EVIDENCE FOR A CYCLIC NUCLEOTIDE-DEPENDANT PHOSPHORYLATION OF RETINAL MYOSIN

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Received 12 June 1978

1. Introduction

In recent years much interest has developed in the possible roles of actin and myosin in non-muscle cells. Counterparts of these once-regarded muscle proteins have been isolated and characterised from many non-muscle tissues (reviewed [1,2]) including the retina [3]; the non-muscle myosins seem to be similar to muscle myosins in possessing both heavy and light chain components, although there are subtle differences between myosin heavy chains of differing origins [4]. Phosphorylation of a muscle myosin light chain component is well documented and is thought to exert some control on the interaction of actin and myosin [5,6]. The phosphorylation of both heavy and light chain components of non-muscle myosins has also been reported [7–9] and there is evidence for both heavy and light chain phosphorylation being mandatory for actin stimulation of myosin ATPase activity [9,10]. In view of the possible regulatory role of phosphorylation in actin–myosin interactions we have studied retinal myosin phosphorylation and its dependance on cyclic nucleotides. In the present paper we report endogenous phosphorylation of retinal myosin heavy chains in a partially purified retinal actomyosin preparation and its stimulation by cAMP.

2. Experimental procedures

Crude actomyosin was prepared [3] from 30–50 bovine retinae by extraction in a 0.6 M KCl buffer, reduction of the ionic strength and ammonium sulphate fractionation of the thus precipitated actomyosin. Protein precipitating between 35% and 60% saturation concentrations of ammonium sulphate was resuspended in 2–3 ml 0.6 M KCl, 15 mM Tris–HCl, 1 mM EDTA, 2 mM dithiothreitol, pH 7.0 (buffer A) and dialysed overnight against 2 l same buffer. The resulting preparation will be referred to as retinal actomyosin.

Phosphorylation was carried out at 37°C in the following medium: 0.2 M KCl, 30 mM Tris–HCl, 12.5 mM $MgCl_2$, 5 mM dithiothreitol, 0.1 mM disodium-ATP (pH 7.5) and sufficient [γ-^{32}P]ATP (25 Ci/mmol, triethylammonium salt from New England Nuclear Ltd) to give 2–4 × 10^6 cpm/incubation (total vol. 500 μl). For analysis of trichloroacetic acid-precipitable ^{32}P incorporation the reaction was stopped by addition of 500 μl 20% trichloroacetic acid, 4% $Na_4P_2O_7$ and the radioactivity associated with the precipitated protein was measured as in [11]. In experiments involving electrophoretic analysis of the phosphorylated mixture the reaction was stopped by addition of 500 μl 2% SDS, 1% $Na_4P_2O_7$ and samples dialysed against 2 l 0.2 M NaCl, then 0.1 M NaCl and finally water for a total 28 h. Samples were then heated for 2 min at 100°C in the presence of 10% mercaptoethanol and 1% SDS and subjected to SDS–polyacrylamide gel electrophoresis (7.5% or 12% polyacrylamide, 0.1% SDS) using Tris–glycine buffer [12]. Gels were subsequently either stained with Coomassie blue, destained and scanned at 650 nm or cut into 2 mm slices and counted by Cerenkov radiation in 4 ml 0.5 M NaOH.

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In certain experiments actomyosin was phosphorylated and the reaction stopped by cooling in ice and addition of 9 vol. ice-cold water. After stirring for 20 min at 4°C actomyosin was collected by centrifugation at 30 000 × *g* for 30 min, resuspended in buffer A (2–3 ml) and chromatographed on a column of Scpharose 4B (40 × 2.5 cm) equilibrated with buffer A. Elution with buffer A was at 8 ml/h and 7 ml fractions were collected. Of each fraction 6 ml was counted by its Cerenkov radiation and suitable aliquots analysed for K^+/EDTA-ATPase activity [3]. Fractions comprising the first peak of radioactivity were pooled, dialysed against water and lyophilised.

3. Results

Incubation of the actomyosin fraction with labelled [γ-^{32}P]ATP led to transfer of the γ-phosphate to proteins as judged by the incorporation of radioactivity into trichloroacetic acid-precipitable material. Maximum incorporation was observed after 15–30 min incubation. Addition of the phosphodiesterase inhibitor isobutyl-methylxanthine resulted in a small increase in this basal phosphorylation (table 1) and subsequent addition of cAMP caused a further stimulation. Maximum stimulation (~100% increase in labelling) occurred with 10 μM cAMP. Addition of 10 μM–1 mM cGMP gave a smaller increase in labelling (30%).

Since the actomyosin fraction is a mixture of many protein species, experiments were performed to investigate which species were phosphorylated. Figure 1 shows the results following SDS–gel electro-

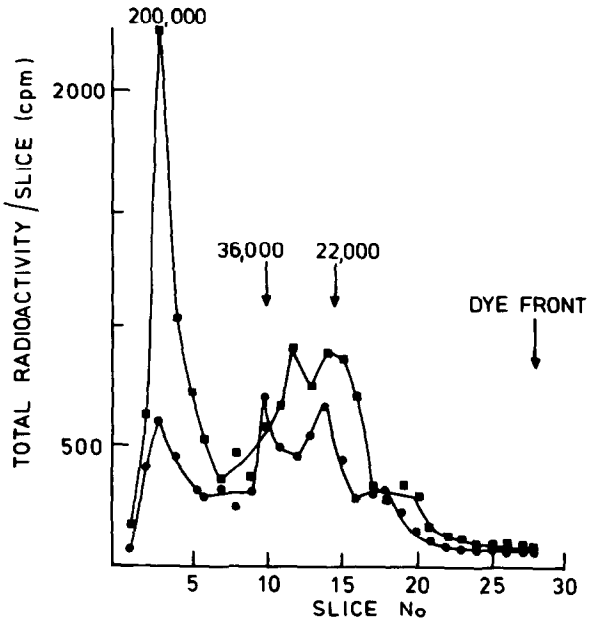


Fig.1. Radioactivity pattern of retinal actomyosin submitted to SDS–polyacrylamide gel electrophoresis. 490 μg protein was incubated with 2 × 10^6 cpm [γ-^{32}P]ATP for 30 min in control medium (—•—) or with 1 mM IBMX and 10 μM cAMP (—▪—). Samples were made 10% with mercaptoethanol and 1% with SDS and heated for 2 min at 100°C. Prior to electrophoresis 330 μg protein was deposited on each gel. Gels were 12% polyacrylamide, 0.1% SDS.

Table 1
Incorporation of ^{32}P into trichloroacetic-acid precipitated protein; effect of cAMP

	Total radioactivity in precipitate (cpm)		% increase compared to control + IBMX	
	exp. 1	exp. 2	exp. 1	exp. 2
control	7994	9335		
+ 1 mM IBMX[a]	9994	11 590		
+ 1 μM cAMP	12 654	11 758	27	1.5
+ 4 μM cAMP	–	14 524	–	35
+ 10 μM cAMP	22 525	20 802	125	80
+ 1 mM cAMP	21 968	22 413	120	93

[a] IBMX, isobutylmethylxanthine

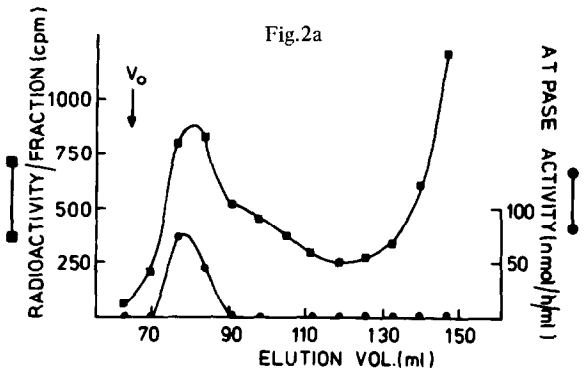
Values given are means of duplicate estimations. Incubation was at 37°C for 30 min with 185 μg (exp. 1) and 295 μg (exp.2) protein/incubation. 2 × 10^6 cpm (exp. 1) and 4 × 10^6 cpm (exp. 2) of radioactive ATP was used per incubation

phoresis of the phosphorylated actomyosin; in the absence of cyclic nucleotides there was phosphorylation of 2 or 3 proteins as manifested by the peaks of radioactivity in the gel. One peak corresponded to mol. wt 22 000, a second to mol. wt 33 000 and a third minor peak, which was not always observed, corresponded to mol. wt ~200 000 (the latter value was estimated from electrophoresis experiments using 7.5% polyacrylamide gels). In the presence of 10 μM cAMP (fig.1) or 100 μM cAMP there was an increase in the phosphorylation of all three components, the most marked being in the high molecular weight peak; average increases with 100 μM cAMP were 500% for the mol. wt 200 000 component and 150% for the 33 000 and 22 000 components. Notably, following incubation with cGMP there was no observable stimulation of phosphorylation of the mol. wt 200 000 molecule.

The association of these three components with myosin was investigated by reducing the ionic strength of the phosphorylated fraction and submitting the precipitated actomyosin to gel filtration on Sepharose 4B. As shown in fig.2, a small peak of radioactive material was present just after the void volume (K_{av} = 0.08) and the fractions containing radioactivity corresponded to those exhibiting K^+/EDTA-ATPase activity, thus suggesting the radioactivity to be associated with the myosin molecule. In addition a large peak of dialysable radioactivity was found towards the exclusion volume. Following dialysis and lyophilisation of the presumptive myosin, SDS–gel electrophoresis showed a single peak of radioactivity in the gel (fig.2b); the mobility of the peak corresponded to that of the high molecular weight component referred to in fig.1. Remarkably, no radioactivity was found in a position corresponding to the light chains of the myosin molecule.

4. Discussion

Incubation of the crude retinal actomyosin with [γ-^{32}P]ATP yielded three phosphorylated species as judged from electrophoresis experiments. Evidence for covalent protein phosphorylation was provided by the incorporation of radioactivity into trichloroacetic acid-precipitable material. The mobilities of the radioactivity peaks suggested mol. wt 22 000, 33 000 and 200 000 for the three components. Phosphorylation of all three species was markedly increased by addition of 10–100 μM cAMP. Chromatography of the phosphorylated actomyosin on Sepharose 4B produced a small peak of radioactive material co-chromatographing with myosin. The phosphorylated myosin component showed a mobility on SDS–gel electrophoresis comparable to that of the mol. wt 200 000 compo-



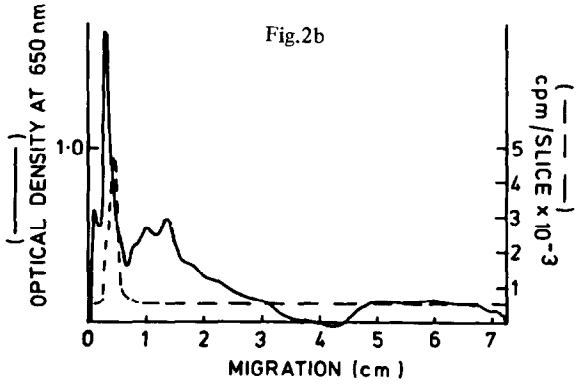


Fig.2. Gel filtration of phosphorylated actomyosin. 2a shows the profile of ATPase activity and radioactivity following chromatography of the phosphorylated actomyosin on Sepharose 4B. 8.3 mg protein was phosphorylated in total incubation vol. 8.5 ml (4 × 10^6 cpm [γ-^{32}P]ATP/ml). Elution was with buffer A at 8 ml/h. 7 ml fractions were collected; K^+/EDTA ATPase activity (—•—) and radioactivity (—▪—) were measured. 2b shows radioactivity pattern and densitometric scan of initial peak of radioactivity eluting from Sepharose 4B column (see fig.2a) after electrophoresis (12% polyacrylamide, 0.1% SDS). One gel was cut in 2 mm slices for estimation of ^{32}P and the other stained with Coomassie brilliant blue and scanned at 650 nm with a Vernon recording spectrophotometer.

nent observed after electrophoresis of actomyosin. Thus, on the basis of estimated molecular weight and co-chromatography with myosin ATPase activity, we identify the phosphorylated mol. wt 200 000 component as the heavy chain of the retinal myosin molecule. Phosphorylation of these presumptive myosin heavy chains was markedly stimulated by cAMP but not by cGMP. During the preparation of this manuscript cAMP-induced phosphorylation of slime mould myosin heavy chains was reported [13].

The position concerning the phosphorylation of a retinal myosin light chain component is not clear; although the size of the mol. wt 22 000 molecule could correspond to that of a light chain [4,8], this component was not observed to co-chromatograph with myosin. Thus either this molecule is a light chain component which is perhaps lost during chromatography or it is a molecular species not associated with myosin. Since others have found a phosphorylated light chain to co-chromatograph with myosin under similar conditions [7,8], the latter possibility may be the more likely. In this context it is interesting that phosphorylation of the mol. wt 22 000 component was stimulated by cAMP whereas purified platelet myosin light chain kinase has been reported not to be affected by cAMP [14]. The mol. wt 33 000 component is as yet unidentified, however it could be related to the mol. wt 30 000 phosphorylated component found in bovine retinal photoreceptor outer segments [15].

In conclusion, the major result from these experiments has been the demonstration of the cAMP-dependent phosphorylation of the heavy chain of retinal myosin. Several reports of phosphorylation of both heavy and light chain components of non-muscle myosins have appeared [7–9] and it seems this phosphorylation is a prerequisite for interaction of myosin (or spectrin) with actin [6,9,16]. Our results show a further example of non-muscle myosin heavy chain phosphorylation and in addition they raise the possibility of cAMP being involved in the control of myosin heavy chain phosphorylation and the interactions of cell cytoskeletal components.

Acknowledgements

We thank Mlle Chantal Hauprich for excellent technical assistance. J. E. H. acknowledges receipt of a grant from INSERM.

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