Studies on the Activation of Phosphorylase in Skeletal Muscle by Contraction and by Epinephrine

George I. Drummond, James P. Harwood, and C. Anne Powell

From the Department of Pharmacology, School of Medicine, University of British Columbia, Vancouver 8, Canada

SUMMARY

The activation of phosphorylase during muscle contraction and in the presence of epinephrine was studied in isolated frog sartorius and rat diaphragm, and in rat gastrocnemius in vivo. In all three tissues, epinephrine-induced conversion of phosphorylase b to a was accompanied by conversion of phosphorylase b kinase to the activated form (as evidenced by increased pH 6.8 to 8.2 activity ratios), and by increased tissue levels of cyclic adenosine 3',5'-monophosphate. In contrast, when glycogenolysis was facilitated by muscle contraction (as induced by electrical stimulation), phosphorylase b to a conversion took place in all three tissues under conditions in which there was no detectable conversion of phosphorylase b kinase to the activated form and no increase in tissue levels of cyclic 3',5'-AMP. It is concluded that the mechanism of activation of phosphorylase by muscle contraction is basically different from that involving adrenergic amines. Activation of the enzyme during muscle contraction is not accompanied by increased cyclic 3',5'-AMP levels nor conversion of phosphorylase kinase to the activated form. The possibility that calcium ion may regulate phosphorylase kinase activity during muscle contraction is discussed.

It is well established that activation of phosphorylase (conversion of phosphorylase b to a) occurs in skeletal muscle when glycogenolysis is facilitated either by electrical stimulation or by epinephrine. From studies on frog and rat muscle, Cori first suggested in 1956 (1) that a correlation might exist between the rate and amount of work performed and the degree to which phosphorylase became activated during contraction. With the use of the excised anterior tibial muscle of the mouse, Rulon, Schottelius, and Schottelius (2) showed that phosphorylase a levels increased rapidly with the onset of electrical stimulation and suggested that phosphorylase was activated at a rate sufficient to mobilize substrate to meet the increased energy demand associated with tetanus. In frog sartorius muscle, Danforth, Helmreich, and Cori (3) reported that phosphorylase a levels rose from near zero to almost 100% of total in 3 sec during rapid electrical stimulation. Phosphorylase activation was attributed to changes in the phosphorylase b kinase activity. Epinephrine was similarly found to cause increased phosphorylase a levels in this tissue, but this occurred much more slowly, requiring minutes instead of seconds to reach peak activity. Danforth et al. (3) suggested that two different mechanisms might be involved in the activation of phosphorylase b by muscle work and by epinephrine. It is well known that epinephrine stimulates the formation of cyclic adenosine 3',5'-monophosphate in many tissues. Studies on phosphorylase b kinase (4) have revealed that like phosphorylase, this enzyme exists in resting tissue in a form that is essentially inactive at physiological pH (nonactivated form). The kinase from skeletal muscle (4) and cardiac muscle (5) can be converted in vovo to an activated form by prior incubation with ATP and Mg++. Cyclic 3',5'-AMP facilitates this activation reaction. The magnitude of the activation in vitro is much greater when the enzyme is assayed at pH 6.8 (where the nonactivated enzyme has very low activity) than at pH 8.2 where the nonactivated enzyme is almost fully active. The activation process is thus characterized by an increase in pH 6.8 to 8.2 activity ratios. With isolated frog sartorius and rat gastrocnemius in situ, Posner, Stern, and Krebs (6) have provided evidence that in these skeletal tissues epinephrine facilitates phosphorylase b to a conversion, by a mechanism which involves increased cyclic 3',5'-AMP levels, and conversion of phosphorylase kinase to the activated form.

The evidence is less clear as to how glycogenolysis is facilitated during muscle contraction. Posner et al. (6) reported that electrical stimulation of frog muscle caused a rapid increase in phosphorylase a levels which was accompanied by increased pH 6.8 to 8.2 phosphorylase kinase activity ratios. Cyclic 3',5'-AMP levels, however, did not change. Similar but smaller changes in the two enzymes were noted on electrical stimulation of rat leg muscle, again with no changes in cyclic 3',5'-AMP levels. This would indicate that during muscle contraction, phosphorylase kinase is converted to the activated form by some process not involving the cyclic nucleotide. In order to clarify this we have studied the effects of electrical stimulation and epinephrine on phosphorylase, phosphorylase kinase, and cyclic 3',5'-AMP in isolated rat diaphragm, frog sartorius, and rat gastrocnemius in vovo. Data are presented which suggest that
during electrical stimulation phosphorylase is activated by a mechanism which involves neither cyclic 3',5'-AMP nor conversion of kinase to the activated form.

**EXPERIMENTAL PROCEDURES**

**Materials**

Rabbit muscle phosphorylase b, glycogen, and glucose 1-phosphate were prepared or purified by methods previously used (7). Solutions of epinephrine bitartrate (1 mg of base per ml) were prepared in 0.1% sodium metabisulfite and stored at -18°.

Preparation of Tissues

**Rat Diaphragm**—Female Wistar rats weighing approximately 200 g were decapitated, and the diaphragm attached to the rib cage was removed and placed in ice-cold Tyrode’s solution. For some experiments, the phrenic nerve was dissected out on each side. The diaphragm was divided into two, trimmed of extraneous tissue, mounted via a segment of the rib cage on a perspex frame by three fine wire hooks, and arranged in a bath containing 100 ml of Tyrode’s solution at 37°. Gasced with 95% N2-5% CO2. A thread from the tip of the hemidiaphragm was attached to a force displacement transducer (Grass Instrument Company, model FT03) and contractions were recorded on a polygraph. An initial tension of 5 g was applied to the muscle and a 15-min equilibration period preceded each experimental procedure. For direct electrical stimulation of the muscle, 5-volt pulses of 2-msec duration were provided by a Grass stimulator. When stimulating the diaphragm via the phrenic nerve, 4-volt pulses of 0.02-msec duration were used. Diaphragms to be treated with epinephrine were equilibrated in a similar manner. Ascorbic acid was added to a final bath concentration of 1.0 mg per ml, followed by epinephrine, 10 μg per ml final concentration. At the appropriate instant during each experimental procedure, the bath was dropped and the tissue was crushed with aluminum tongs previously chilled in liquid N2 (stimulus applied throughout). The preparation was rapidly plunged into liquid N2 and any tissue outside the tongs was chipped away. Control tissues were similarly frozen after the equilibration period. Samples were stored under liquid N2 until use.

**Frog Sartorius**—Leopard frogs (Rana pipiens) were pithed, and the sartorious muscles were dissected out and mounted vertically in bath containing 50 ml of Krebs-Ringer’s bicarbonate solution (adapted for frog muscle (8)) at 20° vigorously gassed with 95% N2-5% CO2. The recording arrangement was the same as that used for diaphragm. Each tissue was allowed to equilibrate for 15 min bearing 0.5 g of tension. The fluid in the bath was then lowered until the muscle was exposed and electrical stimuli at 10 pulses per sec of 2-msec duration (15 volts) were applied through fine wire electrodes in contact with the tissue. At the appropriate instant, while the stimulation was continuously applied, the bath was dropped and the tissue was frozen as above. In other experiments, epinephrine was added to a final concentration of 5 μg per ml (preceded 1 min by the addition of 0.1 mg of ascorbic acid per ml) and after the appropriate interval, the tissue was frozen with clamps.

**Rat Gastrocnemius**—Rats were anesthetized with pentobarbital (60 mg per kg, intraperitoneally), the skin was cut around the ankle and pulled back so as to expose the gastrocnemius; a thread was tied to the Achilles tendon, the tendon was cut distally, attached to a transducer, and placed under 10 g of tension. Fine wire electrodes were inserted into the muscle. After 20 min the muscle was stimulated for 5 sec with 15-volt pulses of 2-msec duration. The entire leg below the knee was then crushed with large aluminum tongs chilled in liquid N2 (while stimulating), the leg was rapidly removed with scissors and plunged into liquid N2. The gastrocnemius was dissected out and stored in liquid N2. In other animals, epinephrine was injected intraarterially in a dose of 5 μg per kg and after 40 sec the prepared leg was frozen as above. Tissues from control animals were similarly prepared. Only one leg was used from each animal.

**Preparation of Tissue Extracts**

Frozen tissues were weighed and ground to a fine powder under liquid nitrogen. Diaphragm and gastrocnemius were pulverized in a suitably constructed stainless steel mortar and transferred to a previously chilled Potter-Elvehjem homogenizer tube. Frog sartorius were conveniently powdered in a small homogenizer tube (immersed in liquid N2) with the aid of a glass rod. The powdered material was thoroughly homogenized in 10 volumes (5 volumes for diaphragm) of a buffer containing 10 mM β-glycero-phosphate, 5 mM EDTA, 20 mM NaF, 30 mM cysteine, pH 6.8, containing 60%; glycerol at -35° (Dry Ice-50%; ethylene glycol bath). Dispersal was conveniently effected using a loose fitting Teflon pestle. When the powder was thoroughly dispersed an equivalent volume of the same buffer containing no glycerol was added, the tube was immersed in an ice-water bath and homogenization was continued for an additional 2 min (120 passes). The homogenate was centrifuged at 12,000 X g for 10 min and the supernatant was removed and assayed immediately.

**Phosphorylase and Phosphorylase Kinase Assays**

The extracts were suitably diluted (usually 10-fold) in 20 mM NaF, 2 mM EDTA and assayed for phosphorylase in the direction of glycogen synthesis by the micromethod described by Mayer, Cotton and Moran (9) using 0.05 ml of assay mix and 0.05 ml of diluted extract. The data are expressed as micromoles of inorganic phosphate released per g of tissue in 10 min at 30°. The activation of phosphorylase b is expressed as the ratio of phosphorylase b to total activity.

The phosphorylase b kinase assay was based on the method described by Krebs et al. (4) with the modifications used previously (5) for the cardiac enzyme. Extracts were suitably diluted (usually 10-fold) in 15 mM neutral cysteine immediately before assay. In the assay of skeletal muscle extracts, 5'-AMP does not accumulate sufficiently to interfere with the phosphorylase a determination and therefore the use of adenyl deaminase (5) was unnecessary. For controls, muscle extract was added to the kinase assay system, phosphorylase b being omitted. After termination of the reaction by dialysis, the appropriate amount of phosphorylase was added, and the assay completed in the usual way. All assays with appropriate controls were performed in duplicate at pH 6.8 and at pH 8.2. One unit of phosphorylase b kinase is defined as that activity which catalyzes the formation of 100 units of phosphorylase a in 5 min under the conditions of the assay. The degree of activation is expressed as the ratio of activity as pH 6.8 to that at pH 8.2.

**Determination of Cyclic 3',5'-AMP**

Boiling water extracts of tissues were prepared as described previously (10). Six hemidiaphragms were pooled to provide
about 1 g of tissue; gastrocnemius were used individually. The cyclic nucleotide was isolated by elution from DEAE-cellulose columns (1 x 10 cm) (10) and the final lyophilized extract was dissolved in 0.5 ml of 25 mM potassium phosphate buffer, pH 7.5. The assay for cyclic 3',5'-AMP used was that of Posner et al. (11) except that the prior incubation step was carried out at 4° for 30 min (10). The extracts of gastrocnemius muscle contained an inhibitor of the activation of phosphorylase kinase by the cyclic nucleotide (11); therefore extracts were incubated with 500 ng of trypsin at 30° for 3 hours (11). Immediately before assay, 100 ng of soybean trypsin inhibitor were added. It has become apparent from the work of Walsh, Perkins, and Krebs (12) that the prior incubation step was carried out at 4° for 30 min (10).

The assay for cyclic 3',5'-AMP used was that of Losner et al. (11) with another enzyme, a protein kinase, which has an absolute requirement for the cyclic nucleotide. In the diaphragm studies reported here, the phosphorylase kinase preparation was supplemented with protein kinase. This latter enzyme was partially purified to the 78,000 x g supernatant stage (12). Rat gastrocnemius muscle essentially in the inactive form in these tissues. Rat gastrocnemius 1:6.8 to 8.2 ratios were especially high.

### Table I

**Tissue levels of phosphorylase and phosphorylase b kinase**

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Phosphorylase</th>
<th>Phosphorylase b kinase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmol/l·g/10 min</td>
<td>unit/g</td>
</tr>
<tr>
<td>Rat diaphragm</td>
<td>25</td>
<td>0.05 ± 0.005</td>
</tr>
<tr>
<td>Rat gastrocnemius</td>
<td>13</td>
<td>0.05 ± 0.005</td>
</tr>
<tr>
<td>Frog sartorius</td>
<td>31</td>
<td>0.044 ± 0.006</td>
</tr>
</tbody>
</table>

Conditions are described in the text. n represents the number of tissues used.

**RESULTS**

**Tissue Levels of Phosphorylase and Phosphorylase b Kinase**—Control levels of phosphorylase and kinase in the three tissues are shown in Table I. In rat diaphragm and frog sartorius, phosphorylase is essentially in the b form as evidenced by the low −AMP: +AMP ratios. These values agree well with those found by Danforth et al. (3) for frog sartorius in which the tissue was homogenized at −35°, which prevents activation of the enzyme during preparation of the extract. The −AMP: +AMP ratio for rat gastrocnemius was considerably higher (Table I). In order to ensure that this was not due to trauma imposed by the surgical procedure, a series of gastrocnemius were assayed from anesthetized animals in which the leg was frozen intact. The ratio did not change. The phosphorylase kinase pH 6.8 to 8.2 activity ratios in each case show that this enzyme is essentially in the inactive form in these tissues. Rat gastrocnemius pH 6.8 to 8.2 ratios were especially low.

**Effect of Electrical Stimulation and Epinephrine**—When rat diaphragm was treated with epinephrine (10 μg per ml) for 5 min, phosphorylase a levels, as expected, rose markedly as evidenced by the increased −AMP: +AMP ratio (Fig. 1, Bar B). Under these conditions the phosphorylase kinase pH 6.8 to 8.2 activity ratio increased approximately 4-fold (Bar G) similar to the results obtained for rat gastrocnemius by Posner et al. (6) and indicative of conversion of this enzyme to the activated form. It should be mentioned that the activated kinase has enhanced activity at pH 8.2 as well as at pH 6.8, as clearly shown in cardiac tissue (10), so the ratio does not reflect the true magnitude of the change at physiological pH. Activity at pH 6.8 actually rose from 235 units per g in the control to 1595 units per g in the presence of epinephrine, a 6.5-fold increase. When contraction was induced in rat diaphragm by electrical stimulation at 50 pulses per sec, phosphorylase was rapidly activated, the ratio rising to 0.69 in 10 sec when the muscle was stimulated directly (Bar D), and to 0.57 when stimulated through the phrenic nerve (Bar E). In fact, phosphorylase was so rapidly activated under these conditions that the ratio after only 2-sec stimulation (the shortest interval that could be conveniently achieved) had risen to 0.50 (Bar C). In contrast to the action of epinephrine, muscle contraction failed to change the pH 6.8 to 8.2 phospho-

![Fig. 1. Effect of electrical stimulation and epinephrine on phosphorylase and phosphorylase kinase in isolated rat diaphragm.](http://www.jbc.org/)

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The rat gastrocnemius preparation represents a relatively intact physiological system with normal nerve and blood supply. When epinephrine was injected intracardially, gastrocnemius phosphorylase, as expected (6), was activated within 40 sec when the tissue was sampled (Fig. 3, Bar B). At this time phosphorylase kinase pH 6.8 to 8.2 ratios had risen from 0.014 to 0.13 (Bar F). This represented an increase in kinase activity at pH 6.8 from 50.2 units per g to 2401 units per g. When this tissue was induced to contract by electrical stimulation at 8 pulses per sec, phosphorylase was slightly activated after 5 sec (Bar C), and when the rate of stimulation was increased to 20 per sec, the phosphorylase ratio rose to 0.77 in 5 sec (Bar D). As in the other tissues, electrical stimulation failed to alter phosphorylase kinase activity as evidenced by pH 6.8 to 8.2 ratios (Bars G and H) essentially identical with the control.

**Effect of Epinephrine and Contraction on Cyclic 3',5'-AMP Levels**—The foregoing data provide evidence that activation of phosphorylase by epinephrine is substantially different from that facilitated by muscle contraction. In the presence of epinephrine, activation was accompanied by a conversion of kinase to the activated form; during electrically induced muscle contraction the latter event did not occur. Epinephrine is known to enhance cyclic 3',5'-AMP levels in several tissues, and Poster et al. (6) have shown increased cyclic 3',5'-AMP levels in epinephrine-treated frog sartorius and rat gastrocnemius. They reported also that electrical stimulation of these tissues failed to increase intracellular levels of the cyclic nucleotide. We have examined intracellular levels of cyclic 3',5'-AMP in rat diaphragm and rat gastrocnemius after epinephrine treat-

A consistent feature of phosphorylase kinase activation occurring in intact tissues in the presence of epinephrine, is the modest degree to which pH 6.8 to 8.2 ratios increase. Ratios above 0.4 have not been reported. Krause and Wollenberger (17), however, have reported a ratio of 0.60 in dog heart during anoxia. It has been calculated that the molar ratio of phosphorylase to phosphorylase kinase in rabbit skeletal muscle is about 10:1 or 15:1 (18). Nevertheless, there seems to be an excessive quantity of kinase in skeletal and cardiac tissue since it is possible essentially to achieve full activation of phosphorylase with only modest increases in kinase activation.

In contrast to the results of Posner et al. (6), indicating activation of phosphorylase kinase on electrical stimulation, we have not detected any increase in pH 6.8 to 8.2 ratios either in isolated rat diaphragm and frog sartorius, or in rat gastrocnemius in situ under conditions which produced a marked increase in phosphorylase a levels. In agreement with Posner et al. we found no increase in cyclic 3',5'-AMP levels during electrical stimulation at a time when phosphorylase was markedly activated. We are led to conclude that phosphorylase must be activated during muscle contraction by a process which involves neither increased levels of cyclic 3',5'-AMP (stimulation of adenyl cyclase) nor conversion of phosphorylase kinase to the activated form.

For some time, calcium ion has been considered as a possible candidate for regulating the action of phosphorylase kinase. Much of this interest has arisen from the fact that this cation causes a rapid conversion of nonactivated kinase to the activated form in vitro (19, 20). However, the activation reaction was irreversible and the Ca++ concentration required for maximal effect was high (3 mM). Activation of phosphorylase kinase by Ca++ required the presence of a soluble protein, kinase-activating factor (20). More recently, kinase-activating factor has been identified as a calcium requiring proteolytic enzyme (21, 22) that effects activation by splitting a peptide or peptides from kinase. Because of the nature of the activation, it is highly unlikely that this reaction plays any role in modulating phosphorylase kinase activity. In spite of this, some evidence exists that Ca++ may still be involved in phosphorylase kinase activity in an entirely different manner. Meyer, Fischer, and Krebs (20) found that both activated and nonactivated kinase were inhibited by 1,2-bis-(2-dicarboxy methylaminoethoxy)ethane and this inhibition was effectively reversed by Ca++. More recently, Ozawa, Hosoi, and Ebashi (23) found the partially purified enzyme to be activated by Ca++ concentration as low as 10^{-7} M; and by complexing the Ca++ with 1,2-bis-(2-dicarboxy methylaminoethoxy)ethane, they were able to demonstrate a partial reversal of the calcium-induced increase in activity. Thus there is evidence that phosphorylase kinase may have an absolute requirement for Ca++.

The work reported here suggests strongly that the mechanism whereby epinephrine facilitates glycogenolysis is basically different from that involved during muscle contraction. The results obtained with epinephrine are consistent with previous evidence from studies in skeletal muscle (6) and cardiac tissue that amine-induced phosphorylase activation results from increased cyclic 3',5'-AMP levels (10, 13-16) concomitant with conversion of phosphorylase kinase to the activated form (10, 16).

From the recent work of Walsh et al. (19), it is apparent that the actual receptor for cyclic 3',5'-AMP is still another enzyme, protein kinase, which activates phosphorylase kinase through a phosphorylation reaction.

\[ \text{Fig. 4. Changes in cyclic } 3', 5'-\text{AMP levels in rat gastrocnemius and rat diaphragm. Bars A and D, controls; B, stimulation at 20 pulses per sec for 5 sec; C, 5 } \mu \text{g of epinephrine per kg, given intracardially for 40 sec; E and F, electrical stimulation at 50 sec per for 2 and 10 sec, respectively; G and H, 10 } \mu \text{g of epinephrine per ml for 1 and 5 min, respectively.} \]
to cause phosphorylase activation. Such a mechanism could, in fact, account for the data in the present experiments. It is tempting to suggest that the release of Ca++ during the electrical event would not only initiate the contractile event, but act catalytically on nonactivated kinase to effect the conversion of phosphorylase \( b \) to \( a \). Thus one biological signal could serve to couple the electrical, mechanical, and metabolic events of muscle contraction. At any rate, it seems clear that the mechanism by which glycogenolysis is facilitated by epinephrine is different from that occurring during muscle contraction. The action of epinephrine clearly involves the stimulation of adenyl cyclase resulting in increased levels of cyclic 3', 5'-AMP with subsequent activation of phosphorylase kinase. The precise mechanism involved during muscle contraction remains unsettled. Calcium ion seems to be a prime candidate but only further work will determine the exact mechanism.

REFERENCES

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J. Biol. Chem. 1969, 244:4235-4240.

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