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Calmodulin and Myosin Light-Chain Kinase of Rabbit Fast Skeletal Muscle

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1. It is confirmed that myosin light-chain kinase is a protein of mol.wt. about 80000 that is inactive in the absence of calmodulin. 2. In the presence of 1 mol of calmodulin/mol of kinase 80–90% of the maximal activity is obtained. 3. Crude preparations of the whole light-chain fraction of rabbit fast-skeletal-muscle myosin contain enough calmodulin to activate the enzyme. A method for the preparation of calmodulin-free P light chain is described. 4. A procedure is described for the isolation of calmodulin from rabbit fast skeletal muscle. 5. Rabbit fast-skeletal-muscle calmodulin is indistinguishable from bovine brain calmodulin in its ability to activate myosin light-chain kinase. The other properties of these two proteins are also very similar. 6. Rabbit fast-skeletal-muscle troponin C was about 10% as effective as calmodulin as activator for myosin light-chain kinase. 7. By chromatography on a Sepharose–calmodulin affinity column evidence was obtained for the formation of a Ca^{2+}-dependent complex between calmodulin and myosin light-chain kinase. 8. Troponin I from rabbit fast skeletal muscle and histone IIAS were phosphorylated by fully activated myosin light-chain kinase at about 1% of the rate of the P light chain.

We have recently described the preparation of myosin light-chain kinase as a single homogeneous protein of mol.wt. about 80000. With myosin or the whole light-chain fraction as substrate, but no further additions, this enzyme possessed high enzymic activity (20–30μg of P transferred/min per mg of enzyme) in the presence of Ca^{2+} (Pires & Perry, 1977). Apparently in contrast with these findings, Yazawa & Yagi (1977) have reported that myosin light-chain kinase preparations from rabbit skeletal muscle could be separated into two fractions both of which were essential for myosin light-chain kinase activity: a Ca^{2+}-binding protein of mol.wt. about 20000 and the kinase proper of molecular weight about 100000. Dabrowska *et al.* (1977) have reported essentially similar results with a myosin light-chain kinase preparation from chicken gizzard muscle. Very recently both groups of workers have concluded that the Ca^{2+}-binding protein component which activates the kinase is similar to the protein calmodulin that is widely distributed in many tissues (Dabrowska *et al.*, 1978; Yagi *et al.*, 1978). This protein was previously known as the modulator protein, troponin C-like protein, cyclic nucleotide phosphodiesterase activating protein or calcium-dependent regulator protein.

The present paper reports a reinvestigation of the nature of the myosin light-chain kinase system of rabbit skeletal muscle. We conclude that the whole myosin light-chain fraction used as substrate in our earlier studies (Pires & Perry, 1977). contained rabbit skeletal-muscle calmodulin in amounts adequate to saturate the enzyme at higher Ca^{2+} concentration. The preparation and properties of a calmodulin-type protein from rabbit skeletal muscle that activates the kinase in equimolar amounts is described. The molecular weight of the myosin light-chain kinase enzyme is confirmed as about 80000. Some aspects of the work have been briefly reported elsewhere (Perry *et al.*, 1978).

Materials and Methods

Preparation of Ca^{2+}-binding proteins

Troponin C and troponin I were prepared from rabbit white skeletal muscle by the method of Perry & Cole (1974) from the troponin complex isolated by the method of Ebashi *et al.* (1971).

Bovine brain calmodulin was obtained by the method of Watterson *et al.* (1976) or by the method of Grand *et al.* (1979).

Myosin light-chain kinase

The enzyme was isolated by the method of Pires & Perry (1977). When 100μg of the preparation was examined by polyacrylamide-gel electrophoresis in sodium dodecyl sulphate it migrated as a single band of mol.wt. about 80000.

The enzyme was assayed as described by Pires & Perry (1977) with either the whole light-chain fraction of myosin or the P light chain as substrate. Initial rates were determined by incubating portions (0.1 ml)

of the reaction mixture for different intervals over a period of 2–10min. The reaction was stopped with 2vol. of 10M-urea/bovine serum albumin (10mg/ml) and the protein immediately precipitated with 2.5ml of cold 5% (w/v) trichloroacetic acid. The incorporation of ^{32}P into the light-chain fraction was determined on the washed precipitate.

In the specificity studies troponin I, histone type IIAS [Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.], 5,5′-dithiobis-(2-nitrobenzoic acid)-treated myosin and 5,5′-dithiobis-(2-nitrobenzoic acid)-extracted P light chain prepared by the method of Weeds (1969) and phosphorylase kinase prepared by the method of Cohen (1973) were incubated with myosin light-chain kinase in the presence of at least 20-fold molar excess of calmodulin. Conditions were otherwise as described above.

Preparation of rabbit skeletal-muscle calmodulin

Freshly minced rabbit white skeletal muscle (300–600g) was extracted in 3vol. of 20mM-sodium phosphate buffer (pH6.0)/4mM-EDTA for 30min at 4°C. After centrifugation at 2000*g* for 30min, the supernatant was filtered through glass wool and applied to a DEAE-cellulose column (3cm×45cm) equilibrated with the extraction buffer. The low ionic strength prevented extraction of troponin C, and EDTA increased the yield of calmodulin (Watterson *et al.*, 1976).

The column was washed with 350ml of extraction buffer and a linear gradient of 0–0.7M-NaCl in the buffer was applied by using mixing chambers of 350ml capacity, and 8ml fractions were collected. The position of elution of calmodulin was determined by gel electrophoresis of samples (50–100μl) in 8M-urea, pH8.6. The fractions containing the protein were eluted at a conductivity of 15–18mmho and were pooled and adjusted to 50% saturation with solid $(NH_4)_2SO_4$ at pH7.0. The supernatant obtained after centrifugation at 10000*g* for 15min was adjusted to pH4.0 with 1M-HCl, and the precipitate collected after further centrifugation at 10000*g* for 15min and dissolved in 2ml of 20mM-NH_4HCO_3 (pH8.6). The solution was applied to a Sephadex G-100 column (2cm×120cm) equilibrated against 20mM-NH_4HCO_3 and 4ml fractions were collected. The proteins were read at 215nm and the largest peak, eluted between 180 and 240 ml, was pooled and freeze-dried (Fig. 1). This consisted of pure skeletal-muscle calmodulin.

Preparation of dephosphorylated myosin P light chain

The whole light-chain fraction of myosin was prepared from rabbit fast skeletal muscle and partially pure P light chain isolated by ethanol fractionation (Pires & Perry, 1977). The ethanol-precipitated

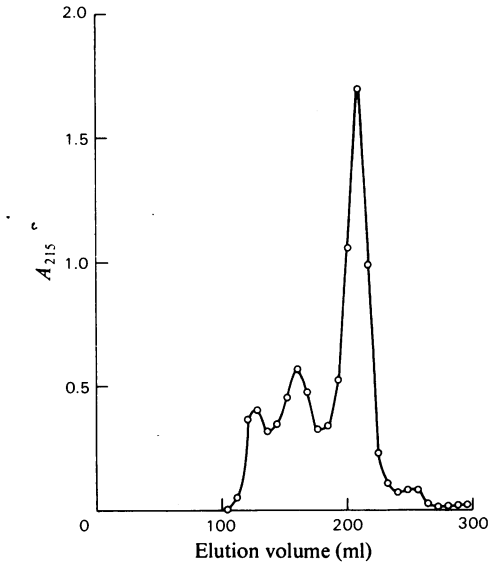

Fig. 1. *Gel filtration of crude calmodulin fraction on Sephadex G-100*

The calmodulin-rich fraction obtained by precipitation in 50%-satd. $(NH_4)_2SO_4$ at pH4.0 (see the Materials and Methods section) was dissolved in 2ml of 20mM-NH_4HCO_3 (pH8.6) and applied to a Sephadex G-100 column (2cm×120cm) and eluted with the same buffer; 4ml fractions were collected. The fractions eluted between 180 and 240 ml contained pure calmodulin and were pooled.

protein (300mg) was dissolved in 15ml of 50mM-Tris/40mM-HCl (pH7.6)/5M-urea/15mM-β-mercaptoethanol and dialysed against 1 litre of the same buffer. The dialysed sample was applied to a column (2cm×100cm) of DEAE-Sephadex A-25 equilibrated with the same buffer. A linear gradient of 0–0.3M-KCl in the buffer was immediately applied by using mixing chambers of 250ml capacity. The eluent was pumped at 0.5ml/min and 5ml fractions were collected. The position of the pure P light chain in the eluate was identified by gel electrophoresis in 8M-urea, pH8.6, and the fractions were pooled, desalted on Sephadex G-25 and freeze-dried.

A stock solution of P light chain was prepared by redissolving the protein (100mg) in 2ml of 50mM-Tris/40mM-HCl (pH7.6)/8M-urea and desalting on a Sephadex G-25 column (2cm×30cm) equilibrated in 20mM-NH_4HCO_3/10mM-β-mercaptoethanol (pH 8.6). The preparation was stored in batches (0.5ml at about 10mg/ml) at −20°C.

Bovine brain cyclic AMP phosphodiesterase

A partially purified enzyme was prepared from bovine brain by the method of Wang & Desaí (1977).

The first fraction obtained from DEAE-cellulose was dialysed against 50mM-Tris/40mM-HCl (pH 7.6)/1mM-$CaCl_2$/10% sucrose and portions were stored at −20°C. Phosphodiesterase activity was assayed by a two-stage process involving production of AMP and release of P_i by using snake-venom 5′-nucleotidase (Watterson *et al.*, 1976). Phosphate was determined by the method of Sannui (1974).

Actomyosin ATPase

Actomyosin was prepared by mixing myosin (3 parts by wt.) prepared by the method of Pires *et al.* (1974) with F-actin (1 part) prepared by the method of Spudich & Watt (1971) in 0.35M-KCl/50mM-β-mercaptoethanol/50mM-Tris/40mM-HCl, pH 7.6. The solution was dialysed against 25mM-Tris/HCl (pH 7.6)/50mM-β-mercaptoethanol for 4h and the precipitate obtained homogenized in an equal volume of water to give a homogeneous suspension.

The Ca^{2+}-sensitivity of the actomyosin in the presence of calmodulin, fast-skeletal-muscle troponin I and tropomyosin was determined in the presence of the latter two proteins prepared as described by Amphlett *et al.* (1976) under the conditions also described by the latter investigators.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was carried out as described by Perrie *et al.* (1973) in the following conditions: (1) 10% polyacrylamide/0.1% sodium dodecyl sulphate in 85mM-Tris/400mM-boric acid, pH 7.0; (2) 10% polyacrylamide/25mM-Tris/80mM-glycine, pH 8.6, in the presence or absence of 8M-urea.

Conductivity

Conductivity measurements were carried out with a Radiometer (Copenhagen, Denmark) conductivity meter type CDM 2E.

Ca^{2+} buffers

Ca^{2+}–EGTA buffers were prepared as previously described (Pires & Perry, 1977) by using the association constant $K_a = 5.76 \times 10^7 M^{-1}$ at pH 7.6 (Charberek & Martell, 1959).

Protein determinations

Protein was assayed by a micro method involving precipitation by tannin (Mejbaum-Katzenellenbogen & Dobryszycka, 1959) by using bovine serum albumin as standard, and by amino acid analysis.

Amino acid analysis

Duplicate samples of protein were hydrolysed in 6M-HCl for 24h and analysed as described by Wilkinson *et al.* (1972). Proteins were analysed for trimethyl-lysine by using system C described by Kuehl & Adelstein (1969).

Results

Effect of bovine brain calmodulin on myosin light-chain kinase

With the whole light-chain fraction of myosin as substrate, the effect of bovine brain calmodulin on the activity of myosin light-chain kinase depended on the Ca^{2+} concentration at which the assay was carried out. At a Ca^{2+} concentration of 0.1μM the kinase activity, which in the absence of calmodulin was about 2–3% of that obtained at maximal activation, was increased 5–6-fold in the presence of 500-fold molar excess of the bovine brain calmodulin

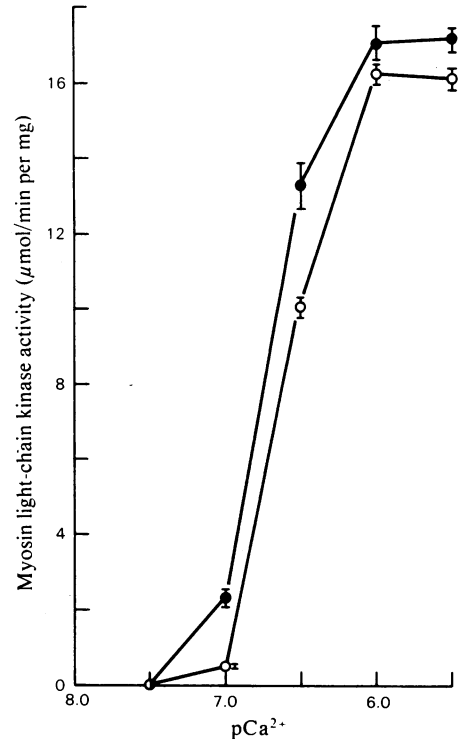

Fig. 2. *Effect of bovine brain calmodulin on the activity of myosin light-chain kinase at various Ca^{2+} concentrations*

Assay mixtures contained: 50mM-Tris/40mM-HCl (pH 7.6), 12.5mM-magnesium acetate, 2mM-EGTA, $CaCl_2$ at concentrations calculated to produce the required pCa^{2+}, 1mM-dithiothreitol, 5.5mM-[γ-^{32}P]-ATP (20μCi/ml), whole light-chain fraction (20mg/ml) myosin light-chain kinase (0.56μg/ml); where indicated bovine brain calmodulin (60.5μg/ml) was also added; temperature 25°C. Procedure was as described in the Materials and Methods section. Each point represents the average of 3–14 measurements of the initial rate, carried out on one preparation of the enzyme. Bars indicate ± S.E.M. ○, Myosin light-chain kinase; ●, myosin light-chain kinase + bovine brain calmodulin.

(Fig. 2). As the Ca^{2+} concentration increased the percentage increase in activity produced by calmodulin decreased as the myosin light-chain kinase activity increased. In the presence of 10μM-Ca^{2+}, calmodulin at a molar concentration 500 times that of the enzyme only increased the activity by about 10% (Fig. 2).

These results indicated that when the whole light-chain fraction was used as substrate the kinase did not require added calmodulin for full activity when saturated with Ca^{2+}, but did at lower, limiting Ca^{2+} concentrations. This suggested that possibly the whole P light-chain fraction used as substrate might contain a trace of calmodulin, which became limiting at lower Ca^{2+} concentrations. Detailed examination by electrophoresis in sodium dodecyl sulphate and 8M-urea indicated that no detectable amounts of calmodulin were present in the kinase preparation. The presence of calmodulin in the substrate could have possibly been an explanation of the apparently high substrate concentration (20mg/ml) required to saturate the enzyme. It is estimated that if the myosin light-chain fraction contained about 1 part of calmodulin per 100000 the kinase would be 80–90% activated. This extent of contamination would not be detected by electrophoresis either in sodium dodecyl sulphate or in the presence or absence of urea.

To test this hypothesis, P light chain isolated from the whole light-chain fraction by ethanol precipitation was further purified by chromatography on DEAE-cellulose in the presence of 5M-urea (see the Materials and Methods section). When P light chain purified in this way was used as substrate for the purified myosin light-chain kinase, activity was negligible in the absence of added bovine brain calmodulin. The increase in activity with increasing bovine brain calmodulin reached a plateau when about 1mol of calmodulin was present per mol of kinase. The activity obtained with equimolar calmodulin and kinase was about 80–90% of that obtained when 65-fold molar excess of calmodulin was present (Fig. 3). The activity did not increase further when more than 65-fold molar excess of calmodulin was added.

Calmodulin isolated from rabbit skeletal muscle (see below) was indistinguishable from the bovine brain protein in activating myosin light-chain kinase (Fig. 3). Slight differences in the activating abilities of the two types of calmodulin were occasionally observed, but these were no greater than the differences in activity obtained between different preparations of the same calmodulin protein. Troponin C isolated from skeletal muscle also activated myosin light-chain kinase, but was about 10% as effective on a molar basis as the calmodulin (Fig. 4). Although the possibility that part of this activity was due to contamination by calmodulin cannot be completely excluded, the latter protein was clearly not present as

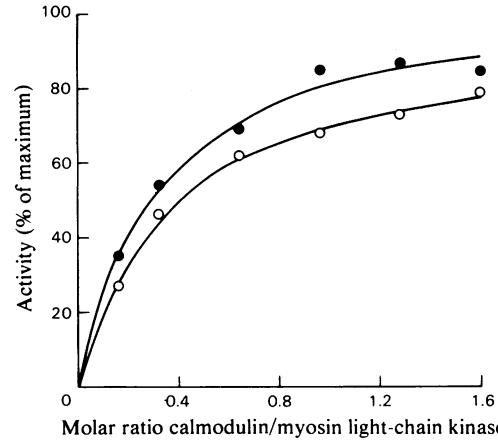

Fig. 3. *Effect of calmodulin on the activity of myosin light-chain kinase*

Assay mixtures contained: 50mM-Tris/40mM-HCl, pH7.6, 12.5mM-magnesium acetate, 0.1mM-$CaCl_2$, 1mM-dithiothreitol, 5mM-[γ-^{32}P]ATP (20μCi/ml), P light chain (1.6mg/ml), myosin light-chain kinase (0.14μg/ml), calmodulin in the molar ratios indicated. (Molecular weights were taken as 77000 and 16700 for the kinase and calmodulin respectively.) ^{32}P incorporation was measured as described in the Materials and Methods section. The enzyme activity is expressed as a percentage of the maximum activity (15–30μmol/min per mg) obtained with a 65-fold molar excess of bovine brain calmodulin. ○, Bovine brain calmodulin; ●, rabbit skeletal-muscle calmodulin.

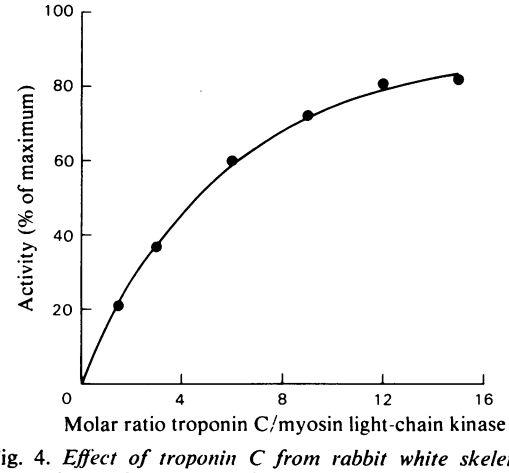

Fig. 4. *Effect of troponin C from rabbit white skeletal muscle on the activity of myosin light-chain kinase*

Assays were carried out as in Fig. 3. Molecular weight of troponin C is taken as 18000. Enzyme activity is expressed as a percentage of maximum activity (15–30 μmol/min per mg) obtained with a 65-fold molar excess of bovine brain calmodulin.

10% of the troponin C preparation. Examination of the troponin C preparation by electrophoresis at pH8.6 in the absence and presence of Ca^{2+} did not reveal the presence of calmodulin, the electrophoretic mobility of which is modified differently from that of troponin C under these conditions (see below, and Grand *et al.*, 1978).

Isolation and properties of rabbit skeletal-muscle calmodulin

The method, which was designed to minimize the extraction of troponin C, gave relatively low yields of calmodulin, about 5mg per kg of minced muscle. The ability of skeletal-muscle calmodulin to activate myosin light-chain kinase as effectively as bovine brain calmodulin strongly suggested that its structure was very similar to that of the latter protein. It migrated as a single band when 10–50μg was electrophoresed on polyacrylamide gel in sodium dodecyl sulphate, pH7.0, and 8M-urea, pH8.6. Under these conditions it had similar mobilities to troponin C and bovine brain calmodulin. It was, however, shown to be a calmodulin by the fact that on electrophoresis in 25mM-Tris/80mM-glycine, pH8.6, in the absence of urea it showed either little change or a decrease in electrophoretic mobility in the presence of Ca^{2+} compared with that in the presence of EGTA. This property is the converse of what is obtained with troponin C (Head & Perry, 1974) and is characteristic of all the calmodulins so far studied (Grand *et al.*, 1978) (Fig. 5).

The amino acid analysis of the skeletal-muscle calmodulin was almost identical with that of bovine brain calmodulin and significantly different from troponin C, especially in the serine/threonine ratio (Table 1). It also contained 1mol of trimethyl-lysine/mol, a feature of all calmodulins so far studied. Further evidence for its great similarity to bovine brain calmodulin was shown by the similarity of the band pattern obtained on electrophoresis of the peptides obtained after digestion of the two proteins with CNBr (R. J. A. Grand, personal communication).

The biological activities of skeletal-muscle calmodulin could not be distinguished from those of the bovine brain protein. Both proteins possessed very similar ability to activate phosphodiesterase (Fig. 6). When added to an equimolar amount of fast-skeletal-muscle troponin I, skeletal-muscle calmodulin neutralized the inhibitory activity of the troponin I on the ATPase of rabbit skeletal-muscle actomyosin (Fig. 7). In the presence of EGTA the neutralizing effect disappeared. In this property of restoring full Ca^{2+}-sensitivity to the system, skeletal-muscle calmodulin behaved exactly like bovine brain calmodulin and not like troponin C (Amphlett *et al.*, 1976). For full restoration of Ca^{2+}-sensitivity in similar systems in which calmodulin is replaced by troponin C, troponin T must also be added.

Specificity of myosin light-chain kinase

In the original studies carried out on the specificity of the myosin light-chain kinase (Pires & Perry, 1977), the requirement of the enzyme for calmodulin was not known. It is possible therefore that the reported inability of the purified kinase to phosphorylate substrates such as casein, histone, phosphorylase *b* or troponin at significant rates was due to the absence of calmodulin from the assay system rather than to the extreme specificity of the enzyme.

When tested under standard assay conditions con-

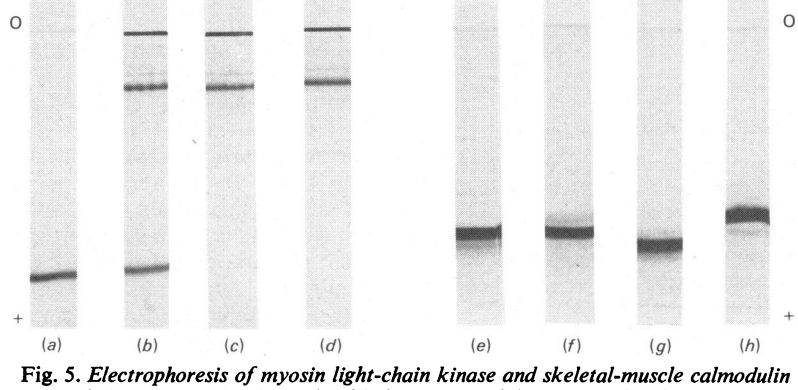

Fig. 5. *Electrophoresis of myosin light-chain kinase and skeletal-muscle calmodulin*

Electrophoresis was carried out on 8% polyacrylamide in 25mM-Tris/80mM-glycine (pH8.6). Proteins were isolated from rabbit white muscle unless otherwise stated. O, Origin. Samples: (*a*) 10μg of bovine brain calmodulin + 2mM-$CaCl_2$, (*b*) 10μg of bovine brain calmodulin + 50μg of myosin light-chain kinase + 2mM-$CaCl_2$, (*c*) 50μg of myosin light-chain kinase + 2mM-$CaCl_2$, (*d*) 50μg of myosin light-chain kinase + 2mM-EGTA, (*e*) 30μg of calmodulin + 2mM-$CaCl_2$, (*f*) 30μg of calmodulin + 2mM-EGTA, (*g*) 30μg of troponin C + 2mM-$CaCl_2$, (*h*) 30μg of troponin C + 2mM-EGTA.

Table 1. *Amino acid analysis of calmodulin from rabbit skeletal muscle*

Analyses (residues/molecule) are averages of four analyses carried out on one preparation and two on another preparation of skeletal-muscle calmodulin. A mol.wt. of 16700 was used to calculate the residue numbers. References: [1] Cohen *et al.* (1978); [2] Vanaman *et al.* (1977); [3] Collins *et al.* (1977).

Amino acid	Skeletal-muscle calmodulin	Phosphorylase kinase calmodulin subunit [1]	Bovine brain calmodulin[2]	Fast-skeletal muscle troponin C [3]
Asp	24.7 (25)	24	23	22
Thr	11.5 (12)	12	12	6
Ser	5.2 (5)	5	4	7
Glu	29.4 (29)	27	27	31
Pro	1.8 (2)	2	2	1
Gly	11.1 (11)	11	11	13
Ala	10.8 (11)	11	11	13
Val	7.0 (7)	7	7	7
Met		8	9	10
Ile	8.5 (9)	8	8	9
Leu	10.3 (10)	9	9	9
Tyr	1.8 (2)	2	2	2
Phe	8.2 (8)	7	8	10
His	1.7 (2)	1	1	1
Lys	7.2 (7)	7	7	9
Arg	6.5 (7)	6	6	7
Trimethyl-lysine	0.6 (1)	1	1	-

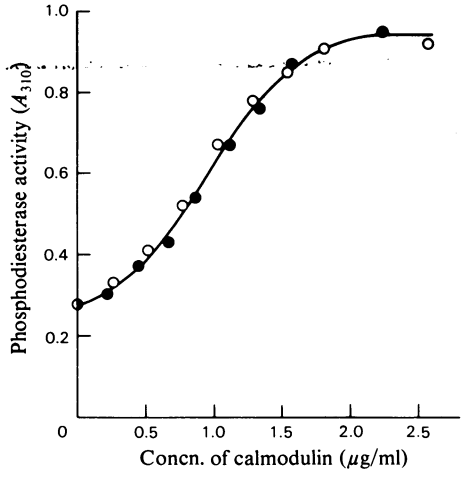

Fig. 6. *Effect of calmodulin from skeletal muscle and bovine brain on the activity of bovine brain cyclic nucleotide phosphodiesterase*

Reaction mixtures (0.5ml) contained 40mM-Tris adjusted to pH8.0 with 11.6M-HCl, 0.4mM-$MnCl_2$, 1.0mM-$CaCl_2$, 2mM-cyclic AMP, bovine brain phosphodiesterase (150μg) and calmodulin as indicated. Incubations were carried out for 5min at 30°C and stopped by boiling. Snake-venom 5′-nucleotidase [10μl, 8 units(μmol/min)/ml] was added and incubated for 10min at 30°C before being stopped by the addition of 0.15ml of 55% (w/v) trichloroacetic acid. After centrifugation at 2000*g* for 10min, P_i was determined in the supernatant. ●, Bovine brain calmodulin; ○, rabbit skeletal-muscle calmodulin.

taining calmodulin (see the Materials and Methods section) histone type IIAS was phosphorylated by the pure kinase at the rate of 0.2–0.3μmol of P/min per mg of enzyme, i.e. about 1% of the rate of phosphorylation of P light chain from rabbit white skeletal muscle under similar conditions.

Rabbit fast-muscle troponin I was phosphorylated very slowly by the myosin light-chain kinase system (about 1% of that obtained with the P light chain as substrate). Preliminary results indicated that myosin light-chain kinase did not phosphorylate phosphorylase kinase.

Although the purified kinase catalysed the phosphorylation of the preparation of myosin without the addition of calmodulin, this was not the case with myosin that had been extracted with 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) partially to remove the P light chain ('DTNB light chain'; Weeds, 1969). The procedure removed about 50% of the total P light chain. In the absence of calmodulin the unextracted P light chain could not be phosphorylated by myosin light-chain kinase. It is considered that the usual preparations of myosin contain adequate calmodulin to activate the kinase, and the extraction with 5,5′-dithiobis-(2-nitrobenzoic acid) removes this protein as well as the P light chain.

Interaction of calmodulin and myosin light-chain kinase

On application of the purified myosin light-chain kinase to a Sepharose–calmodulin column (Watterson & Vanaman, 1976) in 1mM-$CaCl_2$/50mM-Tris (pH

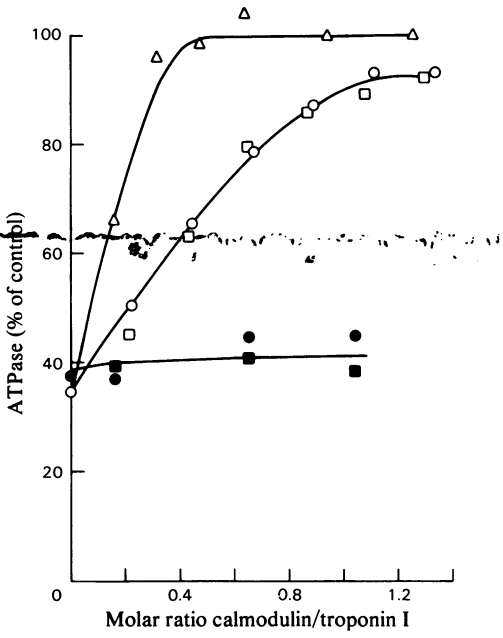

Fig. 7. *Effect of rabbit skeletal and bovine brain calmodulin on Ca^{2+} sensitivity of rabbit skeletal-muscle actomyosin ATPase*

ATPase assays were carried out in 25mM-Tris, adjusted to pH7.6 with 11.6M-HCl, 5mM-ATP, 2.5mM-$MgCl_2$ with 530μg of actomyosin suspension (see the Materials and Methods section), 56μg of tropomyosin and 45μg of troponin I, total volume 2ml, incubated at 25°C for 5min. Activities are expressed as a percentage of that obtained in the absence of troponin I. Rabbit skeletal-muscle troponin C (△), bovine brain calmodulin (□) and rabbit skeletal-muscle calmodulin (○) were added to give the molar ratios indicated. Filled-in symbols represent results obtained after addition of 2mM-EGTA.

8.0)/4mM-dithiothreitol the enzyme was retained on the column. It could be subsequently eluted by the buffer in which the $CaCl_2$ was replaced by 2mM-EGTA. Such an affinity column could be used with advantage instead of the Sepharose-P light-chain column (step 6) in the preparative procedure described by Pires & Perry (1977). In this case step 7 can be omitted. Although the binding of the enzyme to the Sepharose–calmodulin column indicated the formation of a Ca^{2+}-dependent complex between the two proteins, such a complex could not be demonstrated on electrophoresis at pH8.6 (Fig. 5). Under these conditions a complex between calmodulin and troponin I is readily demonstrated (Grand *et al.*, 1978). In the latter case the complex is stable in urea concentrations up to 4M.

Discussion

Our investigations confirm the report of Yagi *et al.* (1978) and Dabrowska *et al.* (1978) that myosin light-chain kinase requires the presence of a calmodulin type of protein for full activity. They also confirm our earlier findings that the purified kinase preparation itself has no intrinsic calmodulin and has a mol.wt. in the region of 80000. A similar molecular weight for the purified kinase from rabbit skeletal muscle has now been obtained by Yagi and collaborators (K. Yagi, personal communication). In the presence of added calmodulin the enzyme has a specific activity of 15–30μmol of P transferred/min per mg of enzyme, which is significantly higher than the values reported by Yagi *et al.* (1978). It is now clear that the whole light-chain fraction of normal rabbit myosin preparations and the P light chain obtained by ethanol fractionation both contain enough calmodulin to activate the kinase fully when assayed at Ca^{2+} concentrations of 10μM or above.

The requirement for calmodulin and its presence in the substrate, possibly in limiting amounts, explains the high substrate concentrations needed to saturate the kinase and the variable values for the K_m that were reported earlier (Pires & Perry, 1977). With cruder enzyme preparations, which presumably contained adequate amounts of calmodulin and made the enzyme less dependent on that present in the substrate, low K_m values of 40–50μM were obtained (Perry *et al.*, 1976; Pires & Perry, 1977). We therefore consider that the true K_m for P light chain is in the region of 40–50μM as originally reported, or possibly even lower. Brain and skeletal-muscle calmodulin appear to be equally effective in activating the enzyme up to 80–90% when present in amounts equimolar with respect to the enzyme. In this they differ markedly from troponin C, which required to be present in a considerable molar excess for significant activation of the kinase. Although it was possible that part of the activity obtained with troponin C could be due to calmodulin contamination, the effect was too great to be solely due to the latter protein, as no calmodulin could be detected in the preparation of troponin C from fast skeletal muscle. Troponin C appears to be much less effective in comparison with calmodulin as an activator of cyclic nucleotide phosphodiesterase (Dedman *et al.*, 1977).

The total amount of calmodulin present in skeletal muscle is clearly of some importance. Calmodulin cannot be detected by electrophoretic analyses in the standard troponin C preparation obtained from whole rabbit white skeletal muscle (Grand *et al.*, 1978). Calmodulin would be expected to be isolated by the procedures used for troponin C preparation.

The calmodulin that we have described is probably identical with the Ca^{2+}-binding protein isolated by

Yagi *et al.* (1978) from rabbit skeletal muscle. Whereas troponin C is associated with the troponin complex in the I filament, most of the calmodulin appears to be extra-myofibrillar. Evidence exists for its association with the phosphorylase kinase complex (Cohen *et al.*, 1978) and with the specific calmodulin-binding proteins that have recently been described (Grand & Perry, 1979). Some calmodulin must also be associated with the myosin light-chain kinase, for the enzyme is active *in vivo* (Barany & Barany, 1977) and in extracts of whole muscle without further addition of calmodulin.

Yagi *et al.* (1978) report that rat skeletal muscle contains about 30mg of calmodulin per kg, which is considerably more than obtained by the procedure described in the present paper. Even if the higher value is taken as the calmodulin content of rabbit skeletal muscle, it only represents 2–3% of the amount of troponin C present (Head & Perry, 1974). In contrast, virtually all the Ca^{2+}-binding protein of smooth muscle is of the calmodulin type (Grand *et al.*, 1978), although the possibility of small amounts of troponin C being present in this tissue cannot be excluded.

The slow rates of phosphorylation of troponin I from rabbit fast skeletal muscle and histone IIAS confirm the high specificity of myosin light-chain kinase. It is possible that the low rates of the phosphorylation are due to interaction of the acidic calmodulin with the basic histone and the troponin I, which leads to the blocking of phosphorylation sites. This effect has been observed with phosphorylation of troponin I catalysed by other kinases in the presence of troponin C (Perry & Cole, 1974) and calmodulin behaves very similarly to troponin C in its interaction with troponin I (Amphlett *et al.*, 1976; Grand *et al.*, 1978; Vanaman & Perry, 1978). Whatever the explanation of the low rates of phosphorylation of these proteins, they are clearly much poorer substrates for myosin light-chain kinase than is the P light chain. In view of the similarity in the rates of phosphorylation of histone and P light chain by the protein kinase described by Waisman *et al.* (1978), it is unlikely that this enzyme is identical with myosin light-chain kinase.

The mode of action of calmodulin probably involves binding to the kinase. The formation of a specific complex is illustrated by the binding of the enzyme to a Sepharose–calmodulin column in the presence of Ca^{2+} and its release when the Ca^{2+} is removed by EGTA. Despite this evidence the complex does not appear to be sufficiently strong for it to migrate on electrophoresis at pH 8.6, under which conditions the troponin I–calmodulin complex migrates as a single well-defined band, even in the presence of 4M-urea (Grand *et al.*, 1978). This suggests that the binding constant for the kinase–calmodulin complex is much lower than that of the troponin I–calmodulin complex. A relatively low binding constant may also explain why a large molar excess of calmodulin with respect to the kinase produces an increase in kinase activity.

A number of common features exist between the function of calmodulin and troponin C. For example, the activation of myosin light-chain kinase by Ca^{2+} mediated by calmodulin is comparable with the activation of the myofibrillar ATPase through Ca^{2+} binding to the homologous protein, troponin C. Recent research indicates that Ca^{2+}-binding proteins may be widely involved in enzyme systems using ATP as substrate and which require Ca^{2+} for activity. Certainly calmodulin appears to be a component of many, if not all, of the Ca^{2+}-requiring protein kinases. In addition to myosin light-chain kinase and the general protein kinase (Waisman *et al.*, 1978), it has also been shown to be essential for the activity of a general protein kinase in brain (Schulman & Greengard, 1978), phosphorylase kinase (Cohen *et al.*, 1978) and glycogen synthetase kinase (P. Cohen, personal communication). Thus it would appear that regulation of the myofibrillar ATPase through the Ca^{2+}-binding protein, troponin C, represents a highly specialized form of a general process of Ca^{2+} regulation in which calmodulin is normally involved.

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