Activity of a gelsolin-like actin modulator in rat skeletal muscle under protein catabolic conditions

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A gelsolin-like actin-modulating protein was isolated from rat skeletal muscle and characterized with respect to its interaction with actin. The protein, with a molecular mass of approx. 85 kDa, forms a stoichiometric complex with two actin molecules and is activated by micromolar concentrations of Ca²⁺. It effectively severs actin filaments and promotes nucleation of actin polymerization. The activity of this protein is detectable already in crude extracts by its capability to reduce the steady state viscosity of actin. Actin-modulating activities were determined in muscle extracts of rats kept under protein catabolic conditions, i.e. as generated by corticosterone treatment and starvation. In both cases we found a marked increase of modulator activity. The possibility is discussed that the increased activity of actin modulator indicates a fragmentation of actin filaments prior to the proteolytic degradation of actin.

INTRODUCTION

Actin is an ubiquitous protein which exists both as a filamentous polymer and in a non-filamentous or monomeric form. Actin filaments are major constituents of the cytoskeleton and the contractile system. A pivotal property of actin for rendering possible its cytoskeletal function in non-muscle cells is a rapid interconversion between a polymeric and monomeric state. Since the ionic conditions in the cytoplasm favour the formation of actin filaments, various actin-binding proteins exist which modify the properties of actin and apparently regulate assembly and disassembly of filaments (for recent reviews: Pollard & Cooper, 1986; Stossel et al., 1985).

In muscle cells actin exists predominantly in a polymeric form and represents the main constituent of thin filaments in the contractile apparatus. Though these filaments are relatively stable it is known that thin filament proteins are continuously degraded and newly synthesized. Since the half-lives of their various constituents differ (Waterlow et al., 1978), degradation of thin filaments in toto is unlikely. Partial or complete disassembly of the filaments may precede proteolytic degradation and it is conceivable that actin-binding proteins are involved in these processes.

Considerable acceleration of protein degradation is known to take place in muscle tissue of animals under catabolic conditions (Rennie, 1985), when an increased muscle protein degradation supplies liver and kidney with substrate precursors for gluconeogenesis (Cahill, 1971). Therefore, it is of interest whether the amount and activity of certain actin-binding proteins would be altered in muscles under catabolic conditions. Actin-modulating proteins are a specific subtype of actin-binding proteins. Their interaction with actin is Ca²⁺-dependent and they nucleate actin polymerization, cap the fast polymerizing end of actin filaments, and are capable of severing F-actin. Actin modulators have been found in various non-muscle cells, e.g. gelsolin in macrophages and blood platelets (Yin & Stossel, 1980; Yin et al., 1980; Kurth et al., 1983), villin in the microvilli of intestinal brush borders (Glenney et al., 1981), and fragmin in Physarum (Hasegawa et al., 1980; Hinssen, 1981a,b), and recently also in different muscle types (Hinssen et al., 1984, 1985b; Rouayrence et al., 1984; D’Haese & Hinssen, 1987). In the present report we describe the isolation and characterization of a gelsolin-like actin modulator protein from rat skeletal muscle and the alterations of actin-modulating activity found in animals which were subjected to experimentally induced protein catabolic conditions.

EXPERIMENTAL

Materials

Corticosterone was obtained from Sigma. All other chemicals were analytical grade. Male Wistar rats were obtained from Winkelmann, Borchen, Germany. Four to six animals were housed together in cages and were fed on standard diet (ssniff from Plange, Soest, Germany).

Extraction of skeletal muscle tissue for determination of actin modulator activity

Extraction and partial purification of actin modulator were based on a protocol for the actin modulator of pig stomach smooth muscle (Hinssen et al., 1984). All procedures were performed at 0–4 °C. Pooled rat hindleg muscle tissue was suspended in a 5-fold volume (v/w)

Abbreviations used: DTE, dithioerythritol; PAGE, polyacrylamide-gel electrophoresis; PMSF, phenylmethanesulphonyl fluoride; f.p.l.c., fast protein liquid chromatography.
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of a solution of 20 mM-KCl/5 mM-EGTA/1 mM-MgCl₂/1 mM-DTE/1 mM-PMSF/1% (v/v) Triton X-100/10 mM-imidazole/HCl, pH 7.0, minced with scissors and homogenized in a glass/Teflon homogenizer. After readjusting the pH value to 7.0 by addition of a 2 M solution of Tris, the homogenate was centrifuged for 30 min at 20000 g. The supernatant was filtered through a layer of glass wool. To the cleared muscle extract a solution of 3.8 M-(NH₄)₂SO₄/20 mM-KCl/5 mM-EGTA/1 mM-MgCl₂/1 mM-DTE/1 mM-PMSF/10 mM-imidazole/HCl, pH 7.0, was added to obtain 35% saturation with respect to the (NH₄)₂SO₄. The mixture was left for 30 min at 4 °C and centrifuged for 20 min at 20000 g. The supernatant was cleared by filtration and (NH₄)₂SO₄ solution was added to 55% saturation. Precipitated proteins were sedimented again and the pellet was dissolved in 50 mM-KCl/1 mM-EGTA/1 mM-MgCl₂/0.5 mM-DTE/20 mM-imidazole/HCl, pH 7.2, to obtain a solution of 50 mg/ml. This crude extract fraction was used for measuring actin-modulating activity of muscles from rats under catabolic conditions taking aliquots of 50, 75 or 100 µl to determine the relative activity as described below.

Purification of the actin-modulating protein

For the preparation of actin-modulating protein about 200 g of pooled hind-leg skeletal muscles were extracted and fractionated as described above. The proteins precipitating between 35 to 55% (NH₄)₂SO₄ saturation were resuspended in a small volume of column buffer (50 mM-KCl/1 mM-EGTA/1 mM-MgCl₂/0.5 mM-DTE/20 mM-imidazole/HCl, pH 7.2) and passed through Sephadex G-25 to remove (NH₄)₂SO₄. This fraction, about 1 g of protein, was applied to a DEAE-Sepharose CL-6B column (2.6 cm × 30 cm), washed with 5 vol. of column buffer and bound proteins were eluted using a linear KCl gradient from 50 to 400 mM (about 1 litre). Fractions with actin-modulating activity were concentrated by (NH₄)₂SO₄ precipitation at 70% saturation and subsequently subjected to gel filtration on a 2.6 cm × 90 cm column of Ultrogel AcA34 (LKB) equilibrated with column buffer. Purified modulator was obtained by processing the collected active fractions from the ion exchange chromatography after equilibration with column buffer on a f.p.l.c. system (Pharmacia). A second ion-exchange chromatography on Mono Q HR 5/5 (Pharmacia) was performed using a linear KCl gradient (100 ml) from 50 to 300 mM for elution. Pooled active fractions were concentrated with Centricon-10 (Amicon) and finally subjected to gel filtration on two Bio-Sil TSK-250 (BioRad) columns (300 mm × 7.5 mm) mounted in series and equilibrated with column buffer.

Purification of muscle actin

Actin from rabbit skeletal muscle was prepared according to Spudich & Watt (1971) with an additional purification by gel filtration on Sephadex G-150 (MacLean-Fletcher & Pollard, 1980).

Viscosity measurements

The actin-modulating activity of the different samples was routinely assayed by their effect on salt-induced polymerization of rabbit skeletal muscle G-actin. To the reaction mixture (2 ml final volume) which contained 1 mg of actin/ml in 0.2 mM-CaCl₂/1 mM-ATP/10 mM-imidazole/HCl, pH 7.4, 50–300 µl of sample solution was added and the polymerization was initiated by 100 µl of 2 mM-KCl/40 mM-MgCl₂. Specific viscosity was determined with an Ostwald-type capillary viscometer with outflow times for water of 25 s. The relative modulator activity (%) was calculated according to the formula \((1 - \eta_p M/\eta_w A) \times 100\) where \(\eta_p M\) is the decreased final specific viscosity of the polymerized actin in the presence of modulator and \(\eta_w A\) the final specific viscosity of the F-actin control. All measurements were made at 25 °C.

Gel electrophoresis

Electrophoresis on 1 mm thick polyacrylamide slab gels in the presence of SDS was carried out according to Laemmli (1970) and Chua (1980) using an acrylamide concentration in the stacking gel of 4% (w/v). In the running gel a linear gradient from 9 to 17% (w/v) was used. Bisacrylamide concentration was 3.5% (w/w) of the acrylamide concentration. Slab gels were stained with Serva Blue G 250 and destained in several changes of 28% (v/v) methanol/5% (v/v) acetic acid.

Experimental protocols for inducing protein catabolic conditions

Starvation. From a group of 24 rats of 119 ± 7 g initial body weight, 20 animals were starved and each day after food removal actin modulator protein was extracted from hind-leg skeletal muscle tissue from each of five animals. Four animals were used as fed controls. During the starvation period the animals continuously lost body weight and those rats fasting 4 days had a final body weight of 85 ± 9 g.

Administration of corticosterone. From a group of 24 rats with about 85 ± 4 g body weight 20 rats received a daily subcutaneous injection of 10 mg of corticosterone/100 g body wt. Four rats were used as control and received a daily injection of vehicle solution only (Tomas et al., 1979). At the times indicated in Fig. 7, four rats were killed and actin modulator activity was measured in extracts from hind-leg skeletal muscle. During the course of the experiment the body wt. of the control rats increased to 119 ± 7, whereas that of the glucocorticoid-treated animals increased to 93 ± 8 g only.

Protein determination

Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as a standard.

Statistics

Mean values ± S.D. were calculated, and for comparison of the means, Student’s t test was used.

RESULTS

Isolation and purification of the actin modulator

Addition of the (NH₄)₂SO₄-fractionated extract of rat muscle to purified skeletal muscle G-actin modulated the polymerization reaction, leading to a reduced steady state viscosity of F-actin. The average reduction under standard assay conditions was about 40% for 2.5 mg of extract protein/ml of assay volume. As the effect was Ca²⁺-activated it is likely to be derived from the presence of an actin-modulating protein. When the extract was
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subjected to ion-exchange chromatography the actinmodulating activity eluted as a distinct peak around 230 mM-KCl (Fig. 1, Fig. 2b). Subsequent gel filtration resulted in the enrichment of an approx. 85 kDa protein as revealed by SDS/PAGE (Fig. 2c). Approx. 1.5 mg of partially purified protein with a 60–100-fold enriched relative activity was obtained from 200 g of hind-leg muscle.

By processing the active fractions from the DEAE column on a f.p.l.c. system, by a second ion-exchange chromatography and subsequent gel filtration, an almost homogeneous fraction of the 85 kDa protein was obtained (Figs. 2d and 2e). The final yield was about 0.15 mg of purified protein with a relative activity of 50%, at a modulator:actin molar ratio of 1:300. The enrichment of the modulator activity calculated was about 500-fold compared with the initial crude extract.

Characterization of the modulator protein and its interaction with actin

To determine the number of actin molecules bound to the modulator, the purified protein was premixed with various amounts of G-actin in the presence of Ca²⁺ and subsequently chromatographed on a calibrated gelfiltration column (Bio-Sil TSK-250, BioRad). The modulator alone eluted as a single peak with an apparent molecular mass of 75 kDa. With a mixture of modulator and actin an additional peak appeared, with an apparent molecular mass of 145 kDa, indicating the formation of an actin–modulator complex. Increasing the amount of actin in the mixture led to a concomitant decrease of the modulator peak and increase in the amount of the actin–modulator complex. When a mixture of modulator and actin at a molar ratio of 1:2 was applied, the 75 kDa modulator peak was absent and a single peak containing the actin–modulator complex was eluted. We therefore conclude that the modulator forms a stoichiometric complex with two actin molecules.

Fig. 1. Ion-exchange chromatography of the modulator extract

The crude modulator extract from 200 g of pooled hindleg rat muscles (about 850 mg of protein) was applied to a DEAE-Sepharose CL-6B ion-exchange column (2.6 cm x 30 cm). One distinct modulator fraction was eluted with 800 ml of a KCl gradient from 50 to 400 mM (broken line) at around 230 mM-KCl. Fractions (5 ml) were collected and the modulator activity of 200 µl aliquots was determined by measuring the effect on actin polymerization. The relative activities (%) were calculated as described in the Experimental section.

Fig. 2. SDS/PAGE of rat skeletal muscle modulator fractions at various steps of the purification procedure

(a) Molecular mass standards; (b) activity peak after separation of crude modulator extract on a DEAE-Sepharose CL-6B ion-exchange column; (c) pooled active fractions from gel filtration on Ultragel AcA34 (partially purified modulator); (d) enrichment of an 85 kDa protein band after second ion-exchange chromatography of the pooled material from the DEAE-Sepharose CL-6B column on Mono Q HR 5/5; (e) purified actin modulator after processing the active fraction from the Mono Q column by gel filtration on TSK-250.

Fig. 3. Nucleation of actin polymerization induced by the actin modulator

Partially purified modulator fraction was dialysed against 0.2 mM-CaCl₂/0.01 mM-ATP/2 mM-imidazole/HCl (pH 7.2) and was added to skeletal muscle G-actin (0.3 mg/ml final concentration in the same buffer) at various ratios (w/w). Polymerization was initiated by the addition of KCl to a final concentration of 0.05 M and monitored by the increase in absorption at 232 nm. The ratios (w/w) of the modulator fraction to actin and the actin control ('Actin') are indicated.
induced on process of polymerized very were polymerization Oosawa, & No ratios of the modulator weight modulator:actin ratio led of micrographs was used it the actin, modulator fraction the action of the modulator was completely dependent on the presence of Ca\(^{2+}\). By the addition of EGTA further interaction with actin was inhibited. The new steady state viscosity of actin after the addition of modulator was attained within only 2–4 s. Electron micrographs of negatively stained samples taken after several time intervals showed a progressive decrease in the average actin filament length from several \(\mu\)m to about 0.3 \(\mu\)m (results not shown). An increase of the modulator:actin ratio led to the formation of concomitantly shorter filaments. The high reaction rate and the resulting short actin filaments implicate a direct seversing of the F-actin by the modulator.

The \(Ca^{2+}\)-dependence of the modulator activity, as determined by its effect on the polymerization of G-actin, is shown in Fig. 5. An almost identical curve was obtained when the influence on the severing activity of the modulator fraction was tested. The modulator was activated within a narrow range of free \(Ca^{2+}\) concentrations between \(5 \times 10^{-7}\) M and \(5 \times 10^{-5}\) M with half-maximum activation at \(5 \times 10^{-8}\) M. Concentrations of free \(Ca^{2+}\) > \(10^{-5}\) M considerably inactivated the modulator. In contrast with the rapid decrease in F-actin viscosity after the addition of modulator in the presence of \(Ca^{2+}\), the decrease of \(Ca^{2+}\) concentration by EGTA to \(< 10^{-7}\) M resulted only in a very slow (within several hours) and incomplete recovery of the initial viscosity.

**Fig. 4. Time course of actin fragmentation by the partially purified modulator**

The modulator fraction was rapidly mixed with F-actin at a weight ratio of 1:25 (1 mg of actin/ml in 1 mm-ATP/0.2 mm-CaCl\(_2\)/100 mm-KCl/2 mm-MgCl\(_2\)/10 mm-imidazole/HCl, pH 7.2). After given time intervals the reaction was stopped by the addition of EGTA to a final concentration of 2 mm and viscosity was measured immediately.

**Fig. 5. \(Ca^{2+}\)-dependence of the modulator–actin interaction**

Skeletal muscle actin (1 mg/ml) was polymerized in the presence of modulator (20 \(\mu\)g/ml) at various concentrations of free \(Ca^{2+}\). Concentrations of free \(Ca^{2+}\) were adjusted using a 2 mm-Ca-EGTA buffer according to Weber (1969). The maximum reduction of viscosity obtained was taken as 100% activity.

**Fig. 6. Actin-modulating activities of crude muscle extracts from fasting rats**

The mean relative actin-modulating activity of the control group was taken as 100% (○). Each value (●) represents the mean relative activity ± s.d. of extracts from the hind-leg muscle of four or five animals after the indicated time of starvation. \(P\) values representing the significance of difference as compared with the mean of the control group are indicated. Abbreviation used: n.s., not significant \((P > 0.05)\).
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smooth muscle it was evident that the amount of modulator is significantly lower in skeletal muscle (H. Hinssen & J. V. Small, unpublished work). Taking into consideration the molar ratio between modulator and actin in smooth and obliquely striated muscle, the amount of modulator present in skeletal muscle is probably less than necessary to cap all existing F-actin filaments. From the fact that the isolated modulator was not associated with actin in form of a stoichiometric complex it is likely to exist in soluble form in the cytoplasm. The Ca\(^{2+}\) concentration necessary for its activation is probably high enough to prevent binding to actin during the contraction–relaxation cycle of the muscle cells.

The protein isolated from rat skeletal muscle has been clearly identified as an actin-modulating protein by its capability to sever F-actin, its Ca\(^{2+}\)-dependence of interaction with actin, low reversibility upon removal of Ca\(^{2+}\), and the formation of a stoichiometric complex with actin. Its properties were almost identical with those reported for the actin modulator from pig stomach smooth muscle (Hinssen et al., 1984, 1985a). By its molecular mass around 90 kDa and its ability to bind two actin molecules the protein resembles gelsolin isolated from macrophages and blood platelets (Yin & Stossel, 1980; Yin et al., 1980, 1981; Kurth et al., 1983) and probably the protein found in cardiac muscle (Rouayrenc et al., 1984). Its effect on the polymer state of actin is also similar to that of the 40 kDa modulators found in Physarum (Hasegawa et al., 1980), sea urchin eggs (Coluccio et al., 1986), and in obliquely striated annelid muscle (D’Haese & Hinssen, 1987).

Though proteins with this type of interaction pattern with actin seemed to be common in muscle and non-muscle cells their physiological function is not yet clear, probably because of the complexity of their effects on actin in vitro. Actin modulators may either serve as capping factors to stabilize the length of F-actin, they may alter the polymerization characteristics of actin, or serve to disassemble actin filaments under conditions where transformations of the polymer state take place. The latter function has been postulated for the extracellular plasma gelsolin which is supposed to fragment actin filaments released into the blood plasma after cell lysis (Harris & Weeds, 1983). It is conceivable that in a similar way actin modulators are involved in intracellular processes where disassembly of actin filaments is required; one of such processes may be the turnover of actin with respect to proteolytic degradation. We have chosen established methods to induce catabolic conditions experimentally in the rat and investigated whether the actin modulator activity in skeletal muscle tissue of these animals is affected by the catabolic stimuli.

In our investigation with starved and corticosterone-treated rats the experimental conditions were identical with those given by Millward & Waterlow (1978) and Odedra et al. (1983), respectively. These authors measured the influence of catabolic conditions on the rates of protein synthesis and breakdown in skeletal muscle tissue. Under both conditions muscle protein synthesis was substantially reduced. However, protein breakdown was considerably increased at days 3 and 4 after food removal and was also transiently increased at days 2 and 3 during the period of treatment with corticosterone, before a decrease occurred again at day 5 of the experiment. In our experiments we have not
measured the degradation of actin or overall protein and it is known that the definite onset of degradation as a response to a catabolic stimulus varies in different animals and depends on their individual physiological state (Goodman & Ruderman, 1980; Goodman et al., 1980; 1984; Santidrián et al., 1981).

Nevertheless, since the actin-modulating activity was increased at day 3 of the fasting period and was elevated at days 2–4 during the period of corticosterone administration before declining at day 5, the data suggest that changes in actin-modulating activity are coincident with changes in muscle protein breakdown. This is corroborated by our earlier findings that alterations of the amount of easily releasable myofilaments, a fraction of actin and myosin filaments that is considered to contain intermediates of the breakdown of myofibrillar proteins (van der Westhuizen et al., 1981; Sims et al., 1978), also run parallel to the changes of the actin modulator activity and the rates of muscle protein breakdown under these different catabolic conditions (Dahmann et al., 1986). In addition, we observed in muscle extracts of rats with streptozotocin-induced diabetes mellitus an increase in the actin-modulating activity (J. D’Haese, M. Rutschmann, B. Dahlmann & H. Hinssen, unpublished work). The activity increased by about 50% within 1 week after onset of diabetes (Rutschmann, 1985) where catabolic protein conditions can be expected to occur (Pain et al., 1983).

The coincidence of biocatalytic activities and metabolic processes does not unequivocally prove the existence of a causal relationship between these phenomena. However, the results obtained in the present experiments provide a reasonable basis for further investigations on a possible involvement of actin modulator proteins in the turnover of skeletal muscle actin.

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