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THE EFFECT OF Ca^{2+} AND FIBRE ELONGATION ON THE ACTIVATION OF THE CONTRACTILE MECHANISM OF INSECT FIBRILLAR FLIGHT MUSCLE

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SUMMARY

1. The relationship between fibre length and the Ca^{2+}-activated ATPase (ATP phosphohydrolase, EC 3.6.1.3) of insect fibrillar muscle has been studied.

2. Low levels of Ca^{2+} and small degrees of fibre extension have been found to increase the ATPase activity. Either factor influences the sensitivity of the material to the other factor.

3. In insect fibrillar muscle, where the A filaments are continuous with the Z line, it is possible to strain the A filaments directly in the absence of permanent actin–myosin interactions.

4. The higher ATPase activity as a result of fibre elongation is accompanied by an increase in Ca^{2+} binding.

5. Small maintained length changes do not affect the rate of ATP breakdown catalyzed by glycerinated fibres from frog sartorius or rabbit psoas muscle.

6. The relevance of the findings to the mechanism of oscillatory contraction of insect fibrillar muscle is discussed.

INTRODUCTION

In glycerinated insect fibrillar muscle length changes under activating conditions are followed by delayed changes of tension, which allow the muscle to sustain oscillatory contractions when connected to a mechanically resonant system[1]. This relationship between length and tension, which at some frequencies amounts to a negative viscosity, is affected by Ca^{2+} concentrations and the degree of elongation of the fibres. High levels of Ca^{2+} or stretch resulted in a greater negative viscous modulus[2], and have further been found to increase the activity of the ATPase (ATP phosphohydrolase, EC 3.6.1.3) in glycerinated fibres of insect flight muscle[3,4]. A more detailed investigation of the change in ATPase activity with varying degrees of stretch and Ca^{2+} levels has now been carried out using detergent-treated fibres from the same material. These fibres offer the important advantage that they are essentially free of

Abbreviation: EGTA, ethylene glycol-bis(β-aminoethyl ether)diaminetetraacetate.

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all non-myofibrillar enzyme activities and that diffusion into the centre of the fibres is greatly facilitated[5]. The present study indicates that actomyosin ATPase activity is a function of fibre length and Ca^{2+} concentration and that ATPase activity and Ca^{2+}-binding show a similar relationship with respect to fibre elongation. The results are discussed in relation to the contractile mechanism of insect fibrillar muscle.

MATERIAL AND METHODS

Glycerination procedure

Dorsal longitudinal muscles of the giant water bugs *Lethocerus cordofanus* and *Hydrocyrius columbiae*, the beetle *Amphimallon solstitialis* and the bumble bee, *Bombus lucorum* have been glycerinated *in situ*, using the schedule given by HUXLEY[6] and stored at −18° for 2–3 months. Experimental material was prepared by treating small bundles of about 20 fibres for 18 h at 2° with the detergent Tween 80 (1 % Tween 80 in 45 mM potassium phosphate buffer (pH 7.5), containing 5 mM $MgCl_2$). This treatment removes the bulk of the non-myofibrillar enzyme activities without affecting the mechanical properties of the fibres[5]. The glycerination procedure of HUXLEY was also used for rabbit psoas and frog sartorius muscle. However, no detergent treatment could be successfully applied in the case of vertebrate skeletal muscles as the large amount of connective tissue present prevents the fibres from floating apart, which is a prerequisite for the Tween 80 effect[5].

Measurements of ATPase activity

Bundles of 10–20 fibres were attached between two glass rods and glued to the glass with cellulose nitrate dissolved in acetone. The position of one of the rods was fixed and the other was connected to an RCA 5734 tension transducer mounted on a micromanipulator movement. A free fibre length of 4–20 mm was used. The initial length of the fibres is the most difficult parameter to set and involves a possible initial error of ± 0.25 mm. The fibres were immersed in the respective solutions by raising around them a bath machined into a perspex block containing 0.5–1.0 ml of the required medium. A continuous channel surrounds the baths with a wall 1/32 inch thick and water at a thermostatically controlled temperature of 23° was pumped through the channel. In the experiments where the effects of quick stretch on the fibre ATPase were investigated the dimensions of the baths were appropriately modified to hold only 0.2 ml solution and the experiments were carried out at room temperature. The muscle fibres were immersed first in rigor solution (see below) for 2 min in order to wash out the glycerol and then transferred to the relaxing solution. The length at which the tension in the relaxing solution is zero (defined as rest length) cannot be determined to better than ± 0.1 % of the total length; this affects the value of applied strain, since for 2 % final extension the actual fibre length may vary between 1.9 and 2.1 % elongation. The mean fibre extension was set by finding the slack length in the relaxing solution and stretching the required amount by slowly moving the force transducer. For fibre extension between 0.5 and 2.5 % the fibres were returned to the relaxing solution between each set of measurements and the fibre length was reduced to the length at which the tension was zero before restretching. In general, the tension was allowed to equilibrate for about 3–4 min at any given fibre length before starting the measurements of ATPase activity in the

respective activating solution. For the determination of the rate of ATP splitting at maintained stretch the fibre bundles were incubated for periods of 10–20 min in the activating solution. In the case of vertebrate skeletal muscle it was found necessary to cycle the fibres several times between the relaxing medium and a Ca^{2+}-containing solution after removal of the glycerol in order to get the fibres to relax (*cf.* ref. 17).

The ATPase activity of fibre bundles was measured by following the release of inorganic phosphate, employing a small-volume adaptation of the MARSH method[7]. The absorption at 310 mμ was read in a Unicam SP 800 ultraviolet spectrophotometer using spectrosil microcells (40-mm pathlength). The *n*-butanol (Analar, BDH) was redistilled twice to reduce the background absorption. The accuracy of this method is $\pm$ 1 mμmole inorganic phosphate.

Ca^{2+} binding with ^{45}Ca

Fibre bundles which had been stretched by the required amount in the relaxing solution were immediately transferred to an activating solution containing $3.55 \cdot 10^{-8}$ M Ca^{2+}, and allowed to equilibrate for 5 min in the solution. Stabilized ^{45}Ca concentrations were obtained by adding ^{45}Ca–EGTA, prepared by combining $^{45}CaCl_2$ (Radiochemical Centre, Amersham, 20 mC/g Ca^{2+}) and EGTA in equivalent concentrations, followed by neutralization with KOH. At the end of the experiment the fibres were washed for 15 sec in a solution containing 1 mM ATP and 1 mM $MgCl_2$, 65 mM KCl, 2 mM EGTA and 20 mM Tris–HCl buffer (pH 7.2). This treatment effectively removes any unbound label from the muscle fibres which may be as much as 2 pmoles/cm fibre. The amount of bound ^{45}Ca was determined as described by SEIDEL AND GERGELY[8]. Special care was taken to reduce the contaminating unlabelled Ca^{2+} present in the reagents used in the ^{45}Ca-binding studies. Solid KCl was washed with methanol–EDTA followed by methanol which effectively removes Ca^{2+} contamination[9]. ATP was treated with Dowex 50 as reported previously[8].

Determination of fibre protein content

The Kjeldahl method, as adapted by KUCH and co-workers[10], was used for the ultramicrodetermination of N_2. As a conversion factor 16.2 % N_2 in the muscle proteins was assumed.

Solutions

The rigor solution contained 70 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, and 20 mM Tris–HCl buffer (pH 7.2). The relaxing medium contained 5 mM $MgCl_2$, 5 mM ATP, 2 mM EGTA, 65 mM KCl and 20 mM Tris–HCl buffer (pH 7.2). The composition of the activating solution was the same except that Ca^{2+}–EGTA was added to obtain a stabilized Ca^{2+} concentration as described by PORTZEHL, CALDWELL AND RUEGG[11]. The relaxing and activating solutions contained additionally as an ATP-regenerating system 1 mM phosphoenolpyruvate and 20 μg pyruvate kinase (ATP:pyruvate phosphotransferase, E.C. 2.7.1.40) as well as small quantities of oligomycin (150 μg/ml) to inhibit any non-myofibrillar ATPase remaining after detergent treatment. Oligogomycin does not affect the actomyosin ATPase[5].

Chemicals

The inorganic reagents were of analytical grade. EGTA was obtained from

the General Chemical Co., Sudbury, Middlesex. ATP (sodium salt), phosphoenolpyruvate (sodium salt) and pyruvate kinase (crystalline) were purchased from Sigma Chemical Ltd., London. The detergent Tween 80 (polyoxyethylene sorbitane monooleate) was supplied by Light and Co., Ltd., Colnbrook, Bucks. Oligomycin (Streptomyces diastata chromogenes) was obtained from Mann Research Laboratories, New York.

RESULTS

Effect of Ca^{2+} and stretch on the ATPase activity of muscle fibres

Glycerinated insect fibrillar muscle fibres, which have been treated with the detergent Tween 80 to remove the bulk of the non-myofibrillar cell constituents and to facilitate diffusion in and out the core of the fibres offer a good opportunity to study the effect of Ca^{2+} on ATP hydrolysis. The use of a Ca^{2+} buffer excludes any variation of the Ca^{2+} concentration inside the fibres due to non-specific binding by other non-myofibrillar components.

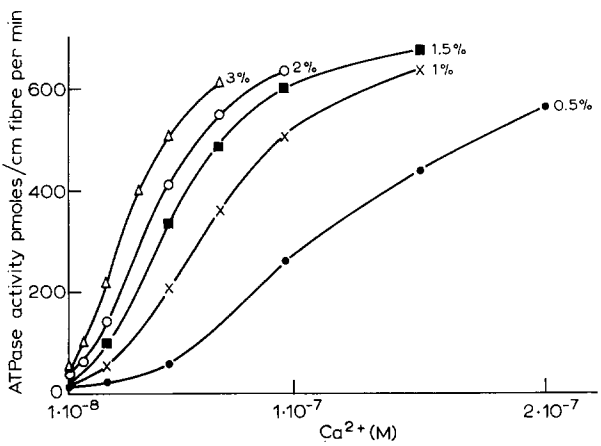

Fig. 1. Effect of varying Ca^{2+} concentrations on the ATPase activity at different fibre extensions. The experimental procedure has been described under MATERIAL AND METHODS. A bundle of 16 muscle fibres (*L. cordofanus*, free fibre length 6 mm) was used. The fibres were initially stretched in the relaxing solution by the required amount given in the figure and then transferred at this fibre length through a series of baths containing activating solutions with increasing levels of Ca^{2+}.

The rate of ATP hydrolysis in insect fibrillar muscle fibres has been found to depend both on Ca^{2+} concentration and the degree of fibre elongation (Figs. 1 and 2). There appears to exist a balanced equilibrium effect of both these parameters on the ATPase activity, as the ATPase activity is the same, whether the experiments are carried out at a fixed fibre length varying the Ca^{2+} level (Fig. 1) or by increasing the fibre length at a given Ca^{2+} level. The sensitivity to Ca^{2+} increases markedly with increasing amounts of fibre elongation; similarly less strain has to be applied to obtain the same degree of ATPase activation at higher Ca^{2+} concentrations.

Reversibility

The effects of stretch and Ca^{2+} on the ATPase activity and tension proved to be fully reversible up to at least 2.5 % stretch in that the fibres reverted to their

original condition at zero length. However, the values of ATPase activity and tension found on returning the fibres to lower degrees of stretch after an initial extension of 2.5 % were lower than those obtained previously for the same amount of stretch on extending the fibres (Fig. 3). The general observation is that the differences in ATPase activity and tension development between the extension and release curve are more marked the greater the maximum fibre extension applied.

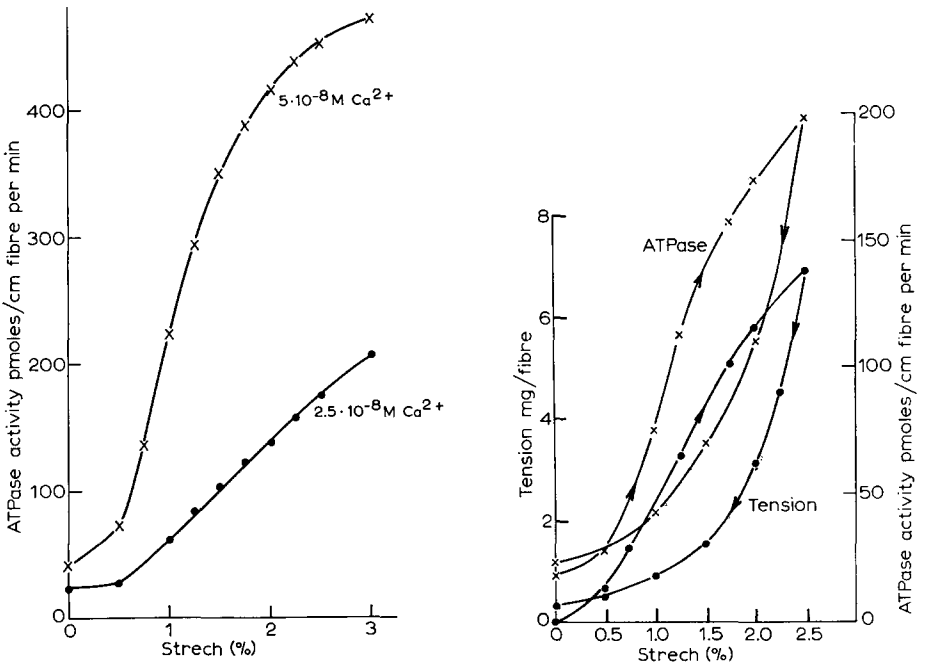

Fig. 2. Stretch-activation of ATPase activity at different levels of Ca^{2+}. The conditions were the same as given in Fig. 1. The fibre length was again set up in the relaxing solution and the ATPase at this fibre length was then determined after transferring the fibres to the activating solution; the Ca^{2+} concentrations are given in the figure.

Fig. 3. Changes in tension and ATPase as a result of extension and reduction of fibre length. The fibres (18 fibres, *L. cordofanus*, free fibre length 7 mm) were gradually extended in relaxing solution and the ATPase activity and tension development were measured in an activating solution, containing $3 \cdot 10^{-8}$ M Ca^{2+}, at the amounts of fibre elongation indicated in the figure. After extending the fibres by 2.5 %, the strain was reduced again in 0.5 % steps. No ATP-regenerating system was present in this experiment.

The relationship between fibre length and Ca^{2+} binding

The effect of fibre length on the ATPase activity in the presence of Ca^{2+} suggests that stretch may result in an increase in the number of Ca^{2+}-binding sites available. Experiments designed to test for this possibility have shown that the amount of Ca^{2+} bound increases with the degree of fibre extension (Fig. 4). Thus, both the Ca^{2+}-activated ATPase activity and the binding of the activating ligand, Ca^{2+}, show the same relationship with respect to fibre elongation. Although not shown in the figure, the bound Ca^{2+} was released again on returning the fibres to zero length. The ^{45}Ca bound to the fibres at any given length completely exchanged with unlabelled Ca^{2+} over a period of 2–3 min.

Comparative aspects of the effects of stretch on ATPase activity

The effect of maintained stretch has been investigated using glycerinated fibres from the bug *H. columbiae*, the beetle and the bumble bee. As these fibres differ in their diameter and myofibrillar content from those of *L. cordofanus* the results are

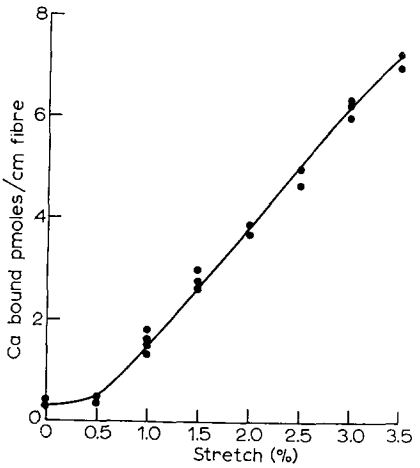

Fig. 4. Ca^{2+} binding as a function of stretch. Bundles of 18 fibres (*L. cordofanus*, free fibre length 6 mm) were used. Each point in the figure represents one particular experiment. The conditions which were kept constant in all experiments were those described in the text.

expressed in terms of protein content. Under comparable conditions, $5 \cdot 10^{-8}$ M Ca^{2+} and 1 % fibre extension, setting the value for ATPase activity of *L. cordofanus* flight muscle as 100, the rates of ATP breakdown obtained for other insect fibrillar muscles were: *H. columbiae* 150, beetle 250–300 and bumble bee 500–600. No effect of maintained stretch could be observed with glycerinated preparations from rabbit psoas or frog sartorius muscle. However, if quick stretch experiments were carried out in activating solution $1.6 \cdot 10^{-7}$ M Ca^{2+} using glycerinated frog sartorius fibres, the level of ATPase activity after 20 sec (which corresponds approximately to the stress-relaxation time of these fibres under the experimental conditions used) was 20–25 % higher following a rapid 1 % length increase than the steady-state value of ATP splitting detectable before or 60 sec after applying the rapid length change. The negative results obtained so far with glycerinated rabbit psoas fibres on applying quick stretches may be due to experimental limitations with this material resulting from a shorter stress-relaxation time of only 10 sec which may make any ATPase increase occurring within this short time interval undetectable with the present techniques used. If rapid 1 % length changes are applied to *L. cordofanus* muscle fibres the ATP-splitting activity of these fibres after 25 sec is about 100 % higher than the rate measured after 2–3 min. However, the total value of ATPase activity remains high even several hours after stretching insect muscle fibres; in insect fibrillar muscle the length and tension changes do not decline steadily to the starting level, as is the case for vertebrate skeletal muscle. A full account of the rapid changes of ATPase and tension observed with insect fibrillar muscle will be presented elsewhere.

DISCUSSION

The results obtained with glycerinated insect flight muscle fibres show that the ATPase activation by Ca^{2+} is similar to that found in actomyosin systems[4]. The activating effect of Ca^{2+} in muscle fibres is additionally influenced by the mechanical state of the fibres; the ATPase activity increases with fibre length. The results suggest that the stretch-activation of ATP hydrolysis may be due to the increased Ca^{2+} binding observed under these conditions (Fig. 4). In vertebrate skeletal muscle under conditions where the muscle fibres were stretched the ATP breakdown has been found either unchanged[12] or greatly reduced[13]. However, the results of WARD, EDWARDS AND BENSON[13] may not be directly comparable as their experimental approach involved the extension of fibres prior to the glycerination procedure and a rigor contraction may have occurred during the extraction. In insect fibrillar muscle there is good evidence for continuity between the A filaments and the Z line[14,15]. The existence of "connecting filaments" offers the opportunity to stress the myosin filaments directly in a Ca^{2+}-free solution, under conditions where no permanent interactions between the actin and myosin filaments are thought to occur[16]. As under the experimental conditions used only the myosin filaments will be strained (assuming only a weak interaction between the A and I filaments), and as Ca^{2+} bound to F-actin is non-exchangeable[18], the Ca^{2+} uptake as a result of stretch would reflect an increase in the number of binding sites on the myosin. The results therefore suggest that the A filaments constitute the length-sensitive transducer element.

The apparent absence of any continuity between the A filaments and the Z line in vertebrate muscle may be responsible for the failure to observe maintained ATPase activation with vertebrate skeletal muscle as a result of small length increases. The observed transient increase in ATPase activity on applying a quick stretch to glycerinated frog sartorius muscle is not inconsistent with this interpretation as strain can be transferred from the I filaments to A filaments through the cross-bridges. However, as under activating conditions cross-bridges are considered to be attaching and detaching continuously[19], tension exerted through the bridges will not lead to maintained length changes within the A filaments. Stress applied to the A filaments through the cross-bridges will therefore only result in transient increases in strain and hence ATPase activity.

Glycerinated insect fibrillar muscle fibres have been shown to carry out oscillatory contractions for prolonged periods in the presence of a Ca^{2+} buffer[1,2,5]. Thus, fluctuations in the level of Ca^{2+} cannot be involved in the actual mechanism of oscillatory contraction, although Ca^{2+} is necessary to activate the system. In contrast, the small sinusoidal length changes, imposed *in situ* by the mechanically resonant system of the wings and pterothorax, are an important part of the oscillatory mechanism[20]. As has previously been shown[21], ADP promotes tension development of glycerinated insect flight muscle fibres. The length changes during the extension phase of the oscillatory cycle, which result in increased ATPase activity would in this way be followed by delayed tension changes.

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