Sarcoplasmic-reticulum biogenesis in contraction-inhibited skeletal-muscle cultures*

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We have previously shown that inhibition of the spontaneous contractile activity of cultured embryonic-chick skeletal-muscle fibres with tetrodotoxin (TTX) leads to decreased sarcoplasmic-reticulum Ca^{2+} -transport rates and steady-state concentrations of the high-energy Ca^{2+} -ATPase phosphoenzyme intermediate [Charuk & Holland (1983) *Exp. Cell Res.* **144**, 143–157]. In the present study we used a monoclonal antibody to the Ca^{2+} -ATPase to show that there is a decreased amount of enzyme accumulated by contraction-inhibited myotubes. Indirect immunofluorescence microscopy using the monoclonal antibody to the Ca^{2+} -ATPase also revealed a disordered subcellular organization of the sarcotubular system in contraction-inhibited myotubes. The biogenesis of sarcoplasmic-reticulum proteins in TTX-paralysed myofibres was studied by labelling cells with [^{35}S]methionine before isolation of the active Ca^{2+} -pump membrane fraction. Protein turnover was selectively increased in that fraction from TTX-treated muscle cultures. Electrophoretic analysis and quantitative fluorography confirmed that decreased accumulation of the Ca^{2+} -ATPase enzyme in contraction-inhibited myotubes was associated with increased turnover of this protein. The present results demonstrate that biogenesis of the sarcoplasmic-reticulum Ca^{2+} -ATPase is regulated by the contractile activity of skeletal-muscle fibres.

INTRODUCTION

The sarcoplasmic reticulum is an intracellular membrane system responsible for the uptake and release of Ca^{2+} during the contraction/relaxation cycle of striated muscle. Sarcoplasmic reticulum isolated from fast skeletal muscle differs from that of slow muscle in its kinetics of Ca^{2+} uptake and its protein composition (Heilmann *et al.*, 1977). Several lines of evidence suggest that innervation influences the amount or functional activity of sarcoplasmic reticulum in skeletal muscle. Cross-innervation of fast and slow muscle fibres alters twitch duration and twitch-to-tetanus ratios through changes in myosin ATPase activity and sarcoplasmic-reticulum Ca^{2+} -transport activity (Mommaerts *et al.*, 1969; Margreth *et al.*, 1973). Furthermore, denervation of rat skeletal muscle decreases steady-state phosphorylation of the Ca^{2+} -ATPase from [^{32}P]ATP (Sreter, 1970), Ca^{2+} uptake rates and Ca^{2+} + Mg^{2+} -dependent ATPase activity in isolated sarcoplasmic-reticulum vesicles (Palexas *et al.*, 1981). Functional denervation of chick-embryo skeletal muscles by injection of curare *in ovo* has also been reported to decrease the accumulation of sarcoplasmic-reticulum Ca^{2+} -ATPase and other muscle-specific proteins (Roufa & Martonosi, 1981).

Other evidence suggesting that muscle contractile activity may influence the sarcoplasmic reticulum has come from experiments in which muscle was subjected to either direct or indirect electrostimulation (Heilmann & Pette, 1979; Heilmann *et al.*, 1981). Depending on the frequency of electrical stimulation, a gradual transformation of fibre phenotypes occurs by a series of temporally ordered biochemical events involving changes in muscle gene expression (for review see Pette & Vrbova, 1985). The earliest detectable changes are decreased sarcoplasmic-reticulum function and parvalbumin contents in chronically stimulated muscles (Klug *et al.*, 1983). These experiments sug-

gested that the contractile pattern is the most important determinant of muscle phenotype.

Motor nerves could influence muscle phenotypes either by supplying trophic factors to the muscle or by determining the pattern of contraction imposed by neuronal synaptic activity. Several investigators have attempted to determine which of these influences is dominant. Wan & Boegman (1981*b*) concluded that axonal transport was more important than contractile activity in the regulation of several soluble enzymes of the rat soleus, including lactate dehydrogenase. In contrast, Ramirez (1984) compared colchicine blockade of axonal transport with surgical denervation and concluded that axonal transport was less important than contractile activity for regulating lactate dehydrogenase activity in skeletal muscle. Using the same approach, Wan & Boegman (1980*a,b*, 1981*a*) compared the effects of neural application of batrachotoxin with those of tetrodotoxin (TTX) on sarcoplasmic-reticulum Ca^{2+} -ATPase activity and Ca^{2+} uptake in rat extensor and soleus muscles. Their results indicated that axonal transport of trophic factors plays a more important role in neural regulation of sarcoplasmic-reticulum function than does contractile activity.

In order to study the effect of contractile activity on the biogenesis and regulation of sarcoplasmic-reticulum function in developing skeletal muscle free from neuronal influences, a methodology for uniformly influencing contractile activity of fibres was required. Embryonic-chicken skeletal myoblasts grown *in vitro* fuse and differentiate to form multinucleated myotubes that spontaneously contract. This aneural tissue-culture system provides the opportunity to examine the effects of contractile activity directly on both the expression and functional activity of muscle-specific proteins. Inhibition of the spontaneous contractile activity of cultured muscle cells with the neurotoxin TTX has been shown to cause (i) decreased accumulation and increased degradation of myosin heavy chain (Walker & Strohman, 1978),

Abbreviation used: TTX, tetrodotoxin.

* This paper is dedicated to the memory of Dr. Elizabeth Zubrzycka-Gaarn.

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(ii) increased accumulation and decreased turnover of phosphorylase (Lawrence *et al.*, 1984) and (iii) increased accumulation of acetylcholine receptors and acetylcholinesterase (Shainberg & Burstein, 1976; Walker & Wilson, 1976). During myogenesis, biosynthesis of the Ca^{2+} -ATPase (Holland & MacLennan, 1976) and other less prominent proteins of the sarcotubular system (Zubrzycka & MacLennan, 1976; Michalak & MacLennan, 1980) results in the development of a microsomal ATP-dependent Ca^{2+} -transport system similar to that of adult muscle sarcoplasmic reticulum (Lough *et al.*, 1972; Martonosi *et al.*, 1977). We have previously shown that contraction-inhibited muscle cultures have decreased rates of ATP-dependent Ca^{2+} uptake into membrane fractions (Charuk & Holland, 1983). Furthermore, this impairment of Ca^{2+} transport was associated with decreased labelling of the phosphorylation site of the Ca^{2+} -ATPase from [^{32}P]ATP. The decreased rates of Ca^{2+} uptake observed in contraction-inhibited muscle cultures could result from a decreased amount and/or altered activity of the sarcoplasmic-reticulum Ca^{2+} -ATPase.

In the present study we have investigated the effects of contractile activity on the expression of Ca^{2+} -ATPase in differentiating chick skeletal-muscle cultures. Both TTX blockade of fast Na^{+} channels and high- K^{+} depolarization of cultured chick myotubes decreased Ca^{2+} -ATPase accumulation. Selective loss of Ca^{2+} -ATPase from paralysed muscle fibres corresponded temporally to decreased myosin heavy-chain accumulation. The ultrastructure of contraction-inhibited myotubes was significantly different from that of contractile fibres with respect to sarcoplasmic-reticulum distribution. Treatment of myotubes with TTX accelerated turnover of the Ca^{2+} -ATPase enzyme and some other polypeptides of the sarcoplasmic reticulum isolated by ATP-dependent loading of microsomes with calcium oxalate. These results demonstrate that accumulation of the sarcoplasmic-reticulum Ca^{2+} -ATPase is regulated by the contractile activity of skeletal-muscle fibres in a manner similar to that previously reported for myosin heavy chain (Walker & Strohm, 1978) and other proteins of the myofibrillar apparatus (Crisona & Strohm, 1983).

MATERIALS AND METHODS

Materials

All tissue-culture reagents, such as minimal essential media and horse serum, were obtained from GIBCO. [^{35}S]Methionine was obtained from Amersham. Cytosine arabinoside, TTX, 3,3'-diaminobenzidine, protein M_r standards and routine laboratory reagents were purchased from Sigma. 4-Chloro-1-naphthol was from ICN. Electrophoresis reagents were obtained from Bio-Rad. Nitrocellulose was from Schleicher and Schuell. Vectastain ABC and biotinylated goat antibody to mouse IgG were Vector Laboratory products purchased from Dimension Laboratories. FITC-labelled goat antibody to mouse IgG was from Flow Laboratories.

Methods

Skeletal-muscle cultures. Embryonic-chick pectoralis major myoblasts were prepared from 13-day embryos and were cultured as previously described (O'Neill & Stockdale, 1972). Cultures were routinely treated with 5 μM -cytosine arabinoside for 24 h, beginning at 48 h in culture, to decrease fibroblast contamination. TTX was added to cultures to a final concentration of 0.14 μM . Muscle cultures were grown in 12 mM- K^{+} -media by the method of Bandman & Strohm (1982).

Metabolic labelling of muscle cultures. Growth medium was removed by aspiration from muscle cultures. Cells were rinsed

with 10 ml of warm Earle's balanced saline solution and methionine-free minimal essential medium was added. Cultures were pulse-labelled for 2 h in the presence of 1 μCi of L-[^{35}S]-methionine/ml. Cells were rinsed twice with 10 ml of phosphate-buffered saline (0.15 M- NaCl /5 mM- KCl /10 mM-sodium phosphate, pH 7.5) and harvested by scraping in phosphate-buffered saline. For turnover studies, cultures were re-fed with complete medium containing 150 mg of L-methionine/l; 24 h later the cells were harvested and membranes isolated.

Ca^{2+} transport. Rates of Ca^{2+} transport were measured with $^{45}\text{CaCl}_2$ by Millipore filtration as previously described (Charuk & Holland, 1983). The rate of ATP-dependent Ca^{2+} uptake was calculated from the difference between the rate of Ca^{2+} uptake in the presence and absence of ATP.

Antibody production. Monoclonal antibodies to the sarcoplasmic-reticulum Ca^{2+} -ATPase were produced as previously described (Karpati *et al.*, 1986). Hybridomas were grown in serum-free medium (Murakami *et al.*, 1982) and immunoglobulins were purified by $(\text{NH}_4)_2\text{SO}_4$ fractionation (Harboe & Ingild, 1973).

Immunofluorescent detection of Ca^{2+} -ATPase in myofibres. Embryonic-chick skeletal-muscle cultures were grown on collagen-coated glass coverslips in the presence or absence of TTX. Fixed cells were incubated with 0.4 μg of mouse monoclonal antibody P3_{x63}-1B₅-1B₆ to Ca^{2+} -ATPase/ml in phosphate-buffered saline containing 3% BSA, followed by 2.5 μg of fluorescein isothiocyanate-labelled goat antibody to mouse IgG/ml. A Leitz fluorescence microscope equipped with a Nikon camera was used to view and photograph cells.

Immunoprecipitation of Ca^{2+} -ATPase. Each 90 mm culture dish of muscle cells was extracted with 250 μl of electrophoresis sample buffer containing 10% SDS. Samples were incubated at room temperature for 30–60 min, rather than boiling, in order to prevent Ca^{2+} -ATPase aggregation (Horgan & Kuypers, 1981). Samples were precipitated in 10 vol. of acetone and protein was determined (Lowry *et al.*, 1951). Samples of 2, 4, 8 and 16 μg of protein and purified rabbit skeletal-muscle sarcoplasmic-reticulum Ca^{2+} -ATPase (MacLennan, 1970) were electrophoresed on SDS/6%-polyacrylamide gels (Laemmli, 1970) and then transferred to nitrocellulose paper (Towbin *et al.*, 1979). Blocked transfers were incubated with a 1:10 dilution of purified mouse monoclonal antibody to chicken Ca^{2+} -ATPase in 5% skim milk/phosphate-buffered saline for 16 h at 4°C. Bound antibodies were detected with affinity-purified biotinylated goat antibody to mouse IgG and Vectastain ABC, followed by staining with either 3,3'-diaminobenzidine or 2-chloro-4-naphthol. Stained immunoblots were scanned with a Zeiss PMQ II spectrophotometer. Peak areas corresponding to the stained 100 kDa Ca^{2+} -ATPase band were measured with a MOP-3 image analyser (Carl Zeiss).

Membrane preparations. Muscle cells were homogenized in 10 vol. of 0.1 M- KCl /0.25 M-sucrose/20 mM-imidazole (pH 7.2), with 30 strokes of a tight-fitting Dounce homogenizer pestle. The homogenate was centrifuged at 10000 g for 15 min and the supernatant layered on 1 ml of 55% sucrose/0.1 M- KCl /20 mM-imidazole (pH 7.2), then centrifuged at 120000 g for 1 h in an SW 41 rotor to sediment vimentin-containing intermediate filaments. The 55%-sucrose interface was carefully removed after centrifugation and incubated at 22°C in 0.5 mM- CaCl_2 /5 mM- NaN_3 /0.5 mM-EGTA/10 mM- MgCl_2 /5 mM-potassium oxalate/0.1 M- KCl /20 mM-imidazole (pH 6.8) for 30 min in the absence or presence of 10 mM-ATP. Samples were chilled in an ice bath, then layered on 10 ml of 55% sucrose/10 mM- MgCl_2 /0.5 mM- CaCl_2 /0.5 mM-EGTA/5 mM- NaN_3 /5 mM-potassium oxalate/0.1 M- KCl /20 mM-imidazole (pH 6.8). The samples were centrifuged at 120000 g for 1 h in an SW 41 rotor.

Fig. 1. Immunoblot analysis of sarcoplasmic-reticulum Ca^{2+} -ATPases

Samples of rabbit skeletal (lanes b, e) and chicken cardiac (lanes c, f) and 19-day-embryonic chick skeletal (lanes d, g) muscle sarcoplasmic reticulum corresponding to 10 μg (lanes b–d) or 1 μg (lanes e–g) of protein were electrophoresed on a SDS/10% polyacrylamide mini gel, then transferred to nitrocellulose paper. Nitrocellulose was stained with Ponceau S (lanes a–d), or blocked and immunoblotted with mouse monoclonal antibody P3_{x63}-1B₅-1B₆ (lanes e–g). Note the antibody detects both skeletal and cardiac isoforms of the Ca^{2+} -ATPase (arrowhead). Lane a represents the standards β -galactosidase, phosphorylase, BSA, ovalbumin, glyceraldehyde-3-phosphate dehydrogenase, trypsinogen, trypsin inhibitor and α -lactalbumin.

The 55%-sucrose interface (unloaded membrane fraction) was removed, and the remainder of the buffered sucrose was aspirated. The pellet (calcium oxalate-loaded membrane fraction) was solubilized directly by addition of 2% SDS/10% glycerol/

62.5 mM-Tris/HCl (pH 6.8) and protein was determined (Peterson, 1977). Mercaptoethanol (1%) was added and unboiled samples were electrophoresed on 8.75–15% acrylamide gradient gels (Laemmli, 1970).

Rabbit skeletal-muscle sarcoplasmic reticulum was isolated by the method of MacLennan (1970). Chicken cardiac-muscle sarcoplasmic reticulum was prepared by the method of Jones & Cala (1981). Embryonic-chick skeletal-muscle sarcoplasmic reticulum was prepared by differential centrifugation of homogenates, followed by calcium oxalate loading of microsomes. Breast muscles from 18-day chicks were homogenized in 5 vol. of 0.1 M-KCl/0.25 M-sucrose/20 mM-imidazole (pH 7.2) in a model CH-6010 Polytron (Kinematica G.m.b.H., Luzern, Switzerland), operating at speed 5, for 1 min. The tissue was further homogenized with 10 strokes of a Dounce homogenizer. Microsomes were prepared by differential and sucrose-density-gradient centrifugation, then loaded with calcium oxalate and fractionated as described above.

Calculation of metabolic labelling indices. The amount of acid-insoluble radioactivity incorporated or retained by proteins after a 2 h pulse label or a 2 h pulse label followed by a 24 h chase period was measured by liquid-scintillation spectrometry. The radioactivity in fractions was normalized to the protein content of the sample, and a fractional metabolic rate was calculated by the formula:

$$\frac{[^{35}\text{S}]\text{methionine d.p.m. (24 h chase)}/\text{mg of protein}}{[^{35}\text{S}]\text{methionine d.p.m. (2 h pulse)}/\text{mg of protein}}$$

The metabolic labelling index of the 100 kDa protein in calcium oxalate-loaded membrane fractions was determined from densitometric scans of gel fluorographs of [^{35}S]methionine-

Fig. 2. Immunofluorescent microscopy of Ca^{2+} -ATPase and myosin

For this 7-day-old control (panels a, c) and TTX-treated (panels b, d) skeletal-muscle fibres were fixed and then incubated with mouse monoclonal antibody to the Ca^{2+} -ATPase, followed by reaction with fluorescein isothiocyanate-conjugated goat antibody to mouse IgG, as described in the Materials and methods section. Note the diffuse staining pattern of Ca^{2+} -ATPase in TTX-treated myotubes (panel b) relative to controls (panel a). Decreased subcellular organization of the sarcotubular system is evident at higher magnification in paralysed myotubes (panel d) relative to controls (panel c). Magnification is $\times 400$ (panels a, b) or $\times 600$ (panels c, d).

labelled proteins. Peak paper weights were obtained and the fractional metabolic rate was calculated by a similar formula:

$$\frac{100 \text{ kDa peak paper wt. for } [^{35}\text{S}]\text{methionine (24 h chase)}}{100 \text{ kDa peak paper wt. for } [^{35}\text{S}]\text{methionine (2 h pulse)}}$$

RESULTS

A mouse monoclonal antibody (hybridoma clone P3_{x63}-1B₆) to the chicken sarcoplasmic-reticulum Ca²⁺-ATPase protein was tested for its cross-reactivity to fast-twitch skeletal and slow-

Fig. 3. Analysis of myosin and Ca²⁺-ATPase in skeletal-muscle cultures

Muscle culture extracts corresponding to 40 µg of protein were electrophoresed on SDS/6%-polyacrylamide gels and stained with Coomassie Blue (lanes a, b). Alternatively, protein samples corresponding to 16 µg were immunoblotted with monoclonal antibody to Ca²⁺-ATPase (lanes c, d). Panel (a), 10-day control (lanes a, c) and high-K⁺-treated (lanes b, d) muscle culture extracts. Panel (b), 14-day control (lanes a, c) and TTX-treated (lanes b, d) muscle culture extracts. The arrowheads represents the 100 kDa Ca²⁺-ATPase protein. Open arrow represents the myosin heavy-chain subunit. Note the decreased amounts of Ca²⁺-ATPase and myosin in both high-K⁺ and TTX-treated fibres relative to contractile cultures. The position of Rainbow (Amersham) prestained molecular-mass (kDa) standards is indicated to the left of lane a for lanes a and b, and to the right of lane d for lanes c and d.

Fig. 4. Accumulation of myosin (a) and Ca²⁺-ATPase (b) in contraction-inhibited muscle cultures

Samples of muscle culture extracts were electrophoresed on SDS/6%-polyacrylamide gels, then either stained with Coomassie Blue or immunoblotted with monoclonal antibody to the Ca²⁺-ATPase. Stained gels and immunoblots were subjected to densitometric scanning with a Zeiss PMQ II spectrophotometer. The relative peak areas corresponding to the 205 kDa myosin heavy-chain band and the 100 kDa Ca²⁺-ATPase band were measured with a MOP-3 image analyser (Carl Zeiss). The accumulation of myosin (a) and Ca²⁺-ATPase (b) in cells grown in high K⁺ (□) or the presence of TTX (◆) relative to untreated fibres represents the average of three experiments. Note the co-ordinate decrease in myosin and Ca²⁺-ATPase in both high-K⁺ and TTX-treated muscle cultures. The decrease in myosin and Ca²⁺-ATPase levels occurs earlier in cells grown in high K⁺ compared with fibres treated with TTX.

twitch/cardiac muscle isoenzymes. In addition to its reactivity with embryonic-chick skeletal-muscle Ca²⁺-ATPase (Fig. 1, lane g), the antibody recognized both isoforms of the 100 kDa enzyme present exclusively in adult rabbit skeletal-muscle and chicken cardiac-muscle sarcoplasmic reticulum respectively (Fig. 1, compare lanes e and f). Previous use of this antibody also confirmed its ability to detect Ca²⁺-ATPase in all fibre types of human skeletal-muscle tissue (Karpati *et al.*, 1986). This particular monoclonal antibody was therefore capable of detecting all of the known sarcoplasmic-reticulum Ca²⁺-ATPases present in developing skeletal-muscle fibres.

The ultrastructural organization of sarcoplasmic reticulum in contraction-inhibited myotubes was examined by indirect immunofluorescence microscopy using the monoclonal antibody to the Ca²⁺-ATPase. An ordered network of sarcoplasmic-reticulum membranes, co-localized with the myofibrillar I-band region, was observed in 7-day untreated myotubes (Fig. 2a). In contrast, 7-day-old TTX-treated cultures showed a less ordered cytoarchitecture as revealed by indirect immunofluorescent

Fig. 5. Composition of calcium oxalate-loaded and unloaded membranes

Samples of calcium oxalate-loaded (lanes b, c) and unloaded (lanes d, e) membrane vesicles, corresponding to 15 μg of protein, isolated from 7-day contractile muscle cultures labelled with [^{35}S]methionine for 24 h, were electrophoresed on a SDS/8.75–15% polyacrylamide gradient gel. The gel was stained with silver (lanes a, b, d) by the method of Sammons *et al.* (1981), then fluorographed (lanes c, e) by the method of Laskey & Mills (1975). Note the high level of incorporation of [^{35}S]methionine into the 100 kDa Ca^{2+} -ATPase protein (arrowhead) and the greater amount of this protein in calcium oxalate-loaded membranes than in the unloaded fraction (compare lanes b and d). Lane a represents the molecular-mass standards myosin, phosphorylase, BSA, ovalbumin and α -lactalbumin.

staining with monoclonal antibody to the Ca^{2+} -ATPase (Fig. 2b). At higher magnification, misalignment of sarcoplasmic-reticulum tubules could be clearly distinguished in 7-day-old TTX-treated muscle fibres relative to controls (Fig. 2 cf. panels c and d). Immunocytochemically, 7-day-old TTX-treated myofibres more closely resembled 5-day untreated muscle cultures than 7-day contractile myotubes with respect to their subcellular distribution of sarcoplasmic-reticulum Ca^{2+} -ATPase (results not shown). This suggested contraction-inhibition may arrest the structural maturation of sarcoplasmic reticulum in embryonic skeletal-muscle fibres.

To assess further the biochemical effects of contraction-inhibition on developing skeletal-muscle cultures, quantitative analysis of the accumulation of myosin heavy chain and the Ca^{2+} -ATPase was performed. The concentrations of sarcoplasmic-reticulum Ca^{2+} -ATPase in extracts of skeletal-muscle cultures were estimated by solid-phase immunoassay of Western blots (Haid & Suissa, 1983). Immunoblots of muscle culture extracts revealed a doublet of immunoreactive bands at the position corresponding to the 100 kDa Ca^{2+} -ATPase (Fig. 3). The existence of a doublet in this region may reflect co-expression of fast and slow isoforms of the ATPase by cultured muscle cells,

Fig. 6. Relative metabolic labelling indices of muscle culture fractions

Muscle cultures were either pulse-labelled for 2 h and then chased for 24 h, or pulse-labelled for 2 h with [^{35}S]methionine before harvest, as described in the Materials and methods section. Cells were homogenized and calcium oxalate-loaded and unloaded membrane vesicles were isolated as described in the Materials and methods section. Samples (5 μg) of SDS-solubilized proteins were spotted on to 3 MM filter-paper discs, and processed for scintillation counting by the method of Mans & Novelli (1961). The fraction of radioactivity remaining after a 24 h chase period relative to the amount incorporated during a 2 h pulse-label was calculated for each sample. Note the similar metabolic labelling indices for homogenate (a) and unloaded microsome (b) fractions isolated from control ($\square$) and TTX-treated ($\blacklozenge$) muscle cultures. However, the metabolic labelling indices of proteins associated with calcium oxalate-loaded membrane fractions (c) of contractile fibres were much higher at later stages of development than for parallel cultures of TTX-treated muscle fibres.

as reported by Kaprielian & Fambrough (1987). Decreased amounts of 100 kDa Ca^{2+} -ATPase were present both in 14-day myotubes treated with TTX (Fig. 3a, arrowhead, lane d) relative to untreated controls (lane c) and cultures grown in high K^+ for 10

Fig. 7. Biosynthetic labelling of calcium oxalate-loaded vesicle proteins

For this experiment 8-day control (lanes b, d, f, h) and TTX-treated (lanes c, e, g, i) muscle cultures were previously pulse-labelled and then chased (lanes b, c, f, g), or pulse-labelled (lanes d, e, h, i), as described in the Materials and methods section. Samples of calcium oxalate-loaded membrane vesicles, corresponding to 2.5 μ g of protein, were electrophoresed on a SDS/8.75–15% polyacrylamide gradient gel. The gel was first stained with silver (lanes a–e) and then fluorographed (lanes f–i) as described in Fig. 5 legend. Note the decreased radioactivity of all proteins after pulse-chase labelling of TTX-treated muscle cultures relative to controls (compare lanes f and g). Lane a represents the molecular-mass markers myosin, phosphorylase, BSA and ovalbumin. Arrowhead is the position of the 100 kDa Ca^{2+} -ATPase protein.

Fig. 8. Relative metabolic labelling indices of the 100 kDa Ca^{2+} -ATPase

Densitometric scans of the 100 kDa region of gel fluorographs were obtained and a metabolic labelling index was determined as described in the Materials and methods section. Note the stabilization of the 100 kDa Ca^{2+} -ATPase protein in calcium oxalate-loaded membrane fractions isolated from contractile myotubes (□) relative to TTX-treated fibres (◆).

days (Fig. 3b, lane d) relative to contractile myofibres (lane c). A correspondingly lower amount of 205 kDa myosin heavy chain was also accumulated by both 14-day TTX-treated (Fig. 3a, open arrow, lane b) and 10-day high- K^{+} cultures (Fig. 3b, lane b) relative to age-matched controls (Fig. 3, lane a, panels a and b respectively). Quantification by densitometric scanning of Ca^{2+} -

ATPase immunoblots and Coomassie Blue-stained gels revealed that the decreased amount of Ca^{2+} -ATPase accumulated by either TTX-paralysed or high- K^{+} -depolarized myotubes at different times in culture paralleled the temporal loss of myosin heavy chain from these cells (Fig. 4, compare panels a and b). Although high K^{+} similarly prevented the spontaneous contractile activity of myofibres, its effect on both myosin and Ca^{2+} -ATPase accumulation tended to occur earlier than TTX paralysis during the post-fusion development of muscle cultures. As in the previous study (Charuk & Holland, 1983), we measured rates of azide-insensitive ATP-dependent Ca^{2+} uptake into the particulate fractions of muscle culture homogenates. Rates of Ca^{2+} uptake by 7–10-day-old muscle cultures averaged 2.8 nmol/min per mg of protein for controls and 1.6 nmol/min per mg of protein for TTX-treated myofibres, a 43% decrease.

In order to study the biogenesis of sarcoplasmic reticulum, purification of the active Ca^{2+} -pump membrane from contractile and TTX-treated muscle cultures was performed by loading microsomes with calcium oxalate in the presence of ATP, followed by sucrose-density-gradient centrifugation. No high-density membrane vesicles were obtained when microsomes were incubated with calcium oxalate in the absence of ATP (results not shown). Calcium oxalate-loaded and unloaded membranes were isolated after a 24 h continuous [^{35}S]methionine labelling of the muscle culture, beginning at 6 days. Fig. 5 shows polypeptide compositions of 7-day untreated skeletal-muscle culture microsomes sedimenting through 55% (lane b) or recovered at the 55%-sucrose interface (lane d). Lanes c and e are the corresponding fluorographs of lanes b and d respectively. A large proportion of the protein recovered in the calcium oxalate-loaded membrane fraction represented the 100 kDa Ca^{2+} -ATPase enzyme (Fig. 4, arrowhead, lane b), as confirmed by parallel immunoblots of samples (results not shown). The calcium oxalate-loaded membrane fraction represents only 10–20% of the microsomal protein, but it contains 20–30 times the concentration of Ca^{2+} -ATPase relative to the unloaded membrane fraction. The Ca^{2+} -ATPase is also the major sarcoplasmic-reticulum protein labelled with [^{35}S]methionine at this stage of development (Fig. 5, arrowhead, lane c). Membrane fractions that failed to become loaded with calcium oxalate possessed a lower concentration of Ca^{2+} -ATPase, but shared several proteins in common with the loaded fraction (Fig. 5, compare lanes b, d and lanes c, e).

The relative metabolic labelling indices of proteins in the calcium oxalate-loaded fraction (Fig. 6c) were compared with those of the unloaded microsomal fraction (Fig. 6b) and the unfractionated homogenate (Fig. 6a). An increased metabolic labelling index indicates stabilization of proteins, since it is a ratio of the amount of radioactivity remaining in proteins after a 24 h chase relative to radioactivity incorporated during a 2 h pulse label. There were no apparent differences in metabolic labelling indices of control and TTX-treated homogenate and unloaded microsomal fractions. In contrast, the relative metabolic labelling indices of calcium oxalate-loaded membrane fractions from controls increased steadily throughout the time course, whereas those of TTX-treated muscle cultures remained low. This indicated that there was a stabilization of protein components in the calcium oxalate-loaded membrane fraction isolated from contractile myofibres that was prevented by TTX treatment.

Analysis of individual proteins of calcium oxalate-loaded membrane vesicles by electrophoresis and fluorography revealed that the 100 kDa Ca^{2+} -ATPase protein (Fig. 7, compare lanes f and g at arrowhead) and several other proteins in this fraction were degraded more rapidly in 8-day TTX-treated muscle cultures relative to controls. However, incorporation of [^{35}S]methionine

Fig. 9. Scanning densitometry of gel fluorographs of [^{35}S]methionine-labelled calcium oxalate-loaded membrane proteins

Control and TTX-treated muscle cultures were previously pulse-labelled with [^{35}S]methionine and then either chased for 24 h or harvested at 7–10 days. Calcium oxalate-loaded membrane vesicles were isolated, proteins electrophoresed and gels fluorographed. Densitometric scans of fluorographs were obtained with a GS300 linear gel scanner (Hoefer Scientific Instruments). The recorder tracings were byte-mapped with a Model 830 imaging system (Xerox Instruments) interfaced to a MacIntosh SE/30 personal computer. Data were converted into a CANVAS line graph (Swati Chung Fairwater, Deneba Systems, 1985–1989), and differences in amounts of radioactivity incorporated into proteins after a pulse-label (upper tracing) or remaining after a 24 h chase (lower tracing) were shaded. Note the greater differences between radioactivities of all proteins in the calcium oxalate-loaded fractions isolated from TTX-treated cultures, especially at 9–10 days of age. The arrowhead represents the position of the 100 kDa Ca^{2+} -ATPase enzyme.

during a 2 h pulse label into membrane proteins was relatively unaffected by TTX treatment (Fig. 7, compare lanes h and i).

To quantify further differences in sarcoplasmic-reticulum protein biogenesis and turnover, samples of calcium oxalate-loaded membrane vesicles were isolated from pulse-labelled and pulse-chased muscle cultures at various stages of development. Proteins were electrophoresed and the resulting fluorographs of gels were subjected to densitometric scanning. The relative metabolic labelling indices were determined for the 100 kDa Ca^{2+} -ATPase protein as described in the Materials and methods section. As Fig. 8 shows, the stability of the Ca^{2+} -ATPase enzyme in contractile myotubes became greater as cultures aged. This was reflected by the higher metabolic labelling indices observed for this protein at 9–10 days. In contrast, the metabolic labelling indices observed for the Ca^{2+} -ATPase in calcium oxalate-loaded membrane fractions isolated from TTX-treated myofibres remained low throughout the time course analysed. This indicated that contraction-inhibition led to increased turnover of the Ca^{2+} -ATPase enzyme in sarcoplasmic-reticulum membranes.

We looked to see whether increased protein turnover was a general phenomenon for calcium oxalate-loaded membrane fractions in TTX-treated muscle cultures. As densitometric scans in Fig. 9 indicate, less radioactivity was incorporated into all proteins of the active Ca^{2+} -pump membrane as cultures matured. The differences between the amounts of radioactivity incorporated into proteins after a 2 h pulse label (upper tracings) and remaining after a 24 h chase (lower tracings) became less distinguishable as control cultures matured (Fig. 9, compare shaded areas). In contrast, significant differences between the radioactivities of pulse-labelled and pulse-chased proteins in calcium oxalate-loaded membrane vesicles from TTX-treated muscle cultures continued to be observed from 8 to 10 days (Fig. 9, compare shaded areas). These findings indicated that increased turnover of the Ca^{2+} -ATPase and other proteins of the functional sarcoplasmic-reticulum membrane accompanied contraction-inhibition of myofibres. The elevated and co-ordinate breakdown of most proteins in the calcium oxalate-loaded membrane fraction of TTX-treated myotubes suggested a mechanism of unit turnover for the functional sarcoplasmic reticulum. Increased turnover of the sarcoplasmic-reticulum membrane is therefore likely to account for the decreased rates of Ca^{2+} transport and lower concentrations of steady-state phosphorylated intermediate formed by the Ca^{2+} -ATPase.

DISCUSSION

Two muscle fibre-specific isoforms of the Ca^{2+} -ATPase (fast and slow/cardiac) have been deduced (Brandl *et al.*, 1986) and their expression during skeletal muscle development has been studied (Brandl *et al.*, 1987). Clonal analysis has demonstrated co-expression of Ca^{2+} -ATPase isoforms during myogenesis, but preferential accumulation of the fast isoenzyme late in embryonic development of the chicken thigh muscle (Kaprielian & Fambrough, 1987). Using a monoclonal antibody that was capable of detecting both isoforms of the Ca^{2+} -ATPase in embryonic skeletal-muscle tissue, we measured the amounts of enzyme accumulated by contractile and paralysed myotubes. To date there has been no direct measurement of the concentration of sarcoplasmic-reticulum Ca^{2+} -ATPase attained by culture embryonic muscle cells. Estimates, based on selective labelling of the active site with ^{32}P , suggested that in aneural chick pectoral-muscle cultures the Ca^{2+} -ATPase accumulates to the extent of only 10% of its concentration *in vivo* (Martonosi *et al.*, 1977). Consequently, failure of the Ca^{2+} -ATPase to accumulate to a

concentration comparable with that observed *in vivo* may be due to the absence of innervation in cultured embryonic skeletal-muscle cells. Using purified rabbit skeletal-muscle Ca^{2+} -ATPase as a standard, we found a maximum of 2.7 μg of enzyme/mg of total cellular protein present on immunoblots of 9-day contractile muscle cultures. This equates to 27 pmol of Ca^{2+} -ATPase/mg of total cellular protein. Previous estimates, based on selective labelling of the active site of the enzyme with ^{32}P , led to an estimate of 40 pmol of Ca^{2+} -ATPase/mg of total cellular protein (Martonosi *et al.*, 1977), in good agreement with our immunochemical quantification.

In aneural chick pectoral-muscle cultures, inhibition of spontaneous contractions of myotubes by TTX results in the biosynthesis of sarcoplasmic reticulum with impaired Ca^{2+} uptake and decreased active-site labelling of the Ca^{2+} -ATPase by ^{32}P (Charuk & Holland, 1983). These deleterious effects of TTX on sarcoplasmic-reticulum function could result from decreased accumulation or activity of the Ca^{2+} -ATPase. Altered biosynthesis, composition or structural integrity of the sarcoplasmic-reticulum membrane itself might also occur during muscle paralysis. In the present study we investigated the effects of both TTX paralysis and high- K^{+} depolarization on the accumulation of the Ca^{2+} -ATPase. We found a lower amount of Ca^{2+} -ATPase in contraction-inhibited fibres that temporally paralleled the decrease in myosin heavy-chain accumulation. A decreased accumulation of the Ca^{2+} -ATPase in contraction-inhibited muscle cultures explains our previous observation of decreased microsomal ATP-dependent Ca^{2+} uptake and Ca^{2+} -dependent phosphoenzyme content in contraction-inhibited muscle cultures (Charuk & Holland, 1983). We do not understand why high- K^{+} depolarization decreased both Ca^{2+} -ATPase and myosin accumulation more dramatically than TTX and earlier during the post-fusion development of myotubes. It is possible that electrophysiological effects unrelated to contractile activity may additionally influence the accumulation of muscle-specific proteins. Alternatively, high K^{+} may block action potentials in young myotubes more effectively, since fewer TTX-sensitive Na^{+} channels are present at this time (Land *et al.*, 1973; Thomson & Dryden, 1981; Lawrence & Catterall, 1981a,b; Frelin *et al.*, 1984).

In addition to the decreased Ca^{2+} -ATPase contents of TTX-treated myotubes, the immunocytochemical distribution of enzyme in these fibres was significantly altered. The results of immunolocalization studies suggested there was a disruption of the normal accumulation of sarcoplasmic reticulum about the myofibrillar apparatus in contraction-inhibited myotubes. The immunofluorescent staining pattern was suggestive of an altered biosynthetic pattern for this membrane system in TTX-treated myotubes. We therefore investigated the synthesis and turnover rates of proteins associated with the function Ca^{2+} -pump membrane by metabolically labelling cells with [^{35}S]methionine and then purifying the sarcoplasmic reticulum by calcium oxalate fractionation. An increased rate of turnover of the Ca^{2+} -ATPase enzyme and some other protein components in calcium oxalate-loaded membrane fractions was observed for TTX-treated muscle cultures relative to controls. This pattern of regulating the accumulation of a muscle-specific membrane protein by contractile activity is therefore similar to that observed for various proteins of the myofibrillar apparatus (Walker & Strohman, 1978; Crisona & Strohman, 1983). This implies the existence of a common mechanism capable of targeting and dismantling various muscle structures responsible for excitation-contraction coupling. Our current hypothesis is that defective Ca^{2+} transport by the sarcoplasmic reticulum in contraction-inhibited muscle fibres leads to elevated resting myoplasmic Ca^{2+} concentrations. This in turn might be responsible for degeneration of the

myofibrillar apparatus by Ca^{2+} -activated neutral proteinase activity (Kasuga & Umazume, 1990). Although it has not been possible to order temporally the exact sequence of biochemical events leading to both the Ca^{2+} -ATPase and myosin deficiency in contraction-inhibited muscle cultures, we have observed that both TTX and high- K^{+} paralysis of fibres produce a co-ordinate effect on the accumulation of these proteins. Experiments designed to both monitor intracellular Ca^{2+} concentrations and measure Ca^{2+} -activated neutral proteinase activity are required to elucidate further the mechanisms responsible for disuse atrophy in this model system.

A major protein component of approx. 50–55 kDa was detected in microsomal fractions during all stages of development and was particularly enriched in younger myotubes. This protein was not a component of the calcium oxalate-loaded membrane fraction and was identified as the intermediate-filament phosphoprotein vimentin both by phosphorylation studies (J. H. M. Charuk & P. C. Holland, unpublished work) and its cross-reactivity with a rabbit antibody to human fibroblast vimentin (Pena *et al.*, 1983). More vimentin was detected in TTX-treated muscle cultures in control cultures (J. H. M. Charuk & P. C. Holland, unpublished work). Vimentin has also previously been reported to accumulate to a greater extent in contraction-inhibited myotubes (Crisona & Strohman, 1983). A role for intermediate filaments regulating assembly of sarcoplasmic reticulum is an interesting possibility. Tubules of sarcoplasmic reticulum initially accumulate near the Z disc of the contractile apparatus during muscle development (Warren, 1973). Vimentin-containing intermediate filaments are removed from this very same intermyofibrillar area during muscle development (Tokuyasu *et al.*, 1984). It was concluded from electron-microscopic studies that intermediate filaments occupy the cytoplasmic space destined to form the fenestrated collar of sarcoplasmic reticulum (Tokuyasu *et al.*, 1985). In the present studies, continued occupation of the intermyofibrillar space by vimentin-containing intermediate filaments in contraction-inhibited myofibres might prevent the insertion of functional sarcoplasmic reticulum into Z-disc regions of fibres, thereby destabilizing this membrane network. Additionally, the failure of the developing contractile and sarcotubular systems to attain structural stability may be due to defective incorporation of α -actinin into the Z discs of myofibrils in TTX-treated myotubes (Jockusch & Jockusch, 1980). How sarcoplasmic-reticulum membranes envelop and remain structurally associated with I- and A-band regions of the myofibrillar apparatus during muscle development and contraction remain unknown.

The present results indicate that the decrease in both the sarcoplasmic-reticulum Ca^{2+} -transport rate and steady-state concentration of the high-energy Ca^{2+} -ATPase phosphoenzyme intermediate observed previously (Charuk & Holland, 1983) can be explained by decreased accumulation of the Ca^{2+} -ATPase in contraction-inhibited muscle cultures. The results further indicate that decreased accumulation of the ATPase in such paralysed myotubes is the consequence of an increased rate of breakdown. The co-ordinate and elevated turnover of other proteins in the calcium oxalate-loaded membrane fraction isolated from TTX-treated muscle cultures supports the concept that the sarcoplasmic reticulum may disassemble as a unit, and may explain the altered subcellular organization of sarcoplasmic reticulum in contraction-inhibited myotubes. The muscle culture system therefore appears to be a suitable model system for studies on the regulatory mechanisms involved in sarcoplasmic-reticulum biogenesis and turnover. Further studies designed to investigate how contractile activity influences the stability of both the sarcotubular and myofibrillar systems are required, however. These studies should extend our knowledge of how

contractile activity regulates the growth and strengthening of skeletal-muscle tissue during embryonic development.

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