THE ROLE OF PHOSPHOCREATINE AND ADENOSINE-TRIPHOSPHATE IN MUSCULAR CONTRACTION

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The whole activity cycle of muscle can be reproduced in glycerol-extracted muscle fibers by suspending them in solutions containing adenosinetriphosphate (ATP) and certain ions, particularly Mg and Ca ions (1). Quantitatively, however, the agreement between the responses of normal and extracted muscle fibers is disappointing. Relaxation induced by ATP in extracted muscle fibers is too slow and is associated with a high rate of P liberation. Also, in the contracted state enzymatic activity is much lower in extracted than in normal muscle (2). In the search for a better reconstruction of muscular activity the action of phosphocreatine (PC) was investigated. Indeed, this substance was found to intensify the actions of ATP to a surprising degree, to increase the tension of contracted fibers, and to accelerate relaxation. The interpretation of these phenomena which will be presented here is based on the accepted view that PC does not directly furnish energy for contraction, but is important for the rapid phosphorylation of adenosinemonophosphate (AMP) and adenosinediphosphate (ADP). Beyond that, the study of the effects of PC has led to the conclusion that contraction requires only a small amount of adenine nucleotide firmly bound to the contractile elements.

Technique

The rabbit’s psoas muscle preserved in 50 per cent glycerol at about -15° was used. The preparations, which had a total cross-sectional area of about 0.2 mm², were suspended in a small chamber as described previously (1). They were divided into at least 12 strands to facilitate diffusion (2). The mechanical responses were recorded on a smoked drum by an isometric lever. Before an experiment the fibers were transferred into a solution which contained 0.16 M KCl and 2 mM MgCl₂ and which will be referred to as saline. All other solutions used contained nearly the same concentrations of KCl and MgCl₂. Solutions of PC were prepared from commercial Na salt (Sigma Chemical Co., St. Louis), those of ATP were prepared from the Na or Ba salt (Nutrition Biochemical Corporation, Cleveland, and Sigma Chemical Company, respectively). These solutions were usually buffered by 0.02 M glycylglycine of pH 6.9, but exactly the same results were obtained without buffer. PC was always used at a concentration of 20 mM per liter, approximately that in normal muscle. Most experiments were carried out at room temperature (21-24°).
RESULTS

1. PC as an Agent Promoting Contraction.—The simplest results were obtained with fibers which had been stored for 2 or more weeks because in such fibers ATP never causes relaxation (1). PC alone did not produce a contraction, but on addition of ATP or AMP in concentrations as low as $10^{-6}$ M per liter a strong contraction occurred. AMP was almost as effective as ATP, but acted more slowly.

It is significant that tension induced by AMP always rose along an S-shaped curve and only after some delay (Fig. 1). Undoubtedly this contraction is due to the conversion of AMP into ATP. A specific enzyme for the direct phosphorylation of AMP has been described by Banga (3), but another explanation, which is in better agreement with accepted views, is possible. If we assume that a trace of ATP is present, myokinase first produces ADP, which subsequently is converted into ATP by the Lohmann reaction. The second of these mechanisms can be expected to proceed autocatalytically. It is, therefore, strongly supported by the S shape of the tension curve.

In contrast to the effect of AMP, tension rose at once nearly linearly if ATP was added to a solution of PC. The rise in tension was very slow at low concentrations of ATP, but ultimately more than half of maximal tension was produced at a concentration of $10^{-6}$ M per liter, as shown by the fact that further additions of ATP raised tension only moderately.

The contraction at low concentrations of ATP merits special consideration. It can be assumed that the energy for contraction under these conditions is furnished almost entirely by PC, because, at the rate of P liberation observed in contractile muscle fibers (2), the total amount of ATP in the solution would be used up within 1 second, if energy were due to the breakdown of ATP. The simplest explanation for the slow rise in tension seems to be the assumption that the liberation of energy from PC depends on the presence of bound nucleotide which is slowly absorbed from the solution.
This assumption is supported by several observations. (1) Most or all of the ATP disappears from the solution during contraction. A solution in which muscle fibers had been allowed to develop a full contraction, when applied a second time to the same preparation, or to a new preparation, produced only a slight contraction or none at all, whereas fresh solutions were effective. (2) If fibers were brought into a PC solution free of ATP, after contraction had partially developed, tension did not rise any further, but was maintained near the highest level already reached. Tension dropped when the PC solution was replaced by saline, indicating that PC supplied the energy for maintaining tension in the absence of free ATP. (3) If tension was suddenly lowered by allowing the contracted muscle fibers to shorten, tension recovered much faster than it had developed at the beginning of the experiment, although much less ATP was present at that time. This indicates that the fibers can release more energy than they could at the beginning of the experiment, presumably because of the presence of absorbed nucleotide.

Assuming complete absorption of nucleotide, it is possible to estimate the amount of nucleotide necessary for a maximal contraction. In preliminary experiments values of about 2 to 4.10^-7 M per gm. muscle were found.

It may be mentioned in passing that the procedure described is suitable for the assay of ATP or AMP in very low concentrations.

If muscle fibers were brought into an approximately optimal concentration of ATP (about 8 mM) and a maximal tension had developed, the addition of PC caused a further rise in tension (Fig. 2). Still more remarkable is the finding...
that PC produced a strong contraction even after ATP had been washed out by saline. In the experiment illustrated in Fig. 3 A ATP first was allowed to produce a constant state of contraction after which ATP was washed out for 1 minute by filling the chamber with saline and changing it twice. Subsequently 20 mM PC caused tension to rise much higher than it did in the solution of ATP. The contraction produced under these conditions is the weaker the longer the period of washing. After washing for about 5 minutes PC no longer gives a response.

For the interpretation of this effect it is important to realize that diffusion equilibrium is established rapidly in the preparations used. This is shown by the fact that the responses caused by ATP alone begin at maximal rate as soon as the solutions are introduced and often are completed in much less than 1

minute (reference 1, see Fig. 5). This question was tested also by applying solutions of PC which had produced a strong response to fresh preparations. If the fibers were washed for 2 to 3 minutes they were found to contain less than 5.10⁻⁷ M ATP. Such solutions give only weak and very slow contractions in fresh preparations. The rapid and strong responses produced by PC after washing out ATP, therefore, indicate the presence of bound nucleotide.

There are indications that the disappearance of bound nucleotide from a contracted muscle also expresses itself mechanically. When ATP is washed out by saline, tension drops at first rather rapidly, later much more slowly (5). It is during the steep phase of relaxation that PC causes strong contractions. That this is more than coincidence is suggested by the observation that the rapid phase of relaxation is absent after contractions induced by solutions of PC containing only a minimal amount of ATP, as shown in Fig. 4. Previous work has shown that relaxation produced by washing out ATP is passive (5, 6). The initial steep part of relaxation, therefore, indicates that at first the fibers are relatively soft due to bound nucleotide and that its disappearance later makes them more rigid.
2. PC as an Agent Promoting Relaxation.—In muscle fibers which have been stored for only a few days, low concentrations of ATP (less than 5 mM) generally produce a weak sustained contraction, higher concentrations usually a brief contraction followed by rapid relaxation (1, 5). In such fibers PC increases the relaxing effect of ATP (7). In the experiment illustrated in Fig. 5 A, 4 mM ATP produced a weak, sustained contraction. When PC was added tension dropped rapidly. A faster relaxation was obtained by the combined action of ATP and PC than could be obtained by ATP of any concentration. That a true state of relaxation was produced by PC is shown by the fact that the addition of a small amount of CaCl₂ caused a strong, sustained contraction.

It is particularly significant that PC can induce and maintain the relaxed state even after ATP has been almost completely washed out from the chamber by saline. In the experiment illustrated in Fig. 6 A a contraction was first produced by a low concentration of ATP. The chamber then was filled with saline, causing a slow drop in tension. After changing this solution twice, a solution of PC produced rapid relaxation. When this solution was replaced by saline, tension rose again, indicating the return to the state of rigor. In a similar experiment illustrated in Fig. 6 B, 1.5 mM CaCl₂ was added to the saline before rigor was complete, causing a rapid and powerful contraction.

During the relaxed state produced by PC the fibers have the properties which have previously been described for fibers in which relaxation has been produced by high concentrations of ATP. The high extensibility of these fibers is particularly striking. These properties evidently depend on the presence of ATP firmly combined with the contractile elements (1).

Because in the experiments just described the muscle fibers were washed in saline for periods of less than 2 minutes, small amounts of ATP remained in the muscle chamber. Whether this ATP was essential for relaxation was tested by renewing the PC solutions thereby further reducing the concentration of ATP.
It was found that after ATP had been virtually eliminated a contraction slowly developed, which could be prevented by adding small amounts of ATP. This may possibly mean that bound nucleotide is slowly inactivated and must be replaced from the outside to prevent rigor.

Quantitative variations which were occasionally observed in the experiments on relaxation should be mentioned. Relaxation produced by some ATP solutions was incomplete and was followed by a slow contraction. Impurities in the ATP used probably were responsible for these variations (2). PC brought about a nearly complete and lasting state of relaxation also in these experiments.

FIG. 6. Sustained relaxation produced by PC. A, after contraction was produced by ATP (first signal), fibers were brought into saline which was changed twice. After 1.6 minutes (second signal) 20 mM PC produced rapid relaxation. 23 minutes later (third signal) muscle was brought into saline (fourth signal), causing rigor. The addition of CaCl₂ (last signal) had no effect. B, a brief contraction was produced by a solution containing 5 mM ATP and 20 mM PC (first signal). This solution was replaced by saline (second signal) which was changed twice. 30 seconds later the fibers were brought into 20 mM PC (third signal). After fibers had remained relaxed for 37 minutes, they were brought into saline (fourth signal). After rigor had begun to develop, the addition of CaCl₂ (1.5 mM per liter) produced a strong contraction (last signal). 24°. Time, 10 minutes. Ordinate, tension in kilos per cm².

Completely unexplained is the observation that in many experiments in which relaxation was maintained by ATP or PC, particularly those in which the onset of relaxation was slow, stirring the solution caused a weak contraction, which usually was followed by relaxation when stirring ceased. A similar observation was made with fibers which were extracted in glycerol for only 1 or 2 days and which gave a prolonged contraction after being transferred from glycerol into saline. Stirring, or changing solution, strongly increased contraction, long after the glycerol had been washed out. These effects can be explained by assuming that an inhibitor is liberated in the fibers. However, the substances which are likely to be produced under these conditions, like phosphate, ADP, AMP, H ions have not been found to have a relaxing effect. The factor involved may be the same as that found by Marsh (8) in muscle homogenates, removal of which caused syneresis and increased ATPase activity.

DISCUSSION

By including PC, a system has been found which seems to reproduce normal muscular contraction more closely than previous systems. Particularly sur-
prising is the finding that striking responses, contraction and relaxation, can be obtained at very low concentrations of nucleotide and that a strong tension can be maintained in the virtual absence of nucleotide. The simplest explanation for these results is the assumption that the activity of muscle requires the presence of only a small amount of nucleotide which is firmly bound and which makes possible the utilization of the energy of PC. Even after dephosphorylation, nucleotide remains combined with the contractile elements as indicated by the fact that PC produces a sustained response several minutes after ATP has been washed out from a contracted muscle. These observations suggest that PC is the normal substrate for the enzymatic activity of the contractile elements and that adenine nucleotide may be considered as a prosthetic group which serves as an energy transfer mechanism.

It has recently been suggested that muscular contraction and ATPase activity are due to phosphorylation and dephosphorylation of nucleotide bound by actin (9-11). If this nucleotide is present in extracted muscle fibers, it is not identical with that which we have assumed to be responsible for energy transfer during contraction, because PC does not cause contraction. However, according to Feuer and Wollemann (12), the nucleotide bound by actin is released when actomyosin is formed and, therefore, may not be present in extracted muscle fibers. These authors (13), moreover, have found that creatine phosphopherase is closely linked with actin and is essential for its polymerization. Therefore, it is possible that actin is responsible for the absorption of nucleotide by the contractile elements in solutions of ATP. Suggestive evidence for this hypothesis is the fact that the amount of nucleotide which is bound by the actin contained in 1 gm. muscle (5.10^-4 M) (10, 11) is of the same order of magnitude as the amount which seems to be absorbed by extracted muscle fibers.

How ATP maintains a contracted state in the absence of PC is uncertain. Because of its firm combination with protein the nucleotide does not seem to be released at once after dephosphorylation. Probably myokinase first converts ADP into ATP and AMP. The latter is inactive and either splits off or is further broken down and finally replaced by ATP. This mechanism is more complex and slower than transphosphorylation by PC. Therefore, it is not difficult to understand why PC increases the strength of contraction and speeds up relaxation. These considerations illustrate that the contractile elements, like other cellular structures, contain a system of enzymes which are firmly attached to each other and act as a unit. The normal response of muscle depends on the coordinated participation of these enzymatic mechanisms and requires the presence of PC.

The observation that PC can induce rapid relaxation is important for the question of the mechanism of muscular relaxation. From previous work it has been concluded that relaxation depends on the reconstitution of an ATP-actomyosin complex (1, 14). The results reported here suggest that this is
accomplished by transphosphorylation of bound nucleotide by creatine phosphorase. That breakdown of PC takes place during relaxation in intact muscle has previously been concluded by Dubuisson (15) from the observation that relaxation is associated with an increase in pH.

SUMMARY

In the presence of 20 mM PC a strong contraction is produced in glycerol-extracted muscle fibers by ATP and AMP in concentrations as low as $10^{-4}$ M per liter. At low concentrations of nucleotide tension rises very slowly. This rise is interpreted as being due to absorption of nucleotide by the contractile elements. AMP gives an S-shaped tension curve, indicating that the conversion of AMP into ATP is an autocatalytic process. Tension is maintained in a contracted muscle even in PC solutions free of ATP. PC alone produces a contraction if applied within 5 minutes after ATP has been washed out from a contracting muscle. It is concluded from these results that PC is the substrate for the enzymatic activity of the contractile elements and that this activity depends on the presence of bound nucleotide which acts as an energy transfer mechanism.

PC accelerates relaxation which is caused by ATP under certain conditions. In the presence of PC even very low concentrations of ATP can produce relaxation. A strong contraction can be produced under these conditions by the addition of Ca ions. These observations support the conclusion that relaxation depends on the rephosphorylation of nucleotide bound by the contractile elements.

REFERENCES