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Neural control of gene expression in skeletal muscle

Calcium-sequestering proteins in developing and chronically stimulated rabbit skeletal muscles

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1. Tissue contents of the sarcoplasmic-reticulum Ca^{2+}-ATPase ($Ca^{2+}+Mg^{2+}$-dependent ATPase), of calsequestrin and of parvalbumin were immunochemically quantified in homogenates of fast- and slow-twitch muscles of embryonic, maturing and adult rabbits. 2. Unlike parvalbumin, Ca^{2+}-ATPase and calsequestrin were expressed in embryonic muscles. 3. Presumptive fast-twitch muscles displayed higher contents of these two proteins than did presumptive slow-twitch muscles. 4. Calsequestrin steeply increased before birth and reached adult values in the two muscle types 4 days after birth. The main increase in Ca^{2+}-ATPase occurred during the first 2 weeks after birth. 5. Denervation of postnatal fast- and slow-twitch muscles decreased calsequestrin to amounts typical of embryonic muscle and suppressed further increases of Ca^{2+}-ATPase. Denervation caused slight decreases in Ca^{2+}-ATPase in adult fast-twitch, but not in slow-twitch, muscles, whereas calsequestrin was greatly decreased in both. 6. Chronic low-frequency stimulation induced a rapid decrease in parvalbumin in fast-twitch muscle, which was preceded by a drastic decrease in the amount of its polyadenylated RNA translatable *in vitro* 7. Tissue amounts of Ca^{2+}-ATPase and calsequestrin were essentially unaltered up to periods of 52 days stimulation. 8. These results indicate that in fast- and slow-twitch muscles different basal amounts of Ca^{2+}-ATPase and calsequestrin are expressed independent of innervation, but that neuromuscular activity has a modulatory effect. Conversely, the expression of parvalbumin is greatly enhanced by phasic, and drastically decreased by tonic, motor-neuron activity.

INTRODUCTION

The $Ca^{2+}+Mg^{2+}$-dependent ATPase (Ca^{2+}-ATPase) of the sarcoplasmic reticulum (SR), the SR membrane-bound Ca^{2+}-binding calsequestrin, and the soluble, cytosolic, Ca^{2+}-binding parvalbumin represent major components of the Ca^{2+}-sequestering system in skeletal muscle. The distribution of these proteins varies in different fibre types. Slow-twitch (type I) fibres contain significantly lower concentrations of Ca^{2+}-ATPase, calsequestrin (Jorgensen *et al.*, 1979) and parvalbumin (Celio & Heizmann, 1982; Heizmann, 1984) than do fast-twitch (type II) fibres. Moreover, the IIA and IIB fast-twitch subgroups display significant differences with regard to these Ca^{2+}-sequestering proteins (Leberer & Pette, 1986*b*; Maier *et al.*, 1986). In view of the specific innervation of these fibre types (for review, see Pette & Vrbová, 1985), the expression of these proteins appears to be under neural control. This suggestion is corroborated by observations in cross-reinnervated (Mommaerts *et al.*, 1969; Sréter *et al.*, 1975; Müntener *et al.*, 1985) and chronically stimulated muscles (Heilmann & Pette, 1979; Wiehrer & Pette, 1983; Klug *et al.*, 1983; Leberer & Pette, 1986*a*). These experimental alterations in neural input have been shown to induce fibre-type-specific changes in the properties and composition of isolated SR preparations.

In addition, the neural control of parvalbumin expression has been established by the finding that innervation is a prerequisite for the pronounced postnatal increase in fast-twitch muscles (Leberer & Pette, 1986*a*). These results, together with data from low-frequency chronic-stimulation experiments, indicate that the expression of parvalbumin in skeletal muscle is under positive control of a phasic neural activity pattern.

This study investigates whether neural control applies to other components of the Ca^{2+}-sequestering system, especially the SR Ca^{2+}-ATPase and calsequestrin. Sensitive immunochemical methods (Leberer & Pette, 1986*a*,*b*) have made it possible to quantify these proteins in muscle homogenates without isolating SR membranes, and therefore to investigate developmental and experimentally induced changes in the cytosolic and SR Ca^{2+}-sequestering proteins at the cellular level. Furthermore, we were interested in correlating changes of these proteins as induced by chronic stimulation with changes in the amounts of their poly(A)$^+$ RNAs translatable *in vitro*. Parts of this work have been communicated in a preliminary form (Leberer *et al.*, 1985).

MATERIALS AND METHODS

Animals

New Zealand White rabbits were supplied by the animal facility of the University of Konstanz.

Embryonic muscles

Pregnant animals were killed at different times of gestation and the embryos were surgically removed. Leg (vastus lateralis, tibialis anterior, semitendinosus, soleus) and psoas muscles were dissected, frozen in liquid N_2 and stored at −75 °C.

Abbreviations used: Ca^{2+}-ATPase, $Ca^{2+}+Mg^{2+}$-dependent ATPase; e.l.i.s.a., enzyme-linked immunoadsorbent assays; poly(A)$^+$ RNA, polyadenylated RNA; SR, sarcoplasmic reticulum.

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Denervation

Denervation of tibialis anterior and soleus muscles in newborn, young and adult animals was performed as previously described (Leberer & Pette, 1986*a*).

Stimulation

Chronic (12 h/day) low-frequency (10 Hz) stimulation was performed as previously described (Seedorf *et al.*, 1986). Animals were killed after different periods of stimulation, and extensor digitorum longus muscles were dissected from stimulated and unstimulated (contralateral) legs and frozen in liquid N_2.

Preparation of muscle extract

A 50–100 mg portion of frozen muscle was pulverized under liquid N_2 in a micro-mortar (Pette & Reichmann, 1982). The muscle powder was then diluted 1:20 (w/v) in 5 mM-Hepes buffer, pH 7.5, containing 250 mM-sucrose, 0.2% (w/v) NaN_3 and 0.1 mM-phenylmethanesulphonyl fluoride and homogenized. After 1:1 dilution with an extraction medium [10 mM-sodium phosphate buffer, pH 7.4, 150 mM-NaCl, 2% (v/v) Triton X-100, 2% (w/v) deoxycholate, 0.2% (w/v) SDS, 0.2 mM-phenylmethanesulphonyl fluoride and 200 kallikrein-inhibitory units of Trasylol/ml], the homogenate was centrifuged at 10000 *g* for 15 min. Protein concentration of the supernatant fraction was determined as described by Lowry *et al.* (1951). The supernatant fraction was frozen in liquid N_2 and stored at −75 °C until analysed. Depending on the contents of the specific antigens, the sample was directly used or appropriately diluted so that the values measured by the 'sandwich' e.l.i.s.a. and the immunoblot analysis were on the linear portion of the calibration curves.

Quantification of Ca^{2+}-ATPase by immunoblotting

Measurements of Ca^{2+}-ATPase in embryonic and developing muscles were performed by immunoblot analysis as previously described (Leberer & Pette, 1986*b*). Proteins were transferred after SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) to nitrocellulose sheets (Towbin *et al.*, 1979). After reaction with the specific sheep anti-rabbit antiserum (Leberer & Pette, 1986*b*) and the horseradish-peroxidase-conjugated rabbit anti-sheep IgG (Nordic Immunological Laboratories, Tilburg, Netherlands), the Ca^{2+}-ATPase band was identified by staining with α-chloronaphthol and H_2O_2 (Hawkes *et al.*, 1982). The staining reaction was calibrated by electrophoresing known amounts of purified Ca^{2+}-ATPase on the same gels and quantified densitometrically with the LKB 2202 Ultroscan laser densitometer. As proposed by Riezman (1984), the linearity of the reaction was verified by applying various dilutions of the extracts.

Quantification of Ca^{2+}-ATPase, calsequestrin and parvalbumin by 'sandwich' e.l.i.s.a.

Enzyme-linked immunoadsorbent assays (sandwich e.l.i.s.a.) were used for measurement in muscle homogenates as described for Ca^{2+}-ATPase and calsequestrin (Leberer & Pette, 1986*b*) and parvalbumin (Leberer & Pette, 1986*a*).

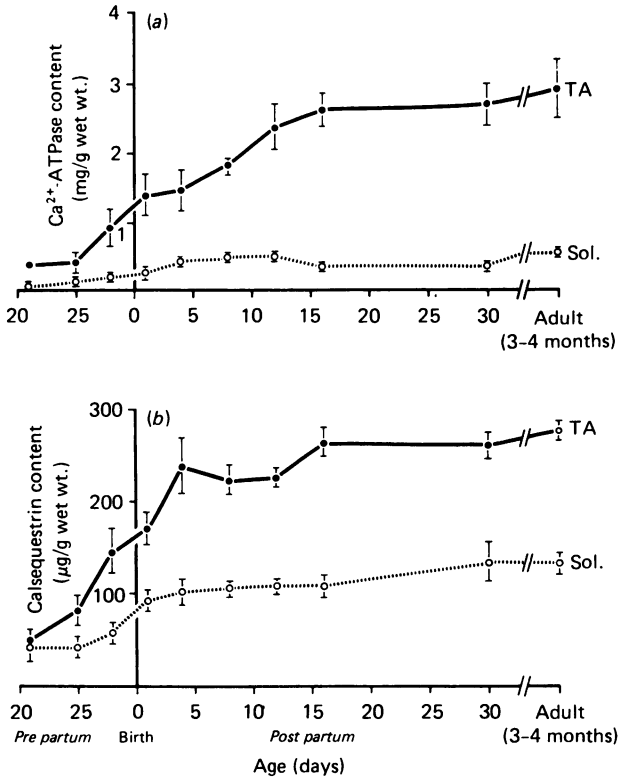

Fig. 1. SR Ca^{2+}-ATPase and calsequestrin contents in fast-twitch tibialis anterior (TA) and slow-twitch soleus (Sol.) muscles of the rabbit during late-embryonic myogenesis and postnatal development

Tissue contents of Ca^{2+}-ATPase were measured by quantitative immunoblot analysis and those of calsequestrin by sandwich e.l.i.s.a. in detergent-solubilized muscle extracts. Values represent means ± S.D. ($n = 3$–5) or means from two independent measurements in different animals.

Determination of specific mRNAs

The mRNAs coding for Ca^{2+}-ATPase and parvalbumin were quantified by translation *in vitro* of poly(A)$^+$ RNA and immunoprecipitation of the [^{35}S]methionine-labelled translation products. The conditions for extraction, purification and translation of poly(A)$^+$ RNA *in vitro*, as well as evaluation of the immunoprecipitates after electrophoresis, were same as described by Seedorf *et al.* (1986).

RESULTS

Developmental studies

Tissue contents of Ca^{2+}-ATPase were 6–7-fold higher in adult fast-twitch (tibialis anterior, vastus lateralis, psoas) than in adult slow-twitch (soleus, semitendinosus) muscles. These differences resulted predominantly from postnatal increases occurring in the fast-twitch muscles, although increases were also evident before birth (Fig. 1*a*). Fast- and slow-twitch muscles differed to a lesser degree with respect to their calsequestrin contents (Fig. 1*b*). Calsequestrin was approx. 2-fold higher in adult fast-twitch than in adult slow-twitch muscles. However, adult contents of calsequestrin were reached earlier than

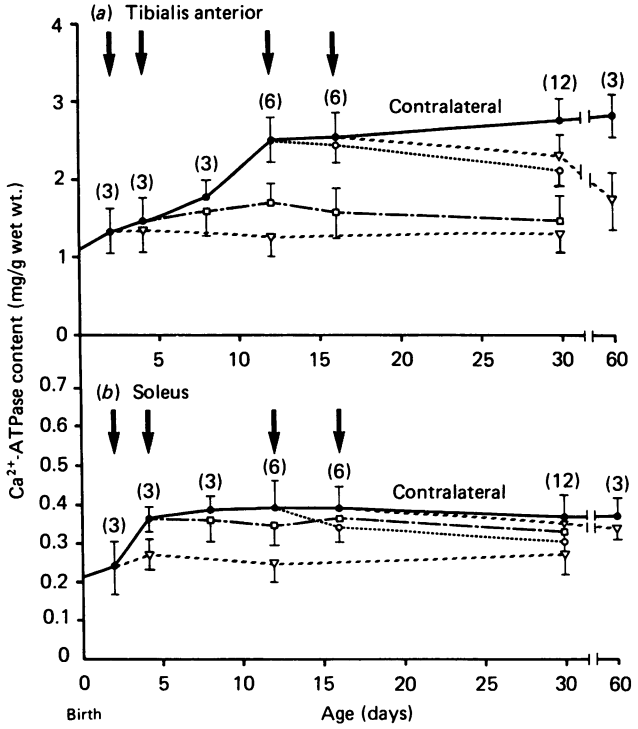

Fig. 2. Effects of denervation at various time points on tissue contents of SR Ca^{2+}-ATPase in maturing (*a*) tibialis anterior and (*b*) soleus muscles of the rabbit

Muscles of one hindlimb were denervated at days 2, 4, 12 and 16 (arrows) *post partum*. The Ca^{2+}-ATPase contents were determined by sandwich e.l.i.s.a. at various time points thereafter. Values given for the denervated muscles are means ± S.D. for three animals each. Values for the contralateral muscles were combined at each time point and represent means ± S.D. with the numbers of animals given in parentheses.

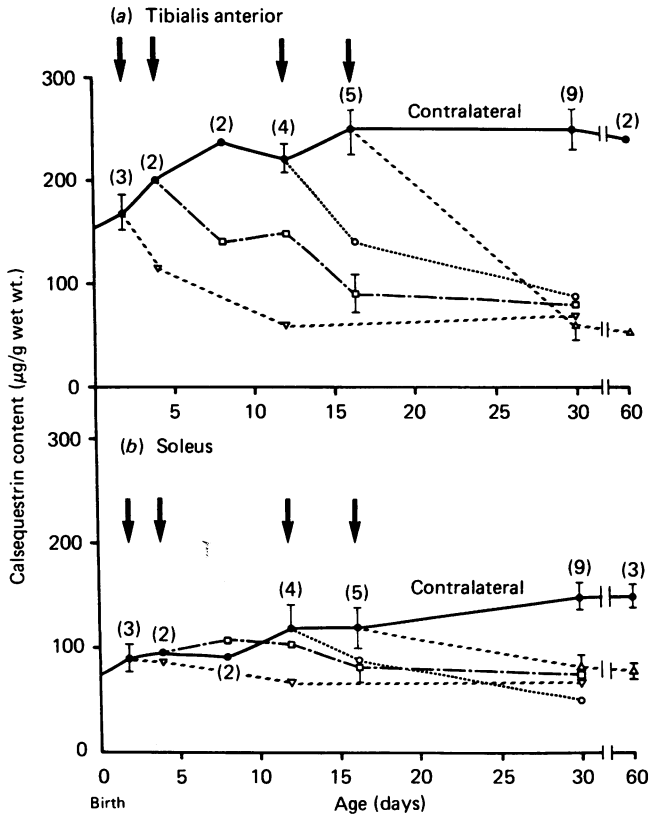

Fig. 3. Effects of denervation at various time points on tissue contents of calsequestrin in maturing (*a*) tibialis anterior and (*b*) soleus muscles of the rabbit

Muscles of one hindlimb were denervated at days 2, 4, 12 and 16 (arrows) *post partum*. The calsequestrin contents were determined by sandwich e.l.i.s.a. at various time points thereafter. Values given for the denervated muscles are means ± S.D. for three animals each, or represent single values or means from two animals. Values for the contralateral muscles were combined at each time point and represent means ± S.D. with the numbers of animals given in parentheses.

adult Ca^{2+}-ATPase values in both muscle types. In addition, calsequestrin appeared to increase at a steeper rate in the embryonic muscles compared with Ca^{2+}-ATPase.

Denervation studies

Denervation had pronounced effects upon the expression of Ca^{2+}-ATPase (Fig. 2) and calsequestrin (Fig. 3) in slow- and fast-twitch muscles during postnatal development. Denervation of developing muscles interrupted the normal increase in Ca^{2+}-ATPase at all time points investigated, and was followed by decreases in calsequestrin to values found before birth. Because this decrease was more pronounced in fast- than in slow-twitch muscles, the two muscle types became similar with regard to their calsequestrin content after denervation. Although denervation of adult fast- and slow-twitch muscles had no significant effect on the amounts of Ca^{2+}-ATPase (Fig. 4*a*), calsequestrin was decreased in both muscle types to approx. 50%, and therefore resembled embryonic values (Fig. 4*b*).

Chronic stimulation

Chronic stimulation for up to 52 days induced only slight decreases in the tissues contents of both Ca^{2+}-ATPase (Fig. 5*b*) and calsequestrin (Fig. 5*c*). However, Ca^{2+}-ATPase and calsequestrin decreased by 32% and 50%, respectively, in one animal which underwent 60 days of stimulation (results not shown). This tendency for a decrease to occur after longer stimulation periods was also evident from changes in the tissue contents of poly(A)$^+$RNA translatable *in vitro*. The amount of mRNA coding for Ca^{2+}-ATPase was decreased by 20% in 52-day-stimulated muscles (Fig. 5*a*).

As previously shown, chronic low-frequency stimulation elicited a drastic decrease in the tissue content of parvalbumin (Klug *et al.*, 1983; Leberer & Pette, 1986*a*). This decrease in parvalbumin began after 6 days stimulation and reached 50% of the control value after 11–12 days. Parvalbumin was decreased to the value normally found in slow-twitch muscles after 3 weeks stimulation (Fig. 6*b*). The decrease in parvalbumin was preceded by a decrease in the amount of its poly(A)$^+$RNA translatable *in vitro*, which began 4 days after the onset of stimulation and reached 50% of the control value in 6-day-stimulated muscles (Fig. 6*a*).

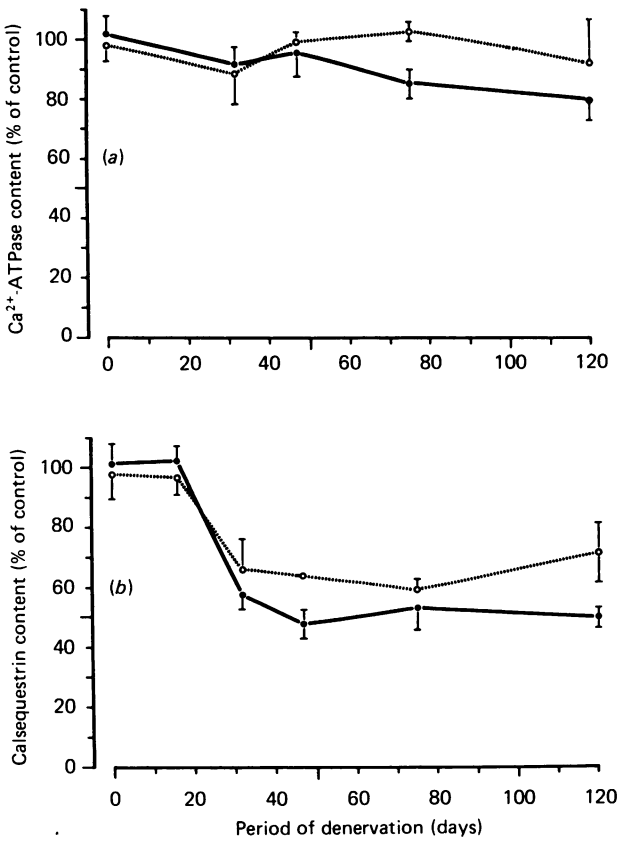

Fig. 4. Effects of denervation on (*a*) Ca^{2+}-ATPase and (*b*) calsequestrin contents in fast-twitch tibialis anterior and slow-twitch soleus muscles of adult rabbits

Ca^{2+}-ATPase and calsequestrin contents were measured by sandwich e.l.i.s.a. in detergent-solubilized extracts from denervated and contralateral fast-twitch tibialis anterior (——) and slow-twitch soleus (· · · · · ·) muscles at different time points after denervation. Values represent means ± S.E.M. for three independent experiments, and are expressed as percentages of the contents in the contralateral muscles.

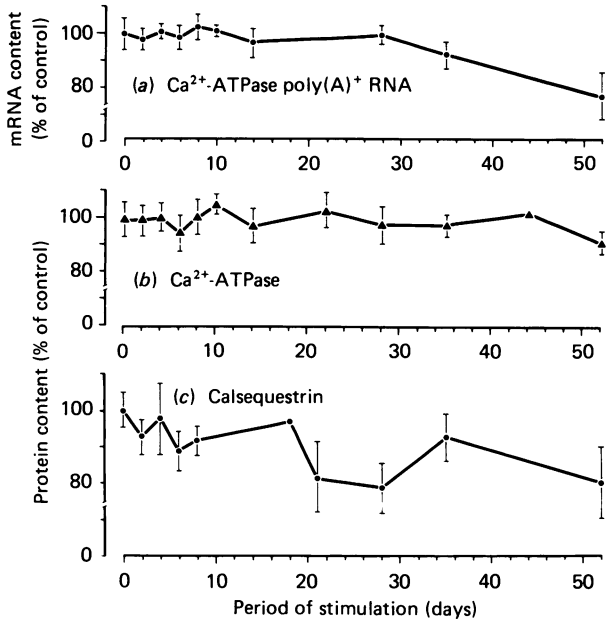

Fig. 5. Effect of indirect low-frequency stimulation on tissue contents of (*a*) poly(A)$^+$ mRNA translatable *in vitro* coding for SR Ca^{2+}-ATPase, (*b*) SR Ca^{2+}-ATPase and (*c*) calsequestrin

Rabbit extensor digitorum longus muscles were indirectly stimulated (10 Hz, 12 h/day) for different time periods. Amounts of poly(A)$^+$ RNA translatable *in vitro* coding for Ca^{2+}-ATPase (*a*) were quantified in stimulated and unstimulated (contralateral) muscles by immunoprecipitation of [^{35}S]methionine-labelled *in vitro* products of translation. Ca^{2+}-ATPase concentrations (*b*) were measured in the same muscles by sandwich e.l.i.s.a. Calsequestrin contents (*c*) were quantified by sandwich e.l.i.s.a. Values represent means ± S.E.M. ($n = 3$–5) or means for two animals and are given as percentages of the contents in the unstimulated contralateral muscles.

DISCUSSION

Quantification of Ca^{2+}-sequestering proteins in whole muscle homogenates

By using immunochemical methods, the present study provides information on the cellular contents of three major Ca^{2+}-sequestering proteins in skeletal muscle. The validity of these measurements is based on the specificity of the antibodies, which have been characterized in two preceding studies (Leberer & Pette, 1986*a*,*b*). Unlike measurements of Ca^{2+}-ATPase and calsequestrin in isolated SR fractions, the present immunochemical quantifications are independent of variations in the yield of the membrane preparations. Therefore the present approach resembles the quantification of Ca^{2+}-ATPase by Martonosi *et al.* (1977), who assessed tissue contents of Ca^{2+}-ATPase by measuring the phosphoprotein intermediate of the enzyme in whole muscle homogenates. Direct measurement of SR proteins in muscle homogenates is preferable because SR preparations are normally contaminated with other membranes. It is also conceivable that the amount of contaminating membranes varies in different muscle types, as well as during development or experimentally induced changes. Indeed, Dux & Martonosi (1984) have shown by electron microscopy of vanadate crystals of the Ca^{2+}-ATPase that differences between SR preparations from fast- and slow-twitch muscles are due to contaminations with non-SR membranes. According to those authors, the density of vanadate crystals of Ca^{2+}-ATPase is identical in SR membranes of fast- and slow-twitch muscles. This applies also to the density of Ca^{2+}-ATPase in the SR of developing muscle (Dux, 1985). Therefore the immunochemically determined increases in Ca^{2+}-ATPase during muscle maturation (Fig. 1) should reflect increases in the fractional surface area primarily of the longitudinal SR. Using the calcium oxalate loading method, MacLennan *et al.* (1985) demonstrated that the protein composition of pure SR vesicles from maturing rabbit skeletal muscles essentially resembles that of adult muscles. This applies to the relative concentration of the Ca^{2+}-pumping ATPase protein, and therefore increases in the Ca^{2+}-ATPase content and/or activity observed in previous studies on isolated microsomal fractions from maturing muscles (Fanburg *et al.*, 1968; Holland & Perry, 1969; Lough *et al.*, 1972; Sarzala *et al.*, 1975) may be explained by decreases in non-SR membranes (Volpe *et al.*, 1982).

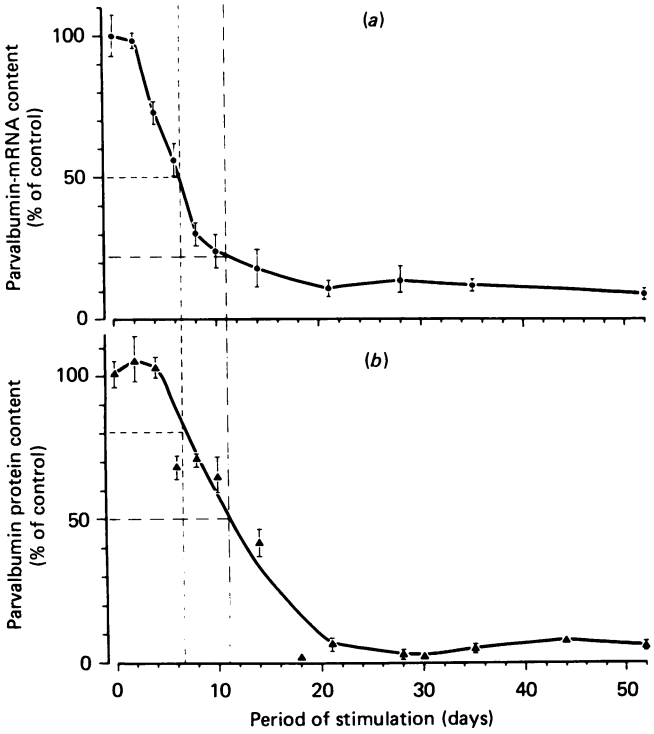

Fig. 6. Effect of indirect low-frequency stimulation on tissue contents of (*a*) poly(A)$^+$ RNA translatable *in vitro* coding for parvalbumin and (*b*) parvalbumin

Rabbit extensor digitorum longus muscles were indirectly stimulated (10 Hz, 12 h/day) for different time periods. Amounts of poly(A)$^+$ RNA *in vitro* coding for parvalbumin were quantified in stimulated and unstimulated (contralateral) muscles by immunoprecipitation of [^{35}S]-methionine-labelled products of translation *in vitro*. Parvalbumin concentrations were measured in the same muscles by sandwich e.l.i.s.a. Values represent means ±S.E.M. (n = 3–11) or means for two animals, and are given as percentages of the contents in the unstimulated contralateral muscles.

Neural control of the expression of Ca^{2+}-ATPase and calsequestrin during development

Unlike parvalbumin (Leberer & Pette, 1986*a*), fast- and slow-twitch muscles express Ca^{2+}-ATPase and calsequestrin during embryonic development. This confirms observations on the early appearance of Ca^{2+}-ATPase in embryonic rabbit (Sarzala *et al.*, 1975) and chick muscles (Fanburg *et al.*, 1968; Boland *et al.*, 1974; Martonosi *et al.*, 1977). The steeper increase in calsequestrin than in Ca^{2+}-ATPase during embryonic development reflects their unco-ordinated synthesis. Studies on embryonic-rat muscle cultures showed that the synthesis of calsequestrin precedes that of Ca^{2+}-ATPase (Holland & MacLennan, 1976; Zubrzycka & MacLennan, 1976; Jorgensen *et al.*, 1977). As shown in embryonic-chick muscle cultures, Ca^{2+}-ATPase and calsequestrin are expressed independent of innervation, but their concentrations remain low as compared with adult muscles (Martonosi *et al.*, 1977; Martonosi, 1982).

During myogenesis *in vivo*, these two membrane proteins attain significantly different contents in presumptive fast- and slow-twitch muscles before birth (Fig. 1). These differences may point to either different intrinsic properties or specific neural influences at this stage. That innervation plays a role in regulating different contents of these two proteins in fast- and slow-twitch muscles clearly follows from the effects of denervation both in maturing and adult muscle. Calsequestrin is decreased to its basal content in immature muscles, whereas the normally occurring postnatal increases of Ca^{2+}-ATPase are repressed. However, fibre-type-specific differences are not abolished. Thus differences between the Ca^{2+}-ATPase contents of fast- and slow-twitch muscles are to some degree independent of innervation and reflect different intrinsic properties. On the other hand, the different increases in Ca^{2+}-ATPase in fast- and slow-twitch muscles during postnatal development suggest that neural input is also involved in the 'fine tuning' of the Ca^{2+}-ATPase expression, as proposed for the regulation of troponin-T isoforms in chicken fast-twitch skeletal muscle (Shimizu & Shimada, 1985). It is interesting that neural activity has a much stronger influence on the expression of parvalbumin in developing and adult fast-twitch muscles. Its expression is primarily under positive control of phasic and under negative control of tonic neuromuscular activity (Leberer & Pette, 1986*a*).

Effect of altered neural activity

The application of a low-frequency (tonic) activity pattern resulted in a rapid decrease in parvalbumin, but only prolonged stimulation periods caused moderate decreases in Ca^{2+}-ATPase and calsequestrin. The rapid decay in parvalbumin appears to be due to a decreased amount of its mRNA, which precedes that of the protein (Fig. 6). Although proteolytic activities have not been studied, it appears unlikely that the decrease in parvalbumin is primarily related to proteolysis. The moderate decrease in Ca^{2+}-ATPase that occurred after prolonged stimulation was also correlated with a decrease in its mRNA (Fig. 5). Similarly, the stimulation-induced decay of the M subunit of lactate dehydrogenase is related to a decrease in its poly(A)$^+$ RNA translatable *in vitro* (Seedorf *et al.*, 1986).

The present finding that the tissue contents of Ca^{2+}-ATPase and calsequestrin are relatively stable in low-frequency-stimulated fast-twitch muscle until 50 days is not in conflict with earlier observations of pronounced changes in the composition of isolated microsomal fractions from short-term-stimulated muscles (Wiehrer & Pette, 1983; Klug *et al.*, 1983). Those studies showed decreases in the relative microsomal concentrations of Ca^{2+}-ATPase and calsequestrin after 1 week of stimulation. These decreases need not reflect changes in tissue contents of these proteins, but are most likely explained by increasing contamination with non-SR membranes in the microsomal preparations.

In summary, these results show that both SR Ca^{2+}-ATPase and calsequestrin are present during late embryonic development in amounts that are higher in presumptive fast- than in presumptive slow-twitch muscles of the rabbit. Differences in the concentrations of these two membrane proteins in maturing and adult fast- and slow-twitch muscles result from different intrinsic properties and, in addition, are caused by modulatory effects of specific motor-neuron activities. Conversely, parvalbumin is expressed only postnatally in fast-twitch muscles and appears to be under direct neural

(phasic) control. Therefore, Ca^{2+}-ATPase and calsequestrin, the two major Ca^{2+}-sequestering proteins of the sarcoplasmic reticulum, and parvalbumin, the major cytosolic Ca^{2+}-binding protein in fast-twitch muscle, are expressed in an unco-ordinated manner.

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