CONVERSION OF CALCIUM-SENSITIVE MYOSIN LIGHT CHAIN KINASE TO A CALCIUM-INSENSITIVE FORM

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1. Introduction

There is a growing body of evidence which indicates that in smooth muscle, actomyosin ATPase activity and, thus by inference, the contractile activity is controlled by a phosphorylation of myosin by a specific myosin light chain kinase (MLCK) and a dephosphorylation by a phosphatase. An understanding of excitation–contraction coupling in smooth muscle and a variety of other non-muscle contractile systems will require a detailed understanding of the regulation of these enzymes.

In smooth muscle of gizzard [1–3], was deferens [4], pig stomach [5] and cow stomach [6] MLCK is found whose activity is controlled by micromolar changes in calcium ion concentration. However, in some other contractile systems such as platelets [7] and proliferative myoblasts [8] the activity of MLCK shows no calcium sensitivity. The only kinases that have been characterized are those from platelets [7] and gizzard [9]. The platelet enzyme appeared to be functional as a single component having mol. wt ~80 000. This kinase was not calcium sensitive. The gizzard kinase, on the other hand, required 2 subunits for activity and was reponsive to changes in calcium concentration. The 2 subunits of this enzyme had mol. wt 105 000 and 17 000.

We have been studying the oxytocin-dependent contractile system in mammary gland. Actomyosin can be extracted in good yield from either lactating or involuted glands [10] and in both cases contains a platelet-like MLCK whose activity is not altered by changes in calcium concentration. We have further found, however, that a calcium-sensitive MLCK loses its calcium control within a few seconds when mixed with mammary actomyosin. It is thus converted to a platelet type of enzyme during the incubation. The MLCK does not appear to lose any activity during this conversion.

2. Materials and methods

2.1. *Protein preparations*

Unless otherwise indicated all preparations were done at 4°C. Actomyosin was extracted from minced mammary tissue washed free of milk and other fluids with 0.15 M NaCl. The washing consisted of stirring the minced residue in about 6 times the residue volume of the salt solution, then sedimenting at 500 × *g* for 5 min. This was repeated 5 times. The washed tissue residue was stored at −20°C for a period of time in 50% glyceril, 60 mM KCl, 20 mM imidazole, pH 7.0, 2 mM dithiothreitol, 0.1 mM phenylmethyl sulfonyl fluoride. There was no detectable difference between actomyosin prepared from fresh tissue and that which had been stored for up to 6 weeks in the freezer. To extract actomyosin the residue was homogenized with a polytron (Brinkmann Instr.) in a medium containing (mM): 20 ATP; 1 EDTA; 1 EGTA; 40 imidazole, pH 7.0; 1 cysteine; 60 KCl [11]. The extraction mixture was centrifuged at 150 000 × *g* for 1 h, then 1 M $MgCl_2$ was added to final conc. 35 mM. Upon setting overnight actomyosin was precipitated and the precipitate was collected and washed in 60 mM KCl, 10 mM imidazole, pH 7.0, each time by centrifugation at 5000 × *g* for 10 min.

Myosin light chain kinase was extracted from

gizzard myofibrils [11] by homogenization in 15 mM $MgCl_2$, 60 mM KCl, 20 mM imidazole, pH 7.0. The supernate of a 5000 × *g* centrifugation was used as a source of kinase and was used without further purification.

The 20 000 dalton (L_{20}) light chain of mammary myosin was isolated by preparative Na-dodecyl sulfate (SDS) gel electrophoresis in a 3 mm polyacrylamide slab using the system in [12]. A small of phosphorylated light chain and was mixed with actomyosin and served as tracer for the light chain purification. The light chain was eluted from the gel slab using the reservoir buffer without SDS. Fractions were counted by Cerenkov counting and those containing the L_{20} were lyophilized. After dialysis against 3 M urea, 1 mM dithiothreitol, 10 mM imidazole, pH 7.0, the residual SDS was removed by adsorption on a Biorad AG 2-X8 ion-exchange column in the Cl^- form. The light chain was eluted from this resin with a gradient of 0.0–1.0 M NaCl in 3 M urea, 10 mM imidazole, pH 7.0, 2 mM dithiothreitol.

2.2. *Phosphorylation assays*

The transfer of ^{32}P from [^{32}P]ATP to the L_{20} was determined by trapping trichloroacetic (TCA) precipitates on glass fiber filters (Whatman GF-A) after incubation. Briefly, the phosphorylation assay was initiated by simultaneously adding radioactive ATP and kinase (or equivalent buffer) to actomyosin. The final concentrations of reactants were 4 mg/ml actomyosin, or 40 μg/ml light chain, 0.1–0.2 mM ATP (74 000–148 000 cpm/nmol), 7 mM $MgCl_2$, 30 mM KCl and 15 mM imidazole, pH 7.0. At various times aliquots were removed and precipitated with ice-cold TCA containing 1 mM ATP and 1 mM NaH_2PO_4. The filter was washed repeatedly with the same solution to remove non-protein-bound radioactivity, then counted by liquid scintillation in a Triton® X-100 based cocktail.

2.3. *Autoradiography*

At the end of some incubations an aliquot was prepared for SDS gel electrophoresis [12]. 12.5% acrylamide was used for the separating gel and 3% for the stacking gel. After running at low current overnight, the 0.75 mM gels were stained with Coomassie brilliant blue G-250 (Miles Lab.) [13,14], dried and exposed to X-ray film (Kodak NS-2T) for 4–48 h to identify protein bands containing radioactivity. The non-protein-bound radioactivity was run off the end of the gel during the electrophoresis.

Table 1
Calcium sensitivity of mammary actomyosin phosphorylation by endogenous mammary kinase and gizzard myosin light chain kinase[a,b]

		Protein bound ^{32}P (cpm)		Kinase activity ratio
Prep.	Kinase	EGTA	Ca^{2+} (10^{-4} M)	EGTA/Ca
1	Mammary	7300	4500	1.62
	Gizzard	72 000	52 000	1.38
2	Mammary	1750	1500	1.17
	Gizzard	55 000	44 000	1.25
3	Mammary	6300	5700	1.11
	Gizzard	49 000	51 000	0.96
4	Mammary	26 000	30 000	0.87
	Gizzard	126 000	165 000	0.76

[a] Phosphorylation measured after a 60 s incubation at 25°C
[b] The mammary kinase was endogenous in the preparation the gizzard kinase was isolated and added to the actomyosin

3. Results

Myosin light chain kinase is a ubiquitous contaminant of actomyosin preparations. In mammary gland actomyosin the endogenous MLCK consistently lacks Ca^{2+} control. This is true for actomyosin from either lactating or involuted glands from both rats and cows. In this respect it is similar to the MLCK in platelets and myoblasts. The activity in mammary actomyosin preparations is considerably lower than that in gizzard muscle and in an attempt to rapidly phosphorylate the mammary light chain we added gizzard kinase to the preparation. Surprisingly, when the Ca^{2+}-sensitive gizzard MLCK was added to the mammary actomyosin nearly the same amount of phosphorylation was detected in the absence (1 mM EGTA) or presence of Ca^{2+} (10^{-4} M). The results obtained with several different preparations of actomyosin are shown in table 1. As can be seen, in some cases there was actually greater phosphorylation in the absence than in the presence of Ca^{2+}. This is also seen in the data [8] with proliferative myoblast kinase.

Two experiments where the kinetics of phosphorylation were followed are shown in fig.1. In one case the activity in the absence of Ca^{2+} is consistently higher than in its presence. In the other, there initially is a slight inhibition in the absence of Ca^{2+} which gradually disappears. Although the amount of phosphorylation occurring when the gizzard kinase is added is greater than that where only endogenous kinase is present the patterns are very similar for the two preparations. When the gizzard enzyme was added the amount of maximum phosphorylation corresponds to 1 mol P/mol light chain.

Since we were measuring the phosphorylation by trapping the TCA precipitates we considered the possibility that we might be detecting the phosphorylation of a different protein. This is not the case as can be seen in fig.2. An aliquot from the experiments in fig.1c,d was prepared for SDS–gel electrophoresis and autoradiography. The only protein which shows any significant phosphorylation is the 20 000 dalton light chain. It is also obvious that there is no Ca^{2+} control. The gizzard myofibrils from which the MLCK was extracted is included to show that in that system the MLCK shows Ca^{2+} regulation.

Among other possibilities we considered that there might be a difference between mammary gland and smooth muscle light chains. This does not appear to be the case as is shown in table 2. When the isolated light chain from mammary actomyosin was tested with gizzard kinase its phosphorylation was calcium sensitive. Likewise Ca^{2+} sensitivity has been seen when light chains from gizzard or stomach muscles were used.

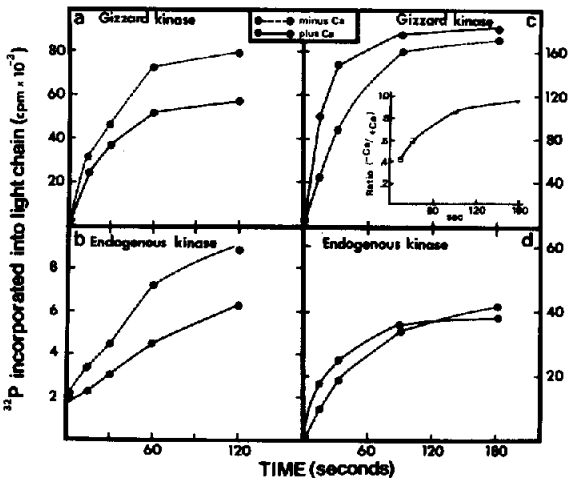

Fig.1. Time dependence of phosphorylation of mammary light chain by endogenous mammary kinase (b,d) and by added gizzard myosin light chain kinase (a,c). The data are from two different mammary actomyosin preparations and show two patterns of activity that have been seen.

4. Discussion

The situation of having myosin light chain kinases which exhibit Ca^{2+} control and others that do not raises questions about the specificity of the control system for Ca^{2+}. It is possible that a system of phosphorylation and dephosphorylation might respond to different intracellular signals than changes in Ca^{2+} concentration. Although our data showing a kinase without Ca^{2+} control agrees with that in platelets and proliferative myoblasts [7,8] such results must be interpreted with care. In view of the rapid loss of Ca^{2+} control when the gizzard enzyme was added to mammary actomyosin it seems likely that in those other cases where no Ca^{2+} control was seen the MLCK lost its Ca^{2+} control during the isolation. In other experiments designed to isolate mammary MLCK

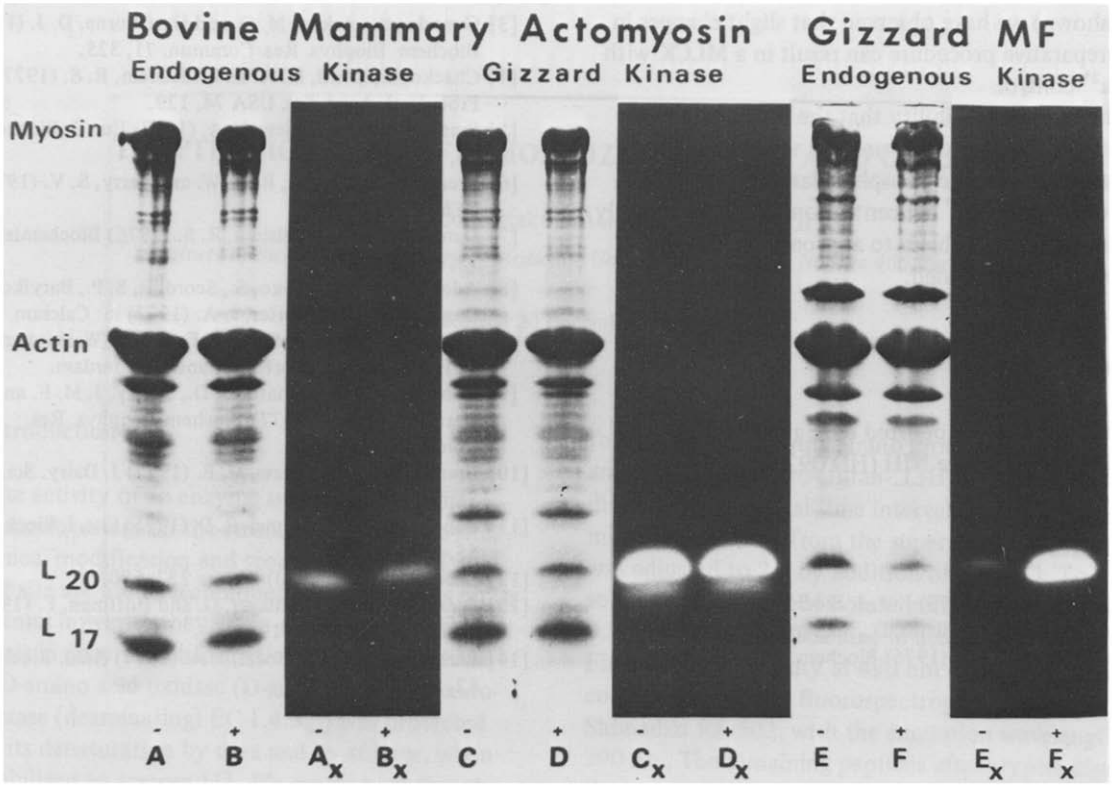

Fig.2. Autoradiography of SDS–slab gels of mammary actomyosin and gizzard myofibrils from which the MLCK was isolated. The dark panels (AX, BX, CX, DX, EX, and FX) are the contact negatives of the X-ray film to which the protein patterns with the same letter had been exposed. The (–) or (+) indicate that the incubation was done in the absence or presence of Ca^{2+}.

Table 2
Calcium sensitivity of phosphorylation of bovine mammary myosin light chain by gizzard light chain kinase[a,b]

		Protein bound ^{32}P (cpm)		Kinase activity ratio
Prep.	Kinase	EGTA	Ca^{2+} (10^{-4} M)	EGTA/Ca
1	Gizzard	112	1592	0.07
2	Gizzard	252	3210	0.08

[a] Notations as in table 1
[b] Light chains were isolated from actomyosin prep. 3, 4 in table 1

(not shown) we have observed that slight changes in the preparative procedure can result in a MLCK with no Ca^{2+} control.

It remains a possibility that the light chain can assume a particular conformation when attached to myosin which can be phosphorylated by the MLCK regardless of the Ca^{2+} concentration. This is technically a more difficult problem to approach and further experiments are needed.

Acknowledgements

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