Regulation of Glycolysis in Muscle

I. THE CONVERSION OF PHOSPHORYLASE b TO PHOSPHORYLASE a IN FROG SARTORIUS MUSCLE*

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Glycogen phosphorylase in skeletal muscle exists as phosphorylase a, active without adenosine 5'-phosphate, and phosphorylase b, active only in the presence of adenosine 5'-phosphate (2, 3). These two forms are interconvertible through the action of a specific kinase (4) which phosphorylates 2 serine residues of the b protein, and of a specific phosphatase (5, 6) which dephosphorylates the serine residues in the a protein. The interconversion is also associated with a change in molecular weight, the a form being a dimer of the b form (2).

Techniques developed for rapid freezing and extraction of thin frog sartorii at low temperature have made possible the determination of the true ratio of phosphorylase b to a in muscle (7). During rest, phosphorylase is present entirely or almost entirely in the b form. With the onset of a tetanic contraction at 30°, rapid conversion to the a form occurs, reaching nearly 100% of the total (phosphorylase a and b combined) within a few seconds. A much slower increase in phosphorylase a is seen in response to epinephrine. Kinetic analysis suggests that changes in activity of phosphorylase b kinase are responsible for changes in phosphorylase a during and after muscle work and that phosphorylase phosphatase activity remains relatively constant (7).

These studies have now been extended. When a frog sartorius muscle contracts isotonically at 20°, there is a lag period before significant amounts of phosphorylase a appear. Variations in the rate of stimulation rather than in external work affect the length of the lag period and the level of phosphorylase a attained at steady state. Membrane depolarization by high concentrations of K+ in the extracellular fluid also causes an increase in phosphorylase a, but membrane depolarization per se is not essential as judged by the rise in phosphorylase a associated with contracture in response to caffeine. These findings indicate that the activation of phosphorylase b kinase and the resultant increase of phosphorylase a are intimately related to events accompanying excitation and contraction of muscle.

EXPERIMENTAL PROCEDURE

Muscle Preparation—Female Rana pipiens from Minnesota were used. The basic methods were the same as those described previously (7). Unless otherwise stated, experiments were performed on winter frogs. The temperature was 20° in all experiments. During the course of this study, the frog Ringer-bicarbonate solution was changed from that used previously (7) to a solution closer to the composition of frog plasma (8), containing NaCl, 0.883 m; NaHCO3, 0.025 m; KCl, 0.002 m; KH2PO4, 0.0012 m; CaCl2, 0.0025 m; and MgCl2, 0.003 m. Muscles kept in this solution contracted more vigorously and gave higher values for phosphorylase a in response to stimulation and to epinephrine. The experiments concerning the effect of potassium ions, caffeine, and fatigue were carried out with this second solution.

All Ringer's solutions were gassed with 95% argon-5% CO2. Each experimental point requires a separate muscle. Since the frogs vary greatly from one lot to another and even within each lot, considerable scatter of the data is to be expected.

Preparation of Enzymes—Phosphorylase b kinase was extracted from rabbit muscle and precipitated at pH 6.1 as described by Krebs and Fischer (9). The precipitate was then taken up in 3 mM disodium EDTA to a volume of that of the original extract and adjusted to pH 7 by the addition of 0.2 m NaHCO3. Insoluble material was removed by centrifugation at 105,000 x g for 30 minutes. Saturated (NH4)2SO4 was added slowly to bring the saturation to 0.33. After standing for 20 minutes, the enzyme was then precipitated at pH 6.1 as described by Trayer et al. (10). This step was repeated once more. Each experimental point requires a separate muscle. Since the frogs vary greatly from one lot to another and even within each lot, considerable scatter of the data is to be expected.

Enzyme Assays—Analyses for phosphorylase a and b were carried out as previously described (7) in a 1:200 to 1:400 dilution based on the wet weight of the muscle. At these dilutions, treatment with charcoal to remove K-AMP made little difference in the relative values obtained for phosphorylase a and b.

Phosphorylase b kinase was assayed according to the method of Krebs, Graves, and Fischer (12) by measuring the appearance of phosphorylase a. Utilization of ATP in this reaction was...
determined in a coupled assay system by measuring DPNH disappearance spectrophotometrically. The ADP formed in the phosphorylase b kinase reaction was allowed to react with pyruvate kinase and phosphoenolpyruvate to form pyruvate and ATP. Pyruvate was then converted to lactate by DPNH and lactate dehydrogenase.

**Materials**—Disodium EDTA, Tris, glucose-1-P, DPNH, phosphoenolpyruvate, ATP, and 5'-AMP were products of Sigma Chemical Company. Rabbit liver glycogen was obtained from Mann Research Laboratories, Inc., and purified as described previously (3). Epinephrine hydrochloride was purchased from Purol, Davis and Company. Caffeine was a product of the Fisher Scientific Company. Crystalline rabbit muscle lactate dehydrogenase and pyruvate kinase were products of Boehringer and Sons. All other reagents were analytical grade.

**RESULTS**

**Lag Period in Rise of Phosphorylase a**—When sartorii are stimulated at a rate of 2 per second, there is a lag period of about 25 seconds before significant amounts of phosphorylase a appear (Fig. 1A). When the rate of stimulation is 6 per second, the lag period becomes shorter and the steady state level of phosphorylase a higher (Fig. 1B). After pretreatment of the muscles with amounts of epinephrine that increased the resting levels of phosphorylase a only slightly, the lag period was abolished and the steady state level of phosphorylase a increased (compare Fig. 1, A and C).

The lag period could reflect a delay in the onset of activity of the phosphorylase b kinase reaction or a delay in phosphorylase a activity. For example, phosphorylation of the two subunits of phosphorylase b might occur stepwise, leading to a less than fully active intermediate, or dimerization of the phosphorylated b chains to form fully active phosphorylase a might be delayed. Since a somewhat similar lag period has been found in vitro (12), the system in vitro was used to examine these possibilities. When phosphorylase b was incubated with phosphorylase b kinase at pH 6.8 in the presence of ATP and Mg++, there was a lag in the utilization of ATP (Fig. 2). An almost identical lag period was observed in this experiment when the appearance of phosphorylase a was measured spectrophotometrically.
is, if the delay were in the activation of phosphorylase $a$ rather than in its phosphorylation, there should have been no lag, or a much shorter one, in the utilization of ATP as compared with the appearance of phosphorylase $a$. Moreover, phosphorylase $b$ kinase preparations can be activated by a variety of procedures, including aging (12). When activated preparations were used at concentrations that gave a rate of ATP utilization similar to that in Fig. 2, the lag period was abolished. Therefore, if conditions are similar in the intact muscle, it would seem that the lag period is the time required to activate the phosphorylase $b$ kinase reaction.

Effect of External Work—The differing response to stimuli at 2 and 6 per second (Fig. 1, A and B) may be associated with the change in rate of contraction per se or with the concomitant change in the rate of work performance. To distinguish between these possibilities, muscles with varying loads were stimulated at a constant frequency. Changes in the amount of work performed per twitch had no effect on the steady state concentration of phosphorylase $a$ (Fig. 3), or on the time course of its appearance (Fig. 4). Resting levels of phosphorylase $a$ were not increased by stretching the muscles.

This also excludes the possibility that the lag period is a property of the auxiliary enzymes used in the coupled assay.
**TABLE I**

*Effect of contracture-producing agents on percentage of phosphorylase a*

The mean and standard errors of the mean are given, with the number of experiments in parentheses.

<table>
<thead>
<tr>
<th>Agent</th>
<th>Muscles</th>
<th>Phosphorylase in a form after addition of agent</th>
<th>%</th>
<th>%</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M K+</td>
<td>Sartorius</td>
<td>%</td>
<td>1.6 ± 0.7 (5)</td>
<td>11.3 ± 2.3 (8)</td>
<td>21 ± 7 (20)</td>
</tr>
<tr>
<td>0.01 M caffeine</td>
<td>Sartorius</td>
<td></td>
<td>19 ± 3 (6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 M K+</td>
<td>Rectus abdominis</td>
<td>%</td>
<td>1.6 ± 0.2 (5)</td>
<td>8.5 ± 0.9*(8)</td>
<td>9.1 ± 3 (8)</td>
</tr>
</tbody>
</table>
| *After 0.75 minute.*

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**Fig. 7.** The effect of external K+ concentration on phosphorylase a. Sartorii of summer frogs were incubated for 10 minutes at 20° with increasing concentrations of K+ in the extracellular medium. To achieve 5 × 10^-2 M K+, 2 M KCl was added in appropriate amounts. The other concentrations were achieved by substitution of K+ for Na+ in the Ringer’s solution. The mean and the standard error of the mean are shown by vertical bars, with the number of experiments in parentheses.

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**Fig. 8.** The response of the phosphorylase system to fatigue and the effect of epinephrine on it. Two trains of single shocks were given, each at the rate of 6 per second. The duration of the first, or fatiguing stimulus, is given on the abscissa. After a 5-minute rest, a second stimulus of 20 seconds’ duration was given to test the responsiveness of the phosphorylase system. O—O, no pretreatment; O—O, pretreatment by soaking in frog Ringer-bicarbonate containing 1.1 × 10^-8 M epinephrine and 5.7 × 10^-4 M ascorbate for 30 minutes preceding the first stimulus.

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2 × 10^-4 to 5 × 10^-2 M by the addition of 2 M KCl, a contracture occurs that reaches a maximum in about 20 to 25 seconds. The muscle then slowly relaxes despite the lowered transmembrane potential. After about 4 minutes, tension can no longer be detected. Such behavior is usual for twitch muscles (14). Table I shows that the phosphorylase a content of the sartorius muscles increased within 0.5 minute and then showed a further slow increase between 0.5 and 10 minutes. This would indicate that, under the influence of K+, sustained activity of the contractile mechanism is not necessary for the maintenance of phosphorylase a. The rectus abdominis, in contrast to frog sartorius, responds to 0.05 M K+ in the extracellular fluid with sustained contraction, but despite the differing contractile response of these two muscles to K+ the changes in phosphorylase a activity followed a similar pattern (Table I).

In frog sartorius, as in many other tissues, the transmembrane potential varies inversely with the logarithm of the concentration of K+ in the surrounding media (15). When the external K+ concentration was varied between 0.035 and 0.115 M, the percentage of phosphorylase a was likewise related to the logarithm of K+ concentration in the medium (Fig. 7).

**Effect of Caffeine**—The association of an increase in phosphorylase a with the rate of stimulation and with external K+ concentration raised the possibility that events associated with a change in transmembrane potential were directly related to the formation of phosphorylase a. It thus seemed important to attempt to dissociate contraction from changes in transmembrane potential.

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2 Adrian (15) has shown that experimental results follow quite closely the Nernst relation,

\[ E = \frac{RT}{F} \ln \frac{K^+}{K^{+\circ}} \]

in which E is the potential difference, K+ and K+ are the concentrations of K+ inside and outside the fiber; R, T, and F are the gas constant, the absolute temperature, and the Faraday, respectively. Since substitution of equimolar amounts of KCl for NaCl does not change the concentration of intracellular K+ significantly (16), K+ is quite constant under the conditions of these experiments.

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![Graph showing the effect of external K+ concentration on phosphorylase a.](https://example.com/figure7.png)

![Graph showing the response of the phosphorylase system to fatigue and the effect of epinephrine on it.](https://example.com/figure8.png)
brane, which occurs in response to electrical stimulation and to
energy utilization (22-24).

The transmembrane potential of the muscles was not significantly different before or during caffeine contracture. In fact, during contracture, action potentials could still be observed in response to electrical stimulation.

Effect of Fatigue—Previous work by G. T. Cori and B. Illingworth suggested that phosphorylase a activity decreases with fatigue and may be restored with epinephrine (18). This problem has been investigated further. Sartorii were given two separate trains of stimuli. First, a fatiguing stimulus was applied, the duration of which is indicated in Fig. 8. This was followed by a rest period of 5 minutes to allow the phosphorylase a to revert to the b form (7). Then a second stimulus of 20 seconds' duration was applied to each muscle to test the responsiveness of the phosphorylase system. In Fig. 8 it can be seen that the response to the test stimulus was dependent on the duration of the preceding fatigue period. The more fatigued the muscles, the less the response to the stimulus. Prior soaking of muscles in epinephrine increased the resistance to fatigue but did not abolish its effect. The unresponsiveness of the phosphorylase system in a fatigued muscle may be part of a regulatory system that prevents complete exhaustion of glycogen (19, 20). The slower fall of phosphorylase a in the fatigued muscle treated with epinephrine could also explain one of the biological actions of epinephrine, namely, its effect in delaying muscle fatigue.

DISCUSSION

The low values of phosphorylase a in resting muscle have on kinetic evidence been interpreted to mean that phosphorylase b kinase is many times less active in resting than in stimulated muscle (7). The physiological events responsible for activation of phosphorylase b kinase appear to be closely connected with contracture. Energy turnover does not seem to be the constant of activation of phosphorylase b kinase in the living muscle have been discussed. The close relationship between rate of stimulation and the percentage of phosphorylase a, the effect of muscle contraction causes an increase in cyclic 3',5'-AMP levels. The rapid appearance of phosphorylase a in stimulated muscles would require an even more rapid formation of cyclic 3',5'-AMP.

SUMMARY

The interconversion of glycogen phosphorylase b ⇄ phosphorylase a in frog sartorii contracting isotonically at 20°C has been studied. When repetitive stimuli were applied, there was a lag period followed by the appearance of phosphorylase a, which increased in amount until a steady state was reached. Increasing the rate of stimulation shortened the lag period and resulted in higher steady state levels of phosphorylase a. Pretreatment of muscles with epinephrine, in amounts that increased the resting level of phosphorylase a only slightly, abolished the lag period completely. Changes in external work affected neither the lag period nor the steady state levels of phosphorylase a.

Lowering the transmembrane potential by increasing the concentration of K+ in the external media was associated with increases in phosphorylase a even after relaxation of the initial contracture. Caffeine (10^{-2} M), which produces contracture of the sartorius in the absence of membrane depolarization, also resulted in increases in phosphorylase a. Possible mechanisms of activation of phosphorylase b kinase in the living muscle have been discussed.

The phosphorylase system of a previously fatigued muscle failed to respond to stimulation with the usual increase in phosphorylase a. Pretreatment with epinephrine increased resistance to fatigue, but did not abolish its effect completely.

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REFERENCES