Coordinated Expression of Phosphorylase Kinase Subunits in Regenerating Skeletal Muscle*

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Kendra C. Cawley, Carol G. Akita, Mark A. Wineinger, Richard C. Carlsen, Fredric A. Gorin, and Donal A. Walsh

From the Departments of Biological Chemistry, Human Physiology, and Neurology, School of Medicine, University of California, Davis, California 95616

The developmental expression of the α, β, and γ subunits of skeletal muscle phosphorylase kinase has been examined in regenerating muscle. Rat extensor digitorum longus (EDL) muscles, treated with bupivacaine, promptly undergo a rapid degeneration of the muscle, followed by regeneration and recovery of essentially normal morphology and physiology by 3–4 weeks post-treatment (Hall-Craggs, E. C. B., and Seyan, H. S. (1975) Exp. Neurol. 46, 345–354). Phosphorylase kinase activity dropped to ~10% of control within 3 days of bupivacaine treatment and remained at this low level for several days but had attained at least 60% of normal levels by day 21. The pH 6.8/8.2 activity ratio was unusually high during the period of low activity, suggesting that the catalytic activity was not under normal regulation at this time. The subunit mRNAs were readily detected in control EDL but were undetectable at day 3 post-bupivacaine treatment. Very small amounts of message for all three subunits were evident by day 6 and began to approach normal levels by day 12–15. The mRNA for both the α and α' subunits of phosphorylase kinase exhibited a similar pattern of recovery, as did also the mRNA for phosphorylase. In contrast to both phosphorylase kinase and phosphorylase, actin mRNA exhibited a quite a different pattern, with a nearly full recovery of message levels by day 6 post-bupivacaine. These data indicate that synthesis of phosphorylase and the α, β, and γ subunits of phosphorylase kinase appears to be coordinately regulated at the level of message accumulation and that the expression of phosphorylase kinase activity is likely to be also regulated post-transcriptionally.

Phosphorylase kinase, a key regulatory enzyme of glycogen metabolism, promotes glycogenolysis by phosphorylating and activating phosphorylase (1). The holoenzyme is a hexadecamer with the structure (αβγδ)6α, in which γ is the catalytic subunit (M6 = 44,673), δ is calmodulin, and α and β are regulatory subunits (Mα ≈ 138,500 and 125,500, respectively). The activity of the enzyme at physiological pH is enhanced up to 20-fold when the α and β subunits are phosphorylated by the cAMP-dependent protein kinase, allowing a rapid response to stimulation by a variety of hormones. Allosteric regulation by Ca2+, via both the δ subunit and “extrinsic” calmodulin (or troponin C), couples neurally invoked muscle activity with glycogenolysis. There are two major forms of α subunit that appear to be derived as a consequence of alternate splicing of the same gene, resulting in the α' form having a deletion of a 59-amino-acid segment in the middle of the polypeptide (2). The α form is the major species in fast glycolytic fibers (FG) whereas the α' form is dominant in cardiac muscle and in skeletal muscle fibers of the slow oxidative (SO) and fast oxidative glycolytic (FOG) types (3–6). Multiple isoforms of β have been observed by Harmann et al. (2) and there is indirect evidence for an alternate form of γ in liver (7), but the same major isoform of both β and γ appears to be present in all muscle fiber types (2, 8). Both the amount of phosphorylase kinase present in muscle, as well as the relative proportion of α' to α, depend upon the particular function of each muscle. The enzyme is much less abundant in SO fibers than in either fast-twitch FG or FOG fibers. The presence of the α' form appears to correlate with oxidative capacity, or “fatigue-resistant” fibers, and appears unrelated to glycolytic capability. The fiber type composition of a given muscle can be altered by changes in muscle use, and metabolic plasticity has been studied extensively, mainly by examination of the effects of chronic nerve stimulation (9). Chronic electrical stimulation of the rabbit tibialis anterior muscle, for example, has been shown to effect a typical fast-to-slow transition of phosphorylase kinase (decrease in total amount, increase percent of α', Ref. 3). Inhibition of spontaneous contraction of muscle cell cultures with lidocaine results in a 3–5-fold increase in the amount of the enzyme, as would occur with a slow-to-fast transition (10). Muscle activity clearly provides an important signal in the regulation of phosphorylase kinase expression.

Since the regulatory subunits of phosphorylase kinase are critical to appropriate function, it would seem likely (though by no means necessary) that the expression of all of the subunits would be coordinated at one or more levels. In rat skeletal muscle developing in vivo, phosphorylase kinase activity is extremely low prenatally, increases slowly in the first 10 days after birth, and then increases dramatically in the next 30 days until adult levels are reached (11, 12). In muscle cells cultured in vitro, phosphorylase kinase activity is extremely low before cell fusion, increases ~10-fold as myotubes are forming (10), but does not reach the level attained in adult muscle. At least one component of phosphorylase kinase expression appears temporally correlated with that of the muscle-specific proteins, but clearly muscle cultures in vitro

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) M92917–M92920.

1 The abbreviations used are: FOG, fast oxidative glycolytic; FG, fast glycolytic; SO, slow oxidative; EDL, extensor digitorum longus; PCR, polymerase chain reaction; kb, kilobase(s); bp, base pair(s).
do not create the conditions necessary for complete phosphorylase kinase expression. Distinctive differences between these conditions include the presence of innervation, resting and active tension, and vascularization. One way to study the expression of phosphorylase kinase, that would provide a more "normal" but also manipulable physiological environment, is to take advantage of the ability of vertebrate skeletal muscle to regenerate following injury. Muscle regeneration following destructive injury involves proliferation and recruitment of intrinsic satellite cells into the myogenic differentiation program. Phosphorylase kinase activity has been studied in autografts of mouse EDL muscle and compared with muscles subjected to denervation, tenotomy, devascularization, or combinations of these procedures (13). All three procedures were implicated as contributing to the decline in phosphorylase kinase activity, but even together resulted in a reduction less than that observed in the grafted muscle. Gorin and coworkers (14) have used regenerating orthotopic free grafts of EDL to examine the expression of glycogen phosphorylase. A significant restoration of phosphorylase activity and glycogen content were coordinately restricted to groups of fibers representing ~20% of the total. Innervation may have been important for induction of phosphorylase expression but was clearly not the sole regulatory signal.

Muscle degeneration can also be brought about by intramuscular injection with the local anesthetic bupivacaine, which is particularly toxic to muscle cells (15). Following bupivacaine-induced degeneration, myofibers are completely removed by macrophage activity. Regeneration from intrinsic muscle satellite cells is rapid and leads to essentially complete restoration of the muscle within 4 weeks of bupivacaine injection (16, 17). The normal fiber distribution for the particular muscle is restored, and innervation and vascular supply remain effectively intact. We have used this system of bupivacaine-induced regeneration to study the expression of phosphorylase Kinase subunits. To accomplish this we have generated probes for the rat skeletal muscle α, β, and γ subunit transcripts and examined potential transcription level coordination.

**EXPERIMENTAL PROCEDURES**

**Preparation of Muscle**

Female Sprague-Dawley rats (200–250 g), from Simonsen laboratories (Gilroy, CA), were anesthetized by intraperitoneal injection of sodium pentobarbital (65 mg/kg). EDL muscle was exposed bilaterally and each was injected with 0.5 ml of 0.5% bupivacaine (Sigma) containing 10 units/ml hyaluronidase (Sigma) essentially according to the procedure of Hall-Craggs and Seyan (17). Bupivacaine was infiltrated into the muscle at a single time point (day 0) at multiple locations in the EDL. At selected post-operative intervals the animals were anesthetized, and treated and control EDL muscles were re-exposed. Muscle extracts were diluted approximately 1/10 or 1/100 (for assay at pH 6.8 and 8.2, respectively) in 10 mM glycerol phosphate, 45 mM 2-mercaptoethanol immediately prior to assay. Unless stated otherwise, pH 8.2. The reaction was determined to be linear (on control muscle extracts) for at least 30 min, and a 20-min reaction time was used in these experiments. Protein determination was by the method of Bradford (20).

**Preparation of RNA**

Total RNA was prepared by the guanidine thiocyanate/acid phenol extraction method of Chomczynski and Sacchi (21). The 4 M LiCl extraction step was modified by the addition of polyethylene glycol 6000 to reduce contamination by carbohydrate. Typical yields of total RNA were approximately 25 µg/control EDL, whereas bupivacaine-treated muscles yielded as much as two to three times more, particularly in the early stages of regeneration. For RNAs to be used in Northern blots, two muscles, usually from the same animal, were pooled to obtain enough RNA for several lanes. For the PCR experiments, RNA was prepared from individual muscles.

cDNA Probes for Northern Blot Analysis of Rat Muscle

To obtain satisfactory signals and low backgrounds, it was found essential to utilize probes that were fully homologous to the rat cDNAs. The procedures to obtain and label these were as follows.

**Phosphorylase Kinase a Subunit**—The 1.4-kb EcoRI fragment containing the entire coding region for the rabbit phosphorylase kinase γ subunit (8) was used to probe Northern blots in these experiments.

**Phosphorylase Kinase γ Subunit**—The full-length cDNA for rabbit phosphorylase kinase α subunit was isolated from a rabbit skeletal muscle library using oligonucleotides with sequence based upon the established sequence (22). A cDNA corresponding to a portion of rat α cDNA was cloned from a rat skeletal muscle cDNA library (Clontech) using this full-length rabbit α-cDNA as a probe. Individual clones from the commercial library contained EcoRI fragments of the rabbit cDNA. Three α fragments, together representing approximately 75% of the coding sequence, were subcloned into pBSKS (Stratagene) and, in some cases, M13, for verification of their identity as phosphorylase kinase α subunit-coding sequences. Sequencing was by the method of Sanger et al. (24), using Sequenase (United States Biochemical). Depicted in Fig. 1 is the relationship of the three rat plasmid clones (pRta4, pRta3, and pRtalO) to the rabbit α-cDNA sequence (23). Regions A, B, and C are the combined segments of each of the three rat clones that were sequenced. The lower panels of Fig. 1 show comparisons of the rat and rabbit sequences for these regions. Sequencing provided both verification of the identity of the rabbit sequences and their alignment. The EcoRI fragment pRta3 was used to probe Northern blots.

**Phosphorylase and γ-Actin**—Northern blots were also probed with the 624-bp KpnI/Smal fragment (corresponding to amino acids 612–820) of the cDNA specific for the rat skeletal muscle isozyme of glycogen phosphorylase (14, 25) and with a 1.3-kb fragment of the coding region for the human fibroblast γ-actin (26). Both were gifts from the Dorin laboratory, University of California, Davis.

**Labeling Procedure for the cDNAs**

*Phosphorylase Kinase α and γ Subunits, Phosphorylase, and γ-Actin*—For probing Northern blots, each of these cDNAs were labeled with 32P by nick translation of random primers. Specific activities at the time of hybridization were approximately 2 × 106 dpm/µg for α- and γ-phosphorylase kinase and phosphorylase, and 3 × 108 dpm/µg for γ-actin.

**Phosphorylase Kinase β Subunit**—The cDNA fragment used to probe for rat phosphorylase kinase β subunit was prepared by PCR. The strategy was to first amplify a small fragment using degenerate primers based on the known rabbit β subunit sequence, then to sequence that fragment in order to define oligonucleotides with which a PCR fragment entirely homologous to rat β could be amplified. Two oligonucleotides (βP1, and βP2, Fig. 2), designed for minimal degeneracy, were synthesized based on the rabbit β subunit amino acid sequence and would be expected to lead to amplification of a 252-bp fragment from the C-terminal end of the β subunit; this is a region where there is minimal amino acid homology with the α subunit (27). PCR was carried out in a 50-µl reaction using as template 1 µg of plasmid DNA purified from transgenic rat skeletal muscle cells used to obtain the α subunit cDNA. The reaction mixture contained 50 mM Tris-chloride, pH 8.3, 1.5 mM MgCl2, 0.2 mM of each dNTP, 1 µM of each primer, and 1.25 units of Taq polymerase
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Fig. 1. cDNA clones and partial sequence for rat phosphorylase kinase α subunit. The solid bar in the upper diagram represents the coding region of rabbit phosphorylase kinase α subunit reported by Zaalder et al. (23). The three clones, pRta4, pRto3, and pRto10, containing partial α-cDNA sequence were isolated from a rat skeletal muscle library and by the combined sequencing of the regions A–C, the EcoRI fragments were aligned to the rabbit sequence. The EcoRI sites in regions B and C of the rat sequence leading to these three fragments are shown in bold. The three lower panels give the cDNA sequence of regions A–C determined for rat cDNA, the changes in nucleotide for rabbit, and the respective amino acid sequences. The numbering of the sequence is based upon that for rabbit. The EcoRI insert of pRta3 was used to probe Northern blots in this study. The αP11 and αP12 oligonucleotides were utilized for the PCR experiment depicted in Fig. 6 and the αP11 and αP12 oligonucleotides were utilized to distinguish between α and α' in Fig. 7. α' deletion denotes the site of deletion that distinguishes α from α'.
synthesized based upon the rat-specific β subunit sequence and used to prepare a labeled 164-bp fragment with exact homology to the rat β sequence (Fig. 2). This PCR reaction was performed as above except that 4 ng of the primary 252-bp PCR product was used in a 25-μl reaction containing 1.27 μM [α-32P]dCTP (2800 Ci/mmol) and 2.5 μM dATP, dGTP and dTTP. To compensate for the reduced concentration of nucleotides, the 72°C extension reaction was carried out for 2 min, the denaturation reactions at 94°C were reduced to 30 s, and the amount of Taq polymerase increased to 1.25 units/25 μl. Following 30 cycles of PCR, approximately 83% of the counts were trichloracetic acid precipitable. Unincorporated nucleotides were removed by ethanol precipitation from 2 M ammonium acetate (using 4 μg of carrier DNA). The specific activity of the probe was approximately 3 × 106 dpm/μg.

Northern Blots and Hybridization of cDNA Probes

Total RNA samples from control and bupivacaine-treated muscles were denatured in 1 M glyoxal, 50% MeSO4, and 10 mM phosphate, pH 7. The RNA was transferred to UV Duralon membrane (Stratagene) in 3 M NaCl, 0.3 M sodium citrate, pH 7.0, and the RNA was UV cross-linked to the membrane and then visualized using methylene blue (28). After destaining, the blots were prehybridized for 1-4 h in the hybridization buffer, pH 7.2, for 1 h at 50 °C, and then electrophoresed through 1% agarose gel. Blots were prehybridized for 1-4 h in the hybridization buffers indicated for each probe. Hybridizations with the cDNAs for the α and γ subunits of phosphorylase kinase, γ-actin, and phosphorylase were carried out in 50% formamide, 3 M NaCl, 0.2 M sodium phosphate buffer, pH 7.4, 20 mM EDTA, 50 × Denhardt’s solution, and 0.1% sodium dodecyl sulfate, overnight at 42°C. The smaller β probe, the formamide was reduced to 25%, and the overnight hybridization carried out at 37°C. All blots were washed in 3 M NaCl, 0.3 M sodium citrate, pH 7.0, 0.1% sodium dodecyl sulfate at 44°C. The probes were used at a concentration of 1-5 × 105 cpm/ml.

Investigation by PCR of Muscle mRNAs for the α, β, and γ Subunits—Total RNA was purified from individual EDL muscles at 1-day intervals following bupivacaine treatment. To prepare radiolabeled cDNA, 200 ng of total RNA was hybridized with 100 pmol of random hexamers and reverse transcribed using Moloney murine leukemia virus-reverse transcriptase (Bethesda Research Laboratories) in 20 μl of a reaction mixture containing 50 mM Tris-chloride, pH 8.3, 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 0.5 mM each of dATP, dCTP, dGTP, and dTTP, plus 0.05 μM [α-32P]dCTP (2.4 μCi/reaction), 8.7 units of RNasin (Promega), and 200 units of reverse transcriptase, at 37°C for 60 min followed by 5 min at 68°C. To control for amplification from DNA which might be present in the RNA preparation, a parallel reaction lacking the reverse transcriptase was prepared. Incorporation of 32P into trichloracetic acid-precipitable material was determined by spotting 3 μl (three times) onto Whatman ET31 paper, washing in 10% trichloroacetic acid (two times), 5% trichloroacetic acid (two times), and once in 95% EtOH. Counts of 32P retained on the filters were compared for each of the cDNA preparations and found to be similar (within 15%). There was no incorporation in the reactions lacking reverse transcriptase. For each PCR reaction the volume of template (cDNA), as well as all other PCR conditions, was identical within a set for each primer pair; the precise conditions, optimized for each primer pair, are given in the figure legend. The primers used for β were βP13 and βP14, as shown in Fig. 2. Primers for α were 5′-TTGATACTACAAGT- TATGGC (αP11) and 5′-CTTTTTAATCGTATTCC (αP12); these are based on the rat nucleotide sequence and should amplify a 257-bp fragment from pRta3, within sequenced region C (Fig. 1). In addition, α primers 5′-GATGATGAGCTGGACTCTGGC (αP1a) and 5′-CTTTTTAATCGTATTCC (αP13); these are based on the rat nucleotide sequence and should amplify a 257-bp fragment from pRta3, within sequenced region C (Fig. 1). In addition, α primers 5′-GATGATGAGCTGGACTCTGGC (αP1a) and 5′-TTATGAAAATCGTATTCC (αP13); these are based on the rat nucleotide sequence and should amplify a 257-bp fragment from pRta3, within sequenced region C (Fig. 1). In addition, α primers 5′-GATGATGAGCTGGACTCTGGC (αP1a) and 5′-TTATGAAAATCGTATTCC (αP13); these are based on the rat nucleotide sequence and should amplify a 257-bp fragment from pRta3, within sequenced region C (Fig. 1). These two α sequences correspond in the rat (Fig. 1) to nucleotide sequences which flank the 177-bp deletion reported by Harmann et al. (2) (beginning at nucleotide 1960), which distinguishes the rabbit α and α’ subunits. These primers should amplify two major species, of approximately 860 bp (for α) and 683 bp (for α’), if the deletion distinguishing α from α’ is essentially the same in rat as in rabbit. For the γ subunit, the primers used were 5′-CGAGGAGATGCTTCACGCTCGC (γP1a) and 5′-CCAGGTAGATTAGTCATG (γP1b), which would amplify a 289-bp fragment corresponding to 59 amino acids at the C terminus plus 110 nucleotides of the 3′-untranslated region (8).

RESULTS

Phosphorylase kinase activity was measured in crude extracts from EDL muscles taken at 3-day intervals following the bupivacaine treatment. Total phosphorylase kinase activity dropped to approximately 10% of the control level by day 3, and remained at this level until after day 12 (Fig. 3B).
Fig. 3. Phosphorylase kinase activity in regenerating EDL. For panel B, phosphorylase kinase activity (at pH 8.2) was determined using between three and seven individual muscle extracts, each with multiple determinations, for each of the time points following treatment of the muscle with bupivacaine (day 0). Procedures for bupivacaine treatment and assays are detailed under "Experimental Procedures." Error bars indicate the S.D. for the multiple determinations. The observed changes in activity were evaluated by a one way analysis of variance and then the Fischer t test applied for intergroup significance. By this criteria the change from control to 3, 6, 9, and 12 days was statistically significant with a p value <0.05. Panel A depicts the phosphorylase kinase pH 6.8/8.2 activity ratio determined with at least two muscle samples.

By day 15 post-bupivacaine, the enzyme activity had clearly started to increase and reached approximately 60% of control values by 18–21 days. The pH 6.8/8.2 activity ratio has been used extensively to characterize phosphorylase kinase. Non-activated holoenzyme typically exhibits a pH 6.8/8.2 ratio of between 0.04–0.1. This is increased to ~0.4 by maximal cAMP-dependent phosphorylation. Dissociation also promotes an increase, with the free γγ subunit and the γδ complex exhibiting ratios of ~0.5–0.6 and ~0.9, respectively (1, 30–32). Following bupivacaine treatment the pH 6.8/8.2 activity ratio is significantly greater during the period of low activity between days 3 and 12 than it is in control muscles or in muscles which have regenerated for longer periods (Fig. 3A).

To examine the regulation of phosphorylase kinase synthesis in regenerating muscle, RNA from muscles was prepared, again at 3-day intervals following bupivacaine injection, and investigated by Northern blot analysis using specific cDNA probes for the rat α, β, and γ subunits. Initial attempts to use cDNA probes based upon rabbit α and β subunit cDNAs were unsatisfactory. Because of this, fragments of the rat-specific cDNAs were obtained, either by cloning (α) or PCR (β), as described under "Experimental Procedures." Sample North ern blots for control muscle and for muscles during regeneration following bupivacaine treatment, showing the reaccumulation of phosphorylase kinase subunit transcripts, are presented in Fig. 4; identical results have been obtained repeatedly. In the experiments presented, a single blot was used for two probes, consecutively, but without stripping between probes, because stripping the blots resulted in a significant decrease in the signal obtained. Thus, the bands for phosphorylase kinase α, at 4.7 and 6.8 kb (panel A), are also seen as fainter bands (because of radioactive decay of the 32P-α-probe) in panel B, in which the bands at 3.6 and 1.75 kb correspond to phosphorylase kinase γ-mRNA. The band (5.1 kb) corresponding to phosphorylase kinase β (panel C) is not seen in panel D because for the 32P-γ-actin probe the autoradiogram was exposed for only 4 h, whereas the bands for the phosphorylase kinase subunits were exposed for 2–3 days. The number and size of transcripts for the α and β subunits seen in this study are similar to those observed previously by Northern blot analysis of rabbit muscle (23, 27, 34). For γ, the major 3.5-kb γ-transcript observed here with rat EDL is larger than the ~2.5 kb reported previously for mouse muscle.

The 2.5-kb band has been detected at day 6 repeatedly but not at any other time points. It is too small to represent full-length β.

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2 J.-H. Lee, S. Maeda, K. L. Angelos, S. G. Kamita, C. Ramachandra, and D. A. Walsh (1992) Biochemistry, manuscript submitted.
but with both mouse and rat there is also a minor transcript of ~1.75 kb (7, 35). Actin was probed in this study as an indicator of contractile protein transcription, and it also served to assess the integrity of the RNA samples used in this study. The complexity of the changes occurring in muscle regeneration makes it inappropriate to use any individual species as a standard for normalization. The major species of actin mRNA, migrating at 1.6 kb, is dramatically attenuated after bupivacaine treatment, and a small amount of a somewhat larger transcript (2.1 kb) is transiently observed. By day 6, however, the 1.6-kb actin transcript was returning to control levels, achieved that level shortly thereafter, and remained at that level for the remainder of the recovery period. Longer exposures of this autoradiogram indicate that the actin RNA was intact in all lanes of this blot.

Although readily detectable in control EDL muscle, no mRNA corresponding to the α, β, and γ subunits of phosphorylase kinase was observable in the Northern blots at day 3 following bupivacaine treatment (Fig. 4). (The same amount of total RNA, by A260, was loaded in each of the lanes for the bupivacaine-treated muscles, and methylene blue staining of the nylon membrane after transfer indicated uniform amounts of rRNA/lane; data not presented.) By 6 days after bupivacaine treatment, trace amounts of all three subunit transcripts were observable. From day 6 the level of mRNA rapidly accumulated for each of the subunits, reaching a maximal value close to that of control EDL by day 15. These results were observed in multiple preparations of RNA from bupivacaine-treated EDL muscles, with no observable change in the mRNA level for any subunit between day 15 and day 21. Although there was some sample-to-sample variability in the amounts detectable at 6 days (and the variability was not always mirrored by variability in the actin signal from the same RNA preparation) the level of transcript for any of the subunits was undetectable at day 3 in all RNA preparations examined. The pattern of phosphorylase kinase subunit mRNAs was quite distinct from that of actin which, as noted, was much reduced at day 3, but restored to the level of the control muscle between days 6 and 9. These data suggest that there is a coordinated mechanism for the accumulation of transcripts for the subunits of phosphorylase kinase, which is different from that of accumulation of actin transcripts.

The pattern of transcript accumulation in regenerating muscle was also examined for the muscle form of glycogen phosphorylase, which is the substrate for phosphorylase kinase (Fig. 5). The levels of phosphorylase transcript are apparently higher than those of the phosphorylase kinase subunits; this difference mirrors the 20-fold difference in the molar levels of the two proteins in normal skeletal muscle. The pattern of accumulation of phosphorylase transcript was the same as observed for the phosphorylase kinase subunits, in that transcripts, readily detectable in control muscle, were undetectable at day 3, detectable in trace amounts at day 6, and returned to control values between 12–15 days. Patterns of phosphorylase regeneration were examined histochemically (Fig. 6), with the results obtained supporting the observed recoveries in messenger level. The nadir was not determined in these latter experiments but a readily observable progression is seen at 7, 14, and 28 days following bupivacaine treatment. A fairly even distribution of enzyme is observed across the fibers in the control EDL. This is as would be expected for rat EDL, which contains nearly equal levels of FG and FOG and almost no fibers. Both FG and FOG contain high phosphorylase levels, whereas SO levels are very low, and in general the levels are higher in FG than in FOG fibers (4). Of particular note, a similar distribution of phosphorylase was seen at 7 and 14 days during recovery and at 28 days (full recovery). This distribution is very similar to the pattern found in untreated, control EDL. This contrasts with our past observation of phosphorylase recovery in free grafts of EDL (14) where, upon maximum observable recovery, phosphorylase (and glycogen) was restricted to contiguous groups of fibers representing only ~20% of the total. The difference in phosphorylase recovery in bupivacaine-treated and free grafts likely reflects the differences in the time courses of reinnervation and resumption of normal muscle activity between the two experimental systems for muscle regeneration. The even distribution of phosphorylase during regeneration following
bupivacaine treatment indicates that this regeneration is occurring quite uniformly throughout the muscle.

To refine the observations of phosphorylase kinase transcript formation further, the relative amounts of message for each of the subunits during the early part of the time course of regeneration after bupivacaine treatment were examined by a protocol based upon PCR amplification. Total RNA was prepared from individual EDL muscles, either untreated or at 1-day intervals following bupivacaine injection; cDNA was prepared from each of these RNA samples by reverse transcriptase using random primers, and then these cDNA preparations were used as templates for PCR with subunit specific oligomer primers. Details of this procedure, and the sequence of the specific subunit oligomer primers used, are given under "Experimental Procedures" and Figs. 1 and 2. This procedure allowed us to eliminate a significant degree of sample-to-sample variation, as all three subunits were amplified out of a single cDNA stock for each RNA preparation. Furthermore, the small amounts of RNA required for this sort of analysis allowed for replicates of the time points, and sampling at more frequent intervals following bupivacaine administration. PCR is not quantitative, but since conditions for all of the reactions across a given primer set were identical, differences between samples within a set will be template-derived. Under conditions of low template concentrations, the changes observed reflect template concentration, thus allowing an examination of changes in transcript level during early phases of transcript production. At high levels of template, differences between template level become obscure.

Results from this analysis are shown in Fig. 7 for an examination of subunit transcript formation at daily intervals during the first 6 days of regeneration after bupivacaine treatment. Using this PCR amplification protocol of reverse-transcribed RNA, transcripts were detectable for each of the subunits at each day of early muscle regeneration. The data clearly suggests that less mRNA is available for each of the subunits in the early days following bupivacaine treatment. For each primer set, there was a marked decrease in transcript level following bupivacaine treatment. The transcript levels increase subtly between day 2 and day 3, and additionally on subsequent days (eventually reaching a point, however, where further elevations in template can no longer be gauged). For all of the subunits, the nadir of transcript level appears to be at day 2, the point of maximal fiber degeneration. The overall time course of transcript accumulation is, however, most accurately represented by the Northern blot experiment presented in Fig. 4. These analyses show patterns of the expression of the phosphorylase kinase α, β, and γ subunits that are strongly indicative of a coordinate regulation of their transcription.

The finding of Harmann and co-workers (2), that α and α' differ by a 177-bp internal deletion (Fig. 1) provides a means by which to evaluate the presence of these isoforms and their regeneration pattern following bupivacaine treatment. The sequences obtained in regions B and C of rat α-cDNA (Fig. 1) would flank the α' deletion identified for the rabbit skeletal muscle α subunit. Primers derived from these segments of the rat nucleotide sequence (Fig. 1), and flanking the expected deletion, were synthesized. These would be expected to amplify ~860- and ~683-bp fragments from the α- and α'-transcripts, respectively (judging from the rabbit sequences). A limited tissue survey with these primers (Fig. 8) confirms this expectation, corroborating the results of Harmann et al. (2). A PCR fragment of ~710 bp was detectable as the sole species in soleus muscle and heart, and as one of two species in EDL. A fragment of ~890 bp was detected in EDL, liver, brain, and testis. These results suggest the correlation of α and α' subunits (2-6). The band that appears at 345 bp in some of the lanes is a PCR artifact and did not label with α subunit probe on the blot (Fig. 8B). This figure also shows that the 890- and 710-bp fragments were only obtained from RNA preparations that were reverse transcriptase-treated (note the absence in lanes C' and 9'), and thus were not due to contamination of the RNA preparations with genomic DNA. Shown in Fig. 8, panel A, is an examination of the α-
Fig. 8. Analysis of transcripts of phosphorylase kinase α and α'-subunits in regenerating EDL, examined using polymerase chain reaction. The experiment was conducted exactly as described in the legend of Fig. 7 except that the PCR was for 35 cycles, the primers used were αPα and αPα', the template was cDNA from 5 ng of reverse transcribed RNA, the annealing temperature was 55°C (panel A) or 58°C (panel B), the reaction contained 0.01 unit/μl of Perfect Match, and the amount loaded per lane was 10 μl of the PCR reaction product. Lanes C' and 9' are controls in which the PCR reactions were undertaken with template prepared identically to that for lanes C and 9 except that no reverse transcriptase was added to the initial reaction with the muscle RNA. Lanes in addition to those of control and bupivacaine-treated muscle are from samples of brain (B), EDL (E), heart (H), liver (L), soleus (S), and testis (T), that were prepared in an identical manner. Panel A, samples were electrophoresed on 2% NuSieve GTG/1% Seakem GTG-agarose (FMC) and stained with ethidium bromide. Molecular weight standards are φX174, digested with HaeII. Panel B, Southern analysis of a PCR performed under essentially identical conditions to that of panel A. Following electrophoresis of the PCR reaction products on 1.5% Seakem-agarose (FMC), the gel was denatured (0.5 M NaOH, 1.5 M NaCl), neutralized (1 M Tris, pH 8.0, 1.5 M NaCl), then transferred to nitrocellulose and probed with α-phosphorylase kinase cDNA exactly as described for Fig. 4.

and α'-derived fragments in samples from bupivacaine-treated rats. The patterns observed for the two isoforms of the α subunit appear very similar. Again, as observed in Fig. 7, there is a clear nadir in the amount of both α and α' subunit transcripts at 2 days following bupivacaine treatment followed by a brisk recovery of both species that are present in the control EDL.

DISCUSSION

We have examined the recovery from bupivacaine-induced degeneration of the rat EDL muscle, focusing on the expression of phosphorylase kinase. As expected, bupivacaine (with hyaluronidase) treatment led to rapid degeneration, which included a dramatic decrease in the level of phosphorylase kinase activity within 3 days of the treatment. The enzyme activity remained low, approximately 10% of the control level, until after day 12, but then quite rapidly returned to ~60% of control values by day 21. Bupivacaine-treated muscle fibers are reinervated soon after fusion of the regenerating myotubes (~5–6 days post-treatment). Both enzymes exhibited a rapid increase in activity by 15 days post-bupivacaine, but phosphorylase kinase levels remained significantly lower than control EDL at day 21. Bupivacaine-treated muscles have regained essentially normal contractile function, including normal resistance to contractile fatigue, by 21 days post-treatment (36). This suggests that a full restoration of phosphorylase kinase activity is not required to support the normal physiological function of fast-twitch muscle, and that restoration of metabolic enzyme activity continues beyond the period of morphological regeneration. Even at day 21, however, the level of phosphorylase kinase would be sufficient to very rapidly and fully activate the amount of phosphorylase present in the muscle.

Interestingly, the ratio of activity at pH 6.8 versus that at pH 8.2 is uncharacteristically high during the several days of low activity in early recovery. For non-phosphorylated phosphorylase kinase in resting muscle, this activity ratio is typically low (~0.05); phosphorylation results in a dramatic increase in the activity at physiological pH, but not at pH 8.2, and the activity ratio is increased to between 0.3 and 0.4. An increase in activity ratio to even higher values (0.5–0.6) can also be accomplished by mild proteolytic digestion of the enzyme (1). Both phosphorylation and mild proteolysis affect primarily the α and β subunits and are believed to reduce the inhibition by α and β on the catalytic γ subunit. When γ is dissociated from the holoenzyme, or expressed in a heterologous system, the activity ratio is again high when γ is present either as the free subunit (0.4–0.6) or as the γβ subunit complex (0.9); (30, 32, 37, 38). Any one of these mechanisms might account for the observed high activity ratio during the early stages of regeneration. Hormonally dependent phosphorylation is a likely candidate to be increased during regeneration, whereas proteolysis would certainly be a component of the initial degeneration. Alternatively, the phosphorylase kinase activity ratio would be high if newly synthesized subunits had yet to achieve a high enough level to allow the formation of the holoenzyme, or some additional control existed for holoenzyme assembly. Another possibility that might account for the early high pH activity ratio would be if the transcription of the δ subunit lags behind that of the other three subunits, leading to a holoenzyme deficient in that subunit or a block in holoenzyme formation. Studies of the coordination of transcription of the δ subunit with the other subunits are hampered by lack of identification of which one(s) of the calmodulin genes produces the polypeptide that becomes incorporated into phosphorylase kinase holoenzyme as the δ subunit.

The sensitivity of phosphorylase kinase to extracellular and intracellular signals depends on the association of catalytic and regulatory subunits in the holoenzyme. It was of interest, therefore, to examine the expression of the subunits of phosphorylase kinase relative to each other, particularly because the γ subunit, when not associated with the α and β subunits, is fully active. By Northern blot analysis of RNAs extracted from EDL muscles at 3-day intervals following bupivacaine treatment, the time courses of accumulation of the transcripts of the α, β, and γ subunits appear to closely parallel each other (Fig. 4). Examining the initial stages of this process in more depth by the PCR approach (Fig. 7) further strengthens this conclusion, in particular with the demonstration that the nadir for each is at 2 days after bupivacaine treatment, followed by a prompt accumulation of transcript in the next days thereafter. These data clearly suggest that subunits may be coordinately regulated at the level of transcription. Message accumulation, as measured in these studies, depends not
only on the relative rates of transcription, but also on message processing and degradation. In the initial differentiation from satellite cells to myotubes, as occurs in regeneration, an increase in transcription is certainly likely to be one key feature. Coordinately regulated transcription is a particularly interesting possibility with phosphorylase kinase because the genes for the α, β, and γ subunits have each been localized to different chromosomes (35, 39, 40). This would imply the existence of a trans-acting mechanism for transcriptional regulation common to these subunits. It is of interest that the time course for the expression of the transcript for phosphorylase, the substrate for phosphorylase kinase, mirrored that of the phosphorylase kinase subunits (Fig. 5). The gene for phosphorylase is on a chromosome that is distinct from that of any of the phosphorylase kinase subunits (33). Possibly, the same trans-acting mechanism is involved in the control of both phosphorylase kinase and phosphorylase.

It appears likely, however, that the regulation of the formation of phosphorylase kinase holoenzyme involves control at more than just the level of transcription. In the experiments presented here, phosphorylase kinase activity remains very low from days 3 to 12 and was first at a detectably higher level at day 15 following bupivacaine treatment. In contrast there is clearly message available for the α, β, and γ subunits several days earlier. This would suggest the regulation of expression at points beyond message accumulation, such as protein synthesis and/or turnover, and holoenzyme assembly. The observation by Harmann and co-workers (2) that the nucleotide sequence for the α isofrom is identical to that of α, except for a 177-bp deletion in the middle of the coding region, is also evidence for additional post-transcriptional regulation. The relative amounts of α and α’ (and changes in these as in fast-to-slow fiber type conversion caused by chronic stimulation, Ref. 3), are likely to be regulated at the level of message processing, in this case alternative splicing of a single transcript. It is of interest, however, that the synthesis of α- and α’-transcripts in regenerating EDL appeared synchronous (Fig. 8). The amount of message for phosphorylase kinase (and phosphorylase) present in normal EDL, a muscle composed primarily of FG and FOG fibers, is much more than that present in soleus,6 which is made up predominantly of SO fibers, matching the different levels of enzyme activity. It seems likely that this is determined, at least in part, by the rates of transcription for these messages. The metabolic plasticity of muscle, which involves changes in the steady state levels of a variety of enzymes and contractile activity, requires that appropriate regulation must exist not only for the establishment of tissue-specific expression, but also for the modulation of expression in response to changing demands. For phosphorylase kinase, in particular, it seems likely that such modulation involves multiple levels of control.

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