Neural regulation of parvalbumin expression in mammalian skeletal muscle

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1. Parvalbumin was purified from rabbit fast skeletal muscle and used to raise antibodies in sheep. 2. Subsequently, a sensitive 'sandwich' enzyme-linked immunoadsorbent assay permitted quantification of parvalbumin in homogenates of embryonic, maturing, innervated, denervated and chronically stimulated skeletal muscles of the rabbit. 3. High concentrations of parvalbumin were detected in various adult fast-twitch muscles of the rabbit (700–1200 µg/g of muscle), whereas slow-twitch muscles contained negligible concentrations (3–5 µg/g of muscle). 4. Parvalbumin was not detectable in embryonic-rabbit muscles (21, 25, 28 days of gestation), either presumptive fast- or slow-twitch. However, parvalbumin concentrations did increase during postnatal development in presumptive fast-twitch muscles. Thus the onset of parvalbumin synthesis appears to be correlated with the neonatal-to-adult transition of motor-neuron activity [Navarrete & Vrbová (1983) *Dev. Brain Res.* 8, 11–19]. 5. The increase of parvalbumin in maturing, presumptive fast-twitch muscle was suppressed by denervation. In the adult rabbit, denervation of the tibialis anterior muscle caused a reduction of parvalbumin to a level normally found in slow-twitch muscles. 6. In contrast, the already low levels of parvalbumin in maturing and adult slow-twitch soleus muscle were unaffected by denervation. 7. Chronic low-frequency stimulation of adult fast-twitch muscle resulted in a rapid reduction of parvalbumin to a level normally found in slow-twitch muscle. 8. These data support the hypothesis that the expression of parvalbumin is under positive control of fast-type motor-neuron activity.

INTRODUCTION

Parvalbumin is a low- M_r cytosolic Ca^{2+} -binding protein (for review, see Demaille, 1982; Wnuk *et al.*, 1982; Heizmann, 1984) that has been identified in mammalian fast-twitch (type IIa, type IIb) skeletal-muscle fibres (Lehky *et al.*, 1974; Blum *et al.*, 1977; Celio & Heizmann, 1982) and in nerve tissue (Baron *et al.*, 1975; Celio & Heizmann, 1981). Blum *et al.* (1977) found no parvalbumin in slow-twitch (type I) soleus fibres of the rabbit, indicating that differences exist in the concentration of parvalbumin between fast and slow muscles. These differences can be extended to the different fibre types (Celio & Heizmann, 1982), as immunohistochemical studies have shown that the concentration of parvalbumin is highest in type IIb fibres, variable in type IIa fibres, and undetectable in type I fibres of rat muscle.

The synthesis and differentiation of various fibre-type-specific protein isomorphs occurs early in postnatal development. In rat muscle, the transition of myosin isomorphs from the neonatal to the adult form (Whalen *et al.*, 1981) and the expression of the adult patterns of troponin (Dhoot & Perry, 1979, 1980) and tropomyosin (Roy *et al.*, 1979) coincide with the appearance of parvalbumin 3–4 days after birth (Celio & Heizmann, 1982; Berchtold & Means, 1985). The concomitant onset of locomotion, development of specific motor-activity patterns (Navarrete & Vrbová, 1983), and the loss of polyneuronal innervation (O'Brien *et al.*, 1978) suggest

that muscle activity and/or neural input may be important at this time in the regulation of the expression of parvalbumin and myofibrillar proteins. In addition, because chronic nerve stimulation of fast-twitch muscle induces fast-to-slow conversions of the major functional systems of the muscle fibre (for review, see Pette, 1984), the activity pattern may control some fibre-type-specific properties in adult muscle also. A decrease in parvalbumin occurs in denervated fast-twitch muscle (Müntener *et al.*, 1985a) and is one of the earliest and most sensitive markers of activity-induced fast-to-slow conversions (Klug *et al.*, 1983a,b). In view of this high degree of sensitivity and the possible role of innervation and activity upon the expression of parvalbumin, we decided to study in more detail the changes in its concentration during development, denervation and low-frequency stimulation.

MATERIALS AND METHODS

Chemicals

Polystyrene microtitre plates with flat bottoms were from Konton AG (Zurich, Switzerland), and nitrocellulose membrane filters (BA-85) from Schleicher und Schüll (Dassel, Germany). HRP-conjugated rabbit anti-sheep IgG was from Nordic Immunological Laboratories (Tilburg, The Netherlands). HRP (grade I) was from Boehringer Mannheim (Mannheim, Germany) and

Abbreviations used: EDL, extensor digitorum longus; e.l.i.s.a., enzyme-linked immunoadsorbent assay; HRP, horseradish peroxidase; PMSF, phenylmethanesulphonyl fluoride; TA, tibialis anterior; k.i.u., kallikrein-inhibitory unit.

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low-fat milk powder from Töpfer (Dietmannsried, Germany). Ponceau S, Triton X-100 and deoxycholate were from Serva (Heidelberg, Germany). Trasylol, PMSF, Tween 20 and α -chloronaphthol were from Sigma (München, Germany). Phenylene-1,2-diamine dihydrochloride was from Fluka AG (Buchs, Switzerland). All other chemicals used were obtained from Merck (Darmstadt, Germany).

Animals

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

Chronic stimulation

Stimulation was performed on adult rabbits (3.0–3.5 kg body wt.) using the previously described telestimulation system (Schwarz *et al.*, 1983). The animals were anaesthetized by intraperitoneal injection of 3.5–4.0 ml (100 mg/ml) of Ketavet (Parke-Davis, München, Germany) plus 0.8 ml of Rompun (Bayer, Leverkusen, Germany). Paired electrodes were implanted lateral to the left popliteal nerve, passed subcutaneously to the back of the animal, and connected to a small receiver secured to the back of the animal. At 1 week after electrode implantation, the animals were stimulated (10 Hz, 0.15 ms pulse duration) for 12 h daily by using alternating periods of 1 h stimulation and 1 h rest. After various periods of stimulation, animals were killed by cervical fracture and exsanguination. EDL muscles were quickly removed from both stimulated and unstimulated, contralateral legs and frozen in liquid nitrogen. Muscles were stored at -75°C until further processed.

Denervation

Animals were anaesthetized as described above. Denervation of TA muscle in adult animals was achieved by extracting a 2 cm piece of the common peroneal nerve at the bifurcation of the sciatic nerve. Soleus was denervated by extracting 2 cm of the branch of the tibial nerve proximal from its entry into the muscle. Denervation in newborn animals was similarly performed, except that the extracted nerve segments were shorter. The denervated and contralateral muscles were dissected at different time points and stored as described above.

Embryonic muscles

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

Preparation of muscles for parvalbumin analysis

A 50–100 mg portion of frozen muscle was pulverized under liquid nitrogen 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-PMSF 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/0.1 mM-PMSF/Trasylol (200 k.i.u./ml)], the homogenate was centrifuged at

10000 g for 15 min. The protein concentration of the supernatant fraction was determined by the method of Lowry *et al.* (1951). The supernatant fraction was frozen in liquid nitrogen and stored at -75°C until analysed for parvalbumin. Depending on the parvalbumin content, the sample was directly used or appropriately diluted so that the values measured by the sandwich e.l.i.s.a. fell on the linear portion of the calibration curve.

Purification of parvalbumin

Parvalbumin was purified from rabbit fast skeletal muscles according to Lehky *et al.* (1974). The purity of the preparation was verified by one- (Laemmli, 1970) and two-dimensional (O'Farrell, 1975) electrophoresis and by comparison of the absorbance at 260 and 280 nm (Lehky *et al.*, 1974). Protein concentration of purified parvalbumin solutions was determined using the relationship $A_{258} - A_{268} = 102 \times N$, where N is the number of phenylalanine residues (Cavé *et al.*, 1982).

Antiserum preparation

Antibodies were raised against rabbit parvalbumin by immunizing sheep. The first injection contained 10 mg of parvalbumin dissolved in sterile phosphate-buffered saline (10 mM-sodium phosphate buffer, pH 7.4, containing 150 mM-NaCl) and mixed with an equal volume of Freund's complete adjuvant. Beginning 2 weeks thereafter, 9 weekly booster injections were given, each containing 5 mg of parvalbumin dissolved in phosphate-buffered saline. The IgG fraction was purified by $(\text{NH}_4)_2\text{SO}_4$ precipitation and chromatography as described by Sober *et al.* (1956). The specificity of the antibody was shown by immunoblotting.

Immunoblotting

Tissue extracts (1:40, w/v) from control soleus and EDL muscles were electrophoresed in a 12.5% gel at 140 V for 4 h as described by Laemmli (1970). The proteins were then transferred to nitrocellulose paper by using a Bio-Rad Trans-Blot cell with a constant voltage of 90 V for 90 min (Towbin *et al.*, 1979). A 25 mM-Tris/200 mM-glycine buffer, pH 8.3, containing 25% (v/v) methanol and 0.02% SDS was used as transfer buffer. After blotting, the nitrocellulose sheets were stained with Ponceau S, photographed and then destained for 5 min in tap water. The sheets were then saturated for 20 min with 5% (1:20, v/v) guinea-pig serum in phosphate-buffered saline. This and all following steps were performed at room temperature. The serum was decanted and replaced by 5% (w/v) milk powder in phosphate-buffered saline for 20 min (Johnson *et al.*, 1984). After removal of the milk powder, the sheets were incubated for 2 h with antiserum (1:2000 in phosphate-buffered saline, containing 1% milk powder), washed twice for 5 min with phosphate-buffered saline containing 0.05% Tween 20 and once with phosphate-buffered saline. The membrane filters were then incubated for 3 h with HRP-conjugated rabbit anti-sheep IgG (1:2000 in phosphate-buffered saline containing 1% milk powder) and washed three times as described above. The bound conjugated IgG was identified by staining with a solution containing 3 ml of 0.3% α -chloronaphthol, 47 ml of phosphate-buffered saline and 0.02 ml of 30% (v/v) H_2O_2 until the bands were visible (Hawkes *et al.*, 1982).

Conjugation of IgG with HRP

IgG was conjugated with HRP by the periodate method of Wilson & Nakane (1978) as described by Boorsma & Streefkerk (1979). The conjugate was stored as 0.1 ml samples in phosphate-buffered saline at -75°C . It was found to be stable for approx. 1 year.

'Sandwich' e.l.i.s.a

In all incubation steps, the wells of the microtitre plates were filled with 0.1 ml of the required solution. Conversely, 0.15 ml of solution was used in each washing step.

The microtitre plates were coated with anti-parvalbumin IgG or control (pre-immune) IgG dissolved in coating buffer (0.1 M-carbonate/bicarbonate, pH 9.6, containing 0.2% NaN_3) by incubating overnight at 4°C in sealed bags (Engvall & Perlmann, 1972; Voller *et al.*, 1976; Engvall, 1980). The coating buffer was decanted and the non-specific protein binding sites were blocked by incubating the plates with 5% (w/v) milk powder in coating buffer for 30 min at room temperature (Johnson *et al.*, 1984) followed by two washings with phosphate-buffered saline/Tween. Several different dilutions of the sample and of purified parvalbumin prepared in incubation buffer [phosphate-buffered saline/Tween containing 1% (w/v) milk powder, 10% (v/v) glycerol, 1 mM-PMSF and Trasylol (100 k.i.u./ml)] were introduced into wells coated with specific IgG. For blank values, the samples were introduced into wells coated with control IgG. The incubation lasted for 2 h at room temperature. After being rinsed three times with phosphate-buffered saline/Tween, the plates were incubated either for 3 h at room temperature or overnight at 4°C with HRP-conjugated anti-parvalbumin IgG in incubation buffer. The plates were then rinsed three times with phosphate-buffered saline/Tween and once with 0.01 M-citrate buffer, pH 5.0. Staining was performed with a substrate solution containing 55 mg of phenylene-1,2-diamine dihydrochloride and 0.03 ml 30% H_2O_2 in 100 ml of 0.1 M-citrate buffer, pH 5.0, for 30 min at 37°C . The staining reaction was stopped by adding 0.05 ml of 2 M- H_2SO_4 . The A_{486} was measured with a Kontron SLT 210 e.l.i.s.a. photometer. The blank values obtained from the incubations with control IgG were subtracted from the sample values.

RESULTS

E.l.i.s.a. technique

The specificity of the antibodies against parvalbumin was tested by immunoblotting. As illustrated in Fig. 1, parvalbumin antibodies did not react with any other proteins present in the muscle extracts.

The optimal IgG concentration for coating the microtitre wells for the sandwich e.l.i.s.a. test was obtained by assaying various coating concentrations of anti-parvalbumin IgG (0.001–1000 $\mu\text{g}/\text{ml}$) with a saturating concentration of parvalbumin (10 ng/ml). A coating concentration of more than 50 μg of IgG/ml resulted in the maximum amount of anti-parvalbumin HRP-IgG bound under these conditions. In the measurements of muscle extracts, a coating concentration of 200 μg of IgG/ml was normally used. The proper dilution of the HRP-conjugated antiparvalbumin IgG was determined

Fig. 1. Immunoblot analysis of the specificity of anti-parvalbumin immunoserum

Extracts of EDL and soleus muscles (60 μg of protein) were transferred to nitrocellulose sheets and stained with Ponceau S (lanes 1 and 3) and immunoreplicated with the antibody (lanes 2 and 4). Purified parvalbumin (PA) stained with Coomassie Blue is shown in lane 5. Arrows indicate the positions of marker proteins.

by varying the concentrations of the conjugated IgG in the above test. A dilution of our conjugated IgG of 1:1280 resulted in the optimal relationship between the maximum sensitivity of the test and minimum background absorbance.

The linearity of the sandwich e.l.i.s.a. technique with respect to purified parvalbumin was determined by incubating increasing parvalbumin in coated wells. With the use of a 1:1280 dilution of the HRP-conjugated anti-parvalbumin IgG, the test was linear within a range of 0–4 ng of parvalbumin/ml. A similar dilution curve was made for muscle extracts so that the amount of parvalbumin present would fall in the linear portion of the standard curve. In the case of rabbit EDL muscle, the concentration necessary for linearity was from 1 to 5 μg of muscle/ml. Separate dilution curves were measured on all muscle types used in these experiments.

Changes during development

Parvalbumin was undetectable in any of the investigated presumptive fast- or slow-twitch muscles during embryonic development (21 days gestation and later, Fig. 2). During postnatal development, there was a progressive increase in parvalbumin in those muscles that, when mature, contain predominantly fast-twitch fibres. However, as seen in Fig. 2(a), the time course of this increase varied in different muscles. In both vastus lateralis and gastrocnemius muscles, parvalbumin was detected at day 1, whereas in TA, psoas and EDL muscles it was first observed 4 days after birth. In addition to the differences

Fig. 2. Parvalbumin contents in various fast- and slow-twitch muscles of the rabbit during embryonic myogenesis and postnatal development

Parvalbumin measurements by sandwich e.l.i.s.a. in extracts of vastus lateralis (VL), gastrocnemius (Gastr.), TA, psoas, EDL, semitendinosus (Sem.) and soleus during pre- and post-natal development. (a) Shows parvalbumin concentrations in early developmental stages, and (b) during maturation to adult levels (the concentrations in adult semitendinosus and soleus were 4.8 ± 0.7 and $4.4 \pm 1.0 \mu\text{g/g}$ respectively). Values represent means $\pm$ S.D. from independent measurements in different animals ($n = 3-10$).

with regard to initial synthesis, there was considerable variation between the time courses of the increases in parvalbumin concentration (Fig. 2b). The values attained for the different muscles in the adult state are similar to those obtained by different techniques (Blum *et al.*, 1977; Heizmann *et al.*, 1982; Klug *et al.*, 1983a,b). In contrast with the results in the presumptive fast-twitch muscles, postnatal maturation did not result in significant increases of parvalbumin in soleus and semitendinosus muscles (Fig. 2).

Denervation studies

In order to investigate the influence of innervation upon the time-dependent increase in parvalbumin, the effect of denervation at different time points during maturation was studied in tibialis anterior and soleus muscles. When denervation was performed in presumptive

Fig. 3. Effects of denervation at various timepoints on parvalbumin contents in developing tibialis anterior muscle of the rabbit

TA muscles were unilaterally denervated at days 2, 4, 12 and 16 (arrows) of postnatal development. Parvalbumin content of the denervated and contralateral muscles was determined at various time points thereafter. Values given for the denervated muscles refer to three animals each (means $\pm$ S.D.). Values for the contralateral muscles were combined at each time point and represent means $\pm$ S.D. with the numbers of animals given in parentheses.

fast-twitch muscles before the onset of the normal rise in parvalbumin, the concentration remained at levels measured in neonatal muscle (Fig. 3). The same low levels were produced in immature muscles already containing parvalbumin when they were denervated at various time points during maturation (Fig. 3). A 60% decrease resulted within 4 days when denervation was performed at day 12. Although the time course after denervation at day 16 was not investigated, a maximum of 14 days was required to reduce parvalbumin to neonatal levels. No effect upon parvalbumin concentration was observed in soleus muscle denervated at identical time points (results not shown).

The time course of the denervation-induced reduction in parvalbumin was studied in more detail in adult TA muscle (Fig. 4). In contrast with the results for developing muscle, the decrease occurred only after a delay of about 2 weeks. At 24 days of denervation, a 50% reduction to approx. $400 \mu\text{g/g}$ of muscle was observed. Longer periods of denervation perpetuated this response until, after 75 days, the parvalbumin concentration of TA was similar to that normally found in soleus muscle. Denervation did not affect the parvalbumin content ($3-5 \mu\text{g/g}$) of adult soleus.

Chronic low-frequency stimulation

Previous studies, using an electrophoretic technique, revealed that chronic, indirect stimulation causes a rapid reduction of parvalbumin in fast-twitch muscle. A 60% decrease in parvalbumin was observed after 14 days (Klug *et al.*, 1983b), and parvalbumin was not detectable in 28-day-stimulated muscles (Klug *et al.*, 1983a). However, the electrophoretic method is not sensitive enough to assess parvalbumin at very low concentrations. Since it was of interest to discover whether or not prolonged stimulation would result in a complete loss of parvalbumin or lead

Fig. 4. Effect of denervation upon parvalbumin content in fast-twitch TA and slow-twitch soleus muscle of adult rabbits

Parvalbumin concentration was determined by sandwich e.l.i.s.a. at different time points after denervation of fast- and slow-twitch muscles. Values represent means $\pm$ S.E.M. of three, or means of two, independent experiments and have been expressed as percentage of the parvalbumin content in the respective contralateral muscles.

Fig. 5. Time course of low-frequency-stimulation-induced decrease in parvalbumin content of fast-twitch rabbit EDL muscle

Parvalbumin was determined by sandwich e.l.i.s.a. in extracts of stimulated (10 Hz, 12 h/day) and unstimulated, contralateral EDL muscles. Values represent means $\pm$ S.E.M. ($n = 3-11$) or means for two animals and are given as percentages of the parvalbumin content in the unstimulated contralateral muscles.

to the very low levels typical of slow-twitch muscle, a more detailed study was performed using the sensitive e.l.i.s.a. technique (Fig. 5).

The reduction in parvalbumin in response to chronic stimulation was earlier and more pronounced than in the adult, denervated fast-twitch muscle (Fig. 4). After 6 days of stimulation, parvalbumin was already reduced below the concentration in the contralateral muscle. A 50%

reduction was reached after 11–12 days, and the concentration was in the range of slow-twitch soleus muscle after 3 weeks of stimulation.

DISCUSSION

The validity of measurements based on immunochemical methods depends upon the specificity of the antibody. As illustrated in Fig. 1, the anti-parvalbumin IgG used in the present study reacted only with parvalbumin. The specificity of the antibody was further established by a competitive e.l.i.s.a. (results not shown). In this test, binding of the specific IgG to microtitre plates, coated with soluble proteins from homogenates from fast-twitch muscle, was completely abolished by incubation with the purified parvalbumin. This high specificity permitted sensitive measurements of parvalbumin without interference from other proteins with similar charge and/or M_r , a possible source of error in the electrophoretic determination. This advantage is of particular importance in experiments such as those in the present study, where it is necessary to incorporate a large spectrum of muscle types and experimental conditions. Another point concerning the specificity of the immunochemical determination of parvalbumin in the present study relates to the possible existence of a parvalbumin isomorph specific for slow-twitch fibres and having a low affinity towards the used antibody. This is highly improbable, since parvalbumin determinations by electrophoresis (Blum *et al.*, 1977; Klug *et al.*, 1983a,b) and by h.p.l.c. (Heizmann *et al.*, 1982) are in accordance with the present results and demonstrate extremely low levels in muscles which are composed predominantly of slow-twitch fibres, such as soleus and semitendinosus.

The results of the present study suggest that neural activity is the major factor in the control of parvalbumin expression. The appearance of parvalbumin during postnatal development seems to correlate with the onset of high frequency, phasic neural activity (Navarrete & Vrbová, 1983) and the loss of polyneuronal innervation (O'Brien *et al.*, 1978) in presumptive fast-twitch muscles. Conversely, presumptive slow-twitch muscles in the newborn display phasic, but low-frequency, activity patterns. The low concentration of parvalbumin in these muscles indicates that this type of activity pattern is not sufficient to elicit its synthesis.

The increase in parvalbumin occurs asynchronously in various presumptive fast-twitch muscles (Fig. 2). It can only be speculated that the successive appearance of parvalbumin is related to sequential transitions from neonatal to adult locomotor activity. A similar sequential appearance during postnatal development has been reported for chicken muscles (LePeuch *et al.*, 1979). Parvalbumin appears immediately after hatching in chick leg muscles, whereas the onset of its synthesis occurs 1 week later in breast muscle. It is conceivable that the earlier expression of parvalbumin in the leg muscles is related to their immediate use after birth.

It is now generally accepted that the expression of specific myofibrillar proteins is under the control of innervation (Rubinstein & Kelly, 1978; Carraro *et al.*, 1981, 1982; Ishiura *et al.*, 1981; Dhoot & Perry, 1982, 1983; Heeley *et al.*, 1985; Pette & Vrbová, 1985). In both developing and adult mammalian muscle, the appearance and maintenance of slow-type myosin light and heavy chains, troponin and tropomyosin subunits depend on

innervation with slow-type motor neurons. Therefore it is remarkable that denervation of fast-twitch muscle results in a reduction of parvalbumin, a marker of fast-twitch fibres. Thus it appears that parvalbumin synthesis is under positive control of fast-type-fibre innervation.

An interesting observation emerges from the comparison of Figs. 3 and 4. Denervation of developing fast-twitch muscles is followed by a rapid reduction in parvalbumin content, whereas there is a delayed decrease in the adult muscle. This difference may be related to the higher turnover of proteins in immature muscles (Lewis *et al.*, 1984). The onset of denervation-induced appearance of fast-type troponin-I in slow-twitch muscle of the rat has also been shown to occur later in older animals (Dhoot & Perry, 1982).

The length of the lag phase in adult muscle is also surprising in light of the rapid reduction of parvalbumin resulting from chronic stimulation (Fig. 5). If the expression of parvalbumin is under positive control of a phasic, high-frequency motor-neuron firing pattern, modulation by a continuous low-frequency pattern should result in reduced synthesis. Indeed, stimulation-induced reduction of parvalbumin is preceded by a decrease in its 'in vitro'-translatable mRNA (Leberer *et al.*, 1985b). Although no similar studies have been performed, the conditions are different in denervated muscle. Chronic stimulation imposes an altered activity, whereas denervation completely abolishes activity.

The specificity of the neural input with regard to the expression of parvalbumin in muscle is further emphasized by cross-reinnervation studies. Müntener *et al.* (1985a,b) observed an increase in parvalbumin content of slow-twitch soleus muscle of the rat after reinnervation with the peroneal nerve, which normally supplies the fast-twitch-peroneal-muscle group. Conversely, reinnervation of EDL with the nerve that normally innervates the slow-twitch soleus decreased parvalbumin. Recent studies performed in this laboratory in collaboration with Dr. T. Lømo, University of Oslo, indicate that these effects may occur independent of innervation, as they could be reproduced in denervated muscles by direct chronic stimulation with different frequency patterns (T. Lømo, E. Leberer & D. Pette, unpublished work).

It is interesting that the stimulation-induced decrease in parvalbumin parallels reductions in the Ca^{2+} -uptake capacity of the sarcoplasmic reticulum (Leberer *et al.*, 1985a). This reduction may lead to an increase in free Ca^{2+} , which negatively affects parvalbumin synthesis. Indeed, increases in free Ca^{2+} have been observed in fast-twitch muscle after short-term low-frequency stimulation (Sréter *et al.*, 1985).

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