

Calcineurin regulates slow myosin, but not fast myosin or metabolic enzymes, during fast-to-slow transformation in rabbit skeletal muscle cell culture

Joachim D. Meißner, Gerolf Gros, Renate J. Scheibe, Michael Scholz
and Hans-Peter Kubis

Zentrum Physiologie, Medizinische Hochschule Hannover, D-30623 Hannover, Germany

(Received 21 July 2000; accepted after revision 15 January 2001)

1. The addition of cyclosporin A (500 ng ml⁻¹) – an inhibitor of the Ca²⁺–calmodulin-regulated serine/threonine phosphatase calcineurin – to primary cultures of rabbit skeletal muscle cells had no influence on the expression of fast myosin heavy chain (MHC) isoforms MHCIIa and MHCIIId at the level of protein and mRNA, but reduced the expression of slow MHCI mRNA.
2. In addition, no influence of cyclosporin A on the expression of citrate synthase (CS) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was found. The level of enzyme activity of CS was also not affected.
3. When the Ca²⁺ ionophore A23187 (4 × 10⁻⁷ M) was added to the medium, a partial fast-to-slow transformation occurred. The level of MHCI mRNA increased, and the level of MHCIIId mRNA decreased. Cotreatment with cyclosporin A was able to prevent the upregulation of MHCI at the level of mRNA as well as protein, but did not reverse the decrease in MHCIIId expression. The expression of MHCIIa was also not influenced by cyclosporin A.
4. Cyclosporin A was not able to prevent the upregulation of CS mRNA under Ca²⁺ ionophore treatment and failed to reduce the increased enzyme activity of CS. The expression of GAPDH mRNA was reduced under Ca²⁺ ionophore treatment and was not altered under cotreatment with cyclosporin A.
5. When the myotubes in the primary muscle culture were electrostimulated at 1 Hz for 15 min periods followed by pauses of 30 min, a partial fast-to-slow transformation was induced. Again, cotreatment with cyclosporin A prevented the upregulation of MHCI at the level of mRNA and protein without affecting MHCIIId expression.
6. The nuclear translocation of the calcineurin-regulated transcription factor nuclear factor of activated thymocytes (NFATc1) during treatment with Ca²⁺ ionophore, and the prevention of the translocation in the presence of cyclosporin A, were demonstrated immunocytochemically in the myotubes of the primary culture.
7. The effects of cyclosporin A demonstrate the involvement of calcineurin-dependent signalling pathways in controlling the expression of MHCI, but not of MHCIIa, MHCIIId, CS and GAPDH, during Ca²⁺ ionophore- and electrostimulation-induced fast-to-slow transformations. The data indicate a differential regulation of MHCI, of MHCII and of metabolism. Calcineurin alone is not sufficient to mediate the complete transformation.

The adult skeletal muscle is able to respond to altered physiological demands by switching from a fast-glycolytic to a slow-oxidative phenotype and vice versa. This high degree of plasticity is one of the most remarkable features of the differentiated muscle. A fast-to-slow transformation involves morphological and biochemical alterations that result in an increased resistance to fatigue. The expression of isoforms of proteins of the contractile apparatus and enzymes of energy metabolism is influenced *in vivo* by the impulse activity as determined by the nerve (Buller *et al.*

1960; Salmons & Sreter, 1976), by imposed electrical stimulation (Salmons & Vrbová, 1969), by the level of physical activity (Salmons & Henriksson, 1981), and by passive stretch (Goldspink *et al.* 1992; Russell & Dix, 1992). Recently, a primary skeletal muscle cell culture derived from newborn rabbit hindlimb muscle has been established and characterized (Kubis *et al.* 1997; Meißner *et al.* 2000). Growing on gelatin bead microcarriers in suspension, the myotubes develop the adult expression pattern of fast myosin heavy chain (MHC) isoforms after having been

cultured for several weeks. When the Ca^{2+} ionophore A23187 is added to the medium, $[\text{Ca}^{2+}]_i$ increases about 10-fold and a fast-to-slow transformation occurs. The Ca^{2+} -induced fast-to-slow transformation of the primary skeletal muscle culture cells is comparable to the effect of low frequency stimulation on fast rabbit skeletal muscle *in vivo* and shows many of the latter's well documented changes of mRNA and protein expression (Pette & Vrbová, 1992). During fast-to-slow transformation genes encoding slow isoforms of myosin heavy (MHC) and light chains (MLC) as well as genes encoding enzymes involved in oxidative metabolism are upregulated, while fast myosin isoform genes and genes encoding enzymes of anaerobic metabolism are downregulated. It has been demonstrated very recently that myotubes in primary culture can also be transformed from a fast to a slow type by electrical stimulation (H.-P. Kubis, R. J. Scheibe, G. Hornung, J. D. Meißner & G. Gros, unpublished observations). Thus, the primary skeletal muscle culture offers the possibility of studying *in vitro* the extracellular factors and intracellular signalling cascades that lead to a fast-to-slow transformation.

The importance of Ca^{2+} for phenotypic adaptations of skeletal muscle has been shown by the finding of a Ca^{2+} ionophore-induced fast-to-slow transformation *in vitro* (Kubis *et al.* 1997). The understanding of how changes in $[\text{Ca}^{2+}]_i$ are translated into signals leading to changes in the expression of genes has been greatly improved by the demonstration of the involvement of a signalling pathway dependent on calcineurin (a Ca^{2+} -calmodulin-regulated serine/threonine phosphatase) in the control of fibre-type specific gene expression in muscle (Chin *et al.* 1998). Activation of calcineurin selectively up-regulates slow type-specific genes. The demonstrated transactivation of the myoglobin and slow troponin I promoter was mediated by a member of the nuclear factor of activated thymocytes (NFAT) transcription factor family. Activated calcineurin dephosphorylates NFAT, which then translocates to the nucleus, where it interacts with target DNA sequences (Rao *et al.* 1997).

In addition to fibre type transformation, calcineurin plays a role in hypertrophy. A calcineurin-dependent transcriptional pathway has been suggested by the finding of a block of the development of cardiac hypertrophy by cyclosporin A in transgenic mice (Molkentin *et al.* 1998). The immunosuppressive drug cyclosporin A is a specific inhibitor of calcineurin (Liu *et al.* 1991). Calcineurin is also involved in overload-induced skeletal muscle hypertrophy in mice (Dunn *et al.* 1999). Furthermore, calcineurin mediates the hypertrophic effects of insulin-like growth factor-1 on skeletal myocytes *in vitro* (Musarò *et al.* 1999; Semsarian *et al.* 1999).

To gain more insight into the involvement of calcineurin in fast-to-slow transformation, we decided to investigate the effects of cyclosporin A on the changes in gene expression of MHC isoforms and metabolic enzymes

during fast-to-slow transformation in the myotubes of the primary culture. This *in vitro* system is particularly useful to exclude systemic effects of the drug cyclosporin A. The effects of cyclosporin A presented in this study demonstrate the involvement of calcineurin-dependent signalling pathways in controlling MHCI, but not MHCIIa, MHCIIc, CS and GAPDH, gene expression, indicating differential regulation during fast-to-slow transformation of MHCI on the one hand, and of fast MHCII isoforms and metabolism on the other. The data indicate that calcineurin is not sufficient to mediate the complete Ca^{2+} ionophore- or electrostimulation-induced fast-to-slow transformation in primary skeletal muscle cell cultures.

METHODS

Material and chemicals

Cell culture material was obtained from Nunc, Roskilde, Denmark. Cell culture media, antibiotics and restriction enzymes were obtained from Gibco Life Technologies, Karlsruhe, Germany. Chemicals and biochemicals were from Merck, Darmstadt, Germany, and from Sigma, Deisenhofen, Germany. Cyclosporin A and A23187 were obtained from Sigma. $[\alpha\text{-}^{32}\text{P}]\text{dCTP}$ and $[\alpha\text{-}^{32}\text{P}]\text{UTP}$ were from obtained New England Nuclear, Boston, MA, USA. Anti-NFATc1 antibody and FITC-labelled anti-goat IgG secondary antibody were from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA.

Culture and harvesting of skeletal muscle cells

Newborn New Zealand White rabbits were killed by decapitation. Hindlimb muscles were cut into small pieces and incubated in bicarbonate-buffered saline solution (BSS), pH 7.0 (4.56 mM KCl, 0.44 mM KH_2PO_4 , 0.42 mM Na_2HPO_4 , 25 mM NaHCO_3 , 119.8 mM NaCl, 50 mg l^{-1} penicillin and 100 mg l^{-1} streptomycin) with 0.125% trypsin with stirring at 37°C for 1 h. The suspension was centrifuged at 800 g for 5 min, the pellet resuspended in Dulbecco's modified Eagle's medium (DMEM) with 10% neonatal calf serum (NCS), and the entire procedure repeated once. The final pellet was suspended in DMEM-10% NCS and then filtered through a sieve with 0.4 mm pores. The filtrate was transferred into culture bottles where the fibroblasts were allowed to settle and attach themselves to the bottom for 30 min. The supernatant suspension was decanted and diluted to a final density of 8×10^5 cells ml^{-1} in DMEM with 10% NCS. A total of 15 ml of this suspension was placed in a 260 ml culture flask and 0.04 g cross-linked gelatin beads with a diameter of 100–300 μm (CultiSpher-GL; Percoll Biolytica, Astorp, Sweden) were added per flask. The flasks were kept at 37°C in air with 8% CO_2 and 95% humidity while being shaken gently to ensure adequate O_2 supply to the cells and to prevent cells and beads from settling. Twenty-four hours later the cell suspension was diluted to a cell concentration of 4×10^5 cells ml^{-1} . Myoblasts attached themselves to the gelatin beads and began to fuse after 3 days in culture. From day 3 onwards cells were grown in supplemented skeletal muscle cell basal medium-5% FCS (PromoCell, Heidelberg, Germany), and then from day 8 onwards again in DMEM-10% NCS. After 2 weeks fusion appeared to be complete and only myotubes were detectable microscopically. To collect the myotubes for protein analysis or for isolation of total RNA after 1–5 weeks of culture, cell-covered beads were allowed to sediment, washed twice in BSS (pH 7.0) with 0.02% EDTA, and resuspended in prewarmed (37°C) BBS (pH 7.9) containing 0.175% trypsin, 1.8 mM CaCl_2 and 0.8 mM MgSO_4 . After incubation for 10–20 min at 37°C with shaking on a rotary shaker in an incubator, the isolated cells were centrifuged at 800 g for 5 min, washed twice in BSS (pH 7.0) with 0.02% EDTA, and then suspended

in BSS (pH 7.0). For the isolation of total RNA, the last two washing steps were omitted.

For immunofluorescence studies, cells were cultured for 14 days, detached from gelatin beads with 0.175% trypsin, pelleted at 800 *g* for 5 min and resuspended in DMEM–10% NCS. Cells were then seeded onto glass coverslips. Cell cultures growing on gelatin beads and glass coverslips were incubated in the absence or presence of the Ca²⁺ ionophore A23187 (4 × 10⁻⁷ M) or cyclosporin A (500 ng ml⁻¹) or the Ca²⁺ ionophore A23187 (4 × 10⁻⁷ M) and cyclosporin A (500 ng ml⁻¹). Other cultures growing on gelatin beads were electrostimulated (1 Hz with a stimulus duration of 2.5 ms for 15 min periods, followed by pauses of 30 min), using an electrostimulator (H.-P. Kubis, R. J. Scheibe, G. Hornung, J. D. Meißner & G. Gros, unpublished results), or electrostimulated and simultaneously treated with cyclosporin A (500 ng ml⁻¹).

Animal experiments were carried out according to the guidelines of the local Animal Care Committee (Bezirksregierung Hannover).

Northern blot analysis

Total cellular RNA was isolated from cells according to the method of Chirgwin *et al.* (1979) including ultracentrifugation of the guanidinium thiocyanate homogenate through a dense cushion of caesium chloride. Alternatively, total RNA was isolated in a single-step procedure by acid guanidinium thiocyanate–phenol–chloroform extraction according to Chomczynski & Sacchi (1987), using the Ultraspec RNA isolation system (Biotecx Laboratories, Inc., Houston, TX, USA). The RNA was size fractionated on 1.2% agarose–formaldehyde gels and transferred to a nitrocellulose filter. After restriction enzyme cleavage of cDNA clones (see below), cDNA probes were purified from agarose gels using the GeneClean kit (BIO 101, Inc., Vista, CA, USA). The cDNA probes were labelled with [α -³²P]dCTP using random hexamers as primers (Feinberg & Vogelstein, 1983) with the prime-a-gene labelling system (Promega Corp., Madison, WI, USA). Filters were prehybridized at 42°C overnight in a solution containing 50% formamide, 4 × SSPE (1 × SSPE = 0.3 M NaCl, 0.02 M NaH₂PO₄, 0.002 M EDTA, pH 7.4), 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin, 0.1% sodium dodecyl sulphate (SDS), and 100 ml of salmon testes DNA. Hybridization was performed for 18 h at 42°C in the same solution containing (1–5) × 10⁶ c.p.m. ml⁻¹ of labelled DNA probes. To minimize cross-reactivity, blots were washed under high stringency conditions (65°C, 0.2 × SSC, 0.5% SDS; 1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7) twice for 30 min. Autoradiography was performed with intensifying screens at –80°C with exposure times from 1 to 5 days.

cDNA probes

To establish muscle type-specific gene expression, we performed Northern blot analysis with probes specific for MHC isoforms I and IId. The probes used are fragments from cDNA clones containing only small parts of the 3' translated and the full sections of the hypervariable 3' untranslated regions. The hypervariable 3' untranslated regions of MHC genes exhibit much greater divergence than the coding regions and are therefore specific for each isoform (Saez & Leinwand, 1986; Schiaffino & Salviati, 1998). For detecting mRNA of slow MHC I mRNA, the 3' terminal 450 bp *Hinf*I fragment from rabbit MHC I cDNA (Brownson *et al.* 1992) was used. The probe for detecting fast MHC IId mRNA was the 3' terminal *Pst*I fragment from the rabbit cDNA (Maeda *et al.* 1987), specific for fast MHC isoform IId (Uber & Pette, 1993).

For investigating changes in enzymes of energy metabolism, gene expression of CS was probed with the 800 bp *Clal*/*EcoRV* fragment of the rabbit cDNA (Annex *et al.* 1991) and that of GAPDH with a 1.3 kb *Pst*I fragment encompassing the complete rat cDNA (Fort *et al.* 1985).

The 18S rRNA was detected with the 5.8 kb *Hind*III fragment of 18S rDNA (Katz *et al.* 1983) for normalization. We found no differences in the total RNA content in our culture system under the different culture conditions in contrast to the reported increase in total RNA in electrostimulated muscles *in vivo* (Brownson *et al.* 1988).

RNase protection assays

The probe used for determination of MHCIIa gene expression by RNase protection assays was derived from the rat MHCIIa cDNA (Wieczorek *et al.* 1985). The 360 bp *Bgl*II fragment from the 3' translated region was cloned into the *Hind*III site of the vector pGEM-7Zf(+) (Promega, Madison, WI, USA). After linearisation of the plasmid at the *Eco*RI site, an anti-sense RNA probe was generated with SP6 RNA Polymerase using the MAXIscript *in vitro* transcription kit (Ambion, Inc., Austin, TX, USA) in the presence of 50 mCi [α -³²P]UTP. For generation of an internal standard, the pTRI- β -actin-mouse control template (Ambion, Inc., Austin, TX, USA), containing a 250 bp *Kpn*I/*Xba*I fragment of the mouse β -actin gene, was *in vitro* transcribed with T7 RNA polymerase. The RNA century marker template set (Ambion, Inc., Austin, TX, USA) was used to determine the fully protected fragments (360 and 250 bp, respectively).

The Multi-NPA kit (Ambion, Inc., Austin, TX, USA) was used for the RNase protection solution hybridization assay according to the manufacturer's instructions. Briefly, 10 mg of total RNA was hybridized with 8 × 10⁴ c.p.m. of labelled RNA probe overnight at 42°C. Digestion of non-hybridized probes and sample RNA was performed with 1 ml nuclease mixture (S1 nuclease) for 30 min at 37°C. The protected fragments were separated on an 8 M urea–5% polyacrylamide gel and visualized by autoradiography with intensifying screens at 4°C with exposure times from 3 to 15 h.

MHC electrophoresis

Collected myotubes were homogenized by sonication (6 × 5 s with 60 W at 0°C) and centrifuged at 100 000 *g* for 1 h. Pellets were extracted with 0.6 M KCl, 1 mM EGTA, 0.5 mM DTT, and 10 mM potassium phosphate, pH 6.8. Extracts were centrifuged at 20 000 *g* for 20 min and supernatants were diluted 1:10 with ice cold water to precipitate the actomyosin. After precipitation at 0°C for 12 h, the suspension was centrifuged at 20 000 *g* for 30 min and the actomyosin pellets were solubilized with the extraction buffer.

SDS–polyacrylamide gel electrophoresis (SDS–PAGE) was performed using the method of Kubis & Gros (1997) with minor modifications. In brief, the myosin extracts were diluted (1:7) with SDS–PAGE sample buffer (Cannon–Carlson & Tang, 1997) and heated for 8 min at 95°C. After heating samples were loaded into the slots of a slab gel (15 × 22 cm) with two stacking gels (3.5 and 6.5%) and two separating gels (6.5 and 8.5%) containing amounts of glycerol increasing from 3 to 35%. Runs were performed at 4°C under constant current conditions at 12 mA for 36 h. After each run, gels were silver stained according to Heukeshoven & Dernick (1985).

Measurement of enzyme activities

The enzyme activity of CS was determined according to Bass *et al.* (1969). Briefly, CS activity was determined photometrically (340 nm) at 30°C. A reaction mixture containing 100 μ M Tris–EDTA, pH 8.0, 5 μ M NAD⁺, 6 μ M malate, 59 μ g ml⁻¹ malate dehydrogenase and 50 μ l of probe was incubated for 5 min before addition of 0.22 μ M acetyl-coenzyme A.

Immunofluorescence studies

Primary myotubes growing on gelatin beads in suspension for 16 days and then for a further 2 days on glass coverslips were washed with phosphate-buffered saline (PBS), fixed with 100% methanol, and washed 3 times with PBS. The cells were permeabilized in 0.1%

Table 1. Electrophoresis of MHC isoforms

MHC isoform	Control	CsA	Iono	Iono + CsA	Electro	Electro + CsA
IIa	42 ± 14	42 ± 21	37 ± 4	50 ± 19	40 ± 14	59 ± 5
IIId	48 ± 16	53 ± 23	22 ± 7	38 ± 13	30 ± 13	38 ± 2
IIb	5 ± 4	3 ± 3	2 ± 4	6 ± 11	0	0
I	5 ± 5	2 ± 4	39 ± 9*	6 ± 6†	30 ± 2*	3 ± 4‡

Densitometric quantification of the content of the SDS-PAGE-separated MHC isoforms (shown as a percentage) from the cultures described in Fig. 1. Values in this table represent the means (\pm S.D.) from three separate cultures. CsA: cyclosporin A (500 ng ml^{-1})-treated cultures; Iono: Ca^{2+} ionophore ($4 \times 10^{-7} \text{ M}$)-treated cultures; Iono + CsA: Ca^{2+} ionophore- and cyclosporin A-treated cultures; Electro: electrostimulated cultures (1 Hz with a stimulus duration of 2.5 ms for 15 min periods, followed by pauses of 30 min); Electro + CsA: electrostimulated and cyclosporin A-treated cultures. Statistical significances of changes in MHCI (Dunn's post test following a Kruskal-Wallis test): *significantly different from Control ($P < 0.05$); †significantly different from Iono ($P < 0.05$); ‡significantly different from Electro ($P < 0.05$).

Triton X-100–PBS and washed again 3 times with PBS. To prevent non-specific binding, the cells were treated for 30 min with 0.2% (w/v) BSA and then incubated with goat polyclonal anti-NFATc1 antibody (Santa Cruz Biotechnology, Inc.). Subsequently, cells were washed with PBS and incubated with FITC-labelled anti-goat IgG secondary antibody (Santa Cruz Biotechnology, Inc.). Stained cells were photographed on an inverted fluorescence microscope (Leitz) at a magnification of $\times 400$.

Statistics

Results were expressed as means \pm S.D. The statistical significance of differences was estimated using Dunn's post test (Dunn, 1964) following a Kruskal-Wallis test (Kruskal & Wallis, 1952, 1953).

RESULTS

Effects of cyclosporin A on MHC isoform expression in Ca^{2+} ionophore-treated cultures

To establish the effect of the calcineurin inhibitor cyclosporin A on changes in MHC expression during a Ca^{2+} ionophore-induced fast-to-slow transformation, SDS-PAGE of MHC isoforms (Fig. 1) with densitometric quantification (Table 1) was performed by the method of Kubis & Gros (1997). In 28-day-old control cultures,

MHCIIId and IIa were the main MHC protein isoforms (Fig. 1, lane 9; Table 1). MHCIIId is the dominating MHC of most fast adult rabbit muscles (Aigner *et al.* 1993). In some cases, small amounts of MHCIIb and MHCI were also found (Table 1). No non-adult isoforms could be detected at the protein level, as has been documented in more detail previously (Kubis *et al.* 1997). The data indicate an adult fast type of myotubes in the primary culture. When cyclosporin A, an inhibitor of calcineurin, was added from day 14 onwards to the medium (500 ng ml^{-1}), the pattern of MHC protein isoforms was the same as under control conditions on day 28 (Fig. 1, compare lane 10 with lane 9; Table 1). When the Ca^{2+} ionophore A23187 ($4 \times 10^{-7} \text{ M}$) was added to the medium from day 14 onwards, raising $[\text{Ca}^{2+}]_i$ about 10-fold (Kubis *et al.* 1997), the expression of MHCI protein increased significantly ($P < 0.05$) (Fig. 1, lane 3; Table 1). The myotubes also express MHCIIa and IIId, but the overall percentage of fast isoforms was clearly reduced (Table 1). Simultaneous treatment with Ca^{2+} ionophore and cyclosporin A led to a significant reduction ($P < 0.05$) in MHCI protein back to control values (Fig. 1, lane 5, compare with lane 9; Table 1).

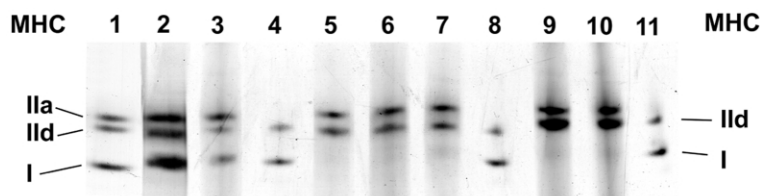


Figure 1. Electrophoresis of MHC isoforms

Cell cultures were grown for 28 days without any treatment (lane 9) or from day 14 of the culture onwards for a further 14 days in the presence of cyclosporin A (500 ng ml^{-1}) (lane 10) or the Ca^{2+} ionophore A23187 ($4 \times 10^{-7} \text{ M}$) (lane 3) or cyclosporin A and Ca^{2+} ionophore (lane 5). Other cultures were electrostimulated (1 Hz for 15 min, stimulus duration 2.5 ms, followed by a pause of 30 min) (lane 2) or electrostimulated and treated with cyclosporin A (lanes 6 and 7) from day 14 onwards for a further 14 days. Myosin extracts of the homogenized cells were separated on SDS polyacrylamide gels (SDS-PAGE). Markers were run in lane 1 (mixture of myosin extracts from m. psoas and m. vastus intermedius, red portion, of the rabbit), and lanes 4, 8, and 11 (mixture of myosin extracts from m. psoas and m. soleus of the rabbit).

To show how these observations at the MHC protein level correlate with the MHC mRNA levels, we performed Northern blot analysis with probes specific for slow MHCI and fast MHCIIId. The specificity of the probe from MHCI cDNA for slow muscle fibres and the specificity of the probe from MHCIIId cDNA for fast muscle fibres was verified by hybridization with total RNA derived from slow soleus and fast extensor digitorum longus muscle of adult rabbits (data not shown).

After 28 days of culture under control conditions, the MHCIIId mRNA level was much stronger (Fig. 3, lane 1) than the low level of MHCI mRNA (Fig. 2A, lane 1), indicating a fast adult type of myotube in the culture at the mRNA level. No non-adult MHC isoform mRNAs were found after 28 days of culture (Meißner *et al.* 2000). When cyclosporin A was added from day 14 onwards to the medium (500 ng ml^{-1}) under control conditions, the expression pattern of MHCI mRNA and MHCIIId mRNA on day 28 of the culture was the same as in the control cultures (Figs 2A and 3, lanes 2). Treatment with the Ca^{2+} ionophore A23187 from day 14 onwards until day 28 resulted in a decrease in the level of MHCIIId mRNA (Fig. 3, lane 3) and an increase in the level of MHCI

mRNA (Fig. 2A, lane 3), indicating a fast-to-slow transformation of the myotubes. Simultaneous treatment of Ca^{2+} ionophore-treated myotubes with cyclosporin A from day 14 onwards led to a marked reduction in the level of MHCI mRNA expression on day 28 (Fig. 2A, lane 4) and did not change the low level of MHCIIId mRNA as seen without cyclosporin A (Fig. 3, compare lane 4 with lane 3).

All these results discussed so far show that cyclosporin A suppresses the upregulation of MHCI protein and MHCI mRNA during fast-to-slow transformation but does not affect the expression of MHCIIId protein and MHCIIId mRNA.

In addition, we examined the expression of MHCIIa mRNA by RNase protection assays. When the cultures were treated from day 14 onwards for 14 days with Ca^{2+} ionophore, the expression of MHCIIa mRNA increased compared with 28-day-old control cultures (Fig. 4, compare lane 3 with lane 1). This increase, according to the fast-to-slow transition sequence of MHC isoforms (MHCIIId > IIa > I) in rabbit muscles (Peuker *et al.* 1998), is compatible with an ongoing fast-to-slow transition in the presence of Ca^{2+} ionophore. Simultaneous treatment

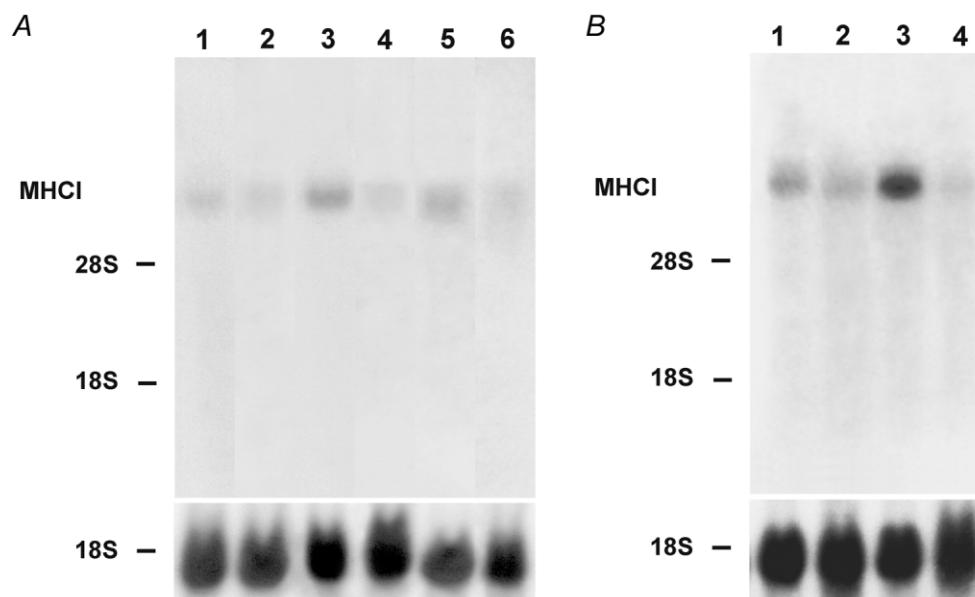


Figure 2. Effect of cyclosporin A, Ca^{2+} ionophore, and electrostimulation on the expression of slow MHCI mRNA

A, cell cultures were grown for 28 days without any treatment (lane 1) or from day 14 of the culture onwards for a further 14 days in the presence of cyclosporin A (500 ng ml^{-1}) (lane 2) or the Ca^{2+} ionophore A23187 ($4 \times 10^{-7} \text{ M}$) (lane 3) or cyclosporin A and Ca^{2+} ionophore (lane 4). Other cultures were electrostimulated (1 Hz for 15 min, stimulus duration 2.5 ms, followed by a pause of 30 min) (lane 5) or electrostimulated and treated with cyclosporin A (lane 6) from day 14 onwards for a further 14 days. B, cell cultures were grown for 16 days without any treatment (lane 1) or from day 14 of the culture onwards or a further 2 days in the presence of cyclosporin A (500 ng ml^{-1}) (lane 2) or Ca^{2+} ionophore ($4 \times 10^{-7} \text{ M}$) (lane 3) or cyclosporin A and Ca^{2+} ionophore (lane 4). Total RNA (20 mg) was isolated from control and treated cultures at the time points indicated, fractionated on a 1.2% formaldehyde agarose gel, and transferred to nitrocellulose. The blots were hybridized with the ^{32}P -labelled 3' terminal *HinfI* fragment of MHCI cDNA or an 18S rDNA probe. The positions of 18S rRNA (1.9 kb) and 28S rRNA (4.8 kb) on the ethidium bromide-stained gel are indicated.

from day 14 onwards with cyclosporin A and Ca^{2+} ionophore for another 14 days did not affect the level of expression of MHCIIa mRNA as observed in cultures that had been treated with ionophore alone (Fig. 4, compare lane 4 with lane 3). After the cultures were treated from day 14 onwards for 14 days with cyclosporin A alone, the level of MHCIIa mRNA was not altered compared with the controls (Fig. 4, compare lane 2 with lane 1). The data demonstrate that cyclosporin A does not affect expression of the fast MHC isoform IIa.

It has been shown previously that changes at the level of gene expression occur very rapidly after the onset of the Ca^{2+} ionophore treatment (Meißner *et al.* 2000). After 16 days of culture under control conditions, the level of MHCIIId mRNA expression was stronger (data not shown) than the level of MHCI mRNA expression (Fig. 2B, lane 1). We note that in 16-day-old controls the

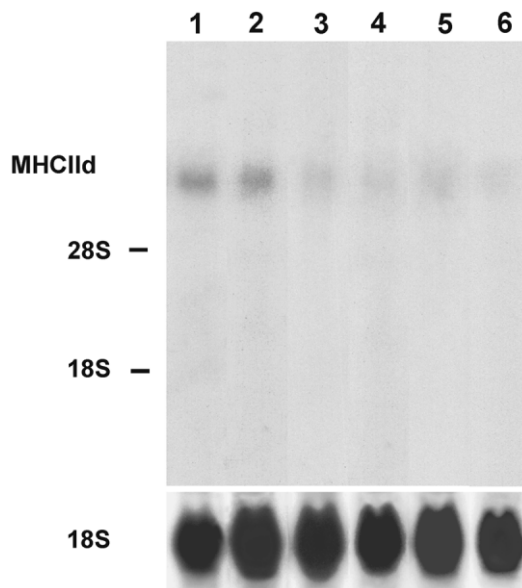


Figure 3. Effect of cyclosporin A, Ca^{2+} ionophore and electrostimulation on the expression of fast MHCIIId mRNA

Cell cultures were grown for 28 days without any treatment (lane 1) or from day 14 of the culture onwards for a further 14 days in the presence of cyclosporin A (500 ng ml^{-1}) (lane 2) or the Ca^{2+} ionophore A23187 ($4 \times 10^{-7} \text{ M}$) (lane 3) or cyclosporin A and Ca^{2+} ionophore (lane 4). Other cultures were electrostimulated (1 Hz for 15 min, stimulus duration 2.5 ms, followed by a pause of 30 min) (lane 5) or electrostimulated and cyclosporin A treated (lane 6) from day 14 onwards for a further 14 days. Total RNA (20 mg) was isolated from control and treated cultures at the time points indicated, fractionated on a 1.2% formaldehyde agarose gel, and transferred to nitrocellulose. The blots were probed with the ^{32}P -labelled 3' terminal *Pst*I fragment of MHCIIId cDNA or an 18S rDNA probe. The positions of 18S rRNA (1.9 kb) and 28S rRNA (4.8 kb) on the ethidium bromide-stained gel are indicated.

expression level of MHCI mRNA is slightly stronger than in 28-day-old controls (Fig. 2B, lane 1, *vs.* Fig. 2A, lane 1), an observation that is in accordance with previous results (Meißner *et al.* 2000). When 14-day-old cultures were treated with Ca^{2+} ionophore for 2 days, the MHCI mRNA expression increased markedly (Fig. 2B, lane 3), even more than after 14 days of Ca^{2+} ionophore treatment (Fig. 2A, lane 3), while the MHCIIId mRNA expression decreased (data not shown). Simultaneous treatment with cyclosporin A and Ca^{2+} ionophore abolished the increase in MHCI mRNA (Fig. 2B, lane 4), but did not change the expression of MHCIIId mRNA (data not shown). Treatment with cyclosporin A alone for 2 days did not alter the expression of MHCIIId mRNA (data not shown), but reduced the expression of MHCI mRNA compared to the control cultures (Fig. 2B, compare lane 2 with lane 1).

The protein and mRNA data indicate that calcineurin is involved in the regulation of gene expression of MHCI, but not of MHCIIa and IId, during the Ca^{2+} ionophore-induced fast-to-slow transformation of the myotubes in primary culture.

Effects of cyclosporin A on MHC expression in electrostimulated cultures

The myotubes in the primary culture can also be transformed from a fast to a slow type by electrostimulation (H.-P. Kubis, R. J. Scheibe, G. Hornung, J. D. Meißner & G. Gros, unpublished observations). We investigated a possible effect of the calcineurin inhibitor cyclosporin A on changes in MHC isoform expression during an electrostimulation-induced fast-to-slow transformation using SDS-PAGE. When the cultures were electrostimulated for 14 days from day 14 onwards at 1 Hz for

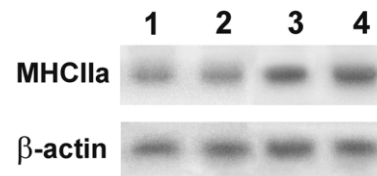


Figure 4. Effect of cyclosporin A and Ca^{2+} ionophore on the expression of fast MHCIIa mRNA

Cell cultures were grown for 28 days without any treatment (lane 1) or from day 14 of the culture onwards for a further 14 days in the presence of cyclosporin A (500 ng ml^{-1}) (lane 2) or the Ca^{2+} ionophore A23187 ($4 \times 10^{-7} \text{ M}$) (lane 3) or cyclosporin A and Ca^{2+} ionophore (lane 4). Total RNA (10 mg) was isolated from control and treated cultures at the time points indicated, hybridized with an *in vitro* transcribed ^{32}P -labelled antisense RNA probe, protecting a 360 bp fragment of MHCIIa mRNA, and an *in vitro* transcribed ^{32}P -labelled antisense probe, protecting a 250 bp fragment of the β -actin mRNA. The protected fragments were separated on an 8 M urea-5% polyacrylamide gel and visualized by autoradiography.

Table 2. Effects of cyclosporin A (CsA) on the activity of citrate synthase (CS) in Ca²⁺ ionophore-treated cultures (days 14–28)

	Control	CsA	Iono	Iono + CsA
CS (units (g protein) ⁻¹)	107 ± 23	131 ± 24	176 ± 25*	285 ± 66*

The activity of CS was determined as described in Methods. The culture conditions were the same as described in Fig. 1. The values in this table represent the means (± S.D.) of three separate cultures. The statistical significance of differences between the values obtained from differently treated cultures and those from controls is indicated (Dunn's post test following a Kruskal-Wallis test): *significantly different from Control ($P < 0.05$).

15 min periods each period followed by a 30 min pause, the expression of MHCI protein increased significantly ($P < 0.05$) (Fig. 1, compare lane 2 with lane 9; Table 1). After electrostimulation, the fast MHC isoforms IIa and IIc were also still present, but the overall percentage of fast isoforms was reduced (Table 1). Cotreatment of electrostimulated cultures with cyclosporin A led to a drastic reduction ($P < 0.05$) in MHCI expression back to control values (Fig. 1, lane 6, compare with lane 9; Table 1).

To investigate the effect of cyclosporin A on electrostimulated cultures at the level of gene expression, we again performed Northern blot analysis with probes specific for slow MHCI and fast MHCIIc. Electrostimulation from day 14 onwards decreased the level of MHCIIc mRNA (Fig. 3, lane 5) and increased the level of MHCI mRNA after 28 days (Fig. 2A, lane 5) compared with the control cultures (Figs 2A and 3, lanes 1, respectively), indicating a fast-to-slow transformation of the myotubes. Simultaneous treatment of electrostimulated myotubes with cyclosporin A from day 14 onwards led to a strongly reduced expression of MHCI mRNA (Fig. 2A, lane 6), and, as in the absence of cyclosporin A, to a weak expression of MHCIIc mRNA on day 28 (Fig. 3, lane 6).

The protein and mRNA data indicate that calcineurin is involved in the regulation of MHCI, but not of MHCIIc, gene expression during the electrostimulation-induced fast-to-slow transformation of the myotubes in primary culture.

Effects of cyclosporin A on the activity and mRNA expression of metabolic marker enzymes in Ca²⁺ ionophore-treated cultures

The metabolic adaptations during low frequency electrostimulation-induced fast-to-slow transformations have been well described *in vivo* (Pette & Vrbová, 1992). A switch from an anaerobic-glycolytic to an oxidative type of energy metabolism has also been demonstrated for the Ca²⁺ ionophore-induced fast-to-slow transformation in the primary skeletal muscle cell culture at the level of enzyme activity and mRNA (Kubis *et al.* 1997; Meißner *et al.* 2000). To investigate a possible role of calcineurin in the regulation of enzymes of energy metabolism during fast-to-slow transformation, Ca²⁺ ionophore-treated

cultures were cotreated with cyclosporin A. Treatment with cyclosporin A alone for 14 days from day 14 onwards had an influence neither on the activity of CS (Table 2) nor on the expression of CS and GAPDH mRNA compared to 28-day-old controls (Figs 5 and 6, compare lanes 2 to lanes 1, respectively). When the cultures were treated with Ca²⁺ ionophore from day 14 onwards, the activity (Table 2) and the level of mRNA (Fig. 5, lane 3) of CS were increased on day 28. In contrast, the level of mRNA of GAPDH (Fig. 6, lane 3) was decreased. Cotreatment with Ca²⁺ ionophore and cyclosporin A did not change the expression of GAPDH mRNA compared to Ca²⁺ ionophore-treated cultures (Fig. 6, lane 4, compare with lane 3). The cotreatment also failed to reduce the activity of CS (Table 2) or to alter the level of gene expression of CS mRNA compared to Ca²⁺ ionophore-treated cultures (Fig. 5, lane 4, compare with lane 3).

These data indicate that calcineurin has no influence on CS and GAPDH gene expression during the Ca²⁺ ionophore-induced fast-to-slow transformation in the myotubes of the primary culture, and that likewise the activity of CS is not influenced. This suggests that calcineurin is not involved in the regulation of the two metabolic marker enzymes investigated.

Translocation of NFATc1 in Ca²⁺ ionophore-treated myotubes

To further characterize the calcineurin-dependent signalling pathway involved in Ca²⁺ ionophore-induced fast-to-slow transformation of the myotubes in primary culture, we analysed the localization of the transcription factor NFATc1 before and during Ca²⁺ ionophore treatment. Calcineurin has a very narrow substrate specificity in contrast to other protein phosphatases (Guerini, 1997), and activated calcineurin can regulate gene expression via dephosphorylation of members of the NFAT transcription factor family. The dephosphorylated transcription factor then translocates into the nucleus, where it directly activates genes (Rao *et al.* 1997). NFATc1 is the NFAT isoform which can undergo nuclear translocation in myotubes (Abbott *et al.* 1998). Immunofluorescence studies using an anti-NFATc1 antibody showed that in 18-day-old control cultures the fluorescence signal was detected in the cytoplasm of the myotubes, while nuclei were not stained (Fig. 7A),

indicating that NFATc1 was localized in the cytoplasm. In contrast, when cultures were treated from day 14 onwards for a further 4 days with the Ca^{2+} ionophore A23187 (4×10^{-7} M), more than 90% of the nuclei of the myotubes were stained positively (Fig. 7B), indicating that the Ca^{2+} ionophore has caused a translocation of NFATc1 into the nuclei of myotubes. When cyclosporin A (500 ng ml^{-1}) was added from day 14 onwards to Ca^{2+} ionophore-treated cultures, the fluorescence signal in the nuclei was reduced to background levels on day 18, whereas the cytoplasm was again positively stained (Fig. 7C). These data indicate that cyclosporin A prevents the Ca^{2+} ionophore-induced translocation of NFATc1 into the nuclei. The cytoplasm only was positively stained after the myotubes had been treated with cyclosporin A alone for 4 days from day 14 onwards (data not shown). Thus, the data from cyclosporin A-treated cultures (see above) and the results obtained by immunofluorescence demonstrate the involvement of a calcineurin-dependent signalling pathway involving NFATc1 in the Ca^{2+} ionophore-induced transformation of the myotubes in the primary culture.

DISCUSSION

The activity of motoneurons plays a central role in determining the fibre type composition of a muscle. Therefore, fast and slow fibres can be transformed into each other by cross-innervation or chronic low frequency stimulation (Buller *et al.* 1960; reviewed by Pette & Vrbová, 1992; Williams & Neuffer, 1996). The involvement of Ca^{2+} in fibre type transformations was suggested by the demonstration of a long lasting elevation of $[\text{Ca}^{2+}]_i$ during the electrically induced fast-to-slow transformation of rabbit fast muscles *in vivo* (Sreter *et al.* 1987). The importance of changes in $[\text{Ca}^{2+}]_i$ was more directly demonstrated by the fact that Ca^{2+} ionophore treatment leads to a reversible fast-to-slow transformation in the myotubes of a primary skeletal muscle cell culture (Kubis *et al.* 1997). This transformation was comparable to that induced by chronic low frequency electrostimulation *in vivo* (Pette & Vrbová, 1992). Furthermore, the myotubes in culture can also be transformed from a fast to a slow type by electrical stimulation (H.-P. Kubis, R. J. Scheibe, G. Hornung, J. D. Meißner & G. Gros, unpublished

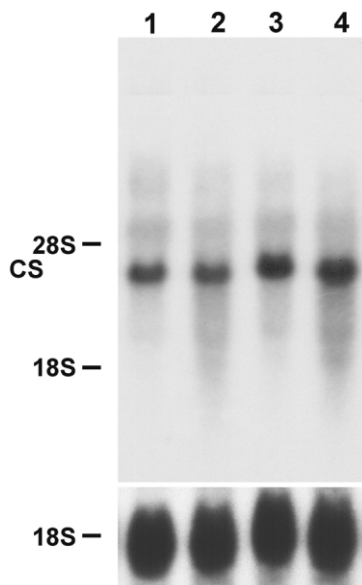


Figure 5. Effect of cyclosporin A and Ca^{2+} ionophore on the expression of citrate synthase (CS) mRNA

Cell cultures were grown for 28 days without any treatment (lane 1) or from day 14 of the culture onwards for a further 14 days in the presence of cyclosporin A (500 ng ml^{-1}) (lane 2) or the Ca^{2+} ionophore A23187 (4×10^{-7} M) (lane 3) or cyclosporin A and Ca^{2+} ionophore (lane 4). Total RNA (20 mg) was isolated from control and treated cultures at the time points indicated, fractionated on a 1.2% formaldehyde agarose gel, and transferred to nitrocellulose. The blots were hybridized with the ^{32}P -labelled 800 bp *Cla*I/*Eco*RV fragment of CS cDNA or an 18S rDNA probe. The positions of 18S rRNA (1.9 kb) and 28S rRNA (4.8 kb) on the ethidium bromide-stained gel are indicated.

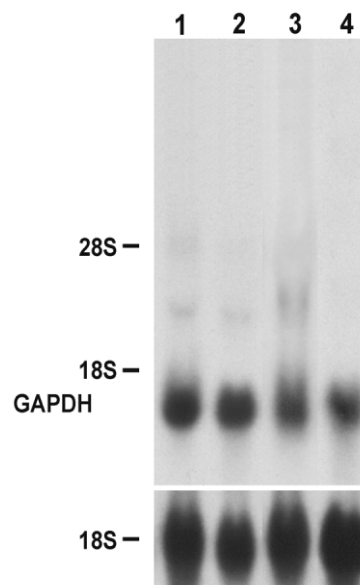


Figure 6. Effect of cyclosporin A and Ca^{2+} ionophore on the expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA

Cell cultures were grown for 28 days without any treatment (lane 1) or from day 14 of the culture onwards for a further 14 days in the presence of cyclosporin A (500 ng ml^{-1}) (lane 2) or the Ca^{2+} ionophore A23187 (4×10^{-7} M) (lane 3) or cyclosporin A and Ca^{2+} ionophore (lane 4). Total RNA (20 mg) was isolated from control and treated cultures at the time points indicated, fractionated on a 1.2% formaldehyde agarose gel, and transferred to nitrocellulose. The blots were hybridized with a ^{32}P -labelled 1.3 kb *Pst*I fragment encompassing the complete GAPDH cDNA or an 18S rDNA probe. The positions of 18S rRNA (1.9 kb) and 28S rRNA (4.8 kb) on the ethidium bromide-stained gel are indicated.

observations). Changes in $[Ca^{2+}]_i$ differing in amplitude and duration have been shown to result in differential activation of transcription factors in lymphocytes (Dolmetsch *et al.* 1997). A low sustained plateau in the level of $[Ca^{2+}]_i$ leads to the activation of NFAT whereas a large transient rise in $[Ca^{2+}]_i$ leads to the activation of NF κ B. It has been postulated that similar mechanisms are involved in the regulation of fibre type-specific gene expression in muscle (Chin *et al.* 1998; Hughes, 1998).

The Ca^{2+} -calmodulin-regulated serine/threonine phosphatase calcineurin plays a central role in Ca^{2+} -dependent signalling pathways in lymphocytes (Rao *et al.* 1997). The data presented in this paper provide clear evidence that the calcineurin inhibitor cyclosporin A has an impact on the fast-to-slow transformation induced by low frequency electrical stimulation or Ca^{2+} ionophore treatment in the myotubes of the primary muscle cell culture. Cyclosporin A abolished the upregulation of MHCI at the protein and mRNA level, but did not change the decreased expression of fast MHC isoform IID. In addition, cyclosporin A treatment alone reduced the expression of the slow MHCI mRNA, but, in contrast, had no influence on the expression of MHCIIa or MHCIIId mRNA and protein. Furthermore,

GAPDH and CS mRNA, and the activity of CS were not affected by cyclosporin A either under control conditions or under Ca^{2+} ionophore treatment in our culture system. The effects of cyclosporin A demonstrate that the phosphatase calcineurin is involved in the changes of MHCI gene expression during fast-to-slow transformation. On the other hand, the inhibition of calcineurin did not restore in all other respects the fast character of the myotubes, indicating that calcineurin is not involved in the downregulation of fast MHC genes during fast-to-slow transformation. The demonstrated effects of cyclosporin A indicate that fast and slow fibre-specific genes are obviously not regulated via the same signalling pathway. Furthermore, the data presented here also indicate that the gene regulation of metabolic marker enzymes during fast-to-slow transformation, like the regulation of fast MHC isoforms, is not influenced by calcineurin signalling pathways. Calcineurin is therefore not sufficient to mediate all changes occurring during Ca^{2+} ionophore- or electrostimulation-induced transformations.

The finding that cyclosporin A treatment, in combination neither with electrostimulation nor with Ca^{2+} ionophore treatment, reverses the decrease in the expression of fast

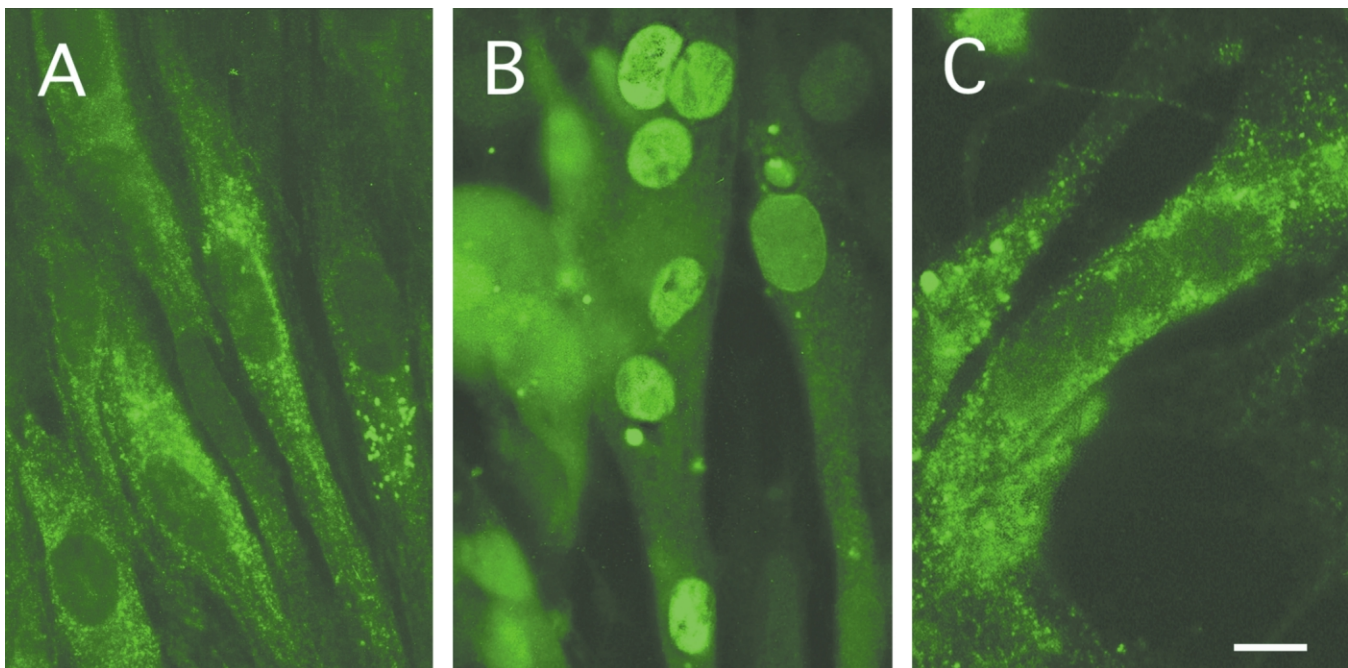


Figure 7. Immunofluorescence analysis of the localization of the transcription factor NFATc1 (nuclear factor of activated thymocytes) in myotubes of Ca^{2+} ionophore-treated cultures

A, cell cultures were grown for 16 days on gelatin beads and then for 2 more days on glass coverslips. *B*, cell cultures were grown for 14 days on gelatin beads and then for 2 more days in the presence of the Ca^{2+} ionophore A23187 (4×10^{-7} M) on gelatin beads. Cells were then transferred to glass coverslips and cultured for additional 2 days in the presence of Ca^{2+} ionophore. *C*, cell cultures were grown for 14 days on gelatin beads and then for 2 more days in the presence of Ca^{2+} ionophore (4×10^{-7} M) and cyclosporin A (500 ng ml^{-1}) on gelatin beads. Cells were then transferred to glass coverslips and cultured for an additional 2 days in the presence of Ca^{2+} ionophore and cyclosporin A. Methanol-fixed and Triton-permeabilized cells were stained with an anti-NFATc1 antibody and fluorescence was detected by using an inverted fluorescence photomicroscope. The scale bar represents $10 \mu\text{m}$.

MHCIIId in the myotubes is in line with the *in vitro* findings of Chin *et al.* (1998) that activation of calcineurin selectively upregulates slow fibre-specific gene promoters. However, Chin and coworkers inferred from *in vivo* data that calcineurin-dependent signalling both activates slow fibre-specific genes and represses the fast fibre-specific programme. But it cannot be excluded from their immunohistochemical data that the relative increase in fast fibres in the soleus of the rat under cyclosporin A treatment might be explained solely by downregulation of MHCI and a concomitant decrease in the number of slow fibres, rather than by upregulation of fast MHCII gene expression. The *in vitro* data presented in our paper clearly demonstrate that inhibition of calcineurin does not reverse the repression of the fast MHCIIId gene during fast-to-slow transformation.

Dunn *et al.* (1999) have demonstrated that cyclosporin A is able to prevent overload-induced skeletal muscle hypertrophy in mice. In the long-term condition of overload a fibre transformation occurred in addition to hypertrophy, which in contrast to our *in vitro* data could be reversed by treatment with cyclosporin A. On the other hand, very recently it has been shown that activated calcineurin stimulates slow skeletal muscle fibre gene expression, but not hypertrophy, in fast hindlimb muscle in transgenic mice, which express a Ca^{2+} -independent, constitutively active calcineurin under the control of the muscle creatine kinase enhancer (Naya *et al.* 2000). In accordance with this latter finding, visual inspection and electron microscopy in the present culture system revealed that no hypertrophic response, only transformation, was induced in the myotubes during Ca^{2+} ionophore treatment or electrostimulation (B. Decker, H.-P. Kubis & G. Gros, unpublished observation). In the transgenic mice, the transformation of fast muscle in response to activated calcineurin was incomplete. These data led the authors to suggest that other calcium-dependent signalling systems may be required in addition to mediate the complete programme of slow and fast fibre gene expression. These conclusions are in line with the present data showing that inhibition of calcineurin has no influence on the expression of fast MHC isoforms and metabolic enzymes. The outlined differences between *in vitro* and *in vivo* data may be explained either by systemic drug effects occurring *in vivo* or by a more complex action of calcineurin-dependent signalling pathways in the organism *in vivo*. In addition, different regimens like electrostimulation or overload may involve different interactions between signalling pathways and induce different patterns of gene expression, leading to transformation and/or hypertrophy. In contrast to the fibre transformation associated with overload, which is not the primary effect of overload and occurs only in the long-term condition, changes of MHC mRNA levels associated with electrostimulation occur early after the start of the treatment in rabbit fast-twitch muscles *in vivo* (Brownson *et al.* 1988) as well as after Ca^{2+} ionophore

treatment or electrostimulation of the present primary skeletal muscle cell culture (Meißner *et al.* 2000; H.-P. Kubis, R. J. Scheibe, G. Hornung, J. D. Meißner & G. Gros, unpublished observations). This is compatible with a different pattern of signalling events under overload and under electrostimulation.

A consistent picture of the action of calcineurin in regulating muscle-specific gene expression has not yet emerged from the available data. Calcineurin has a very narrow substrate specificity with a small number of substrates in contrast to other protein phosphatases (Guerini, 1997). One downstream target of calcineurin in muscle, NFATc1, has been identified (Abbott *et al.* 1998; Chin *et al.* 1998). We have demonstrated the nuclear translocation of NFATc1 in the myotubes of the primary culture after treatment with a Ca^{2+} ionophore. This effect could be suppressed by addition of cyclosporin A to the medium. Very recently it has been demonstrated that calcineurin-dependent gene regulation in skeletal myocytes is also mediated by the transcription factor myocyte enhancer factor 2 (MEF-2) (Wu *et al.* 2000). These data do not exclude a possible role for other transcription factors in the regulation of fibre type-specific gene expression. During IGF-1-induced skeletal muscle hypertrophy the transcription factor GATA-2 is involved in calcineurin-dependent signalling (Musarò *et al.* 1999). An *in vitro* model of cardiac hypertrophy indicates the activation of NFAT3, GATA-4 and MEF-2C (Xia *et al.* 2000). In general, the mechanism by which the calcineurin action is mediated at the level of promoter activity may be a direct or an indirect one.

Further possible interactions of the calcineurin signalling pathway with other signalling pathways have to be taken into account. NFATc1 can interact with other transcription factors, which are in turn activated by different pathways (Crabtree, 1999). In T-cells, the calcineurin signalling pathway is activated independently of, but is integrated with, the Ras–mitogen-activated protein (MAP) kinase and protein kinase C pathways, leading to an interaction between the transcription factors NFAT and activation protein 1 (AP-1) (Rao *et al.* 1997). A role for Ras–MAP kinase signalling in the nerve activity-dependent regulation of slow fibre genes *in vivo* was demonstrated by Murgia *et al.* (2000). In addition, a Ca^{2+} -sensitive protein kinase C-dependent pathway has been shown to be involved in the Ca^{2+} ionophore-induced increase in cytochrome c (nuclearly encoded) expression in L6E9 myotubes (Freysenet *et al.* 1999). The possible interactions of the calcineurin-dependent and other Ca^{2+} -dependent signalling pathways and their role in regulating changes in gene expression during fibre transformation remain to be determined. The present primary skeletal muscle culture is well suited for such studies.

- ABBOTT, K. L., FRIDAY, B. B., THALOOR, D., MURPHY, T. J. & PAVLATH, G. K. (1998). Activation and cellular localisation of the cyclosporine A-sensitive transcription factor NF-AT in skeletal muscle cells. *Molecular Biology of the Cell* **9**, 2905–2915.
- AIGNER, S., GOHLSCH, B., HÄMÄLÄINEN, N., STARON, R. S., UBER, A., WEHRLE, U. & PETTE, D. (1993). Fast myosin heavy chain diversity in skeletal muscles of the rabbit: heavy chain II_d, not II_b predominates. *European Journal of Biochemistry* **211**, 367–372.
- ANNEX, B. H., KRAUS, W. E., DOHM, G. L. & WILLIAMS, R. S. (1991). Mitochondrial biogenesis in striated muscles: rapid induction of citrate synthase mRNA by nerve stimulation. *American Journal of Physiology* **260**, C266–270.
- BASS, A., BRODICZKA, D., EYER, P., HOFER, S. & PETTE, D. (1969). Metabolic differentiation of distinct muscle types at the level of enzymatic organization. *European Journal of Biochemistry* **10**, 198–206.
- BROWNSON, C., ISENBERG, H., BROWN, W., SALMONS, S. & EDWARDS, Y. (1988). Changes in skeletal muscle gene transcription induced by chronic stimulation. *Muscle and Nerve* **11**, 1183–1189.
- BROWNSON, C., LITTLE, P., JARVIS, J. C. & SALMONS, S. (1992). Reciprocal changes in myosin isoform mRNAs of rabbit skeletal muscle in response to the initiation and cessation of chronic electrical stimulation. *Muscle and Nerve* **15**, 694–700.
- BULLER, A. J., ECCLES, J. C. & ECCLES, R. M. (1960). Interactions between motoneurons and muscles in respect of the characteristic speeds of their responses. *Journal of Physiology* **150**, 417–439.
- CANNON-CARLSON, S. & TANG, J. (1997). Modification of the Laemmli sodium dodecylsulfate-polyacrylamide gel electrophoresis procedure to eliminate artifacts on reducing and nonreducing gels. *Analytical Biochemistry* **246**, 146–148.
- CHIN, E. R., OLSON, E. N., RICHARDSON, J. A., YANG, Q., HUMPHRIES, C., SHELTON, J. M., WU, H., ZHU, W., BASSEL-DUBY, R. & WILLIAMS, R. S. (1998). A calcineurin-dependent transcriptional pathway controls skeletal muscle fiber type. *Genes and Development* **12**, 2499–2509.
- CHIRGWIN, J. M., PRZYBYLA, A. E., MACDONALD, R. J. & RUTTER, W. J. (1979). Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry* **18**, 5294–5299.
- CHOMCZYNSKI, P. & SACCHI, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.
- CRABTREE, G. R. (1999). Generic signals and specific outcomes: signaling through Ca²⁺, calcineurin, and NF-AT. *Cell* **96**, 611–614.
- DOLMETSCH, R. E., LEWIS, R. S., GOODNOW, C. C. & HEALY, J. I. (1997). Differential activation of transcription factors induced by Ca²⁺ response amplitude and duration. *Nature* **386**, 855–858.
- DUNN, O. J. (1964). Multiple comparisons using rank sums. *Technometrics* **6**, 241–252.
- DUNN, S. E., BURNS, J. L. & MICHEL, R. N. (1999). Calcineurin is required for skeletal muscle hypertrophy. *Journal of Biological Chemistry* **274**, 21908–21912.
- FEINBERG, A. P. & VOGELSTEIN, B. (1983). A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. *Analytical Biochemistry* **132**, 6–13.
- FORT, P., MARTY, L., PIECHACZYK, M., EL SABROUTY, S., DANI, C., JAENTEUR, P. & BLANCHARD, J. M. (1985). Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. *Nucleic Acids Research* **13**, 1431–1442.
- FREYSSINET, D., DI CARLO, M. & HOOD, D. A. (1999). Calcium-dependent regulation of cytochrome c gene expression in skeletal muscle cells. Identification of a protein kinase C-dependent pathway. *Journal of Biological Chemistry* **274**, 9305–9311.
- GOLDSPINK, G., SCUTT, A., LOUGHNA, P. T., WELLS, D. J., JAENICKE, T. & GERLACH, G. F. (1992). Gene expression in skeletal muscle in response to stretch and force generation. *American Journal of Physiology* **262**, R356–363.
- GUERINI, D. (1997). Calcineurin: not just a simple protein phosphatase. *Biochemical and Biophysical Research Communications* **235**, 271–275.
- HEUKESHOVEN, J. & DERNICK, R. (1985). Simplified method for silver staining of proteins in polyacrylamide gels and the mechanism of silver staining. *Electrophoresis* **6**, 103–112.
- HUGHES, S. M. (1998). Muscle development: Electrical control of gene expression. *Current Biology* **8**, R892–894.
- KATZ, R. A., ERLANGER, B. F. & GUNTAKA, R. V. (1983). Evidence for extensive methylation of ribosomal RNA genes in rat XC cell line. *Biochimica et Biophysica Acta* **739**, 258–264.
- KRUSKAL, W. H. & WALLIS, W. A. (1952). Use of ranks in one-criterion variance analysis. *Journal of the American Statistical Association* **47**, 583–621.
- KRUSKAL, W. H. & WALLIS, W. A. (1953). Use of ranks in one-criterion variance analysis. *Journal of the American Statistical Association* **48**, 907–911.
- KUBIS, H.-P. & GROS, G. (1997). A rapid electrophoretic method for separating rabbit skeletal muscle myosin heavy chains at high resolution. *Electrophoresis* **18**, 64–66.
- KUBIS, H.-P., HALLER, E.-A., WETZEL, P. & GROS, G. (1997). Adult fast myosin pattern and Ca²⁺-induced slow myosin pattern in primary skeletal muscle cell culture. *Proceedings of the National Academy of Sciences of the USA* **94**, 4205–4210.
- LIU, J., FARMER, J. D. JR, LANE, W. S., FRIEDMAN, J., WEISSMAN, I. & SCHREIBER, S. L. (1991). Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. *Cell* **66**, 807–815.
- MAEDA, K., SCZAKIEL, G. & WITTINGHOFER, A. (1987). Characterization of cDNA coding for the complete light meromyosin portion of a rabbit fast skeletal muscle myosin heavy chain. *European Journal of Biochemistry* **167**, 97–102.
- MEIBNER, J. D., KUBIS, H.-P., SCHEIBE, R. J. & GROS, G. (2000). Reversible Ca²⁺-induced fast-to-slow transition in primary skeletal muscle culture cells on the mRNA level. *Journal of Physiology* **523**, 19–28.
- MOLKENTIN, J. D., LU, J.-R., ANTOS, C. L., MARKHAM, B., RICHARDSON, J., ROBBINS, J., GRANT, S. R. & OLSON, E. N. (1998). A calcineurin-dependent transcriptional pathway for cardiac hypertrophy. *Cell* **93**, 215–228.
- MURGIA, M., SERRANO, A. L., CALABRIA, E., PALLAFACCHINA, G., LOMO, T. & SCHIAFFINO, S. (2000). Ras is involved in nerve-activity-dependent regulation of muscle genes. *Nature Cell Biology* **2**, 142–147.
- MUSARÒ, A., MCCULLAGH, K. J. A., NAYA, F. J., OLSON, E. N. & ROSENTHAL, N. (1999). IGF-1 induces skeletal myocyte hypertrophy through calcineurin in association with GATA-2 and NF-ATc1. *Nature* **400**, 581–585.
- NAYA, F. J., MERCER, B., SHELTON, J., RICHARDSON, J. A., WILLIAMS, R. S. & OLSON, E. N. (2000). Stimulation of slow skeletal muscle fiber gene expression by calcineurin in vivo. *Journal of Biological Chemistry* **275**, 4545–4548.

- PETTE, D. & VRBOVÁ, G. (1992). Adaptation of mammalian skeletal muscle fibers to chronic electrical stimulation. *Reviews of Physiology, Biochemistry and Pharmacology* **120**, 115–208.
- PEUKER, H., CONJARD, A. & PETTE, D. (1998). Alpha-cardiac-like myosin heavy chain as an intermediate between MHCIIa and MHCI beta in transforming rabbit muscle. *American Journal of Physiology* **274**, C595–602.
- RAO, A., LUO, C. & HOGAN, P. G. (1997). Transcription factors of the NFAT family: regulation and function. *Annual Reviews of Immunology* **15**, 707–747.
- RUSSELL, B. & DIX, D. J. (1992). Mechanisms for intracellular distribution of mRNA: in situ hybridization studies in muscle. *American Journal of Physiology* **262**, C1–8.
- SAEZ, L. & LEINWAND, L. A. (1986). Characterization of diverse forms of myosin heavy chain expressed in adult human skeletal muscle. *Nucleic Acids Research* **14**, 2951–2969.
- SALMONS, S. & HENRIKSSON, J. (1981). The adaptive response of skeletal muscle to increased use. *Muscle and Nerve* **4**, 94–105.
- SALMONS, S. & SRETER, F. A. (1976). Significance of impulse activity in the transformation of skeletal muscle type. *Nature* **263**, 30–34.
- SALMONS, S. & VRBOVÁ, G. (1969). The influence of activity on some contractile characteristics of mammalian fast and slow muscles. *Journal of Physiology* **201**, 535–549.
- SCHIAFFINO, S. & SALVIATI, G. (1998). Molecular diversity of myofibrillar proteins: isoform analysis at the protein and mRNA level. *Methods in Cell Biology* **52**, 349–369.
- SEMSARIAN, C., WU, M.-J., JU, Y.-K., MARCINIEC, T., YEOH, T., ALLEN, D. G., HARVEY, R. P. & GRAHAM, R. M. (1999). Skeletal muscle hypertrophy is mediated by a Ca²⁺-dependent calcineurin signalling pathway. *Nature* **400**, 576–580.
- SRETER, F. A., LOPEZ, J. R., ALAMO, L., MABUCHI, K. & GERGELY, J. (1987). Changes in intracellular ionized Ca concentration associated with muscle fiber type transformation. *American Journal of Physiology* **253**, C296–300.
- UBER, A. & PETTE, D. (1993). PCR-based assignment of two myosin heavy chain cDNA clones to biochemically and histochemically defined single type IIB and IID fibers of rabbit muscle. *FEBS Letters* **331**, 193–197.
- WIECZOREK, D. F., PERIASAMY, M., BUTLER-BROWNE, G. S., WHALEN, R. G. & NADAL-GINARD, B. (1985). Co-expression of multiple myosin heavy chain genes, in addition to a tissue-specific one, in extraocular musculature. *Journal of Cell Biology* **101**, 618–629.
- WILLIAMS, R. S. & NEUFER, P. D. (1996). Regulation of gene expression in skeletal muscle by contractile activity. In *Handbook of Physiology*, section 12, *Exercise: Regulation and Integration of Multiple Systems*, ed. ROWELL, L. B. & SHEPHERD, J. T., pp. 1124–1150. Oxford University Press, New York, Oxford.
- WU, H., NAYA, F. J., MCKINSEY, T. A., MERCER, B., SHELTON, J. M., SHIN, E. R., SIMARD, A. R., MICHEL, R. N., BASSAL-DUBY, R., OLSON, E. N. & WILLIAMS, R. S. (2000). MEF2 responds to multiple calcium-regulated signals in the control of skeletal muscle fiber type. *EMBO Journal* **19**, 1963–1973.
- XIA, Y., McMILLIN, J. B., LEWIS, A., MOORE, M., ZHU, W. G., WILLIAMS, R. S. & KELLEMS, R. E. (2000). Electrical stimulation of neonatal cardiac myocytes activates the NFAT3 and GATA4 pathways and up-regulates the adenylosuccinate synthetase 1 gene. *Journal of Biological Chemistry* **275**, 1855–1863.

Acknowledgements

We are grateful to Drs C. Brownson, R. Guntaka, P. K. Umeda, D. F. Wieczorek, R. S. Williams and A. Wittinghofer for their generous gift of plasmids. We thank E.-A. Haller, A. Jacobs and K.-H. Reichmuth for excellent technical assistance. We wish to thank Dr W. H. Müller for helpful discussions.

Corresponding author

J. D. Meißner: Zentrum Physiologie, Medizinische Hochschule Hannover, D-30623 Hannover, Germany.

Email: meissner.joachim@mh-hannover.de