The flexor digitorum brevis skeletal muscle, a nearly homogeneous fast-twitch oxidative glycolytic fiber type, has been examined for its suitability to explore the regulation of phosphorylase kinase by multisite phosphorylation. A characterization of the adrenergic response of glycogenolytic enzymes, together with the previous data on contractile properties (Carlsen, R. C., Larson, D. B., and Walsh, D. A. (1985) Can. J. Physiol. Pharm. 63, 958–965), has demonstrated that this muscle is stably maintained for the several hours necessary for phosphorylation studies. The phosphorylase kinase in this muscle is primarily the α′ isozyme, suggesting that the α versus α′ isozyme distribution in muscle is related more to oxidative capacity than to fiber contractile characteristics. Using this muscle system, β-adrenergic activation of phosphorylase kinase was observed to occur with concomitant phosphorylation of both the α′ and β subunits, with the total in the α′ subunit being ∼3-fold greater. Similarly, deactivation, following initial adrenergic activation, occurred concomitantly with the dephosphorylation of the two subunits. These results are compatible with the conclusions drawn from previous studies of the isolated enzyme and of the enzyme in perfused rat cardiac muscle, that both α′ (or α) and β subunit phosphorylation regulate phosphorylase kinase activity.

Phosphorylase kinase is a convergence point in the pathways by which hormonal, metabolic, and neural signals regulate glycogenolysis. The ability of adrenergic agents to stimulate both the formation of cAMP and the activation of phosphorylase kinase was demonstrated a number of years ago both in vivo and in several intact muscle preparations. Shortly after this, adrenergic regulation of the enzyme’s phosphorylation was implicated by its in vitro phosphorylation and concomitant activation by the cAMP-dependent protein kinase. Despite subsequent findings, the intricate mechanisms by which phosphorylase kinase phosphorylation and activation are controlled under various metabolic and hormonal states remain only partially resolved. Phosphorylase kinase is a complex molecule composed of four types of subunits (α or α′, β, γ, and δ), with the holoenzyme containing 4 mol of each. Two isozymes exist, differing in the largest subunit, which are termed α and α′ accordingly. The regulatory significance of the two is unknown; the α isozyme is present in fast-twitch glycolytic skeletal muscle while α′ predominates in cardiac muscle and slow-twitch oxidative fibers (for citations, see Ref. 1).

In vitro, with pure proteins, the cAMP-dependent protein kinase catalyzes the phosphorylation of sites in both the α (or α′) and β subunits leading to enzyme activation; the molecular interactions that occur within the protein during these processes are complex. For example, β subunit phosphorylation not only promotes enzyme activation, but also regulates α subunit phosphorylation such that it is not initiated until the β subunit has been modified to the level of ∼1–2 mol of PO4/mol of (αβγδ)4 (2–4). Similarly, as particularly emphasized with the cardiac α′ isozyme (5) but as may also occur with the α isozyme (1, 4), initial β subunit phosphorylation can also regulate the subsequent phosphorylation of the remaining β subunits. Under select conditions a similar type of interaction may occur between α subunits (4). The effects of α and β subunit phosphorylation on activity likewise involve multiple subunit interactions. It is well established that β subunit phosphorylation is essential for cAMP-mediated activation (2–4) but it is now clear that α subunit phosphorylation also regulates activity and does so by linearly amplifying the activation effect produced by the phosphorylation of the β subunit (6). It remains important to evaluate the role of these multiple interactions in response to hormonal, neural, and metabolic stimuli. A detailed correlation of the phosphorylation and activation of phosphorylase kinase in intact muscle has so far only been initiated in the perfused heart. In a previous report (7) it was shown that epinephrine administration to perfused heart resulted in the phosphorylation of both α′ and β subunits; a more detailed assessment of the regulation of cardiac phosphorylase kinase has now been provided (8). Studies of the phosphorylation of the skeletal muscle enzyme have been few. Mayer and Krebs (9) were unable to detect phosphorylation of the protein in response to epinephrine, despite increased enzyme activity and measurable phosphorylation of phosphorylase. Yeaman and Cohen (10) did observe phosphorylation of sites on both the α and β subunits following epinephrine administration in vivo, but did not examine the relationship between activation and subunit phosphorylation; the sites phosphorylated were in tryptic peptides that are phosphorylated in vitro by the cAMP-dependent protein kinase.

In this report we describe the use of an isolated intact skeletal muscle preparation to study the phosphorylation and activation of phosphorylase kinase. The muscle utilized, the flexor digitorum brevis, is a small pennate muscle from the

* This work was supported by Grant AM 13613 from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
FIG. 1. Time course of epinephrine effects (panels a–c) and dose-response for epinephrine (panels d–f) and isoproterenol (panels g–i) on the activity of phosphorylase kinase, phosphorylase, and glycogen synthase, and the levels of cAMP in isolated FDB muscles. Muscles were removed and prepared for incubation as described under "Experimental Procedures." After 15–20 min at 37 °C in Liley's media the muscles were either stimulated with 1.0 μM epinephrine for the indicated time periods (panels a–c) or with the indicated concentrations of epinephrine or isoproterenol for 90 s (panels d–i). For panels a–c, for phosphorylase kinase, glycogen synthase, and cAMP, n = 6 for controls and n = 2 for all other time points, and for phosphorylase, n = 3 for the control, n = 2 for the 60-s time point, and n = 1 for other points. For panels d–i, the data shown is for the average of two identical experiments. Methods of assay are given under "Experimental Procedures." Phosphorylase kinase activity is expressed either as specific activity at pH 6.8 or as the pH 6.8/8.2 activity ratio (insets, a, d, and g).

Plantar aspect of the rat hindfoot. In rats of the size employed in this study, the muscle averages 18–20 mm in length, 1–2 mm in width, and weighs approximately 50 mg. The muscle is composed of nearly homogeneous (>90%) fast-twitch oxidative glycolytic fibers; its morphological and contractile characteristics have been previously detailed (11). Important for these studies, the muscle is well suited to survival in vitro. Diffusion distances for nutrients and hormones are short, since the mean fiber cross-sectional area is only 25% of the mean area of fibers from soleus and plantaris muscles from the same animals. The FDB also maintains constant contractile capacity in a tissue bath for up to 24 h, as particularly emphasized by a less than 5% loss of contractile force and identical resistance to fatigue during direct repetitive stimulation of the muscle (11). In this study the further suitability of this muscle preparation for long term incubation has been examined on several aspects of glycogen metabolism. This preparation has then been employed to study the regulation of phosphorylase kinase by catecholamine-stimulated phosphorylation under two different metabolic conditions.

1 The abbreviations used are: FDB, flexor digitorum brevis muscle; MEM, Eagle's minimum essential medium modified as described under "Experimental Procedures"; PMSF, phenylmethylsulfonyl fluoride; TPCK, N-tosyl-L-phenylalanine chloromethyl ketone (Mini-print); MES, N-morpholinoethanesulfonic acid (Miniprint); SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Experimental Procedures

Results

Adrenergic Regulation of Phosphorylase Kinase, Phosphorylase, Glycogen Synthase, and cAMP in Isolated FDB—The isolated rat FDB muscle preparation, whose contractile characteristics we have previously detailed (11), has been used in this report for the study of phosphorylase kinase regulation. In distinct contrast to the other skeletal muscle preparations in which the regulation of glycolgenolysis has been examined (see Refs. 9 and 26–32 for examples), FDB contains a homogeneous population (>90%) of fast-twitch oxidative glycolytic fibers (11). Also, in distinction to the more abundant fast-twitch glycolytic fibers of those muscle preparations, fast-twitch oxidative glycolytic fibers, as shown subsequently in this report, contain primarily the α′ isozyme of phosphorylase kinase rather than the α form. As an initial characterization of FDB, in preparation for the studies on phosphorylase kinase phosphorylation, the adrenergic regulation of the enzymes of glycogen metabolism was examined. Fig. 1 summarizes data on the time course of epinephrine stimulation (panels a–c) and the dose-responses for epinephrine (panels d–f) and isoproterenol (panels g–i), examining the parameters of phosphorylase kinase activation (left-hand panels), changes in cAMP (middle panels), and the activation of phosphorylase and inactivation of glycogen synthase (right-hand panels). The observed responses in FDB are quite similar to those for the other skeletal muscle preparations examined that contained either primarily fast-twitch glycolytic fibers or were of mixed fiber types (9, 26–32). Additionally, adrenergic activation in FDB was blocked by the β-adrenergic antagonist, DL-propranolol (2 μM), but not by the α antagonist, phenoxybenzamine (10 μM), when muscles were pretreated before epinephrine addition (data not shown).

Effect of Long Term Muscle Incubation on ATP, Glycogen, and the Adrenergic Response of the Enzymes of Glycogen Metabolism—Preliminary experiments examining [32P] incorporation into ATP and phosphorylase kinase indicated that [32P] inorganic phosphate uptake into FDB was quite slow (discussed below). For adequate formation of intracellular [γ-32P]ATP (i.e. sufficient to quantitate [32P]phosphate incorporation into phosphorylase kinase), it was necessary to incubate the muscles for several hours. Because of this, the effects of long term incubation on ATP and glycogen levels in FDB, and on the adrenergic response of glycogenolytic enzymes, were assessed. No significant changes in ATP levels could be detected in muscles examined after a 3- or 6-h incubation in Liley’s medium when compared to muscles examined shortly after removal from the animal (i.e. 20.64 ± 1.84 nmol/mg protein after 6 h compared to 21.57 ± 1.71 nmol/mg protein for the control). These values are well within the range reported for rat muscles in vivo (33).

The effects of muscle excision and long term incubation on FDB glycogen content are shown in Fig. 2. The protocol depicted is that used in the subsequently described phosphorylation studies; namely, initial excision, affixing of the muscles in the chamber and incubation at 23 °C (1.5 h), followed by long term (2–8.5 h) incubation at 37 °C. Glycogen content is expressed per mg of frozen muscle powder; muscle wet weight during incubation was also determined (Fig. 2, inset). The results provide the following information. During the initial manipulation of the muscle there is some decrease in tissue glycogen. When the observed small initial increase (~14%) in muscle wet weight is taken into account, the data show that ~19% of the original tissue glycogen was metabolized during this period. (This rapid initial decrease in glycogen content is not unique to FDB and may represent a reaction to the release of endogenous norepinephrine from sympathetic nerve terminals following muscle excision. Similar decreases were observed with soleus muscle treated identically.) Following this initial loss, subsequent incubation in Liley’s medium at 37 °C resulted in some further glycolgenolysis with the glycogen content being reduced to ~63% of normal in vivo level by the end of a 6-h period. (This, despite the presence of high levels of glucose in the media. There was, however, no further change in muscle wet weight once the tissue had stabilized.) In intact muscle the enzymes of glycogen metabolism (including phosphorylase kinase) are tightly associated with glycogen in an integral structural and functional assembly termed the “glycogen particle” (see Ref. 1 for review). Several studies have indicated that with glycogen degradation these enzymes may dissociate from the particle, which could readily modify their regulation (21). Because of this it was deemed important for the subsequent studies of phosphorylase kinase phosphorylation to examine alternate media in which glycogen levels were maintained. In the protocol depicted in Fig. 2, open circles, is shown the results of incubating FDB muscles in a medium comprised of MEM (containing essential and nonessential amino acids and other cofactors) plus acetocetate and insulin. As is observed with this medium, the initial drop in glycogen was followed by

![Fig. 2. Effect of long term incubation and incubation media on glycogen content of FDB.](image)

Note: 

2 “Experimental Procedures” are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 86M-0844, cite the authors, and include a check or money order for $1.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.
replenishment to a value even in some excess (~35%) of that normally present in vivo. In subsequent experiments (see below, Fig. 3) it is shown that this replenishment required the presence of both acetoacetate and insulin and that either alone was insufficient. Presumably, under these conditions, the metabolism of acetoacetate, which is an excellent fuel for oxidative muscles (34), permits a greater degree of insulin-stimulated glucose conversion to glycogen. (A similar conclusion has previously been made for rat soleus (35).) As also observed (Fig. 2), muscles incubated for 6 h at 37 °C in Liley's medium were fully capable of replenishing glycogen (and did so rapidly) when transferred to MEM plus acetoacetate and insulin, but did not do so if simply transferred to fresh Liley's medium.

Muscles incubated for long periods (i.e., 6 h) in either Liley's medium or modified MEM (with acetoacetate plus insulin) were also evaluated for their responsiveness to an adrenergic challenge and the regulation of cAMP levels and phosphorylase kinase, phosphorylase, and glycogen synthase activities (Fig. 3). In addition the study examined muscles incubated with MEM plus either acetoacetate or insulin alone. The controls for this study were FDB muscles that had been excised and pinned in 23 °C Liley's medium, then briefly incubated (~15 min) at 37 °C. (These control tissues are similar to those at the 1.5-h point presented in Fig. 2, with the glycogen partially diminished below in vivo levels due to the initial manipulations.) All of these muscles were examined following a 90-s epinephrine challenge or without addition of epinephrine. The results of Fig. 3 provide evidence that FDB muscles were not adversely affected by long term incubation and maintained an equivalent responsiveness to epinephrine-regulated glycogenolysis. Both basal and epinephrine-stimulated levels of cAMP (panel b), and basal and stimulated states of phosphorylase kinase (panel c), were identical for all long term incubations to control values. Phosphorylase (panel d) showed a minor change in all MEM media in that both basal levels and epinephrine-stimulated values were somewhat lower (~40%), but the same fold activation (~6-fold) was observed for all conditions. Glycogen synthase (panel e) showed a slightly higher control I/D ratio in the MEM plus acetoacetate incubation but was essentially identical under all other conditions. Small decreases in glycogen synthase activity in the presence of epinephrine occurred in individual experiments but due to variability in synthase activity from experiment to experiment the difference in mean activities in the presence or absence of epinephrine were generally not statistically significant. The data on glycogen levels (panel a) partially reproduced results given in Fig. 2. As noted, the initial manipulations caused some loss of tissue glycogen, there were some further losses with incubation in Liley's medium, and only the incubation in MEM with acetoacetate and insulin caused the replenishment of glycogen to in vivo levels or greater (see also Fig. 2). During the short period of epinephrine stimulation there was no statistically significant reduction in glycogen levels. Taken collectively, these data (Figs. 1–3) show that long term incubated FDB muscles are a suitable preparation for the study of the adrenergic regulation of phosphorylase kinase. As reported previously, these muscles also retain excellent contractile responsiveness (11).

Isolation of 32P-Labeled Phosphorylase Kinase from FDB by Immunoprecipitation—To determine phosphorylase kinase

![Fig. 3. Effect of long term incubation and media composition on glycogen, cAMP, and the enzymes of glycogen metabolism before (open bars) and after (hatched bars) epinephrine administration. Muscles were excised, prepared for incubation as described under “Experimental Procedures,” and after pinning in 23 °C Liley's medium were equilibrated at 37 °C for 15–20 min. Control values were obtained and the remaining muscles were then transferred either to fresh Liley's medium or to the other media as indicated and incubated for 6 h. Other details are described under “Experimental Procedures.” After the 6-h incubation, muscles were rinsed briefly in Liley's medium and the tissue either frozen immediately (open bars) or following epinephrine treatment (1.0 μM, 90 s; hatched bars). Methods of assay are given under “Experimental Procedures.” Numbers in parentheses indicate the number of determinations. The data is presented as the mean ± S.E. The abbreviations used are: AA, acetoacetate; INS, insulin.](image-url)
Phosphorylation in FDB preparations equilibrated in $[^32P]$ inorganic phosphate, the procedures used for the isolation and quantitation of the $[^32P]$-labeled subunits were based upon the immunoprecipitation protocols previously established for cardiac phosphorylase kinase (7, 8) and glycogen synthase (15, 36). The $[^32P]$-labeled protein was isolated from muscle extracts using guinea pig anti-rabbit skeletal muscle phosphorylase kinase IgG and co-precipitated with Staphylococcus aureus-protein A cells. The subunits were then separated by SDS-gel electrophoresis and the individual protein bands corresponding to the subunits cut from the gels and counted. The variety of controls for this procedure to ensure recovery of the protein, specificity of the antibody precipitation, and the absence of modification of the protein during isolation by phosphorylation, dephosphorylation, or proteolysis, have been presented previously (7, 8, 15, 36) and under "Experimental Procedures," and/or are illustrated by the electrophoretic experiments depicted in Fig. 4. As illustrated (Fig. 4a, lane 1; see also Figs. 6–8), radiography of SDS gels of immunoprecipitates of $[^32P]$phosphorylase kinase from FDB maximally stimulated with epinephrine, showed primary labeling of the $\alpha'$ and $\beta$ subunits (with that of the $\alpha'$ predominating), and a low level of phosphorylation of $\alpha$. (As discussed below, FDB contains primarily the $\alpha'$ isozyme of phosphorylase kinase; details of the kinetics of phosphorylation are shown in subsequent experiments.) In addition to the observed phosphorylation of phosphorylase kinase subunits, the washed immunoprecipitates also contained some lower molecular weight $[^32P]$-labeled proteins. These, most likely, are due to nonspecific precipitation (or adsorption to the S. aureus cells) as they were also present when either normal IgG plus protein

A (Fig. 4a, lane 2) or protein A alone (lane 3) were used; conditions which did not result in phosphorylase kinase precipitation. (With protein A cells alone (lane 3), the amount of these lower molecular weight proteins was somewhat higher, possibly due to the absence of IgG, which may prevent some nonspecific adsorption.) Importantly, as illustrated by both of these controls (Fig. 4a, lanes 2 and 3), the background radioactivity in the region of the gels corresponding to the $\alpha'$ and $\beta$ subunits was quite minimal. As one of the controls, standard immunoprecipitates were made from extracts of FDB incubated with nonradioactive medium, but with $[\gamma-^3P]$ATP added to the extraction buffer. The amount of $[\gamma-^3P]$ATP added was enough to achieve a specific activity in the extract (including the ATP contributed by the muscle) equal to that obtained by incubating the tissue with $[^32P]$inorganic phosphate. This protocol was followed for tissue incubated in either the absence (Fig. 4a, lane 4) or presence (lane 5) of epinephrine. In neither was phosphorylated phosphorylase kinase detected, thus providing evidence that the labeling of the enzyme observed with $[^3P]$-incubated tissue is a consequence of phosphorylation occurring within the cell and not during the extraction procedure. Dephosphorylation during the immunoprecipitation protocol has also been demonstrated not to occur ("Experimental Procedures" and Ref. 7). With Coomassie staining of the gels (Fig. 4b), prominent protein bands for the FDB $\alpha'$ and $\beta$ phosphorylase kinase subunits, and for the IgG heavy and light chains and the proteins derived from the S. aureus-protein A cells, were evident (lanes 1–3, as in panel a; lane 4, pure skeletal muscle phosphorylase kinase). Two minor bands of protein in the region of the phosphorylase kinase $\alpha'$ and $\beta$ subunits were also seen. These

![Fig. 4. SDS-gel electrophoresis profile and immunoblot analysis of immunoprecipitated phosphorylase kinase of FDB. Panels a and b, lanes 1-3: FDB muscles were excised from rats and incubated for 6 h in Liley's medium containing $[^3P]$inorganic phosphate and at the end of this period treated with 1 mM epinephrine for 120 s. The frozen muscle powder was extracted and the immunoprecipitation procedure followed using: lane 1, antiphosphorylase kinase IgG; lane 2, normal IgG; or lane 3, no IgG. All samples had S. aureus-protein A added. For panel a, lanes 4 and 5, the muscles were incubated in nonradioactive media, either without (lane 4) or with (lane 5) an epinephrine stimulation (2 min, 1 mM). The standard extraction and immunoprecipitation procedures (i.e. with antiphosphorylase kinase IgG) were employed, except that the extraction buffer contained $[\gamma-^3P]ATP$, added to give a specific activity of 400 dpm/pmol inclusive of the ATP contributed by the tissue. This is equal to the level achieved by the standard incubation of the intact tissue with $[^3P]$inorganic phosphate, see Fig. 5. Lane 4, panel b, is of 2 mg of pure skeletal muscle phosphorylase kinase. Panel a is the autoradiogram, panel b is the Coomassie protein stain. For panel c, either 1.3 mg of pure rabbit skeletal muscle phosphorylase kinase (lane 1) or standard immunoprecipitates from 50 mg of FDB (lane 2), 250 mg of rat cardiac muscle (lane 3), or 15 mg of rat medial white gastrocnemius muscle (lane 4), prepared by the standard procedure, were electrophoresed. At the termination of the electrophoresis, the gels were immunoblotted using antiphosphorylase kinase IgG and bands detected with $[^32P]$-labeled S. aureus-protein A. Details are provided under "Experimental Procedures."
were present when normal IgG was used (lane 2) and most likely represent nonspecific adsorption to the IgG-protein A-S. aureus cells complex; neither, however, was phosphorylated (panel a, lanes 1 and 2). Consistent with the observations of subunit phosphorylation indicated by the autoradiograms, densitometric scans of the Coomassie-stained gels showed that FDB contained primarily (>90%) the α' isozyme of phosphorylase kinase, with the α' and β subunits being present in essentially identical amounts. This latter observation also provides further evidence that the lower level of αβ-phosphate in the β subunit was not due to selective proteolysis; proteolysis would also have given rise to marked enzyme activation (1), which did not occur during the extraction.) As a further examination of isozyme distribution and of the specificity of the antibody immunoprecipitation, gels of the immunoprecipitates of FDB were examined by immunoblotting (Fig. 4c, lane 2). Comparisons were made to standard rabbit skeletal muscle enzyme (lane 1) and to immunoprecipitates prepared from rat cardiac muscle, which also contains primarily the α' isozyme (5, 8) (lane 3), or from rat gastrointestinal muscle, which contains predominantly the α isozyme but also substantial amounts of α' (lane 4). These data showed that FDB contained predominantly the α' isozyme, with small amounts of α subunit. The immunoblot analysis provided further evidence that the IgG preparation was specific for phosphorylase kinase and that other protein bands evident on the gels were not a consequence of cross-reactivity or due to impurities in the initial antigen giving rise to nonspecific IgG.

[32P]Phosphate Uptake Into Flexor Digitorum Brevis Muscle—Labeling of the intracellular ATP of FDB with [32P] was accomplished by incubating muscle preparations with [32P] inorganic phosphate; the characteristics of this process are presented in Fig. 5. As with both cardiac (22, 36) and diaphragm (32, 37) muscle, the uptake of phosphate into FDB is quite slow. The rate of uptake was linear for at least 10 h (shown up to 6 h in Fig. 5a) and at the end of a typical 6-h incubation the specific activity of [γ-32P]ATP was 25–35% of that of extracellular [32P] inorganic phosphate. During this time the specific activity of the medium decreased ~30% (Fig. 5a), whereas total extracellular phosphate remained constant (not illustrated), indicating an exchange of phosphate between the muscle cell and the extracellular medium. The rate-limiting step appears to be phosphate transport, whereas the equilibration between intracellular phosphate and the γ-P of ATP is rapid (22). The Kₘ for phosphate uptake was found to be 0.18 ± 0.04 mM (Fig. 5a, inset); this has allowed the use of a low phosphate medium and hence has provided a means to increase the specific activity of the medium [32P]phosphate, and thus the specific activity of intracellular [γ-32P]ATP, while using the same level of radioactivity. Although full equilibration into ATP (γ-phosphate) is not achieved in 6 h, in the subsequent experiments examining phosphorylase kinase phosphorylation, a 6-h incubation with 0.5 mM extracellular [32P] phosphate has been used as the standard condition of incubation. Longer periods of incubation were found to increase the variability of the glycogenolytic enzyme's responsiveness to adrenergic agents, perhaps reflecting some variation with longer times of incubation in the utilization of glycogen as a metabolic fuel.
The uptake of [32P]inorganic phosphate into FDB was not significantly enhanced if the muscles were electrically stimulated nor by several alterations in the incubation medium, including low (6.8–7.0) or high (8.0) pH, preincubation without phosphate, hypertonic media (made 3 times isosmolar with K₂SO₄ or sucrose), or replacement of HCO₃⁻ or Cl⁻ with hippurate (as a possibly less favored anionic transport ion). Phosphate transport was 4.5-fold faster at 37 °C (the standard incubation condition) than at 23 °C (data not shown).

During a 6-h incubation of FDB, even in the absence of hormonal stimulation, [32P]phosphate was incorporated into the α', β, and α subunits of phosphorylase kinase (Fig. 5b). During this time period, phosphorylase kinase activity remained constant, suggesting that the 32P incorporation observed represents the turnover of phosphate in the protein and not net phosphorylation. The exchange of phosphate into the α' subunit considerably exceeded that of the β subunit; the exchange into the α' and α subunits roughly reflected the

![Graph](image)

**Fig. 6.** Time course of subunit phosphorylation and activation of phosphorylase kinase in epinephrine-treated FDB. Muscles were incubated for 6 h in MEM with acetoacetate and insulin (panels a and b) or in Liley's medium (panels c and d) containing [32P]phosphate before exposure to 1.0 μM epinephrine for the indicated times. Muscle powders were extracted and portions of the extracts used for determination of enzyme activity (○) and 32P incorporation into the α' + α (▲) and β (●) subunits, as described under "Experimental Procedures." In a and b, n = 6 for controls, n = 5 for 120-s point, and n = 3 for all other points. Data are presented as the mean ± S.E. In c and d, n = 4 for controls and n = 2 for all other points. The insets show the autoradiographs for one set of data from which the values on the graphs were obtained.
relative amounts of the two isozymes in the muscle. In subsequent measurements, data for the phosphorylation of the α' and α subunits has been combined, with the latter always being only a small component (~10%). By visual inspection, the patterns of phosphorylation of the α subunit reflected those seen with the α' subunit.

Subunit Phosphorylation of Phosphorylase Kinase in Response to Adrenergic Agents: Correlation with Activation—Epinephrine caused a time-dependent increase in the \(^{32}\)P content of the α', α, and β subunits of phosphorylase kinase as shown in Fig. 6. The specific activity of intracellular [γ-\(^{32}\)P]ATP was not affected by epinephrine over the time course shown, nor was it affected by placement of the muscles into fresh media, thus indicating that the increased \(^{32}\)P content is indeed due to enhanced phosphorylation.

Since muscles incubated in Liley’s medium appeared to represent a different metabolic state from those incubated in MEM with acetoacetate and insulin (as evidenced by different muscle glycogen levels), epinephrine-stimulated subunit phosphorylation and enzyme activation were examined under both conditions. In muscles incubated in either medium, the \(^{32}\)P content of the α' + α and β subunits increased approximately 2-fold during a 2-min exposure to 1.0 µM epinephrine. Both α' + α and β subunit phosphorylation appeared to commence simultaneously, in contrast to the pattern of phosphorylation generally observed with the cAMP-dependent protein kinase in vitro (i.e. phosphorylation of the α' + α subunits did not appear to lag appreciably behind phosphorylation of the β subunits, Refs. 2-4), although time periods earlier than 15 s were difficult to evaluate. Phosphorylation of the β subunits was maximal by 30-60 s with the [\(^{32}\)P]phosphate content remaining stable for at least 60 s thereafter. Phosphorylation of the α' + α subunits occurred most rapidly during the first 60 s of epinephrine exposure after which a variable degree of continued phosphorylation was observed. In some experiments, α' + α subunit phosphorylation appeared to be near maximal after 60 s while in others phosphorylation continued, but at a slower rate. Phosphorylase kinase activity also appeared to increase most rapidly during the first 60 s of epinephrine exposure. Variability between individual experiments similar to that observed for phosphorylation of the α' + α subunits was observed between individual experiments in the later phase of the activation time course. Although the patterns of subunit phosphorylation observed in muscles with depleted glycogen levels (i.e. Liley’s incubation, Fig. 6, panel c) and those observed in muscles with elevated glycogen levels (i.e. MEM with acetoacetate and insulin incubation, panel a) were very similar, the data suggests a somewhat higher initial phosphate content of both α' + α and β subunits in the glycogen-depleted state. However, a similar quantitative in-
crease in subunit phosphate content resulting from epinephrine exposure was seen for muscles in the two metabolic conditions.

The epinephrine dose dependence of $\alpha' + \alpha$ subunit phosphorylation and that of $\beta$ subunit phosphorylation were similar (Fig. 7). In agreement with the dose dependence of cAMP elevation and phosphorylase kinase activation (see Fig. 1), half-maximal phosphorylation of both types of subunits occurred at a concentration between $10^{-8}$ and $10^{-7}$ M epinephrine. The type of incubation medium utilized (and metabolic differences resulting therefrom) did not appear to affect the dose dependence of either subunit phosphorylation or activation. The maximal quantitative change in both phosphorylation and activation due to epinephrine stimulation was approximately the same under either incubation condition.

Table I summarizes data obtained from a number of experiments wherein the activity and subunit phosphorylation of phosphorylase kinase were examined in both untreated and epinephrine-treated muscles. A 90-s exposure to 1.0 $\mu$M epinephrine, which appeared to produce maximal stimulation of $\beta$ subunit phosphorylation and near-maximal stimulation of $\alpha' + \alpha$ subunit phosphorylation and activation, clearly resulted in a significant increase (i.e., p < 0.01 or p < 0.001) in all three parameters. Similar changes in response to epinephrine were observed in muscles from either incubation medium. The difference between the control phosphate content of both $\alpha + \alpha'$ and $\beta$ subunits in unstimulated muscles incubated in Liley’s medium and muscles incubated in MEM (with acetocacetate and insulin) also proved to be statistically significant. Activity was measured both as specific activity measured at acetate and insulin) also proved to be statistically significant.

An estimate of the molar stoichiometry of phosphate incorporation was obtained after densitometrically quantitating the amount of phosphorylase kinase present on SDS gels of immunoprecipitates (as described under “Experimental Procedures”). This value could then be used to convert picomoles of phosphate/mg of soluble protein to a molar ratio of phosphorylase kinase. Thus the molar stoichiometry of phosphate incorporation in phosphorylase kinase (for muscles incubated in Liley’s) was estimated at 0.8-1.9 mol/mol ($\alpha' + \alpha$), and 0.2-0.5 mol/mol $\beta$, with a maximal epinephrine stimulation. It must be pointed out that these values are at best estimates which may be subject to significant error. In addition, the assumption has been made that specific activity of phosphate incorporated into phosphorylase kinase is equal to the determined specific activity of $[\gamma-32P]ATP$ in the muscles.

In a report by Mayer and Krebs (9) phosphorylase kinase appeared to be phosphorylated from a pool of ATP of higher specific activity than the total cellular pool. The latter might also account for apparent differences in the degree of subunit phosphorylation between the MEM and Liley’s media.

The potential correlations between epinephrine-induced activation and subunit phosphate content of phosphorylase kinase for the experiments shown in Figs. 6 and 7 are presented in Table II. These were examined by linear least squares regression analysis. As observed, activity appeared to correlate somewhat better with $\alpha' + \alpha$ subunit phosphorylation than with $\beta$ subunit phosphorylation in a majority of experiments, but also correlated equivalently with the level of total phosphorylation.

The potential correlations between phosphorylase kinase subunit phosphorylation and activation have been examined with two other conditions. In the first approach (Fig. 8, panel a), subunit phosphorylation and enzyme activation were de-

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**Table I**

*Subunit phosphate content and phosphorylase kinase activity in FDB incubated in the presence or absence of epinephrine*

The data are taken from experiments depicted in Figs. 6 and 7 and other identical experiments not shown. Tissue was incubated in either MEM (plus acetocacetate and insulin) or Liley’s medium as described in the legends of those figures. Control values are the zero time points (i.e. determined at the end of a 6-h incubation). Stimulated values refer to 90-s exposure to 1.0 $\mu$M epinephrine. Phosphate content is expressed as picomoles of phosphate/mg soluble protein; activity is expressed either as micromoles/min/mg soluble protein (listed as units/mg) or as the pH 6.8/8.2 activity ratio. Numbers in parentheses indicate the number of separate extracts examined. Data are from six separate experiments and are presented as the mean ± S.E.

<table>
<thead>
<tr>
<th>Incubation medium</th>
<th>Parameter</th>
<th>Control</th>
<th>+Epinephrine</th>
<th>p (control versus epinephrine)</th>
<th>Average fold increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>MEM</td>
<td>$\alpha' + \alpha$</td>
<td>0.85 ± 0.10 (9)</td>
<td>2.12 ± 0.16 (6)</td>
<td>&lt;0.001</td>
<td>2.49</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>0.30 ± 0.03 (9)</td>
<td>0.65 ± 0.04 (6)</td>
<td>&lt;0.001</td>
<td>2.16</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>3.03 ± 0.44 (9)</td>
<td>7.72 ± 1.01 (6)</td>
<td>&lt;0.001</td>
<td>2.55</td>
</tr>
<tr>
<td></td>
<td>Units/mg</td>
<td>0.13 ± 0.02 (9)</td>
<td>0.24 ± 0.03 (6)</td>
<td>&lt;0.01</td>
<td>1.85</td>
</tr>
<tr>
<td></td>
<td>6.8/8.2 activity ratio</td>
<td>Phosphate content</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liley’s</td>
<td>$\alpha' + \alpha$</td>
<td>1.41 ± 0.18 (7)</td>
<td>2.91 ± 0.25 (6)</td>
<td>&lt;0.001</td>
<td>2.06</td>
</tr>
<tr>
<td></td>
<td>$\beta$</td>
<td>0.58 ± 0.07 (7)</td>
<td>1.08 ± 0.15 (6)</td>
<td>&lt;0.001</td>
<td>1.86</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>3.29 ± 0.33 (8)</td>
<td>7.33 ± 0.96 (6)</td>
<td>&lt;0.01</td>
<td>2.23</td>
</tr>
<tr>
<td></td>
<td>Units/mg</td>
<td>0.11 ± 0.02 (8)</td>
<td>0.18 ± 0.02 (6)</td>
<td>&lt;0.05</td>
<td>1.64</td>
</tr>
<tr>
<td></td>
<td>6.8/8.2 activity ratio</td>
<td>p Value (MEM versus Liley’s)</td>
<td>$\alpha' + \alpha$</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>Activity</td>
<td>NS*</td>
<td>NS</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* NS, no significant difference.
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terminated in response to the β adrenergic agonist, DL-isoproterenol. The patterns obtained were similar to those seen with epinephrine; both subunits were rapidly phosphorylated in coordination with changes in phosphorylase kinase activity and with the α' subunit being phosphorylated to a higher maximal level. In a second approach (Fig. 8, panel b), dephosphorylation and deactivation of FDB phosphorylase kinase were examined following the removal of an epinephrine stimulation. As is observed, all three parameters appeared to follow a fairly similar time course in return to basal conditions suggesting that, as with activation, deactivation appears to be coordinated with the dephosphorylation of both subunits.

**TABLE II**

Correlations between phosphorylase kinase activation and specific subunit phosphorylation

Data is taken from the experiments of Figs. 6 and 7 and other identical experiments for incubations in either MEM (plus acetoadetate and insulin) or Liley's medium. Correlation coefficients were determined from linear regression analyses.

<table>
<thead>
<tr>
<th>Experimental conditions</th>
<th>Incubation medium</th>
<th>No. of time or dose points</th>
<th>Correlation coefficients, phosphorylation versus activation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epinephrine (1.0 μM)-time course</td>
<td>MEM</td>
<td>7</td>
<td>β α' + α Total α + α' + β</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td>0.64 0.87 0.82</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td>0.87 0.89 0.90</td>
</tr>
<tr>
<td>Experiment 3</td>
<td></td>
<td></td>
<td>0.76 0.90 0.87</td>
</tr>
<tr>
<td>Epinephrine (1.0 μM)-time course</td>
<td>Liley's</td>
<td>7</td>
<td>0.82 0.93 0.91</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td>0.87 0.89 0.90</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
<td>0.92 0.94 0.95</td>
</tr>
<tr>
<td>Epinephrine (90 s)-dose response</td>
<td>MEM</td>
<td>7</td>
<td>0.97 0.91 0.96</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
<td>0.97 0.91 0.96</td>
</tr>
</tbody>
</table>

**DISCUSSION**

Fast- and slow-twitch muscle fibers may be distinguished on the basis of their contractile characteristics, correlated experimentally with which isozyme of myosin ATPase they contain. Within the fast-twitch category, there is a continuum of metabolic profiles ranging from largely glycolytic to having an extensive oxidative capacity. Most skeletal muscles contain fibers of more than one type, but the most prominent type will provide the dominant contribution to the overall biochemical profile. White, or fast-twitch muscles that have been used in the past for contractile-metabolic studies have contained a high percentage of glycolytic fibers and a minority of fast-twitch red, or oxidative glycolytic fibers. The rat FDB, characterized by us here and in a previous report (11), is almost exclusively fast-twitch oxidative glycolytic and thus provides an excellent opportunity to study the metabolic and related contractile characteristics of this fiber type. Importantly for such studies, the FDB preparation is stable in an in vitro muscle bath for several hours of incubation, illustrated here by the profile of hormonal responsiveness of glycogen and energy metabolism, and detailed previously with contractile measurements (11). A major probable contributing factor to this stability is its sheet-like cross-section, such that the central innermost fibers are no more than 10 fibers distant from the outside surface. This, together with a small cross-sectional fiber area serves to minimize diffusion distances. Thus, the nearly homogeneous fiber composition of FDB, and its metabolic and contractile stability, make it highly suitable for in vitro studies. Other on-going investigations with FDB in this laboratory include an examination of the appearance of α-adrenergic receptors with age (2-3 year) (38) and a

![FIG. 8](image-url)

**Fig. 8. Panel a**, time course of isoproterenol-stimulated phosphorylase kinase subunit phosphorylation and activation. Muscles were incubated in Liley's medium with [32P]phosphate under standard conditions prior to treatment with 1.0 μM DL-isoproterenol. Symbols are as in Fig. 6. The inset shows sample autoradiographs for the zero and 60-s time points. Panel b, dephosphorylation and inactivation of phosphorylase kinase following removal of epinephrine. Muscles were incubated in Liley's medium as described in the legend to Fig. 6 and treated with 1.0 μM epinephrine for 120 s. Following this the epinephrine solution was removed and replaced with media containing 1.0 μM DL-propranolol; muscles were frozen at the indicated time points. Symbols are as indicated in Fig. 6. The initial activity and phosphate content, prior to epinephrine treatment is indicated on the left-hand side. The inset shows sample autoradiographs for the basal (B), epinephrine stimulated (E), and 60-s dephosphorylated (D) time points.
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comparison of the sources of Ca\textsuperscript{2+} that regulate contraction and phosphorylase kinase activity.\textsuperscript{4} Of interest in FDB is the predominance of the \(\alpha'\) isozyme of phosphorylase kinase (Fig. 4). Previous descriptions of phosphorylase kinase distribution have attributed the presence of the \(\alpha\) isozyme to fast-twitch fibers and the \(\alpha'\) isozyme to slow-twitch muscles (39, 40). This report indicates that rather than being related to contractile characteristics, it now seems more reasonable to correlate phosphorylase kinase isozyme distribution with metabolic competency. Thus, fast-twitch oxidative glycolytic fibers, slow-twitch oxidative fibers, and cardiac muscle, each of which contain the \(\alpha'\) isozyme but exhibit markedly different contractile properties, all possess substantial oxidative metabolic capacity. Fast-twitch glycolytic fibers, in contrast, which rely on glycolytic energy production, predominantly contain the \(\alpha\) isozyme. A similar isozyme distribution has very recently also been described by Lawrence \textit{et al.} (41). Why such a relationship might exist is unknown; the major differences in properties between the two isozymes that have so far been recognized are some quantitative variations in regulation by phosphorylation (in \textit{vitro} data) (5, 42, 43) and a difference in regulation by exogenous calmodulin (45).

One primary objective of our current studies has been to examine in intact muscle the regulation of phosphorylase kinase activity by multisubunit phosphorylation. The studies presented here with FDB have initiated this for skeletal muscle containing the \(\alpha'\) isozyme. (Additional future studies will be necessary for skeletal muscle containing the \(\alpha\) isozyme.) The accompanying report (8) details the pattern of multisubunit phosphorylation and associated subunit phosphorylation in the perfused rat heart; it too contains almost exclusively the \(\alpha'\) isozyme. Currently it is not known if the cardiac \(\alpha'\) isozyme and skeletal muscle \(\alpha'\) isozyme are identical. When compared from a single species (beef), the \(\alpha'\) subunits from cardiac and skeletal muscle exhibited identical electrophoretic mobilities (in SDS-PAGE) (46); studies with the purified enzymes, however, have suggested that there could be some differences (42–44). In the intact tissue studies described here with FDB and in the accompanying paper with perfused rat heart (8), several similarities and some apparent differences were observed. In both, there was phosphorylation of both the \(\alpha'\) and \(\beta\) subunits associated with enzyme activation. With the cardiac perfusion, similar to what is observed with the purified enzyme in \textit{vitro}, there was a lag between the phosphorylation of the \(\alpha'\) and \(\beta\) subunits in response to a CAMP-mediated hormonal signal. Such a lag was not apparent in the study of FDB phosphorylase kinase phosphorylation, but it could have been masked by limitations in the experimental system. With phosphorylase kinase phosphorylation in the perfused heart, the \(\alpha'\) and \(\beta\) subunits were phosphorylated in response to CAMP-mediated hormonal stimuli to very nearly the same level. In contrast to this, in FDB there was clearly a much greater level of hormonally stimulated phosphorylation of the \(\alpha'\) than the \(\beta\) subunit. This quite closely mirrors what has been observed for the phosphorylation of purified beef heart phosphorylase kinase by the CAMP-dependent protein kinase, where likewise the \(\alpha'\) subunit is phosphorylated to a level 2–4 times that obtained for the \(\beta\) subunit. The reasons for the differences seen between cardiac muscle and FDB are not known and await further investigation. Clearly the two represent quite different experimental and physiological situations; the purpose of activation in each could well be subtly different, as could other components and conditions within the two cell types that may influence phosphorylase kinase regulation. We have previously emphasized (4) that the multisite phosphorylation of phosphorylase kinase is a highly complex reaction with the potential for several different routes of phosphorylation, even when considering only those catalyzed by the CAMP-dependent protein kinase. In an accompanying paper (6), we have shown in \textit{in vitro} studies that both \(\alpha\) subunit phosphorylation and \(\beta\) subunit phosphorylation regulate enzyme activity, but they play different roles, with \(\beta\) subunit phosphorylation being essential for activation and \(\alpha\) subunit phosphorylation modulating the extent to which this occurs. The ramifications of these complexities are not readily recognized at this stage, but it is probable that a molecule with a structure as complicated as that of phosphorylase kinase, which contains four species of subunits, must have cellular roles or interactions that are yet to be elucidated. Multisite phosphorylation clearly plays a pivotal role in the regulation of phosphorylase kinase, which is most likely also modulated by different conditions within the cell. These studies of FDB (reported here), and cardiac muscle (8), have begun to enumerate which of the characteristics that have been observed in the extensive \textit{in vitro} phosphorylation studies (see Ref. 1 for further review) are reflected in the regulation of the enzyme in the intact tissue.

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Bioc. Chem. 244, 4235-4240

Phosphorylase Kinase Phosphorylation in Skeletal Muscle

by (Inky) A. Adenisi, Richard C. Jordan (Inky), J. Anderson, Karen L. Angles and Donal A. Walsh

EXPERIMENTAL PROCEDURES

Sodium Metat and Phosphate

The two media used for cell incubations were: 1% NaCl solution, pH 7.4 (1 mL) and 2% bovine serum albumin (BSA) solution (1 mL). Both were incubated at 37°C for 1 h in a humidified incubator. After the incubations, the cells were washed twice with PBS (1 mL). PBS was used for the final wash. The cells were then homogenized with a teflon homogenizer. The homogenates were centrifuged at 10,000 rpm for 30 min at 4°C. The supernatants were then collected and stored at -80°C until use.

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was performed on a 10% gel using the method of Laemmli (1970). Electrophoresis was performed at 80 V for 2 h. After staining, the gels were photographed using a gel documentation system.

RESULTS

A. Effect of Phosphorylase Kinase Phosphorylation on Glycogen Synthesis

Glycogen synthesis was assessed using the isolated perfused extensor digitorum longus (EDL) muscle of the rat. The EDL muscle was perfused at a constant pressure of 90 mm Hg. The perfusate contained glucose, insulin, and phenol red. The perfusion solution was recirculated through the muscle, and the glycogen content of the muscle was determined by the method of Maurer and Konrad (1968). The glycogen content was increased by 20% in the phosphorylated muscle compared to the non-phosphorylated control.

B. Effect of Phosphorylase Kinase Phosphorylation on Glycogenolysis

Glycogenolysis was assessed using the isolated perfused EDL muscle of the rat. The perfusate contained 5 mM glucose, 1 mM insulin, and 1 mM phenol red. The perfusion solution was recirculated through the muscle, and the glycogen content of the muscle was determined by the method of Maurer and Konrad (1968). The glycogen content was decreased by 20% in the phosphorylated muscle compared to the non-phosphorylated control.

C. Effect of Phosphorylase Kinase Phosphorylation on Glycogen Phosphorylase Activity

Glycogen phosphorylase activity was assessed using the isolated perfused EDL muscle of the rat. The perfusate contained glucose, insulin, and phenol red. The perfusion solution was recirculated through the muscle, and the glycogen phosphorylase activity was determined by the method of Maurer and Konrad (1968). The glycogen phosphorylase activity was increased by 20% in the phosphorylated muscle compared to the non-phosphorylated control.

DISCUSSION

The results of this study indicate that phosphorylation of phosphorylase kinase increases glycogen synthesis and decreases glycogenolysis. This effect is consistent with the hypothesis that phosphorylation of phosphorylase kinase is involved in the regulation of glycogen metabolism in the skeletal muscle.

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