Actin mediated calcium dependency of actomyosin in a myxomycete

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A fraction obtained from Physarum polycephalum by differential centrifugation displays magnesium adenosine triphosphatase activity; at low ionic strength (0.07 M KCl) the rate at which ATP* is split in 0.1 mM $CaCl_2$ is from 1.5 to 6.6 times the rate in 1 mM EGTA*. Both actin and myosin are present in this fraction. On SDS gels several polypeptide bands are present in the range of 39,000 daltons to 14,000 daltons as well as those of actin and myosin. The addition of desensitized rabbit muscle actin to the fraction increased the rate of ATP splitting in EGTA, thereby decreasing the EGTA inhibition 30-50%. We conclude that actomyosin regulation by calcium in this acellular slime mould is, at least in large part, mediated through actin.

Calcium sensitive actomyosins have been reported from several non-muscle tissues, including leukocytes, brain and platelets (Shibata et al., 1972; Fine et al., 1973; Cohen et al., 1973). Furthermore Cohen, Kaminski and DeVries (1973) have utilized the actin competition test recently devised by Lehman, Kendrick-Jones and Szent-Gyorgyi (1972) to show that in human platelets calcium sensitivity of the actomyosin appears to be regulated by sites associated with actin. This test is based on the idea that actins from different sources can compete for activation of the ATPase activity of myosins. Therefore, when a calcium dependent

*Abbreviations: EGTA, ethylene glycol-bis(aminoethyl)-tetraacetic acid. SDS, sodium dodecyl sulfate. ATP, adenosine triphosphate.

actomyosin is found, if the addition of desensitized actin increases the rate of ATP splitting in the presence of a strong calcium chelator, this result implies that the myosin in the system is not itself inhibited by the low levels of calcium but is free to react with the added desensitized actin. Therefore the system in question must have originally been sensitive to calcium by mediation of sites associated with actin. In the case of vertebrate muscle (Ebashi and Endo, 1968) the sites are on, and activated by, the tropomyosin troponin proteins, and in platelets (Cohen et al., 1972) their non-muscle analogues appear to be involved.

From extensive comparative studies with muscles (Lehman et al., 1972) it has been concluded that actin-linked regulation evolved later than myosin-linked regulation, since muscles from animals near the base of the phylogenetic scale appear to have myosin-linked systems. Two phyla have dually regulated systems (Lehman et al., 1972). Platelets might be considered highly evolved cells, since they occur in specialized forms only in higher vertebrates. However, we present evidence here that in Physarum, a primitive acellular slime mould, all or a large part of calcium regulation of the actomyosin is mediated through actin.

In order to use the actin competition test, one must first isolate a calcium sensitive actomyosin. Actomyosin as conventionally prepared from Physarum is desensitized, even when all precautions for preservation of sulfhydryl groups are taken. An extract from Physarum which could confer calcium sensitivity to sesensitized muscle actomyosin (but only by superprecipitation) has been described (Tanaka

and Hatano, 1972). Although we could confirm their results by superprecipitation, we could not obtain significant calcium sensitivity by enzymatic assay with this preparation. It seemed preferable in any case, to work directly with proteins derived entirely from Physarum. We discovered that direct differential centrifugation of extracts of Physarum yields a calcium dependent pellet and we present evidence that the enzymatic activity and the calcium dependency is due to actomyosin in the fraction.

Physarum was maintained as microplasmodia on semi-defined medium by the method of Daniel and Baldwin (1964), transferred to oatmeal after 2 days' growth, and harvested from plastic wrap in contact with plasmodia. Only clean, migrating plasmodia free of slime were collected. From 20-70 grams of plasmodia were homogenized in all glass homogenizers in an equal volume of a low salt buffer (LSB) containing 0.05 M KCl, 0.01 M imidazole, 5 mM dithiothreitol, pH 7.0. The homogenate was centrifuged for 1 hour at 100,000 g (mean value). The clear yellow supernatant was filtered through glass wool and centrifuged for 2 hours at 100,000 g. The transparent yellow pellet, referred to henceforth as the pellet, estimated as 3-5 mg per original 10 grams of myxomycete, was gently dissolved in 0.5 ml of a high salt buffer (HSB) containing 0.5 M KCl, 0.05 M imidazole, 5 mM dithiothreitol, pH 7. It was used directly for assay of adenosine triphosphatase activity at 24° C alone or with addition of actin. Table 1 shows the results of two such preparations. The rabbit muscle actin (in 0.04 M imidazole) that was added to such preparations had been extracted at 0° C and treated with 0.6 M KCl (Spudich

Table I

Calcium sensitivity in a high speed pellet fraction from *Physarum* and its reversal by added muscle actin

Conditions: 0.07 M KCl, 0.01 M imidazole, pH 7 in either 10 mM $MgCl_2$, 1 mM EGTA or 10 mM $MgCl_2$, 0.1 mM $CaCl_2$.

Expt. 1 - actin : pellet, 1 : 13 (actin conc., 0.06 mg/ml; pellet, 0.78 mg/ml)

		nmoles Pi/ml reaction mixture			
pellet	min	EGTA	Calcium	Ca^{++}/EGTA	% inhibition*
	5	79.2	120.2	1.52	34
	10	113.0	163.4	1.45	30.6
	15	154.1	221.0	1.43	30.3
pellet plus actin**	5	108.7	77.0	0.71	---
	10	161.3	163.4	1.01	1.2
	15	204.5	223.2	1.09	8.0

* defined as $100\left[1-\frac{EGTA}{Ca}\right]$

** corrected for a 1-2% contribution from actin

Expt. 2 - actin : pellet, 3 : 4 (actin conc., 0.3 mg/ml; pellet, 0.4 mg/ml)

		nmoles Pi/mg high speed pellet fraction*			
pellet	min	EGTA	Calcium	Ca^{++}/EGTA	% inhibition
	5	16.5	90.5	5.48	81
	10	52.7	159.7	3.03	67
	15	74.1	212.3	2.87	65
pellet plus actin**	5	77.4	113.6	1.47	32
	10	111.9	171.2	1.53	35
	15	138.3	241.9	1.75	43

* myosin estimated as 2-4% of the protein in a comparable pellet by complement fixation. This yields a calcium value of 0.3 - 0.6 μmoles Pi/min/mg myosin.

** corrected for small actin blank.

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and Watt, 1971) so that it was only 3% contaminated with tropomyosin and troponin on a molar basis as measured by densitometry (Plate I, a). Table I shows that the % inhibition by EGTA was different for the two preparations, but that in both cases the addition of desensitized actin

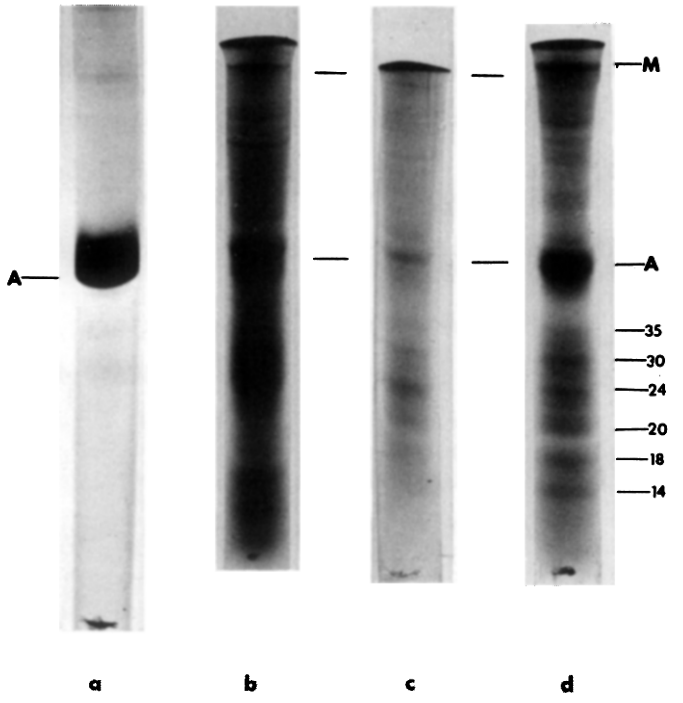

PLATE 1

increased the rate of ATP splitting in the presence of EGTA so that the extent of inhibition was sharply reduced, and in experiment 1, approached zero. In Plate I, b, c and d are shown the SDS gels from pellet fractions. Plate I<u>b</u> shows the entire pellet, <u>c</u> shows the pellet after super-precipitation and <u>d</u> shows the pellet reprecipitated with added muscle myosin. Bands corresponding to the molecular weights of actin, myosin and several bands in the range expected from tropomyosin or troponin factors are present both before and after reprecipitation, but we cannot yet distinguish which of these are essential for the effect.

Although the pellet fraction (estimated as 30-40S material) is an unusually soluble source of actomyosin, the evidence that it contains both actin and myosin and that they are responsible for the ATPase is conclusive. First,

as shown above (Plate I) bands on SDS gels correspond to the molecular weights expected from the reduced polypeptides of actin and myosin. Second, the bands corresponding to actin

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Plate I. Sodium dodecyl sulfate gel electrophoresis of 7.5% gels according to Fairbanks et al. (1971)

(a) Actin, prepared by shortened modification of the Spudich and Watt method. Densitometry reveals a 3% or less molar contamination from tropomyosin-troponin.

(b)-(d) High speed pellet fraction. Gels show presence of myosin and actin bands as well as persistent bands below actin with approximate molecular weights of 55,000 (faint and not marked on Plate I); 35,000; 30,000; 24,000; 20,000; 18,000 and 14,000 daltons.

(b) Entire pellet, 70 µg.

(c) Superprecipitate from pellet ATPase assay.

(d) Low speed myosin precipitate of pellet. 0.1 ml of 9.5 mg/ml rabbit muscle myosin in 0.25 M KCl was added to pellet resuspended 0.05 M KCl, 0.01 M imidazole, 5 mM DTT and spun for 10 minutes at 12,000 xg to bind selectively any actin present in the supernatant. Some enhancement of bands below actin due to myosin light chains (25,000, 18,000 and 16,000) may be present even though the loading of myosin heavy chain is very light.

and myosin persisted when the redissolved pellet was superprecipitated and the precipitate run on gels (Plate I, c and d). This precipitate retained calcium sensitivity. Third, when rabbit actin was added at high concentrations to the high speed pellet, actin activation was sometimes observed (cf. Table I, expt.2) as would be expected from an ATPase of the actomyosin type (Eisenberg and Moos, 1967). Physarum myosin has been previously shown to be activated twofold (Hatano and Ohnuma, 1970) or 6-10 fold (Nachmias, 1974) by rabbit actin at high concentrations. Fourth, the ATPase activity was reduced to half the original level by increasing the KCl concentration from 0.04 M to 0.12 M. Strong inhibition by increased ionic strength in this range is characteristic of actomyosin types of ATPase (Eisenberg and Moos, 1967). Fifth, a highly specific test for myosin was carried out: Previously prepared myosin-specific antibody was combined with the pellet fraction and the combination tested for its ability to fix complement, a sensitive assay for the presence of the specific antigen, in this case myosin. The complement-fixation test was positive and indicated 2-4% myosin content in the pellet. This would be sufficient reasonably to account for the ATPase activity observed in Table I. Other tests on the pellet indicated the presence of calcium inhibited pyrophosphatase activity and the absence of glycogen.

From this evidence we believe that although the pellet is a crude fraction, all or the major part of the ATPase activity of this fraction can be ascribed to actomyosin. The successful preservation of calcium sensitivity may be due to the direct and gentle preparative procedure. Therefore the release

of EGTA inhibition by desensitized actin, shown in Table I indicates that the relative inhibition of the ATPase in EGTA is due to factors associated with Physarum actin. It is interesting to note however that when the EGTA inhibition was strongest (cf. expt. 2), the reversal was incomplete even at a much higher concentration of added actin than in expt. 1. Since Lehman, Bullard and Hammond found (1974) that in insect muscle myosin mediated calcium dependency is synergistic with actin mediated calcium dependency, it is possible that labile myosin mediated, as well as actin mediated calcium dependence may occur in Physarum. Further study will be needed to determine whether or not this is true.

Physarum is, we believe, the most lowly organism in the phylogenetic scale yet shown to possess actin-linked actomyosin regulation. Perhaps it is the case that this type of regulation was invented in nature even before muscles were evolved.

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