Effect of denervation on the expression of glycogen phosphorylase in mouse skeletal muscle

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After sciectomy of the left hind-limb of C57BL/J mice, a denervation-induced muscular atrophy ensued and was accompanied by a decrease in the specific activity of glycogen phosphorylase to approx. 25% of control values. The cofactor of phosphorylase, pyridoxal 5'-phosphate, was used as a specific label in the determination of the degradation rate of the enzyme following nerve section. After a delay of 3–4 days, phosphorylase was degraded approx. twice as rapidly in the denervated gastrocnemius (0.20 day^{-1}) as in the control muscle (0.12 day^{-1}). The effect of denervation on phosphorylase mRNA was measured by quantitative Northern-blot analysis using a rat skeletal-muscle phosphorylase cDNA probe. After an initial rapid decline, phosphorylase mRNA levels stabilized in denervated muscle at 50% of the value measured in the contralateral control muscle.

INTRODUCTION

A primary influence on protein expression in skeletal muscle is the pattern of innervation. The steady-state levels of many proteins are strongly influenced by neural stimulation, and when the signal is removed, for example, by surgical intervention or trauma, the overall pattern of proteins in the muscle can change dramatically (Leberer, 1984). Denervation is selective, inasmuch as some proteins are affected much more dramatically than others, and unilateral section of the sciatic nerve provides a convenient model to examine the dynamics of change in the protein profile of specific muscles. In this paper, we present a detailed examination of the effect of denervation on the expression of glycogen phosphorylase.

There is substantial evidence for neurotrophic influences on skeletal-muscle glycogen phosphorylase. Denervation of skeletal muscle results in a marked decrease in phosphorylase levels (Turner & Manchester, 1972; Butler *et al.*, 1985a). In denervated chick breast muscle the levels of several glycolytic enzymes, including phosphorylase, decreased by 50% compared with the innervated control; this new steady state was established within 2 weeks of nerve section (Shackelford & Leberer, 1981). The levels of two glycogenolytic enzymes, glycogen phosphorylase and phosphorylase kinase, were investigated in rat epitrochlearis, hemidiaphragm and soleus muscle (muscles of different fibre-type compositions) after motor-nerve section. Both enzymes were markedly decreased in the denervated muscles as compared with levels measured in the contralateral control muscles (Lawrence & Smith, 1990). Chronic stimulation of fast-twitch fibres with a frequency pattern characteristic of slow-twitch fibres induces major changes in the enzymes of oxidative and glycolytic energy metabolism, which reflects the adaptive response to the new conditions of use and which results in a marked decrease in phosphorylase levels (Henriksson *et al.*, 1986). A 14-fold increase in the low level of the muscle-specific isoenzyme expressed in human muscle fibres cultured aneurally followed innervation by foetal-rat spinal cord (Martinuzzi *et al.*, 1988).

Glycogen phosphorylase, the key regulator of glycogenolysis, is a major sarcoplasmic protein in fast muscle. The intracellular

concentration of the enzyme declines in response to denervation in mice (Butler *et al.*, 1985a), chickens (Shackelford & Leberer, 1981) and rats (Lawrence & Smith, 1990). However, it is not clear whether the decay in phosphorylase levels is elicited via changes in the rate of synthesis and/or degradation of the enzyme. We have developed a technique to measure phosphorylase degradation, based on the specificity of labelling attainable by the cofactor, pyridoxal 5'-phosphate (PLP) (Butler *et al.*, 1985b; Cookson & Beynon, 1989). Over 95% of the PLP in skeletal muscle is associated with phosphorylase, and we have demonstrated that, after administration of [^3H]pyridoxine to C57BL/6J $^{+/+}$ mice, free pools of the vitamer decline quickly, but the phosphorylase pool declines slowly, at the same rate as the turnover rate of this enzyme derived from measurement of the rate of synthesis (Beynon *et al.*, 1986). We show that after denervation the degradation of glycogen phosphorylase increases and the phosphorylase mRNA pool declines.

MATERIALS AND METHODS

Antibodies

A monoclonal antibody to PLP, E6(4)1, prepared by Viceps-Madore *et al.* (1983), was generously provided by Dr. J. A. Cidlowski, Department of Physiology, Biochemistry, and Nutrition, University of North Carolina, Chapel Hill, NC, U.S.A. Ascites fluid was used as a source of IgG without further purification. Polyclonal antisera to mouse phosphorylase was raised in rabbits housed in the Departmental animal unit. Rabbit anti-mouse horseradish peroxidase (HRP)-conjugated immunoglobulins and swine anti-rabbit HRP-conjugated immunoglobulins were purchased from Dako Ltd., High Wycombe, Bucks., U.K. Cytidine 5'-[α - ^{32}P]triphosphate (110 TBq/mmol, uridine 5'-[α - ^{35}S]thiotriphosphate (15.2 TBq/mmol) and [^3H]pyridoxine hydrochloride (37 MBq/mmol) were from Amersham International, Amersham, Bucks., U.K. Liquid-scintillation counting of radioisotopes used OptiPhase 'safe' obtained from Pharmacia-LKB, Milton Keynes, Bucks., U.K. Autoradiographic detection of ^{32}P -labelled DNA probes used X-ray film (NIF-RX) purchased from Fuji, London, U.K. *Pst*I restriction endo-

Abbreviations used: PBS-T, 10 mM-phosphate/0.15 M-NaCl/0.2% (v/v) Tween-20, pH 7.4; HRP, horseradish peroxidase; PLP, pyridoxal 5'-phosphate.

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nuclease was purchased from BCL, Uxbridge, Middx., U.K. Hybond-N nylon membranes were from Amersham International. Nitrocellulose (pore size 0.2 μm) was from Schleicher and Schuell, D-3354, Dassel, W. Germany. Sephadex G-25 (fine) and G-50 (medium) and 5'-AMP-Sepharose 4B were from Pharmacia. All other chemicals were of analytical grade.

Determination of the degradation rate of glycogen phosphorylase

Adult C57BL/6J inbred mice of either sex were maintained on a 12 h-light/12 h-dark cycle and allowed free access to food and water. The animals received a subcutaneous injection of 3.7 MBq (100 μCi) of [^3H]pyridoxine in 0.9% (w/v) NaCl (0.1 ml). On day 13 after administration of label, the animals were anaesthetized with 2% halothane in O_2/CO_2 (19:1) and a section of the sciatic nerve was removed. In all cases healing was excellent. The right gastrocnemius served as the innervated control for the denervated muscle.

At various times after denervation (2–15 days) the left and right gastrocnemius muscles were dissected out, and each was homogenized in 1.5 ml of ice-cold 5 mM-Hepes/1 mM-EDTA/14 mM-2-mercaptoethanol, pH 7.5. The homogenate was centrifuged at 14800 g for 15 min. A portion of the supernatant was reserved for scintillation counting and protein and phosphorylase assays, and the remainder (~ 0.8 ml) was chromatographed on 5'-AMP-Sepharose.

AMP-Sepharose affinity chromatography of muscle supernatants was at room temperature on a column ($V_t = 2.5$ ml) previously equilibrated in a buffer consisting of 5 mM-Hepes, 1 mM-EDTA, 14 mM-2-mercaptoethanol, pH 7.5. The column was developed at a flow rate of 12 ml/h; 1 ml fractions were collected. After seven fractions had been collected the buffer was modified to include 10 mM-AMP, and elution was continued. Radioactivity in each fraction was determined by counting 0.8 ml of each fraction in 10 ml of scintillant. Recoveries of protein/radioactivity from the column were $> 95\%$.

The degradation data (time, radioactivity) defining the decay of labelled pools were analysed by non-linear curve fitting as a single exponential. Three parameters (A_0 , the initial pool size; A_t , the final pool size; k , the first-order rate constant for decay of the pool) were optimized by the Patternsearch algorithm (Colquhoun, 1971; Beynon, 1985), to a parameter accuracy of better than 0.1%, and using several sets of starting values to minimize the possibility of the algorithm locating a sub-minimum on the error surface.

Assays

Phosphorylase was assayed in the direction of glycogen synthesis by monitoring the release of orthophosphate from glucose 1-phosphate (Carney *et al.*, 1978a). Protein was assayed by a dye-binding assay (Bio-Rad Laboratories, Watford, Herts., U.K.).

SDS/PAGE

This was conducted by the method of Studier (1973). The 1.5 mm-thick gels consisted of a 10% resolving gel and a 5% stacking gel. Electrophoresis was for 3–4 h at 40 mA. The gels were stained for protein with 0.1% Fast Green in 45% (v/v) methanol/7% (v/v) acetic acid or electroblotted on to nitrocellulose (Towbin *et al.*, 1979). Under the conditions of SDS/PAGE, PLP dissociates from phosphorylase, preventing subsequent detection with the anti-PLP monoclonal antibody. The Schiff-base linkage between PLP and the enzyme can be stabilized for SDS/PAGE/immunoblotting with sodium cyanoborohydride (NaCNBH_3). Muscle supernatants (100 μl) were placed on ice and 50 μl of 0.8 M-imidazole/citrate, pH 6.0,

was added. NaCNBH_3 (10 μl of a solution at 100 mg/ml in ice-cold water) was added to the sample. After 5 min at 4 $^\circ\text{C}$ further addition was made from a newly prepared solution of NaCNBH_3 , and the sample was incubated on ice for 10 min. SDS/PAGE sample-loading buffer (300 μl) was then added, and the sample was boiled for 2 min.

Western blotting

Proteins separated by SDS/PAGE were electroblotted overnight on to nitrocellulose at a current of 80 mA in a buffer comprising 25 mM-Tris/HCl, 192 mM-glycine, 20% (v/v) methanol, pH 8.3, and 0.1% (w/v) SDS. The gel was subsequently stained for protein to confirm that all the protein had been transferred. The nitrocellulose was then probed for material cross-reactive with a rabbit antiserum to mouse phosphorylase or a monoclonal antibody to PLP. All incubations were at room temperature with continuous shaking. The nitrocellulose was first incubated for 1 h in 10 mM-sodium phosphate/0.15 M-NaCl, pH 7.4, containing 0.2% (v/v) Tween 20 (PBS-T) and antiserum diluted 1:1000 for anti-phosphorylase or 1:250000 for anti-PLP. The nitrocellulose was then washed three times in PBS-T (5 min/wash). The second antibody, HRP-conjugated goat anti-rabbit Ig, diluted 1:1000 in PBS-T, was incubated with the nitrocellulose for 1 h, followed by a further three washes in PBS-T (5 min/wash). Second antibody was detected by incubating the nitrocellulose in PBS-T, containing 0.2% (w/v) diaminobenzidine and 0.06% (w/v) H_2O_2 . After thorough washing in water, the stained bands were intensified by washing in 0.5% (w/v) CuSO_4 in 0.15 M-NaCl for 5 min, before rinsing in water and drying in air.

Isolation of RNA

At intervals of 2, 5, 9, 14 and 21 days after sciactomy of the left-hind limb two animals were killed. The left (denervated) and right (control) gastrocnemius muscles were dissected out and frozen in liquid N_2 . The innervated and denervated muscles from both animals were combined. Total RNA was isolated from the innervated and denervated muscles by the single-step method described by Chomczynski & Sacchi (1987). RNA recoveries were monitored by addition to the homogenate of RNA labelled with uridine 5'-[α - ^{35}S]thiotriphosphate by transcription *in vitro* of a 382 bp *Xenopus laevis* histone sequence cloned into a pSP64 vector.

Electrophoresis of RNA samples

Sample preparation and electrophoresis of RNA through formaldehyde/agarose gels were as described by Maniatis *et al.* (1982). Gels were capillary-blotted overnight on to nylon membranes, by using 5 $\times$ SSPE (1 $\times$ SSPE = 0.9 M-NaCl, 0.05 M- NaH_2PO_4 , pH 7.7, 0.5 mM-EDTA). The membrane was illuminated (RNA side down) on a transilluminator (peak wavelength 302 nm) for 3 min to immobilize the RNA.

Rat muscle phosphorylase cDNA probe

The 447 bp rat muscle phosphorylase cDNA encodes from Val⁷⁶¹ into the 3' untranslated region of the mRNA (Hwang *et al.*, 1985). The cDNA, cloned into the *Pst*I site of pUC7 (pAB6/4), was generously given by Dr. M. Crerar, York University, Ontario, Canada. The *Pst*I insert was recovered and used to prime synthesis of a ^{32}P -labelled probe by random-primed DNA labelling (Feinberg & Vogelstein, 1983). The mouse skeletal-muscle actin cDNA, cloned into pBR322, was recovered by digestion with *Pst*I (the 1419 bp clone was kindly provided by Dr. D. Leader, University of Glasgow). The specific radioactivity of cDNA probes thus prepared was $(1\text{--}6) \times 10^8$ c.p.m./ μg of DNA.

Northern-blot hybridization

The Northern blots were prehybridized for at least 4 h at 37 °C in a heat-sealed polythene bag, in 5 ml of a solution comprising 5 × SSPE, pH 7.7, 50% (w/v) formamide, 0.5% (w/v) SDS, 5 × Denhardt's solution [2% (w/v) BSA, 2% (w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone]. Hybridization was at 37 °C overnight in the above solution to which radiolabelled probe was added. After hybridization, the membrane was given three 30 min washes in 5 × SSPE and a final wash in 1 × SSPE (100 ml/wash), all at 37 °C. The membrane was air-dried and autoradiographed on X-ray film, by using intensifying screens, at -70 °C for 12–24 h.

Quantification of membrane-bound probe

The portion of membrane corresponding to the region of hybridization was cut out and counted for radioactivity in scintillant. This was achieved by superimposing the nylon membrane over the autoradiograph which was positioned on an illuminated light box. This enabled the hybridization band to be marked out on the membrane. The amount of probe bound to the membrane was determined for cDNA probes of known

specific radioactivity. Quantification results expressed as the amount of probe bound (pg) were corrected for background.

RESULTS AND DISCUSSION

Effect of denervation on phosphorylase

After denervation, the wet weight of the gastrocnemius falls gradually, such that after 21 days the muscle weighs about 50% of the contralateral (non-operated) control muscle. The activity of phosphorylase also falls throughout the 21 days after denervation (Fig. 1). The decline of phosphorylase is faster than that of total muscle mass and, as the sarcoplasmic protein content of the denervated muscle does not vary during this period, the specific activity of phosphorylase declines after denervation. Thus phosphorylase is lost in preference to other muscle proteins. After 21 days, the specific activity of phosphorylase is approx. 30% that of the contralateral control (results not shown).

The pattern of total sarcoplasmic proteins changes after 21 days of denervation (Fig. 2). Sarcoplasmic proteins from denervated and control muscles were separated on SDS/PAGE, electroblotted on to nitrocellulose and probed with a polyclonal antibody to phosphorylase and a monoclonal antibody to the cofactor, PLP. The cofactor of phosphorylase is linked to the protein as a Schiff base that is labile under the conditions of SDS/PAGE. Therefore, before electrophoresis, the supernatant proteins were treated with NaCNBH₃ to stabilize the aldimine linkage between cofactor and protein. This reduction is accompanied by a shift in mobility of the enzyme on SDS/PAGE to a position of apparently higher molecular mass, possibly as a result of altered binding of SDS by the treated holoenzyme (Fig. 2). From the protein-stained SDS/PAGE gel and both Western blots it is apparent that the decrease in activity of phosphorylase is accompanied by a marked decrease in phosphorylase protein.

Fig. 1. Changes in gastrocnemius wet weight and phosphorylase activity following unilateral sciactectomy

A portion of the sciatic nerve was extirpated from the left hind-limb of a group of C57BL/6J mice. At the times indicated the denervated (□) and contralateral control (■) gastrocnemii were dissected and weighed. Each muscle was homogenized at 4 °C in 1.5 ml of 5 mM-Hepes/1 mM-EDTA (pH 7.5)/14 mM-2-mercaptoethanol. A low-speed supernatant (14800 g, 15 min) was prepared and phosphorylase activity was assayed. Each datum is the mean value from two animals.

Fig. 2. SDS/PAGE and Western-blot analysis of sarcoplasmic proteins isolated from denervated and control gastrocnemii 21 days after denervation

The sciatic nerve was sectioned in the left hind-limb of a group of four mice. After 21 days the denervated and control muscles were dissected out and pooled. Each group was homogenized in 10 vol. of 5 mM-Hepes/1 mM-EDTA (pH 7.5)/14 mM-2-mercaptoethanol at 4 °C. A 14800 g supernatant was prepared, and a portion of this was treated with NaCNBH₃ before electrophoresis. Part of the gel was stained for protein and the rest was electroblotted on to nitrocellulose and probed with antibodies to both phosphorylase and PLP. Molecular-mass markers are indicated in kDa (97, phosphorylase; 68, albumin; 45, ovalbumin; 31, carbonic anhydrase).

There is no evidence for induction of an inhibitor of phosphorylase in denervated muscle, nor of a shift in holo- to apoenzyme ratio. Phosphorylase is largely in the holoenzyme form in the normal or operated muscle, as evidenced by the shift in mobility after NaCNBH_3 treatment (Fig. 2). Scanning densitometry of a protein-stained gel indicated that phosphorylase declines from 4.3% to 1.2% of total soluble protein after 21 days of denervation (results not shown). This approx. 70% decrease in phosphorylase protein matches the decrease in phosphorylase activity.

Western blotting with the monoclonal antibody to PLP also identifies other soluble PLP-binding proteins that are putatively aminotransferases (Fig. 2). Most of the PLP is still associated with phosphorylase in either muscle, which validates the use of the cofactor as a specific label with which to measure the rate of degradation of the enzyme.

Phosphorylase degradation

The rate of degradation of phosphorylase was measured as the rate of clearance of total phosphorylase-bound radioactivity (Butler *et al.*, 1985a). By 12 days after administration of $[^3\text{H}]$ pyridoxine, free pools of label have declined to low levels and the phosphorylase pool is fully labelled (Cookson & Beynon,

1989). Sciactectomy was performed 13 days after administration of label, and the decay of muscle pools of label was measured over the next 15 days.

At 12 days after administration of $[^3\text{H}]$ pyridoxine a large proportion (80–90%) of radiolabel in skeletal muscle was associated with glycogen phosphorylase. Phosphorylase was resolved from the low level of radioactivity associated with other PLP-binding proteins and with free forms of the cofactor by AMP-Sepharose affinity chromatography (Cookson & Beynon, 1989). Fig. 3 shows the AMP-Sepharose elution profiles of radioactivity for supernatants prepared 2 and 15 days after denervation. At 15 days, radioactivity has declined in both the unbound and AMP-eluted pools, although the decline is more dramatic in the AMP-eluted phosphorylase pool in denervated muscle. The decline of radioactivity from the phosphorylase pool was used to determine the first-order degradation rate constant for phosphorylase. In the control innervated gastrocnemius, the first-order rate constant for the degradation of phosphorylase was 0.11 day^{-1} ($t_{1/2} = 6.3$ days), compared with 0.20 day^{-1} ($t_{1/2} = 3.5$ days) in the denervated gastrocnemius. The lines corresponding to the fitted data are superimposed on the data in Fig. 4. The enzyme is degraded approximately twice as rapidly in the denervated as in the control gastrocnemius muscle.

These values are in close agreement with those found by Butler *et al.* (1985a), who obtained a rate constant of 0.198 day^{-1} ($t_{1/2} = 3.5$ days) for phosphorylase in the denervated gastrocnemius and 0.116 day^{-1} ($t_{1/2} = 6.0$ days) in the contralateral control between 14 and 28 days after nerve section. In contrast with the previous study, which examined the turnover of phosphorylase in a new and denervated steady state, we have determined the rate of degradation as phosphorylase moves towards the new steady state. At the time of label administration both gastrocnemius muscles were innervated, and thus it can be assumed that both left and right muscles received the same exposure to circulating label and that the turnover of phosphorylase was the same in both muscles. Fig. 4 shows that during the early phase of denervation (between 2 and 4 days after nerve section, corre-

Fig. 3. 5'-AMP-Sepharose chromatography of muscle supernatants

A group of C57BL/6J mice were injected subcutaneously with $100 \mu\text{Ci}$ of $[^3\text{H}]$ pyridoxine; 13 days later, sciactectomy of the left hind-limb was performed. At various times after denervation ($\circ$, 2 days; $\bullet$, 15 days) a pair of animals were killed, and the left and right gastrocnemius muscles were dissected out and each was homogenized in 1.5 ml of 5 mM-Hepes/1 mM-EDTA (pH 7.5)/14 mM-2-mercaptoethanol. A 14800 g supernatant was prepared, and this was chromatographed on 5'-AMP-Sepharose ($V_i = 2.5$ ml), equilibrated and eluted in 5 mM-Hepes/1 mM-EDTA (pH 7.5)/14 mM-2-mercaptoethanol, as described in the Materials and methods section.

Fig. 4. Degradation of glycogen phosphorylase in control and denervated muscle

Experimental animals received a subcutaneous injection of $100 \mu\text{Ci}$ of $[^3\text{H}]$ pyridoxine. On day 13 sciactectomy of the left hind-limb was performed. At various times after denervation (2–15 days) the animals were killed, and the left ($\square$) and right ($\blacksquare$) gastrocnemius muscles dissected out and homogenized at 4°C in 1.5 ml of 5 mM-Hepes/1 mM-EDTA (pH 7.5)/14 mM-2-mercaptoethanol. Low-speed supernatants (14800 g, 15 min) were prepared and applied to an 5'-AMP-Sepharose column as described above. Radioactivity was determined in the phosphorylase pool and expressed as $\ln$ (c.p.m./g wet wt. of muscle). Each symbol represents a single animal.

sponding to 15 and 17 days on Fig. 4) the degree of labelling of phosphorylase is similar in both the innervated and the denervated muscle. The increased rate of catabolism of phosphorylase occurs in the denervated muscle after about 3–4 days of denervation. Moreover, this increased rate is maintained for at least 4 weeks (Butler *et al.*, 1985a).

Regression of phosphorylase mRNA after denervation

Total RNA was isolated from innervated and denervated gastrocnemii and size-fractionated on formaldehyde/agarose gels. The gels were capillary-blotted on to nylon membranes and probed with radiolabelled probes generated by random priming of a rat muscle phosphorylase cDNA clone (Hwang *et al.*, 1985). The region of hybridization was identified by autoradiography and cut out for scintillation counting. These data were corrected for background radioactivity that was present on the membrane. Fig. 5(a) is representative of the signal-to-noise ratio obtained during probe quantification; the membrane was sectioned along the length of one lane, and radioactivity was measured by scintillation counting of the membrane slices. The area centred around membrane slice 11 was used for quantification of bound probe, expressed as probe bound (pg) per amount of RNA loaded (μg), since the specific radioactivity of the radiolabelled probes was measured by Čerenkov counting. The linearity of the

Fig. 5. Quantification of membrane-bound cDNA probe for phosphorylase mRNA quantification

(a) After Northern-blot analysis and autoradiography the nylon membrane was sectioned into small slices ($0.5 \text{ cm} \times 0.8 \text{ cm}$) along the length of one lane. The membrane slices were counted for radioactivity for 20 min in scintillant. The area centred around membrane slice 11 was used for determination of probe bound after correction for background radioactivity. (b) The radioactivity (c.p.m.) associated with each region of hybridization was expressed as probe bound (pg), and this was plotted against the amount of RNA [isolated from denervated' ($\square$) or control ($\blacksquare$) gastrocnemii] applied to the original gel.

relationship between the amount of RNA loaded on the original gel and the amount of probe bound was confirmed by Northern-blot analysis of RNA from normal and 21-day-denervated gastrocnemii. A linear response was achieved for RNA isolated from denervated muscle over the range studied. A linear response between the amount of RNA loaded and probe bound was obtained for normal muscle for up to $7 \mu\text{g}$ of RNA applied to the gel. The abundance of phosphorylase mRNA as a percentage of total RNA is clearly greater in the innervated muscle (Fig. 5b). Linear regression of the data gave a slope of 0.11 and 0.39 pg of probe per μg of RNA for denervated and normal RNA respectively. After 21 days of denervation the abundance of phosphorylase mRNA as a percentage of total RNA in denervated muscle is 30 % of that in the innervated gastrocnemii.

RNA was isolated from innervated and denervated gastrocnemii at various times after denervation (2–21 days) and the phosphorylase mRNA was quantified as described above. The amount of probe bound to RNA isolated from denervated muscle was expressed as a percentage of that bound to the corresponding contralateral control RNA sample (Fig. 6). A constant amount of RNA ($3 \mu\text{g}$; within the linear response region) was applied to each lane. A similar blot was probed with a mouse skeletal-muscle actin cDNA sequence. Denervation caused no dramatic change in the abundance of actin mRNA as a percentage of the total mRNA. By contrast, the expression of phosphorylase mRNA is responsive to denervation. There is an initial rapid decline in phosphorylase mRNA in the denervated muscle, which stabilizes around 9 days after nerve section at approx. 50 % of the abundance of innervated muscles; this value is maintained until 21 days after sciactectomy. mRNA, like protein, is subject to continuous turnover to allow constant re-adjustment of the total mRNA pool. The processes of modulation of mRNA levels are poorly understood; however, the rate of synthesis of any protein is in general proportional to the quantity of its corresponding mRNA (Ross, 1989). At present, it is not possible to discriminate between decreased synthesis and/or accelerated degradation of the phosphorylase mRNA.

The sequence of events following denervation is complex, and is best illustrated with a comparison of the mRNA abundance, and protein abundance (specific enzyme activity), where the denervated limb is expressed as a percentage of the contralateral control value. Up to 4 days after denervation the decrease in phosphorylase specific activity is slight, but mRNA levels decline

Fig. 6. Northern-blot analysis of phosphorylase mRNA isolated from control (c) and denervated (d) gastrocnemius muscles

The sciatic nerve was sectioned in the left hind-limb of a group of C57BL/6J mice. At the times indicated two animals were killed and both gastrocnemius muscles dissected. The innervated and denervated muscles were pooled, and total RNA was isolated as described in the Materials and methods section. RNA was separated by agarose-gel electrophoresis and was capillary-blotted on to a nylon membrane before probing with a rat muscle phosphorylase cDNA probe or a mouse skeletal-muscle actin clone.

Fig. 7. Regression of phosphorylase mRNA and activity levels in mouse gastrocnemius muscle after denervation

A portion of the sciatic nerve was extirpated from the left hind-limb of a group of C57BL/6J mice. At the times indicated a pair of animals were killed and the gastrocnemius muscles were dissected out. Both denervated muscles were pooled for the isolation of total RNA, as were the two control muscles. The levels of phosphorylase mRNA (●) were determined by quantitative Northern-blot analysis (the results represent the combined data from four blots). Phosphorylase specific activity (○) was calculated from the data presented in Fig. 1; protein concentrations were determined by a dye-binding assay.

much more rapidly from the onset of denervation (Fig. 7). At 4 days after nerve section the specific activity of phosphorylase regresses more rapidly. From about 9 days onwards phosphorylase mRNA levels in denervated muscle have stabilized, and this level, which is about 50% of that measured in the contralateral control muscle, is maintained until at least 21 days after denervation.

The results suggest that an early event following sciactomy is an initial decrease in phosphorylase synthesis, consequential to the rapid decline of phosphorylase mRNA. Phosphorylase activity declines very gradually up to 4 days after denervation; this period of stability is also observed in the decay of phosphorylase-bound radioactivity. The curves describing the decay of phosphorylase-bound radioactivity in normal and denervated muscle intersect at a time corresponding to day 4 after nerve section (17 days after injection of label). This indicates that the rate constant for the decay of phosphorylase-bound label is the same in both normal and denervated muscle over the first 4 days. Subsequently, from day 4 after denervation the curves diverge as phosphorylase is degraded at the accelerated rate characteristic of denervated muscle. It is of interest that a similar lag is seen in the decline of parvalbumin, a cytosolic Ca^{2+} -binding protein abundant in fast-twitch skeletal muscle fibres. Denervation of adult fast-twitch muscle decreased parvalbumin levels (after an initial lag phase) to values normally found in slow-twitch muscle (Leberer & Pette, 1986).

The mechanisms for the increase in protein degradation in denervated muscle are not known. Denervation is associated with an increase in the activities of a number of lysosomal enzymes (Goldspink *et al.*, 1971; Pennington, 1977). Increased intracellular Ca^{2+} levels are also a feature of denervated muscle (Joffe *et al.*, 1981). Elevated Ca^{2+} may mediate an increase in the activation of Ca^{2+} -dependent proteinases in addition to stimulating phospholipase A_2 , which in turn can stimulate prostaglandin production. Prostaglandin E_2 enhances protein degradation by lysosomal activation (Rodemann & Goldberg, 1982), although acute inhibition of prostaglandin E_2 production had no effect on protein degradation (Turinsky, 1986).

The increase in protein catabolism that occurs in denervated

muscle necessitates the activation of one or more degradative pathways. Moreover, the preferential degradation of phosphorylase suggests that this protein is somehow targeted for degradation. The two interconvertible forms of phosphorylase (*a* and *b*) differ in their proteolytic susceptibility. Phosphorylase *a* is inherently more stable to proteolytic attack than is phosphorylase *b* (Carney *et al.*, 1978b). The absence of contractile activity in a denervated muscle elicits a decreased energy demand and requirement for glycogenolysis. Thus the enzyme would be present in the proteolytically susceptible *b* form. Such a model would not, however, be consistent with the delay in onset of increased phosphorylase degradation, and measurement of phosphorylase *a/b* ratios would be needed to develop this idea.

Under physiological conditions, a substantial proportion of phosphorylase in the cell is bound to glycogen particles along with most of the other enzymes associated with glycogen metabolism. Glycogen particles are localized in the sarcoplasm close to the sarcoplasmic reticulum at the level of the I band of the sarcomere, and are often associated in ordered arrays near the transverse tubule system (Johnson *et al.*, 1989). This necessarily puts compartmentalization constraints on phosphorylase degradation. Calder & Geddes (1989) reported that lysosomes isolated from rat skeletal muscle contained 3–4% of the tissue glycogen content. This observation reveals that in normal adult skeletal muscle a proportion of glycogen is stored and presumably degraded intralysosomally. Lysosomal proteolysis, as a consequence of lysosomal glycogen degradation, must also be considered as a candidate for degradation of phosphorylase.

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REFERENCES

- Beynon, R. J. (1985) *Comput. Appl. Biosci.* **1**, 111–115
- Beynon, R. J., Fairhurst, D. & Cookson, E. J. (1986) *Biomed. Biochim. Acta* **45**, 1619–1625
- Butler, P. E., Cookson, E. J. & Beynon, R. J. (1985a) *Biosci. Rep.* **5**, 567–572
- Butler, P. E., Cookson, E. J. & Beynon, R. J. (1985b) *Biochim. Biophys. Acta* **847**, 316–323
- Calder, P. C. & Geddes, R. (1989) *Int. J. Biochem.* **21**, 561–567
- Carney, I. T., Beynon, R. J., Kay, J. & Birkett, N. (1978a) *Anal. Biochem.* **85**, 321–324
- Carney, I. T., Beynon, R. J., Kay, J. & Birkett, N. (1978b) *Biochem. J.* **175**, 105–113
- Chomczynski, P. & Sacchi, N. (1987) *Anal. Biochem.* **162**, 156–159
- Colquhoun, D. (1971) *Lectures in Biostatistics*, Clarendon Press, Oxford
- Cookson, E. J. & Beynon, R. J. (1989) *Int. J. Biochem.* **9**, 974–982
- Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
- Goldberg, A. L. (1969) *J. Biol. Chem.* **244**, 3223–3229
- Goldspink, D. F. (1976) *Biochem. J.* **156**, 71–80
- Goldspink, D. F., Harris, J. B., Park, D. C., Parsons, M. E. & Pennington, R. J. (1971) *Int. J. Biochem.* **2**, 427–433
- Henriksson, J., Chi, M.-Y., Hintz, C. S., Young, D. A., Kaiser, K. K., Salmons, S. & Lowry, O. H. (1986) *Am. J. Physiol.* **251**, C614–C632
- Hwang, P. K., See, Y. P., Vincentini, A. M., Powers, M. A., Fletterick, R. J. & Crerar, M. M. (1985) *Eur. J. Biochem.* **152**, 267–274
- Joffe, M., Savage, N. & Isaacs, H. (1981) *Biochem. J.* **196**, 663–667
- Johnson, L. N., Hajou, J., Ravindra Acharya, K., Stuart, D. I., McLaughlin, P. J., Oikonomakos, N. G. & Barford, D. (1989) in *Allosteric Enzymes* (Herve, G., ed.), pp. 81–127, CRC Press, Boca Raton, FL
- Lawrence, J. C. & Smith, R. L. (1990) *Muscle Nerve* **13**, 133–137
- Leader, D. P., Gall, I., Campbell, P. & Frischauf, A.-M. (1986) *DNA* **5**, 235–238
- Leberer, E. & Pette, D. (1986) *Biochem. J.* **235**, 67–73

- Lebherz, H. G. (1984) *Int. J. Biochem.* **16**, 1201–1205
- Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning*, pp. 202–203, Cold Spring Harbor Laboratory Press, New York
- Martinuzzi, A., Askanas, V., Kobayashi, T. & Engel, W. K. (1988) *Neurosci. Lett.* **89**, 216–222
- Pennington, R. J. T. (1977) in *Proteinases in Mammalian Cells and Tissues* (Barrett, A. J., ed.), pp. 515–543, Elsevier North-Holland, Amsterdam
- Rodemann, H. P. & Goldberg, A. L. (1982) *J. Biol. Chem.* **257**, 1632–1636
- Ross, J. (1989) *Sci. Am.* **260**, 28–35
- Shackleford, J. E. & Lebherz, H. G. (1981) *J. Biol. Chem.* **256**, 6423–6429
- Studier, F. W. (1973) *J. Mol. Biol.* **79**, 237–248
- Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4350–4354
- Turinsky, J. (1986) *Am. J. Physiol.* **251**, R165–R173
- Turner, L. V. & Manchester, K. L. (1972) *Biochem. J.* **128**, 789–801
- Viceps-Madore, D., Cidlowski, J. M., Kittler, J. M. & Thanassi, J. W. (1983) *J. Biol. Chem.* **258**, 2689–2696

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