removal capacity, unless the slow isoform of Ca^{2+} -ATPase is expressed or even stimulated in response to the different Ca^{2+} load, implying a different $[\text{Ca}^{2+}]_i$ optimum. This question deserves further study.

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