A RELATIONSHIP BETWEEN Ca^{2+} SENSITIVITY AND PHOSPHORYLATION OF GIZZARD ACTOMYOSIN

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SUMMARY

Ca^{2+} regulated actomyosin from chicken gizzard is phosphorylated to a significant extent ($\sim$2.5 moles P per 10^6g. actomyosin) only in the presence of Ca^{2+}. The Ca^{2+} dependence of phosphorylation is similar to that shown by the ATPase activity. When the actomyosin is desensitized to the effects of Ca^{2+} the phosphorylating system is removed. Reconstitution of Ca^{2+} sensitivity is accompanied by an increase in the extent of phosphorylation. These results suggest a relationship between phosphorylation and the Ca^{2+} sensitive ATPase activity of gizzard actomyosin.

INTRODUCTION

All of the muscle systems which have been analyzed to date are regulated by changes in the Ca^{2+} concentration. The most extensively examined system is skeletal muscle in which regulation is achieved via the thin filament proteins, troponin, tropomyosin and actin. In smooth muscle our knowledge of the regulatory mechanism is at a much more fundamental level. For example a major point which remains to be established is whether or not a troponin-like protein is involved in the process. Ebashi and co-workers (1,2) and Carsten (3) are of the opinion that troponin is involved, whereas Bremel (4) has recently suggested that regulation by Ca^{2+} is a function of the myosin molecule.

In general our results (5) supported the contention of Bremel (4) that regulation in smooth muscle is myosin-linked. It was difficult, however, to rationalize all of our results on the basis of regulation by the myosin molecule alone, and it was suggested that an additional factor might be involved (5). The possibility that this might be troponin was considered unlikely, and it was tentatively suggested that a phosphorylating system could be implicated. Bremel and Sobieszek (6) have in fact demonstrated the phosphorylation of smooth muscle

myosin and they have suggested that the Ca^{2+} sensitive response is linked to a phosphorylating system.

It was our intention to analyze this hypothesis in more detail, and to investigate the relationship between phosphorylation and Ca^{2+} sensitivity. It is possible to desensitize smooth muscle actomyosin to the effect of Ca^{2+}, using a method developed by Sparrow and van Bockxmeer (7). This actomyosin can be reconstituted simply by the addition of proteins removed during the desensitization procedure (7,8). These preparations therefore offer a convenient way of testing whether or not phosphorylation is a requirement for Ca^{2+} sensitivity. The results presented below indicate that a connection does exist between the regulation by Ca^{2+} of smooth muscle actomyosin and phosphorylation.

MATERIALS AND METHODS

Gizzard actomyosin was prepared as described previously (8), and desensitized to the effect of Ca^{2+} by the procedure of Sparrow and van Bockxmeer (7). The protein eluted from the actomyosin during the first three dialysis stages was collected as the supernatant following centrifugation at approximately 10,000xg for 10 mins., combined and dialyzed against 0.5mM dithiothreitol, 10mM tris-HCl (pH 7.6) and its effect on the phosphorylation of desensitized actomyosin was assayed. This fraction will be referred to as Eluate I. The native tropomyosin-like fraction as described by Ebashi et al. (1) was also prepared. The original procedure was modified slightly as we used the fraction obtained between 37 and 55 percent ammonium sulfate saturation. This preparation was assayed for its effect on desensitized actomyosin and will be referred to as the P_{37-55} fraction.

ATPase activity was assayed under the conditions described previously (8).

Phosphorylation of actomyosin was measured using a procedure similar to that described by Daniel and Adelstein(9). The stock ATP solution consisted of 10mls 50mM ATP plus 100 μCi of γ-labelled [^{32}P] ATP (specific activity about 28 Ci per m.mole). The reaction mixture contained 1 to 5mg actomyosin in 10mM $MgCl_2$, 25mM tris-HCl (pH 7.6), 50mM KCl in a final volume of 2.0ml. Other additions are given in the legends. The effect of Ca^{2+} was monitored in this solution and in one containing in addition 1mM 2,2'-ethylenedioxybis[ethyliminodi(acetic acid)], EGTA. The solution was pre-incubated for 10 mins. at 25°C and the reaction started by the addition of stock ATP solution. For most experiments the final concentration of ATP was 2.5mM, i.e. 1.0 μCi[^{32}P] ATP per assay. The reaction was stopped after 10 or 15 minutes by the addition of 2ml 10% trichloracetic acid, 2% sodium pyrophosphate. The samples were then heated at 90°C for 20 mins., cooled and filtered through Millipore filters (1.2μ porosity). Each assay tube was washed 3 times with several volumes of 5% trichloracetic acid, 1% sodium pyrophosphate and care was taken to transfer all the precipitated protein to the filter. Each filter was then washed 5 times with this acid-pyrophosphate solution, and 5 times with distilled H_2O. The filters were transferred to scintillation vials and dried at 50°C for 3 hours. The extent of ^{32}P incorporation was estimated using the scintillation fluid described by Fricke (10). A Nuclear Chicago Isocap 300 scintillation counter was used.

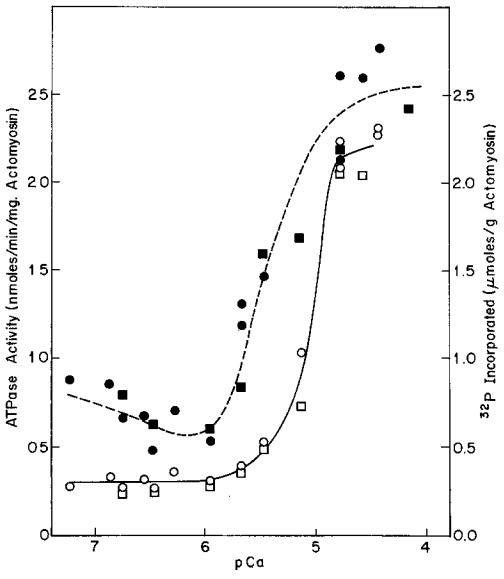

Fig. 1. The Ca^{2+} dependence of the Mg^{2+} activated ATPase activity and phosphorylation of gizzard actomyosin. Solid symbols indicate ^{32}P incorporation, and open symbols the ATPase activity. Two different experiments are represented by the circles and squares. Assay conditions for both ATPase activity and ^{32}P incorporation as follows: 10mM $MgCl_2$, 50mM KCl, 25mM tris-HCl (pH 7.6), actomyosin approximately 1.0mg/ml. CaEGTA and EGTA varied. The total concentration of EGTA was 1mM. The apparent binding constant for CaEGTA was taken to be $1.7 \times 10^7 M^{-1}$.

RESULTS AND DISCUSSION

The Mg^{2+} activated ATPase activity of gizzard actomyosin over a range of Ca^{2+} concentrations is shown in Fig. I. At low Ca^{2+} concentrations ($<10^{-6}M$) the ATPase activity was low and as the Ca^{2+} concentration was increased the ATPase activity was activated. Also shown in this figure is the extent of phosphorylation of the actomyosin over the same Ca^{2+} range. It is significant that a similar Ca^{2+} dependence was seen for both the phosphorylation and ATPase activity. At Ca^{2+} levels where maximum ATPase activity was observed about 2.5 moles phosphate was incorporated per 10^6g actomyosin. This is equivalent to approximately 1.5 moles phosphate per mole myosin (taking the molecular weight of myosin as 4.6×10^5 daltons and assuming that the content of myosin in the actomyosin is 75%).

The time dependence of phosphorylation of Ca^{2+}-sensitive gizzard acto-

Table I

^{32}P incorporation into desensitized actomyosin and the effect of reconstitution of Ca^{2+} sensitivity

The assay conditions for phosphorylation and ATPase activity are as described under Materials and Methods.

Sample*	^{32}P incorporation (μmoles/g. actomyosin)		Mg^{2+}-ATPase activity (nmoles/min./mg actomyosin)	
	$+Ca^{2+}$	$-Ca^{2+}$	$+Ca^{2+}$	$-Ca^{2+}$
GDAM	0.22	0.22	9.9	8.3
GDAM	0.28	0.16	7.4	5.7
GDAM	0.36	0.24	6.4	5.3
GDAM (3.0mg) plus eluate I (0.15mg)	0.72	0.22	12.8	8.4
GDAM (2.2mg) plus eluate I (0.19mg)	1.22	0.33	9.5	5.6
GDAM (3.0mg) plus P_{37-55} (0.33mg)	1.55	0.14	17.1	5.6

*Abbreviations used: G DAM, gizzard desensitized actomyosin; eluate I, protein removed during the desensitization process; P_{37-55}, fraction corresponding to the native tropomyosin from gizzard as described by Ebashi et al. (1).

myosin showed that after approximately 2 minutes a maximum level was attained, and this remained constant for up to 20 minutes. These results were obtained at 2.5mM ATP.

When the Ca^{2+} sensitivity of the actomyosin was reduced using the procedure of Sparrow and van Bockxmeer (7) the extent of phosphorylation also was significantly reduced (Table I). It is interesting that the extent of incorporation into the desensitized actomyosin is less than that for the Ca^{2+} sensitive actomyosin in the absence of Ca^{2+}. This suggests that the kinase responsible for the phosphorylation is not completely inhibited in the absence of Ca^{2+}. Under identical conditions the extent of ^{32}P incorporation into rabbit skeletal natural and desensitized actomyosin was less than 0.2 mole per 10^6g. actomyosin.

It is known that the reconstitution of gizzard desensitized actomyosin can be achieved by the addition of those proteins removed during the desensitization process (7,8). This is illustrated in Table I. The important point, however, is that increase in Ca^{2+} sensitivity was accompanied by a higher extent

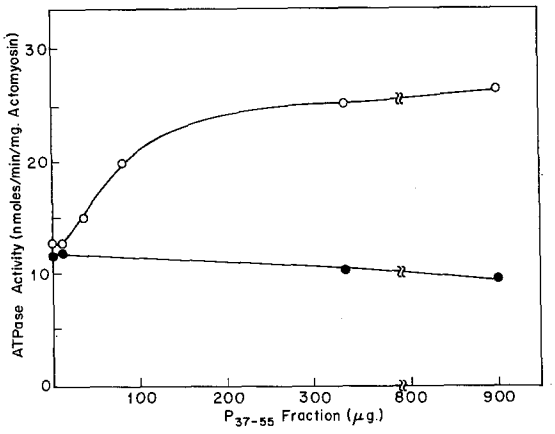

Fig. 1 The effect of the P37-55 fraction (gizzard native tropomyosin) on the Ca^{2+} sensitivity of desensitized gizzard actomyosin. Assay conditions as in Fig. 1, (O) absence of EGTA, (●) presence of EGTA, 1mM. 1.6mg actomyosin/ml.

of ^{32}P incorporation. The reconstitution of Ca^{2+} sensitivity was found also when the native tropomyosin from gizzard (the P_{37-55} fraction) as described by Ebashi et al. (1), was used. This is shown in Table I and in more detail in Fig. 2. It is clear that the addition of the P_{37-55} fraction to desensitized gizzard actomyosin resulted in a significant increase of Ca^{2+} sensitivity and thus confirms the observations of Ebashi et al. (2). It is also apparent (Table I, Fig. 3) that this effect is accompanied by an increase in the level of phosphorylation of actomyosin, but only in the presence of Ca^{2+}. The reconstitution of Ca^{2+} sensitivity is reflected, therefore, in both the ATPase activity and the extent of ^{32}P incorporation.

The data presented above shows that Ca^{2+} sensitive actomyosin from chicken gizzard can be phosphorylated and that this follows a similar Ca^{2+} dependence to the Mg^{2+} activated ATPase activity. The removal of the phosphorylating system on desensitization of the actomyosin and its recovery on reconstitution of Ca^{2+} sensitivity implies a link between phosphorylation and the Ca^{2+} regulated ATPase activity.

The simplest and most attractive hypothesis which is suggested from these results is that phosphorylation of a component of the actomyosin (probably myosin)

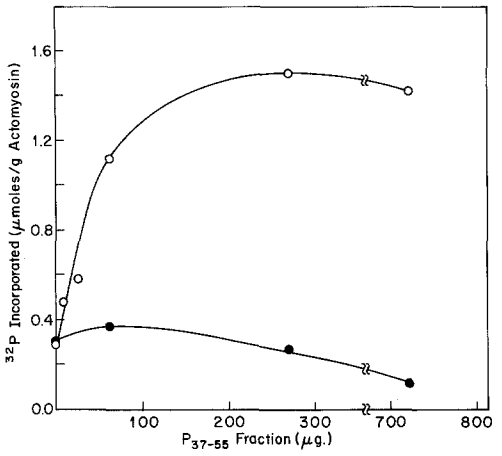

Fig. 3. The effect of the P37-55 fraction (gizzard native tropomyosin) on the phosphorylation of desensitized gizzard actomyosin. Assay conditions as in Fig. 2.

is an essential feature of the regulatory mechanism. This cannot be concluded however, until it is established that the apparent increase of ATPase activity associated with the increased extent of phosphorylation is not a reflection of a cyclic kinase-phosphatase system. It might be proposed that Ca^{2+} activates a kinase which phosphorylates the actomyosin and that this serves as the substrate for a phosphatase. This "pseudo" ATPase activity therefore would not be associated with the true actomyosin ATPase activity (c.f. 11). There is no good evidence at present to favor either possibility and this is an important point which must be clarified.

The protein most likely to be phosphorylated in the gizzard actomyosin is myosin (6) and there are several instances where this has been described using different systems (see review, 11). One interesting report by Adelstein and Conti (12) which is pertinent to this discussion describes the phosphorylation of platelet myosin by a kinase found in platelets. This is one of the few examples where a functional alteration accompanies phosphorylation, and it is shown that the actin activated ATPase activity of the platelet myosin is increased on phosphorylation of the myosin molecule. No Ca^{2+} requirement is demonstrated.

In another article by Pires et al. (13) a Ca^{2+} dependent light chain kinase is described although its function is not clear. In our results elements of these two reports are combined in that the phosphorylation is Ca^{2+} dependent and accompanied by an apparent activation of ATPase activity.

Our findings do not resolve the controversy of whether or not troponin is present in smooth muscle, but they do raise the interesting point that the fraction suggested by Ebashi et al. (1) to be native tropomyosin does in fact contain a kinase. This kinase appears to be responsible for the apparent Ca^{2+} sensitivity of gizzard actomyosin. Whether or not this is the only regulatory factor involved remains to be established.

ACKNOWLEDGMENTS

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