

Some Properties of Glycerinated Skeletal Muscle Fibres Containing Phosphorylated Myosin

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Abstract. The structural changes of phalloidin-rhodamin labelled F-actin at relaxed and contracted skeletal muscle fibre containing phosphorylated myosin and at contracted state after dephosphorylation were investigated by measuring of polarized fluorescence of the fluorophore. The mechanical properties (isometric tension development) of fibre were studied in parallel. At submaximal concentration of Ca ions (0.6 $\mu\text{mol/l}$) the isometric tension was decreased after dephosphorylation of fibre myosin. The changes in polarization of fluorophore bound to actin filament were correlated with isometric tension developed by the muscle fibre. The angles between the actin filament long axis and the absorption and emission dipoles for contracted and relaxed fibre were different, suggesting changes in the organization of the actin monomers in thin filament, dependent on the physiological state of the fibre. The flexibility of the thin filaments during transition of the fibre from relaxed to "contracted" state increases as indicated by greater average angle between the F-actin long axis and the fibre axis.

Key words. Phosphorylated myosin — Glycerinated fibres — Fluorescence polarization

Introduction

It is widely accepted that the regulation of skeletal muscle contraction is based on calcium dependent conformational changes of the tropomyosin-troponin complex involving interaction of actin with myosin cross-bridges. However, an increase of the concentration of free calcium ions in muscle cell activates the Ca^{2+} and calmodulin dependent myosin light chain kinase (Pires and Perry 1977). Moreover, with increased concentration of Ca ions in muscle cell, magnesium ions bound with myosin are likely to be replaced by calcium ions (Morimoto and Harrington 1974; Bremel and Weber 1975). Although several findings showed, that myosin phosphorylation (Morgan et al. 1976; Butler et al.

1983) and replacement of magnesium ions bound to myosin light chains by calcium ions (Bagshaw and Reed 1977) cannot play any essential role in the regulation of contraction, both myosin phosphorylation and exchange of bound divalent cations modulate the interaction of F-actin with myosin heads (Perrick 1980; Michnicka et al. 1982; Stępkowski et al. 1985a, b). More recent, studies of fluorescence polarization of intrinsic and extrinsic fluorophores bound with F-actin in ghost fibres, showed that the degree of polarization of fluorophore in the acto-heavy meromyosin complex depends both on the kind of divalent ions bound with myosin heads and on whether the myosin regulatory light chains are phosphorylated or dephosphorylated (Borovikov et al. 1987; Kąkol et al. 1987; Szcześna et al. 1987a).

The aim of the present paper was to compare the fluorescence polarization of phalloidin-rhodamin specifically (Dancker et al. 1975) bound to F-actin in glycerinated fibre containing phosphorylated myosin and in the same fibre after myosin dephosphorylation. Changes of fluorescence polarization were correlated with physiological state (relaxation and isometric contraction) of the fibres. Isometric tension developed by glycerinated skeletal muscle fibre containing phosphorylated myosin and after myosin dephosphorylation was compared at low (0.6 $\mu\text{mol/l}$) and high (10 $\mu\text{mol/l}$) concentration of calcium ions.

Materials and Methods

Preparation of glycerinated psoas muscle fibres. Immediately after dissection rabbit psoas muscles were cooled at 0° C for 20–30 min in a solution containing 100 mmol/l KCl, 1 mmol/l MgCl_2 , 67 mmol/l phosphate buffer, pH 7.0. Then bundles of fibres from the psoas muscle were isolated at 0° C and tied to the ends of glass sticks. The fibre bundles were then incubated in a solution containing 50% glycerol, 5 mmol/l ATP, 0.1 mmol/l CaCl_2 , 100 mmol/l KCl, 12.5 mmol/l MgCl_2 , 67 mmol/l phosphate buffer, pH 7.0, and 0.5% Triton X-100 to allow endogenous myosin light chain kinase to phosphorylate myosin regulatory light chains. The incubation time was 48 h at 0° C with two replacements of the incubation solution. For details concerning preparation of single glycerinated fibres containing myosin with phosphorylated or dephosphorylated light chains see a previous report (Lebedeva et al. 1987). Dephosphorylation of myosin light chains in glycerinated psoas fibres was performed by 30 min incubation at 20° C with "crude" phosphatase (protein concentration 4 g/l), obtained by the method of Morgan et al. (1976), in a solution containing 1 mmol/l EGTA, 10 mmol/l magnesium acetate and 50 mmol/l Tris-HCl buffer, pH 7.6. The dephosphorylated fibres were occasionally phosphorylated to check the reversibility of the effects of dephosphorylation. To do the check-up tests, the fibres were incubated 15 min at 20° C with myosin light chain kinase (0.3 mg/ml) in a solution containing 42 nmol/l calmodulin, 50 mmol/l KCl, 10 mmol/l MgCl_2 , 0.1 mmol/l CaCl_2 , 5 mmol/l ATP, 1 mmol/l DTT and 20 mmol/l phosphate buffer, pH 8.0. Myosin light chain kinase was prepared from rabbit skeletal muscles by the method of Nunnally et al. (1985).

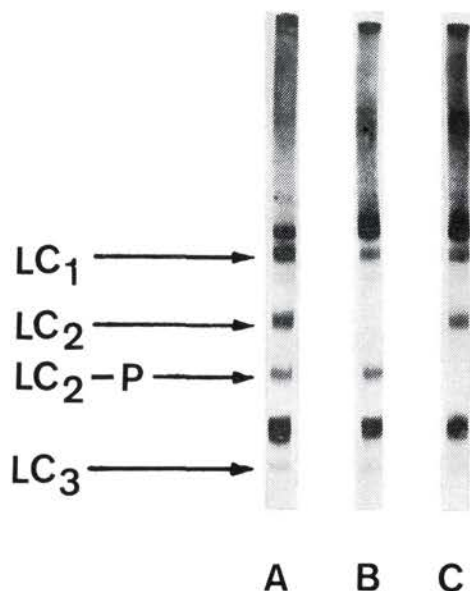


Fig. 1. Electrophoretic patterns of glycerinated muscle fibres: (A) — before incubation of the muscle bundle for myosin phosphorylation, (B) — after incubation for myosin phosphorylation, (C) — phosphorylated fibres after incubation with “crude” phosphatase. LC₁, LC₂, LC₃ — myosin light chains; P-LC₂ — phosphorylated myosin regulatory light chain.

Electrophoretic control of phosphorylation of fibre myosin light chains. Electrophoresis was performed according to Perrie and Perry (1970) in 4% polyacrylamide gel containing 8 mol/l. The amount of phosphorylated and dephosphorylated myosin light chains in glycerinated fibres was checked electrophoretically after dissection of a single fibre from the bundle. The fibres were incubated with “crude” phosphatase to obtain fibres containing dephosphorylated myosin light chains. To obtain myosin with phosphorylated light chains, fibre was incubated with myosin light chain kinase.

Labelling of F-actin in the fibres with phalloidin-rhodamin. Prior to their use for experiments muscle fibres were labelled by specific binding of phalloidin-rhodamin to F-actin (Dancker et al. 1975). The fibres were incubated in a solution containing 100 mmol/l KCl, 1 mmol/l CaCl₂, 6.7 mmol/l phosphate buffer pH 7.0 and 4 μmol/l phalloidin-rhodamin during 20 min. Subsequently, the unbound reagent was removed (Kąkol et al. 1987).

Measurement of isometric tension development. The isometric tension development was measured as described by Borovikov and Lebedeva (1987). One end of single fibres was mounted to a tensiometer and the other end to a lever with a distance between the attachment points of approx. 1.5 mm. The fibre was bathed in a solution containing 50 mmol/l KCl, 10 mmol/l Tris-Acetate buffer pH 7.0, 5 mmol/l MgCl₂, 4 mmol/l ATP, 5 mmol/l EGTA and a proper aliquot of 100 mmol/l stock solution of CaCl₂ calculated as described by Persechini et al. (1985) to obtain nominal free Ca²⁺ concentration of 0.6 or 10 μmol/l.

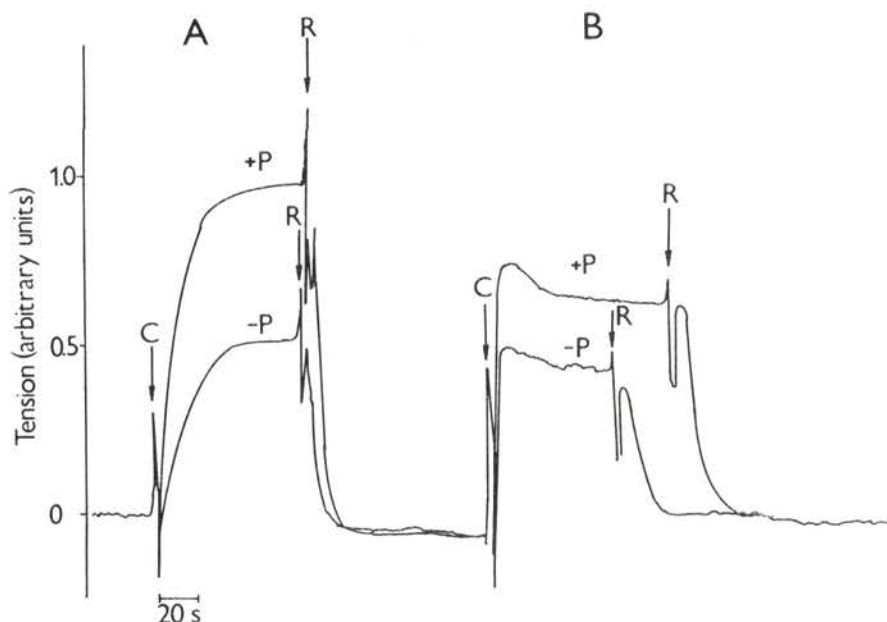


Fig. 2. The effect of dephosphorylation of phosphorylated myosin in muscle fibre on the isometric tension development by glycerinated rabbit psoas muscle fibre at $0.6 \mu\text{mol/l CaCl}_2$ and $10 \mu\text{mol/l CaCl}_2$. A typical example: A — isometric tension development at $0.6 \mu\text{mol/l free Ca}^{2+}$. B — isometric tension development at $10 \mu\text{mol/l free Ca}^{2+}$.

Measurements of polarized fluorescence of phalloidin-rhodamin bound to F-actin in glycerinated muscle fibre. The fluorescence of phalloidin-rhodamin bound to F-actin in glycerinated muscle fibre was excited at $478 \pm 5 \text{ nm}$ and recorded at $550 - 650 \text{ nm}$ using a microfluorimeter. Four intensities of polarized fluorescence were measured in parallel (I_{\parallel} , I_{\perp}) and perpendicular (${}_{\perp}I_{\perp}$, ${}_{\perp}I_{\parallel}$) direction of the fibre axis to polarization plane of the exciting light. "I" denotes intensities of four components of polarized fluorescence. The direction of the polarization planes of the exciting and the emitted light relative to the fibre axis are indicated on the left and right side respectively. The degree of fluorescence polarization P_{\parallel} and P_{\perp} was determined from the equations:

$$P_{\parallel} = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$$

$$P_{\perp} = ({}_{\perp}I_{\perp} - {}_{\perp}I_{\parallel}) / ({}_{\perp}I_{\perp} + {}_{\perp}I_{\parallel})$$

The angle of absorption (ϕ_A) and emission (ϕ_E) dipoles of the fluorophore relative to the F-actin axis and $\sin^2\theta$ (where θ is the angle between the long axis of F-actin filament and the fibre axis) were obtained by the least squares method from experimental ratios I_{\parallel}/I_{\perp} , ${}_{\perp}I_{\perp}/I_{\parallel}$.

Experimental data were analysed with mathematical models as described by Traeger and Mendelson (1975), Yanagida and Oosawa (1978) and Kałkol et al. (1987).

Results and Discussion

A fibre sample processed in parallel was used for electrophoresis to check the

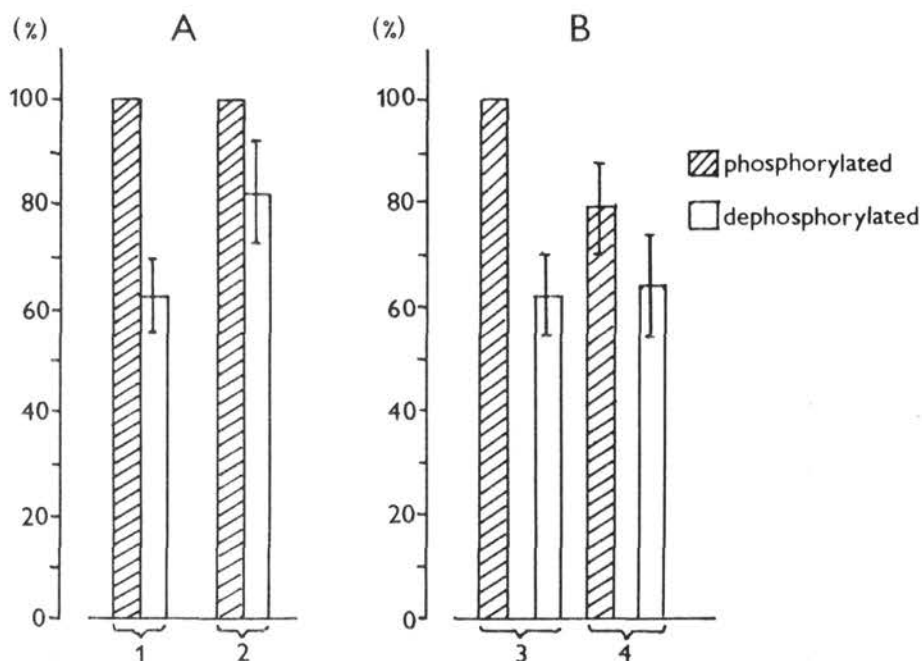


Fig. 3. The effect of dephosphorylation of phosphorylated myosin in muscle fibre on the isometric tension development by glycerinated rabbit psoas muscle fibre at $0.6 \mu\text{mol/l}$ CaCl_2 and $10 \mu\text{mol/l}$ CaCl_2 . Summarized results of 14–15 experiments. *A* — Percentual values related to isometric tension developed by the fibres containing phosphorylated myosin. *B* — Percentual values related to isometric tension developed by the phosphorylated fibres at $0.6 \mu\text{mol/l}$ free Ca^{2+} . 1,3 — at $0.6 \mu\text{mol/l}$ free Ca^{2+} ; 2,4 — at $10 \mu\text{mol/l}$ free Ca^{2+} .

amount of phosphorylated regulatory light chains in the fibre dissected from bundles of the psoas muscle incubated to activate endogenous myosin light chain kinase. The amounts of dephosphorylated regulatory light chains are determined after incubation of the parallel sample, containing phosphorylated myosin with exogenous phosphatase (see Methods). Fig. 1 shows a typical electrophoretic pattern of 8 mol/l urea gel electrophoresis. It can be seen that before incubation for myosin phosphorylation the muscle bundles contain partly (Fig. 1*A*), and after incubation almost entirely (Fig. 1*B*), phosphorylated myosin regulatory light chains. The electrophoretic pattern of the fibre containing myosin with entirely phosphorylated light chains (after incubation with phosphatase) is shown on Fig. 1*C*. After incubation with phosphatase, all regulatory light chains in myosin were dephosphorylated.

Fibres with phosphorylated myosin were used for measurements of tension development in the presence of $0.6 \mu\text{mol/l}$ and $10 \mu\text{mol/l}$ CaCl_2 in conditions as

Table I. The effect of dephosphorylation on parameters of polarized fluorescence of phalloidin-rhodamin bound to F-actin in contracted glycerinated psoas muscle fibre containing phosphorylated myosin

The values of fluorescence polarization P_{\parallel} and P_{\perp} are expressed as $(I_{\parallel} - I_{\perp}) / (I_{\parallel} + I_{\perp})$ and $(I_{\perp} - I_{\parallel}) / (I_{\perp} + I_{\parallel})$ respectively, where I denotes intensities of four components of polarized fluorescence. The directions of polarization planes of the exciting and the emitted light relative to the fibre axis are indicated on the left and right side, respectively. Φ_A and Φ_E are the angles of absorption and emission dipoles respectively. The angle between the absorption and emission dipoles. $\gamma = 46^\circ$. $\sin^2\theta$ — characterizes the flexibility of thin filaments, where θ is the angle between the filament axis and the fibre axis. The data are mean \pm SE from 35–55 measurements in 14–15 series. Free Ca^{2+} concentrations are given in $\mu\text{moles/l}$.

Fibre state	Myosin form	Free Ca^{2+}	P_{\parallel}	P_{\perp}	Φ_A	Φ_E	$\sin^2\theta$
relaxed	+P	—	0.485 ± 0.002	-0.221 ± 0.005 (55)	40.1 ± 0.2	40.1 ± 0.2	0.064 ± 0.002
contracted	+P	0.6	0.494 ± 0.003	-0.153 ± 0.008 (55)	39.7 ± 0.2	38.8 ± 0.2	0.108 ± 0.003
contracted	+P	10.0	0.508 ± 0.009	-0.172 ± 0.010 (35)	41.5 ± 0.3	39.4 ± 0.2	0.107 ± 0.003
contracted	-P	0.6	0.511 ± 0.005	-0.183 ± 0.006 (55)	39.0 ± 0.2	39.0 ± 0.2	0.102 ± 0.005
contracted	-P	10.0	0.540 ± 0.006	-0.163 ± 0.005 (35)	40.1 ± 0.1	37.8 ± 0.3	0.136 ± 0.005

described by Persechini et al. (1985) but without ATP regenerating system. The ATP regenerating system was omitted to simplify conditions of the fibre treatment with phalloidin-rhodamin. The optimal ATP concentration used in relaxation and contraction solutions was chosen experimentally, and control experiments did not show any effect of higher ATP concentrations on the tension level.

The isometric tension generated at $0.6 \mu\text{mol/l Ca}^{2+}$ concentration by fibres containing phosphorylated myosin decreased significantly when regulatory light chains of myosin were dephosphorylated. This decrease was more pronounced at $0.6 \mu\text{mol/l}$ than at $10 \mu\text{mol/l Ca}^{2+}$ (Figs. 2A, 2B).

The isometric tension generated by glycerinated psoas fibres after myosin dephosphorylation was reduced in all 15 experimental series as compared to that generated by fibres with phosphorylated myosin before dephosphorylation (Fig. 3A, 3B) both at $0.6 \mu\text{mol/l}$ and $10 \mu\text{mol/l Ca}^{2+}$ the difference was more pronounced at $0.6 \mu\text{mol/l Ca}^{2+}$. Our findings support the report of Persechini et al. (1985) suggesting an increase of isometric tension at submaximal Ca^{2+} concentrations by myosin phosphorylation. However, no increase of tension was observed upon increasing Ca^{2+} concentration from $0.6 \mu\text{mol/l}$ to $10 \mu\text{mol/l}$; fibres containing phosphorylated myosin, even showed a significant decrease of tension at $10 \mu\text{mol/l Ca}^{2+}$ (Fig. 3B). This behavior is probably due to the fact that phosphorylation is a more sensitive modulator of tension development at low than at high calcium concentrations. It seems also that fibres with phosphorylated myosin are more sensitive to changes in calcium concentration. In parallel experiments the changes of polarized fluorescence of phalloidin-rhodamin bound to F-actin in glycerinated muscle fibres were investigated. Table 1 summarizes the results obtained for fibres containing phosphorylated myosin in relaxed and contracted state, and for the same fibres after dephosphorylation in contraction at $0.6 \mu\text{mol/l Ca}^{2+}$ and $10 \mu\text{mol/l Ca}^{2+}$. Upon the transition of glycerinated fibre from relaxed to contracted state significant changes of the parameters of fluorescence polarization of phalloidin-rhodamin attached to actin were observed. The changes of phalloidin-rhodamin polarized fluorescence, both with fibres oriented parallel (P_{\parallel}) and perpendicular (P_{\perp}) to the polarization plane of exciting light, depended on free calcium ions concentration and on phosphorylation of myosin regulatory light chains. At low Ca^{2+} concentration ($0.6 \mu\text{mol/l}$) the changes of P_{\perp} were more expressed when myosin regulatory light chains were phosphorylated. Upon increasing free Ca^{2+} concentration from $0.6 \mu\text{mol/l}$ to $10 \mu\text{mol/l}$ changes of P_{\parallel} upon transition of the fibre from relaxation to contraction were also enhanced both with myosin regulatory light chains phosphorylated and dephosphorylated (Table 1). The changes of the degree of polarization P_{\parallel} and P_{\perp} are associated with structural alterations of actin filaments with the attached fluorophore due to the statistica-

lly averaged changes in orientation of actin monomers and the flexibility of actin filaments (Borovikov and Gusev 1983). Thus the structural change of actin filaments upon the transition of the fibre from relaxed to contracted state depend on the phosphorylation of myosin regulatory light chains and on concentration of free Ca^{2+} . It should be noted that the parameters of polarized fluorescence of the phalloidin-rhodamin bound with the fibre containing phosphorylated myosin in contraction solution remained practically unchanged upon placing the fibre in relaxation solution following phosphatase treatment (data not shown). This observation requires further studies.

More information about the kind of the structural change of the actin filament was obtained from mathematical analysis of experimental data (Traeger and Mendelson 1975; Yanagida and Oosawa 1978; Kąkol et al. 1987). Table 1 shows values of the angle between the absorption (ϕ_A) and emission (ϕ_E) dipoles and the long axis of actin filament, and those of $\sin^2\theta$ (where θ is the angle between the actin filament long axis and the fibre axis) for phalloidin-rhodamin labelled actin filaments in relaxed fibres containing phosphorylated myosin and contracted fibres containing phosphorylated or dephosphorylated myosin.

Fibres containing dephosphorylated myosin regulatory light chains had lower values of ϕ_A and ϕ_E in contracted state. The values of $\sin^2\theta$ were higher at $10 \mu\text{mol/l}$ than at $0.6 \mu\text{mol/l}$ Ca^{2+} .

The changes of the actin filament structure at $0.6 \mu\text{mol/l}$ and $10 \mu\text{mol/l}$ Ca^{2+} seem to be a consequence both of conformational changes of the tropomyosin troponin complex (Borovikov and Gusev 1983) and of conformational changes of myosin heads (Borovikov et al. 1987; Stepkowski et al. 1985a).

Assuming that muscle fibre contraction is accompanied by more actin monomers pasting to the "on" state the effect of phosphorylation of myosin regulatory light chains seems to be connected with an increased ability of phosphorylated myosin to form strong bonds with actin filaments at low Ca^{2+} concentrations. In conditions of contraction used in our experiments (free Mg^{2+} 1 mmol/l , free Ca^{2+} $0.6 \mu\text{mol/l}$) the myosin heads are mainly saturated with Mg^{2+} and therefore the affinity to actin of phosphorylated myosin heads is higher than that of dephosphorylated myosin (Szczęsna et al. 1987b). Moreover, as shown previously (Kąkol et al. 1987), in contrast to dephosphorylated heavy meromyosin the binding of phosphorylated heavy meromyosin in absence of ATP to actin in ghost fibre at low Ca^{2+} concentrations increased actin filament flexibility. Therefore it seems reasonable to assume that phosphorylation and binding of calcium ions to myosin heads modulate the interactions between thick and thin filaments by changing the number of cross-bridges strongly attached to actin. The increased tension development observed at $0.6 \mu\text{mol/l}$ calcium with phosphorylated myosin as compared to that in the same conditions

by fibres containing dephosphorylated myosin heads may be explained by increased numbers of phosphorylated myosin heads attached strongly to actin.

As mentioned above our observations are in agreement with the findings of Persechini et al. (1985) who showed, that phosphorylation of myosin heads in skinned fibres of vertebrate skeletal muscle increases the force level at sub-maximal calcium concentrations.

Thus phosphorylation of myosin heads seems to be a more sensitive modulator of muscle contraction at low than at high calcium concentrations, representing a higher step of regulation than the regulation by calcium.

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