Neural regulation of the formation of skeletal muscle phosphorylase kinase holoenzyme in adult and developing rat muscle

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Neural influences on the co-ordination of expression of the multiple subunits of skeletal muscle phosphorylase kinase and their assembly to form the holoenzyme complex, $\alpha_4\beta_4\gamma_4\delta_4$, have been examined during denervation and re-innervation of adult skeletal muscle and during neonatal muscle development. Denervation of the tibialis anterior and extensor digitorum longus muscles of the rat hindlimb was associated with a rapid decline in the mRNA for the γ subunit, and an abrupt decrease in γ-subunit protein. The levels of the α- and β-subunit proteins in the denervated muscles also declined rapidly, their time course of reduction being similar to that for the γ-subunit protein, but they did not decrease to the same extent. In contrast with the rapid decline in γ-subunit mRNA upon denervation, α- and β-subunit mRNAs stayed at control innervated levels for approx. 8–10 days, but then decreased rapidly. Their decline coincided very closely with the onset of re-innervation. Re-innervation of the denervated muscles, which occurs rapidly and uniformly after the sciatic nerve crush injury, produced an eventual slow and prolonged recovery of the mRNA for all three subunits and parallel increases in each of the subunit proteins. A similar co-ordinated increase of both subunit mRNA and subunit proteins of the phosphorylase kinase holoenzyme was observed during neonatal muscle development, during the period when the muscles were attaining their adult pattern of motor activity. The phosphorylase kinase holoenzyme remains in a non-activated form during all of these physiological changes, as is compatible with the presence of the full complement of the regulatory subunits. These data are consistent with a model whereby the transcriptional and translational expression of phosphorylase kinase γ subunit occurs only with concomitant expression of the α and β subunits. This would ensure that free and unregulated, activated γ subunit alone, which would give rise to unregulated glycogenolysis, is not produced. The data also suggest that control of phosphorylase kinase subunit expression and the formation of the holoenzyme in skeletal muscle is provided by the motor nerve, probably through imposed levels or patterns of muscle activity.

INTRODUCTION

Phosphorylase kinase is a crucial enzyme in the control of glycogen metabolism and is the molecular site for the co-ordination of metabolic regulation by a diversity of signal transduction mechanisms [1]. The enzyme, as a possible consequence of the heterogeneity of signals that it must process, has a complex structure, consisting of four each of the four subunits α, β, γ and δ. The γ subunit contains the site for phosphotransferase catalysis and at least two calmodulin-binding sites [2,3]. The γ subunit alone, in the absence of the other subunits, has an endogenous catalytic competence equal to, or even exceeding, that of the most activated form of holoenzyme obtainable [4,5]. In either the non-activated holoenzyme, or the partial $\alpha\gamma\delta$ and $\gamma\delta$ complexes, the other subunits clearly act as inhibitors of γ subunit catalytic activity [6–9]. With enzyme activation, produced physiologically by protein phosphorylation or Ca^{2+} stimulation, or non-physiologically by limited proteolysis, the inhibitory effects of the α, β and δ subunits on the γ subunit are lessened, leading to an increase in enzyme activity. The δ subunit is identical with calmodulin and clearly serves as one means whereby phosphorylase kinase activity can be regulated by increases in cytosolic Ca^{2+} [10]. Unlike most other calmodulin-dependent enzymes, the δ subunit is an endogenous component of the phosphorylase kinase holoenzyme (and also of the $\alpha\gamma\delta$ and $\gamma\delta$ complexes), whether or not Ca^{2+} is present. Both the α and β subunits are also key regulatory subunits [11,12]. Each are sites of both auto- and cAMP-dependent-phosphorylation, which individually or in concert lead to enzyme activation. Regulatory cAMP and Ca^{2+}-independent phosphorylation of these subunits is also probable [13]. Both α and β subunits contain high-affinity calmodulin-binding sites [14,15] and these sites are probably where extrinsic calmodulin and/or Troponin C promotes enzyme activation [16]. These sites are also involved in the signalling that occurs as a consequence of cAMP-dependent phosphorylation of the α and β subunits. Transduction of these signals, leading to enzyme activation, may well be a consequence of some component of δ subunit interchange between the different calmodulin-binding sites on the distinct subunits [14]. The mode by which calmodulin interacts with these sites varies between the sites [14,17,18], allowing for a further diversity of signalling.

It is apparent that a complex network of interactions occurs between the subunits involved in orchestration of the regulation of phosphorylase kinase activation. This network of interactions is also important to ensure that catalytic competence is depressed in the absence of the regulatory signals. It is essential that there is a close co-ordination between subunit expression and holoenzyme assembly. Were free γ subunits produced, rapid and unregulated glycogenolysis would ensue. The expression and activity of metabolic pathways in skeletal muscle, however, are, in large part, regulated by its motor nerve [19–21]. In the studies presented here, the influence of motor innervation on subunit mRNA expression and translation and the assembly of the phosphorylase kinase holoenzyme has been examined in adult animals following muscle denervation and during re-innervation. In addition, regulation of holoenzyme assembly and activity was

Abbreviations used: TA, tibialis anterior; EDL, extensor digitorum longus.

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also examined in the context of the development of motor behaviour in hindlimb muscles.

EXPERIMENTAL

Animal and tissue preparation

For the denervation/re-innervation studies, adult female Sprague–Dawley rats, 200–230 g body weight and approx. 2–3 months of age, were anaesthetized by intraperitoneal injection of Ketamine (52 mg/kg) and Rompum (46 mg/kg). The common peroneal branch of the sciatic nerve was exposed bilaterally and the two nerves crushed using number 5 watchmaker forceps. Crush pressure was maintained for 30 s and the nerve was crushed twice at separate sites within 1 mm of each other. At the indicated days after denervation, an animal was anaesthetized by intraperitoneal injection of chloral hydrate C-IV (350 mg/kg). The tibialis anterior (TA) and extensor digitorum longus (EDL) muscles from both limbs were excised and freeze-clamped with Wollenberg clamps precooled in liquid N_2. The frozen muscles were stored at -70 °C until being analysed. The frozen muscle was pulverized at -70 °C, and separate aliquots of the muscle powder were used for analysis of protein and mRNA. Controls for the denervation studies were untreated or sham-operated animals. For the latter, the peroneal nerve was exposed as described, but the incision was closed without injuring the nerve. Contralateral control muscles were not used because phosphorylase levels have been shown to exhibit compensatory change with increased muscle use as a consequence of denervating muscles in the opposite limb [21,22]. All of the sham-operated controls exhibited phosphorylase kinase activity, subunit protein and mRNA levels that were not statistically different from the untreated adult controls. The developmental studies used animals that were between 9 and 45 days of age. Pups were weaned at 21 days. All animal protocols were approved by the Office of the Campus Veterinarian and were in accord with the NIH Guide for the Care and Use of Laboratory Animals.

Quantitative Western blot analyses and measurement of enzyme activity

Frozen muscle powder (200–300 mg) was homogenized in 0.5 ml of 30 mM Tris/Cl, pH 7.5, 30 mM KCl, 5 mM EDTA, 100 mM NaF, 45 mM β-mercaptoethanol, 1 mM PMSF, 2 mM benzamidine, 0.1 mM L-1-chloro-4-phenyl-3-toluene-*p*-sulphonamidobutan-2-one and 20 units/μl aprotinin at 4 °C, using a stainless steel–glass homogenizer at 300 rev./min. The homogenate was centrifuged for 15 min at 12000 ***g*** and 4 °C. The supernatant (200 μl) was mixed with 200 μl of 2% (w/v) SDS, 50 mM Tris/Cl, pH 6.8, 10% (v/v) glycerol and 75 mM β-mercaptoethanol, and then immediately heated at 95 °C for 5 min for subsequent Western Blot analysis. An aliquot of the remaining supernatant was diluted in 10 mM β-glycerophosphate, pH 6.8, 5 mM EDTA, 100 mM NaF and 4.5 mM β-mercaptoethanol for immediate measurement of phosphorylase kinase activity. Total phosphorylase kinase activity was determined at pH 8.2, and the activity ratio at pH 6.8 and pH 8.2 was determined based upon the incorporation of ^{32}P into pure skeletal muscle phosphorylase *b* using the assay conditions detailed previously [23,24]. At least three animals were used per time point and each tissue extract was assayed in triplicate at each pH. Responses within the linear ranges of the assay were obtained with diluted extracts that gave reaction rates of less than 40 and 5 pmol of ^{32}P incorporated into phosphorylase per min at pH 8.2 and pH 6.8 respectively. Protein concentration of muscle extracts were measured with extracts

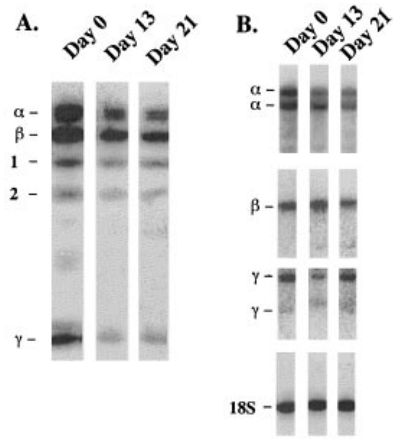

Figure 1 Sample chemiluminescence images for Western blot analysis (A) and ^{32}P images of Northern blot analysis (B) obtained by the phosphorimager for muscle extracts from control animals and 13 and 21 days following denervation

Procedures for tissue preparation and Northern and Western blot analyses are detailed in the Experimental section. The α, β and γ subunits of phosphorylase kinase on Western blot were localized with reference to purified phosphorylase kinase (**A**). Bands 1 and 2 are trace signals of phosphorylase and glycogen synthase that are trace contaminants in the phosphorylase kinase holoenzyme preparation ($< 0.5\%$) to which some polyclonal antibodies are generated. The 6.8 kb and 4.7 kb α subunit transcripts, the 5.1 kb β transcript and the 3.5 kb and 1.75 kb γ subunit transcripts, identified by Northern blot analyses (**B**), are as previously characterized for rat muscle [31]. The 18 S ribosomal RNA marker is also indicated.

further diluted in deionized water using Bio-Rad Dye (Catalogue No. 500-0006) with BSA as the standard.

For quantitative Western Blot analysis, samples were electrophoresed according to Laemmli [25] using 2 cm 4% (w/v) stacking and 8 cm 8% (w/v) resolving gels. Following electrophoresis, the gels were soaked briefly (approx. 1 min) in transfer buffer [25 mM Tris/92 mM glycine, pH 8.3, in 10% (v/v) methanol] according to Towbin et al. [26] and placed against PVDF membrane pre-wetted in 100% methanol. The gel and membrane were then sandwiched between Whatman 3MM papers, and electrophoresed at 4 °C and 0.2 A for 30 min and then at 0.4 A for 2 h using a Hoefer Transphor apparatus. Prestained standards were used to monitor gel resolution and 100% transfer efficiency. Coomassie staining of the transferred gel also confirmed the complete transfer of all muscle proteins. Following protein transfer, the PVDF membrane was incubated at 4 °C on a rocker for 1 h in blocking solution [0.5% (w/v) casein and 0.2% (v/v) Tween in 25 mM Tris/Cl, pH 7.5, 137 mM NaCl, 2.7 mM KCl, as modified from Gillespie and Hudspeth [27], and then for 16 h with fresh blocking solution containing a 1:4000 dilution of guinea-pig anti-(rabbit skeletal muscle phosphorylase kinase) polyclonal antibody [28]. After the antibody treatment, the membrane was washed 3 times for 10 min each at 22 °C in a wash solution (0.1% casein and 0.2% Tween 20 in 25 mM Tris/Cl, pH 7.5, 137 mM NaCl) and then incubated at 22 °C for 2 h with biotinylated donkey anti-(guinea pig) IgG (Jackson Immuno Research Laboratories, Inc.). The membrane was washed 3 times for 10 min each at 22 °C in the wash solution and then incubated for 30 min at 25 °C with Streptavidin–alkaline phosphatase (Boehringer Mannheim Catalogue No. 1089-161). Following the alkaline phosphatase incubation, the membrane was washed twice, 10 min each, in 0.95% (v/v) diethanolamine with 1 mM $MgCl_2$ (DM solution), incubated in Tropix Nitroblock® (1:20, v/v dilution in DM solution) for 10 min, washed twice in DM solution as before, and

developed with 0.116 mg/ml Tropix substrate CSPD [disodium 3-(4-methoxyspiro-1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.1-^{3,7}]decan-4-yl)phenyl phosphate] in DM solution for 5 min.

Quantification of the signal was obtained from the chemiluminescence using 15 min to 4 h exposure to Bio-Rad Molecular Imager screens (length depending upon signal intensity), and then the screens were scanned and the signals were quantified using Phosphoanalyst 1.1 software. To validate the actual linear range of the Western blot, serial 2-fold dilutions of untreated muscle extracts were processed to formulate a dilution curve and then compared with serial dilutions of standard, pure skeletal muscle phosphorylase kinase. The range of the sample muscle extracts loaded was 0.4–32 mg of total extract protein. The standard curve for each subunit was close to linear with a modest deviation from linearity at the lower signal levels. Quantification of each subunit was based upon the standard curve generated for each subunit and also the defined stoichiometry of pure skeletal muscle phosphorylase kinase of $\alpha_4\beta_4\gamma_4\delta_4$ [29,30]. Unless otherwise noted subunit content in muscle extracts was determined using 20 mg of soluble muscle protein per experimental lane. The extract from each animal was analysed in triplicate and a minimum of three animals was used for each determination. The high specificity of the guinea-pig antiphosphorylase kinase antibody used in the current studies has been previously documented [23,28]. Examples of the chemiluminescence image obtained by the phosphorimager of the Western Blot analysis for muscle extracts from control animals and 13 and 21 days following denervation are presented in Figure 1(A).

Quantitative Northern blot analyses for the α, β and γ subunits of phosphorylase kinase

Oligonucleotides corresponding to 308 bp (227 bp of the 5′-untranslated region plus the first 81 amino-terminal-coding nucleotides) of the rat skeletal α subunit ([31] and D. C. Ng and D. A. Walsh, unpublished work), the 165 bp (coding nucleotides 1062–1350) of the rat skeletal β subunit [31] and the 288 bp (the carboxy-terminal 81 coding nucleotides plus 107 nucleotides of the 3′-untranslated region) of the rat skeletal γ subunit [32] were prepared by PCR. For each, 50 ng of the corresponding template cDNAs were primed using 0.5 μM sense and antisense 17–22-mer oligonucleotides with 2.5 units/100 μl of Amphi-Tag® polymerase and using 30 cycles of 1 min at 95 °C, 1 min at 48 °C and 3 min at 72 °C. Single-stranded antisense-labelled probes for Northern blot analyses were then prepared from each of these templates by PCR using 20–50 ng of each oligonucleotide templates, 2 μM of the antisense primers, 25 μM each of dATP, dGTP and TTP, 0.83 μM [α-^{32}P]dCTP (S.A. 6000 Ci/mmol), 2.5 units/100 μl Amphi-Tag® polymerase in a buffer of 50 mM Tris/Cl, pH 8.3, 1.5 mM $MgCl_2$ and 10 mM KCl_2, with 20 cycles of 1 min at 95 °C, 1 min at 48 °C and 10 min at 72 °C. Each probe incorporated 50–70 % of [^{32}P]dCTP, yielding specific radioactivities of $(1–4) \times 10^9$ c.p.m./μg of DNA. Each probe was specific for their respective subunit mRNAs of: α, 6.8 and 4.7 kb; β, 5.1 kb; and γ, 3.6 kb.

For Northern Blot analyses, 100–200 mg of powdered freeze-clamped muscle was homogenized at 4 °C in 1.0 ml of TRIzol® reagent (Gibco-BRL), which contains guanidinium isothiocyanate and acidic phenol [33]. The homogenate was incubated for 5 min at 22 °C, 0.2 ml of chloroform was then added with vigorous shaking, the sample incubated for a further 3 min and then centrifuged at 12000 ***g*** for 10 min at 4 °C. The RNA was precipitated from the upper phase, obtained by this centrifugation, by the addition of 0.5 ml of propan-2-o1. The RNA pellet obtained from this procedure was washed once with 1 ml of 75 % (v/v) ethanol, prepared using diethylpyrocarbonate-treated deionized distilled water. The washed pellet was vacuum dried for 5–10 min and then resuspended in 0.5 % SDS in diethylpyrocarbonate-treated water. Each RNA sample (5 μg) was glyoxylated by incubation at 50 °C for 1 h with 15 μl of a reaction mixture containing 5.87 % (v/v) glyoxal, 50 % (v/v) DMSO and 10 mM sodium phosphate, pH 7.0. At the end of this incubation 4 μl of 50 % glycerol/10 mM sodium phosphate (pH 7.0)/0.4 % (v/v) Bromophenol Blue was added to each of the samples which were then electrophoresed on 1.2 % (w/v) Seakem GTG–agarose gel in 10 mM sodium phosphate buffer at 8 V/cm until the Bromophenol Blue dye front reached 70–80 % of the gel length. The RNA on the gel was then transferred overnight by capillary action on to Stratagene Duralon-UV® membrane with 3.0 M NaCl/0.3 M sodium citrate, and then cross-linked by UV. Each blot was first pre-hybridized at 68 °C for 30 min in Stratagene QuikHyb® and then hybridized by incubation at 68 °C for 2 h with $(8–11) \times 10^6$ c.p.m./ml of each of the specific α, β and γ antisense probes in Stratagene QuikHyb® solution. After hybridization, the membranes were first washed for two 15 min periods with 0.3 M NaCl/30 mM sodium citrate at 22 °C. Because of the difference in the sizes of the probes, the blots were then washed twice at 68 °C for 60 min and 15 min with either 0.6 M NaCl/60 mM sodium citrate, for detection of α and γ, or 0.15 M NaCl/15 mM sodium citrate, for detection of β.

For quantification, the blots were exposed on phosphorus screens and quantified using a Bio-Rad Molecular Phosphorimager. The integrity of the mRNA was examined by staining with 0.02 % (v/v) Methylene Blue dye. Equal loading of RNA to each lane was determined by scanning and quantification of the Methylene Blue staining with a Molecular Dynamics Computing Densitometer (Model 300A), and also by quantification of the hybridization to ribosomal RNA of a ^{32}P-labelled 25-mer oligonucleotide probe specific for 18 S rat ribosomal RNA (5′-GCCATTCGCA GTTTCACTGT ACCGG). This probe was prepared by direct synthesis and labelled using T4 polynucleotide kinase to a specific activity of $(1–2) \times 10^8$ c.p.m./μg of DNA. To validate the range of the Northern blot that would allow for accurate quantification of each subunit mRNA, serial dilutions of untreated adult skeletal muscle extracts were processed to formulate a dilution curve for each subunit mRNA. The range of the sample muscle extracts loaded was 0.5–5 μg of total RNA, and signals for each subunit equivalent to 0.5 μg of muscle RNA from untreated adults were readily detectable. The standard curve for each subunit deviated modestly from each other and quantification for each was based upon the standard curves generated for the specific subunit. Data are expressed in comparison with the amount of each mRNA in an adult untreated animal. The extract from each animal was analysed in triplicate and two to five animals were used for each determination. Examples of the ^{32}P image obtained by the phosphorimager of the Northern blot analysis for muscle extracts from control animals and 13 and 21 days following denervation are presented in Figure 1(B).

Reagents

Rabbit phosphorylase kinase was prepared as described by Pickett-Gies and Walsh [34]. General molecular biology methods and solutions were performed or prepared according to Ausubel et al. [35] and Sambrook et al. [36]. RNA and protein gels were standardized with BRL RNA standards of 9.5, 7.5, 4.4, 2.4, 1.4 and 0.24 kb and prestained protein standards respectively obtained from Bio-Rad.

Statistical methods

Experimental data are means ± S.D. Statistical comparisons were made between control values and values at different time points using ANOVA. Differences were accepted as statistically significant at $P \leqslant 0.05$.

RESULTS

Neural regulation of phosphorylase kinase expression in skeletal muscle

We used a nerve-crush model to explore both the consequences of denervation and the relationship of re-innervation to changes in the expression of the subunits of phosphorylase kinase. Nerve-crush produces complete muscle denervation, but re-innervation is rapid and relatively precise. The characteristics of axon regrowth and neuromuscular re-innervation have been well documented [37–40]. After a delay of approx. 36 h following the injury, axonal regeneration in rat peripheral nerves proceeds linearly at a rate of approx. 4 mm/day. Thus the time to muscle re-innervation following the injury is predictable. The time course of recovery of subunit expression and enzyme activity may be viewed from the time of re-innervation and evaluated from the perspective of the recovery of trophic support from the motor nerve (rapid), or the re-establishment of normal motor activity (slow). Crushing the common peroneal nerve at a distance of 30 mm from the EDL and TA muscles left the muscles without innervation for a period of approx. 9 days. Re-innervation began at approx. 10 days and all fibres could be expected to be re-innervated in the next 3–5 days, i.e. motor nerve terminals were present at all of the neuromuscular endplates. Recovery of normal muscle contractile function, on the other hand, may take as long as 2–3 weeks from the start of re-innervation [41,42].

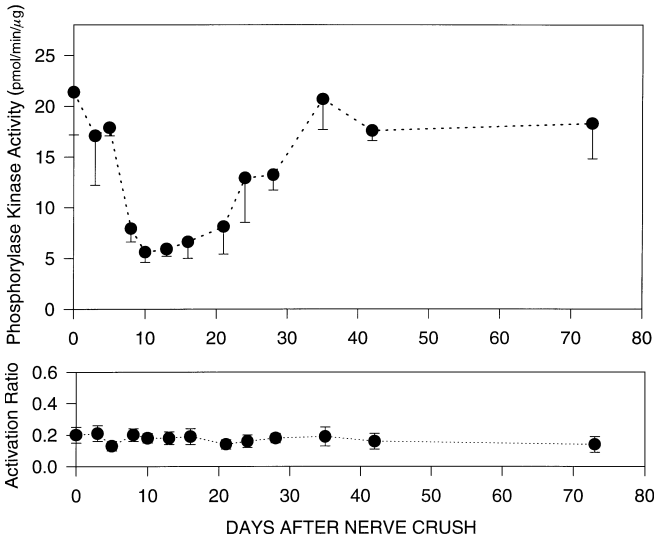

Figure 2 Time course of changes in total skeletal muscle phosphorylase kinase activity (upper panel) and the pH 6.8/pH 8.2 phosphorylase kinase activation ratio (lower panel) during denervation and subsequent re-innervation following nerve crush

TA and EDL were excised from rats at the days indicated following a crush of the sciatic nerves at the peroneal branch points. Procedures for animal treatment, muscle excision and enzyme activity determination are detailed in the Experimental section. A minimum of three animals was used for each time point, and each assay was determined in triplicate. ANOVA comparison of the experimental phosphorylase kinase levels with the control showed significant differences of $P < 0.005$ at the time points of 8, 10, 13, 16 and 20 days post-nerve-crush. The ratio of activities of the muscle extracts assayed at pH 6.8–8.2 is an indicator of the state of enzyme activation. The mean activation ratio = 0.17 ± 0.03.

The predominantly fast-twitch (80–95%) EDL and TA muscles were pooled to obtain enough material for analysis in triplicate. There was a rapid decrease in phosphorylase kinase activity following nerve crush, reaching a nadir of approx. 20% of the normal adult activity level by day 10 (Figure 2). There was no significant change in phosphorylase kinase activity between 10 and 21 days post-denervation, which constitutes the period of re-innervation and re-establishment of neuromuscular transmission. A progressive rise in enzyme activity occurred between 21 and 35 days, and normal tissue activity was restored by 35 days. This is the period associated with the recovery of normal motor behaviour in rats following peroneal nerve crush [42]. Average enzyme activities in re-innervated muscles were statistically indistinguishable from untreated animals from days 35 to 72. The restoration of phosphorylase kinase activity to normal adult levels was accompanied by a restoration of the normal-muscle-fibre-type pattern in EDL and TA. Fibre types were determined from a histochemical evaluation of myosin ATPase, succinic dehydrogenase and phosphorylase activities (results not shown). These observations, where both phosphorylase kinase levels and the original muscle fibre type composition were restored in the re-innervated muscles, were similar to those previously obtained in regenerating muscles following bupivacaine treatment [31], and in muscles recovering from tenotomy [43]. The results, however, are in contrast with measurements in regenerating fibres in free-muscle grafts or in muscles denervated by nerve section, where phosphorylase kinase activity is not restored to normal levels [43]. Thus nerve crush produced the decrease associated with denervation, but did not evoke the permanent depression in activity that occurs in a free graft or in a muscle re-innervated by a transected nerve.

The pH 6.8/pH 8.2 activity ratio of phosphorylase kinase is a commonly used indicator of the state of activation of phosphorylase kinase and is reflective of phosphorylation-dependent activation [1,23,34] and/or the absence of one or more of the inhibitory subunits (α, β or δ) [4,5,8,9,44]. Throughout the entire period of denervation and re-innervation phosphorylase kinase remained in the non-activated state (Figure 2, lower panel) at an activity ratio level (0.17 ± 0.03) similar to the control values determined in previous studies [23,31,45].

Phosphorylase kinase subunit protein levels during the periods of denervation and re-innervation were determined from Western blot analyses. The quantification of each protein on the blots was undertaken as described in the Experimental section using a standard curve generated by serial dilutions of pure rabbit skeletal muscle phosphorylase kinase. Data is expressed in comparison with the level of the subunits in adult rat skeletal muscle controls and is consistent with a control stoichiometry of $\alpha_4\beta_4\gamma_4\,\delta_4$. As might be expected, the change in the amount of the γ-subunit protein following nerve crush (Figure 3) closely mirrored the changes observed in catalytic activity (Figure 2). There was a rapid decline in the level of the γ-subunit protein following nerve crush, reaching a level of approx. 20% of that of normal adult muscle by day 10. In accord with the measurements of catalytic activity, the amount of γ subunit present between days 10 and 20 remained at this reduced level and then showed a progressive return to the normal adult level by day 35. Changes in both α- and β-subunit levels were also similar to those observed for γ. They, like γ, reached a low level by day 10, remained at close to that level for the next 10 days and then returned to normal adult levels by day 35. Although the data for the α and β subunits were not statistically different from those obtained for γ, a comparison between the sets of data suggested a trend

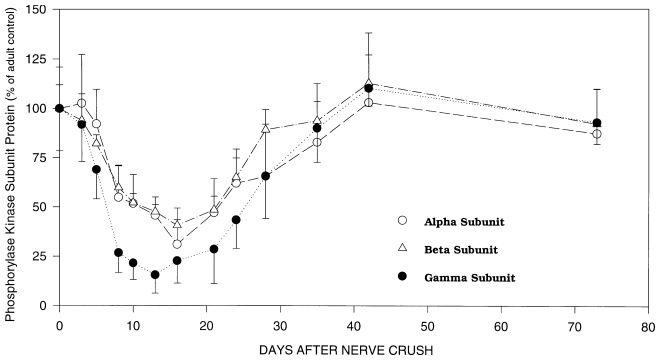

Figure 3 Quantification of the levels of phosphorylase kinase subunit protein during the time course of muscle denervation and subsequent re-innervation following nerve crush

The levels of α, β and γ subunits of skeletal muscle phosphorylase kinase were determined by quantitative Western blot analyses detailed in the Experimental section. The procedure for nerve crush was identical with that employed for Figure 2. Each subunit level was determined from pairs of muscles from a minimum of three animals, with each determined in triplicate, and the profile was determined for each, with two different levels of protein applied to the gels. Average signals ($\pm$S.D.) were normalized as percentages in comparison with the average values for untreated animals. Sham-treated controls gave results equivalent to untreated animals.

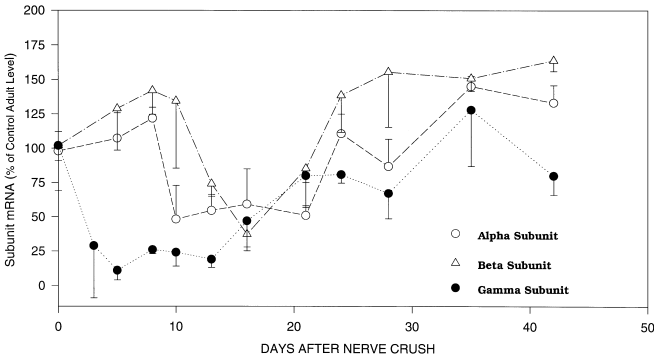

Figure 4 Quantification of the levels of phosphorylase kinase subunit mRNAs during the time course of muscle denervation and subsequent re-innervation following nerve crush

The levels of α, β and γ mRNAs of skeletal-muscle phosphorylase kinase were determined by quantitative Northern blot analyses detailed in the Experimental section. The procedure for nerve crush was identical with that employed for Figure 2. Each subunit mRNA level was determined in triplicate from pairs of muscles from at least three animals, except that two animals were used for the 24- and 42-day time points. Average signals ($\pm$S.D.) were normalized as percentages in comparison with the average values of untreated animals. Sham-treated controls gave results equivalent to untreated animals.

whereby the amounts of α- and β-subunit protein remained relatively higher than the amount of γ throughout the transitions induced by denervation and innervation. This might ensure that the regulatory relationship between the α and β subunits and the γ subunit would be maintained, and that free γ subunits, which would promote unregulated glycogenolysis, were not present.

An indication of possible differences in subunit expression or stability following denervation was clearly evident from comparisons of the levels of their transcripts. The amount of mRNA for each subunit was quantified from Northern blot analyses as described in the Experimental section, using a standard curve generated with serial dilutions of control adult rat skeletal muscle. The Northern blots were standardized for RNA loading by using both Methylene Blue staining and an 18 S ribosomal oligonucleotide-labelled probe. Following denervation by nerve crush, the level of γ subunit mRNA promptly decreased, reaching a nadir at approx. 20 % of normal levels by day 5 (Figure 4). The decrease in γ subunit mRNA preceded changes in both γ-subunit protein (Figure 3) and holoenzyme activity (Figure 2). Following the denervation-induced decline, γ-subunit mRNA remained low during the period of re-innervation, and then increased to adult levels as normal motor activity patterns were re-established (Figure 4). As might be anticipated, the increase in γ subunit mRNA levels preceded the increases in both new γ-subunit protein and phosphorylase kinase activity (Figures 2 and 3).

The pattern of change of the α and β subunit mRNAs was notably different from that of the γ subunit mRNA. Following nerve crush, the mRNA levels for both α and β subunits remained at control levels for 8–10 days, whereas the mRNA of the γ subunit decreased significantly. The α- and β-subunit proteins, in contrast with the γ-subunit protein, declined before any change in their mRNA levels. The mRNA levels of the α and β subunits, however, declined significantly at 10 days even though at this point in time re-innervation would be occurring [41,46]. The α- and β-subunit mRNA levels were reduced to approx. 50 % of the normal adult level between days 10 and 16, and then slowly returned to control values. Unlike the denervation-induced decline in mRNA levels, the restoration of α and β mRNA occurred in co-ordination with restoration of the γ-subunit mRNA, and preceded the return of subunit protein.

Developmental regulation of phosphorylase kinase expression

Rat skeletal muscle phosphorylase kinase activity is very low at birth and takes approx. 30 days to reach the adult level [47,48]. Neuromuscular development encompasses a series of events that are recapitulated in regenerating muscle following injury, and during the re-innervation of denervated adult muscle [49–52]. Given these similarities, we have explored the regulation of phosphorylase kinase expression during neonatal muscle development using the same analysis procedures that we have employed to examine the consequences of denervation and re-innervation. Phosphorylase kinase activity is barely detectable in rat TA and EDL 9 days after birth, but then shows a linear increase with age, reaching the adult level by postnatal days 35–40 (Figure 5A). This is consistent with previous reports [47,48]. During the developmental increase in phosphorylase kinase activity, the resting muscle enzyme exhibited the low of the pH 6.8/pH 8.2 activity ratio (Figure 5B) characteristic of the non-activated holoenzyme form containing the normal complement of regulatory α, β and δ subunits. Consistent with these findings, Western blot analyses showed that the regulatory α and β subunits appeared at the same time as the γ subunit (Figure 5C). There was some suggestion from the data, although not at a level of statistical significance, that at the earliest time points examined (8–15 days) the relative levels of the regulatory α- and β-subunit proteins might be higher than that of the γ-subunit protein. Additional increases in the subunit proteins occurred in parallel from 30 to 45 days after birth, reaching their adult levels for all of the subunits by approx. day 45. Northern blot analyses showed closely parallel changes in the mRNA messages for all three subunits from 9 days until adult levels were reached at 30 days (Figure 5D). Total protein production also appeared to increase with development after the maximum level of measured activity had been achieved, but by approx. day 45 it had reached a plateau. This may reflect some additional developmental

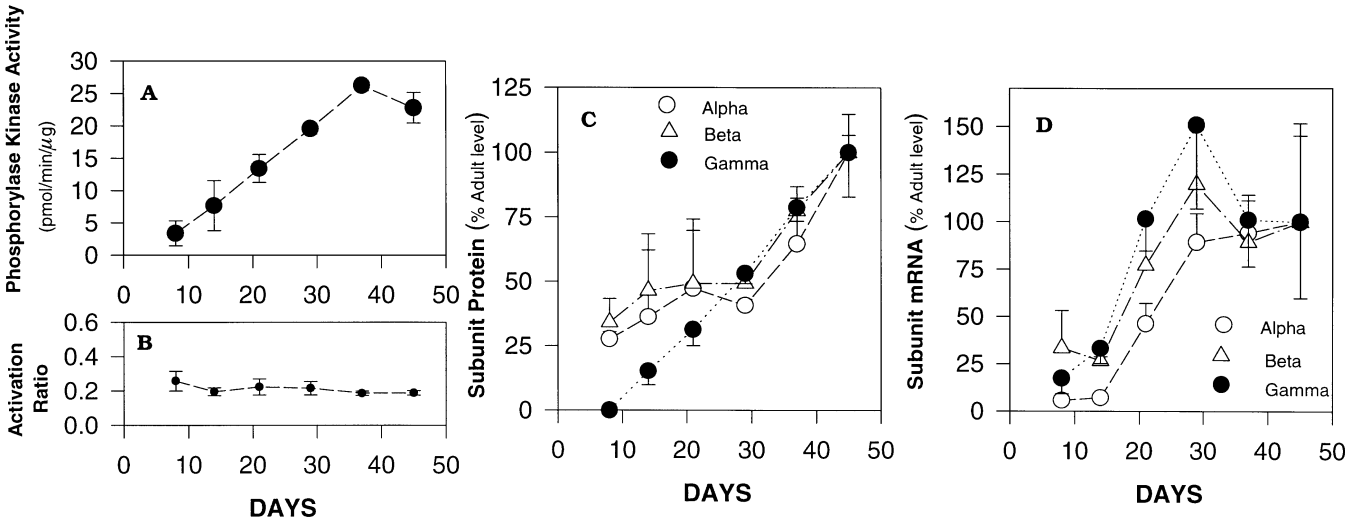

Figure 5 Developmental profiles of phosphorylase kinase activity (A), phosphorylase kinase activation ratio (B), subunit content (C) and subunit mRNA levels (D)

Procedures for each of these determinations were as defined in the Experimental section and as employed for Figures 1–4. Three animals, with triplicate analyses for each, were used for each determination.

changes, such as a change in membrane association of the enzyme, that may modulate the level of expressed enzyme activity.

DISCUSSION

In confirmation of what had been suggested by previous studies using a variety of approaches [21,31,43], the current investigation demonstrates unambiguously that the expression of skeletal muscle phosphorylase kinase is regulated by neural input. It is further implied by these data that there is close integration in the expression of the catalytic and regulatory subunits of phosphorylase kinase, so that free γ subunit does not arise. Free γ subunit is highly active [5,53]. If it were to arise in the absence of the regulatory subunits it would result in the rapid phosphorylation of phosphorylase and lead to unregulated and rapid glycogenolysis. The α-, β- and γ-subunit genes are each located on a different chromosome [54–56] and thus their co-ordinate expression must be complex. The δ subunit is identical with calmodulin [57,58]. There are three different genes for calmodulin, giving rise to five forms of mRNA, each of which, however, codes for an identical protein [59–61]. No single calmodulin gene provides uniquely for the δ subunit of phosphorylase kinase [62], but rather it appears that the different calmodulin genes give rise to a pool of calmodulin from which is derived that used to assemble phosphorylase kinase holoenzyme complex. This pool also provides the calmodulin for its other diverse cellular functions. Because of the clear divergence in the regulation of calmodulin expression and that of the other subunits of phosphorylase kinase, this current study has focused on the co-ordination of expression of the α, β and γ subunits.

Neural signalling appears to be critical for γ subunit mRNA expression, as is indicated by the prompt decrease in γ-subunit mRNA and protein following nerve crush, and their subsequent return as the muscle is re-innervated (Figure 3). The gene for the γ subunit has already been shown to have several key regulatory elements ([63]; A. O'Mahony and D. A. Walsh, unpublished work). Which, if any, of these elements may be obligate for neural control is under current study. How co-ordination of the neural regulation of the γ subunit is coupled to the control of the expression of the α and β subunits also remains to be elucidated, but it is apparent from the current studies that there is a close co-ordination between the regulation of the levels of mRNA for all three subunits. mRNA levels for all three subunits having declined following nerve crush with different time courses, they are then restored in parallel as an end consequence of re-innervation (Figure 4). Similarly, there appears to be co-ordinate regulation of subunit mRNA expression during development (Figure 5). Whether the co-ordinated initiation of RNA synthesis occurs because all three genes contain similar regulatory sequences, or because a γ gene product promotes α and β expression, are both possibilities. These changes in mRNA were mirrored by equivalent changes in subunit protein. In noted contrast, there was a temporal disparity between the decrease in α-, β- and γ-subunit mRNA levels and the decrease in the subunit proteins following denervation of adult muscle. The γ subunit mRNA rapidly declined following nerve crush but those for the α and β subunits remained elevated for as long as 10 days (Figure 4). The decline of the α- and β-subunit mRNA occurred, in fact, during the period of initial re-innervation of the muscle [41,46]. The differences that are observed in the patterns of subunit mRNA change with denervation could be a consequence of a difference either in the regulation of the transcription of the three subunit genes or in the stability of their mRNAs. The decline in α and β protein in the face of elevated levels of their mRNAs is not an unusual finding. It has been well established, for example, that a principal means whereby the level of regulatory and catalytic subunits of the cAMP-dependent protein kinase remain equal is a stabilization of the regulatory subunit when present as a component of the holoenzyme [64,65]. Thus diminished levels of α- and β-subunit protein following nerve crush, although their mRNAs remained elevated, may be indicative of the α and β subunits being more stable when present as a component of the holo-phosphorylase kinase. Whatever the reason for these changes, the physiological consequence is the presence of sufficient amounts of the α and β subunits to ensure the absence of free and unregulated γ subunit. This ensures that,

when produced, phosphorylase kinase is always present as a regulated complex of the subunits.

Neural influences have been implicated in the control of skeletal muscle phosphorylase kinase expression in several previous studies. Evaluations of free-muscle grafts of mouse muscle, in which a muscle is removed from the animal and then re-inserted, showed that phosphorylase kinase activity is almost undetectable in new, regenerating muscle fibres until approx. 42 days after the procedure, and does not return to control values by 100 days after grafting [43]. Free-muscle grafts customarily recover no more than 50 % of their original contractile capacity and fibre cross-sectional area [66], and show a substantial deficit in glycolytic metabolic capacity [19]. In contrast, the induction of muscle degeneration and regeneration using the myotoxic local anaesthetic, bupivacaine, which spares the muscle nerves and blood supply, produces an abrupt reduction in phosphorylase kinase activity during the period of fibre degeneration, but a rapid recovery as the regenerating fibres are innervated and become active [31]. The regenerated fibres show normal contractile and metabolic activity by 21–28 days after the injection. The relationship between phosphorylase kinase activity and innervation is more clearly established in denervated muscles, where activity is significantly reduced within a few days of nerve section. The post-denervation recovery of phosphorylase kinase activity is slow and incomplete following the transection of muscle nerves, but, as noted in the present study, returns to control levels within 35 days of a nerve crush. Re-innervation following transection is far slower and less precise than after nerve crush, and the recovery of normal motor activity is limited.

It is clear from these studies that neural influences play a role in the regulation of phosphorylase kinase, but it is not clear how the influence is manifest. Neural regulation of skeletal-muscle gene expression is provided both by the control of muscle electrical activity and by the delivery of neurotrophic factors to the muscle. It has been difficult to separate the contributions of these two regulatory systems to the expression of specific muscle genes. Nonetheless, current evidence suggests that the glycolytic pathways in skeletal muscle, including regulation of expression of the enzyme glycogen phosphorylase, are associated with nerve-imposed patterns of electrical activity [20]. Previous reports concerning the neural regulation of phosphorylase kinase also suggest that muscle activity may play a prominent role in the process. Reducing muscle activity by transecting the muscle tendon (tenotomy), while maintaining an intact innervation, has been shown to reduce phosphorylase kinase activity to a level similar to that following nerve transection [43]. Recovery of phosphorylase kinase activity following tenotomy, however, is essentially complete by 100–110 days. Chronic electrical stimulation, using a continuous, low-frequency (10 Hz) stimulus pattern [67] also leads to a significant reduction in phosphorylase kinase activity. The fact that both reduced and increased muscle activity can produce a reduction in phosphorylase kinase activity may appear contradictory, but, in fact, it emphasizes the importance of the pattern of muscle activation in regulating gene expression. This is apparent from the present data, where phosphorylase kinase activity does not begin to recover until well after the period of re-innervation, when normal patterns of neural activation are restored [42]. Similarly, phosphorylase kinase activity appears in developing TA and EDL during the period when the firing patterns of their motor neurons are acquiring their adult pattern [68]. It appears therefore that the expression and assembly of the subunits of phosphorylase kinase in skeletal muscle is regulated by the muscle's motor nerve through the imposition of specific patterns of electrical activity. The manner in which electrical activity is translated into the intracellular signals responsible for gene regulation remains to be determined.

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