A Protein Factor Inhibiting the Magnesium-Activated Adenosine Triphosphatase of Desensitized Actomyosin

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1. The preparation and properties of a myofibrillar protein factor which inhibits the Mg^{2+}-activated adenosine triphosphatase of desensitized actomyosin is described. 2. This factor had negligible effect on the Mg^{2+}-activated adenosine triphosphatase of natural actomyosin and on the Ca^{2+}-activated adenosine triphosphatases of desensitized actomyosin and myosin. 3. The Mg^{2+}-activated inosine triphosphatase activity of desensitized actomyosin was not affected by the factor. 4. The inhibitory effect was sensitive to ionic strength. In addition to their ionic effects Mg^{2+} and Ca^{2+} appeared to have a specific action in reducing the effect of the inhibitor. 5. F-actin reduced the inhibition whereas Bailey-type tropomyosin had little effect. 6. As far as can be judged from the reported experiments this factor is different from any of the previously described myofibrillar components.

It was noted by Perry, Davies & Hayter (1966) during investigations on the factor responsible for the sensitivity of the Mg^{2+}-activated ATPase‡ of natural actomyosin to EGTA, that on some occasions a different factor was obtained which inhibited the enzymic activity of actomyosin in the absence of EGTA. Certain properties of this inhibitory factor (IF) have been described by Hartshorne, Perry & Davies (1966), who reported that in addition to its action on the ATPase the factor also inhibited the superprecipitation of actomyosin suspensions. The inhibitory factor was shown to be specific for the Mg^{2+}-activated ATPase and effective only on synthetic actomyosin or on actomyosin which had been previously treated by a procedure (Perry *et al.* 1966; Schaub, Hartshorne & Perry, 1967*a*) which rendered its Mg^{2+}-activated ATPase insensitive to EGTA (desensitized actomyosin, DAM).

The present paper describes in detail the preparation and properties of the inhibitory factor, confirms its protein nature and provides evidence suggesting non-identity with the recognized protein components of the myofibril.

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‡ Abbreviations: ATPase, adenosine triphosphatase; EGTA, ethylenedeoxybis(ethyleneamino)tetra-acetic acid; DAM, desensitized actomyosin; IF, inhibitory factor.

METHODS

Preparation of the inhibitory factor. A myofibril suspension (20–30 mg./ml.) in 0·1 M-KCl–0·039 M-borate buffer, pH 7·0, prepared as described by Perry & Zydowo (1959), was extracted for 15 hr. at 0° with an equal volume of 1·2 M-KCl–0·08 M-$NaHCO_3$–0·02 MNa_2CO_3, pH 8·7. The whole extract was then exhaustively dialysed against 0·1 M-KCl–20 mM-tris–HCl buffer, pH 7·6. Dialysis against this buffer rather than that of lower ionic strength originally reported (Hartshorne *et al.* 1966) facilitated the subsequent $(NH_4)_2SO_4$ fractionation. The precipitate which formed on dialysis was removed by centrifugation at 20000*g* for 15 min., and the concentration of the supernatant obtained was adjusted if necessary with the dialysis solution to give $E^{1\,cm.}_{280}$ 4–6. This supernatant could be freeze-dried and stored at −20° for up to 2 months without appreciable loss of activity. However, the supernatant was always brought to 30% saturation by addition of solid $(NH_4)_2SO_4$ at 0° and the precipitate, which represented about 50% of the total protein in the supernatant, collected by centrifugation at 30000*g* for 15 min. The precipitated protein was dissolved in about 20 vol. of 10 mM-tris–HCl buffer, pH 7·6, and exhaustively dialysed against this solvent. After centrifugation at 100000*g* for 1 hr. the dialysed protein solution containing the inhibitory factor was used for the experiments described below. These preparations retained activity for up to 14 days at 0° and could be stored at −20° for up to 4 weeks. On thawing solutions frozen at −20°, up to 50% of protein was found to be insoluble and removed by centrifugation before use, but the protein in solution retained unchanged its inhibitory activity when related to total N.

Actomyosin preparations. Natural actomyosin was extracted by the method of Perry & Corsi (1958) and converted into desensitized actomyosin as described by Schaub *et al.* (1967*a*).

Myosin. Myosin was prepared by the method of Perry (1955) and converted into heavy meromyosin by chymotryptic digestion as described by Perry & Cotterill (1964).

Tropomyosin was prepared by the method of Bailey (1948). The method for the preparation of a low-ionic-strength extract of myofibrils (the 'soluble fraction') is described elsewhere (Perry & Corsi, 1958; Schaub *et al.* 1967*a*).

Actin. Actin was extracted from acetone-dried fibre prepared by the method of Straub (1943) as modified by Leadbeater & Perry (1963).

The acetone-dried fibre was extracted with 0·2mM-tris–ATP, pH 7·0, for 45min. at 0° and the clear extract separated by filtration was brought to pH 4·7 with 10mM-sodium acetate buffer. The precipitate was separated by centrifugation, dissolved by the addition of sufficient saturated $NaHCO_3$ solution to bring the pH back to 7·0 and the protein concentration adjusted to about 10mg./ml. with water. After clarification by centrifugation for 15min. at 18000*g*, 0·6mM-$MgCl_2$ was added. The solution was kept for 2hr. at 0° and F-actin sedimented by centrifugation for 90min. at 100000*g*. The pellet of F-actin was dispersed in 10mM-tris—HCl buffer, pH 7·6, with a hand-operated homogenizer and dialysed overnight against the same buffer. This actin preparation was free from EGTA-sensitizing factor.

Enzyme assays. ATPase activities were determined in a total volume of 2ml. at 25° in 2·5mM-tris–ATP–2·5mM-$MgCl_2$–25mM-tris–HCl buffer, pH 7·6. After preincubation for 5min. the reaction was started by the addition of approx. 0·6mg. of actomyosin. Unless otherwise stated activities were expressed as μg. of inorganic phosphorus liberated by 1mg. of actomyosin in 5min.

Estimation of protein concentration. The protein content of actomyosin solutions was determined by the biuret reaction (Layne, 1957), which was standardized by micro-Kjeldahl estimations of N. For other protein solutions $E_{280}^{1\,cm.}$ 1 was taken to represent 1mg./ml. for inhibitory factor and actin, 1·68mg./ml. for myosin, 1·55mg./ml. for heavy meromyosin and 3·0mg./ml. for tropomyosin.

Determinations of ATP and ADP. These were carried out by chromatographic separation as described by Hurlbert (1959).

Reagents. Tris–ATP was prepared by the method of Schwartz, Bachelard & McIlwain (1962) from the disodium salt supplied by C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. All other reagents were of analytical grade. Distilled deionized water was used throughout.

RESULTS

Origin of the inhibitory factor. Since the normal procedure for the isolation of the IF involved fairly rigorous conditions, the possibility existed that the IF was a modified form of one of the recognized myofibrillar proteins. In an attempt to throw some light on this aspect the effects of changing the extraction conditions on the yield of the factor were investigated. It was found that active inhibitor preparations could be obtained by extraction over the range pH 7·0–9·0, with no detectable differences in specific activity. The higher pH values, however, gave a more efficient extraction of the myofibril. Variations in the time of extraction of the myofibrils between 10min. and 24hr. also resulted in active preparations at all stages. Extraction times up to 12hr. improved the yield. For extraction times of less than 2hr., it was necessary to precipitate the actomyosin by dilution rather than by dialysis. This procedure produced large volumes of supernatant of low protein concentration and resulted in a considerable loss of protein. Shorter extraction times and lower pH lowered the possibility of the oxidation of protein thiol groups, but this did not appear to be a factor in IF preparation for the addition of 0·5mM-dithiothreitol (Cleland, 1964) to all the solutions used had no effect on the activity of the factor. Subsequent treatment of the isolated IF for periods of up to 24hr. with either 0·5mM-dithiothreitol or 0·1M-β-mercaptoethanol likewise did not change the inhibitory activity.

Since extraction of the myofibrils by solutions of high ionic strength was found to be essential for the isolation of the IF the possibility that this treatment in some way modified a known myofibrillar component to produce the IF could not be completely eliminated. This explanation seems unlikely, however, as active inhibitor could not be prepared from any of the following preparations: F and G forms of actin prepared by extracting muscle fibre at both 0° and 25°, myosin, tropomyosin and DAM.

The IF could not be isolated from sarcoplasm or from a crude granular fraction obtained by centrifugation at 100000*g* for 1hr. of a muscle medium homogenate in 0·025M-potassium chloride–0·039M-sodium borate buffer, pH 7·0, from which the myofibrillar fraction was initially removed by centrifugation at 600*g* for 15min. This suggests that it is associated with the myofibril rather than other parts of the muscle cell.

Generally inhibitor preparations could not be isolated from natural actomyosin although occasionally preparations of a very low activity were obtained. This was considered to be due to IF carried over from the myofibrils as a result of inadequate washing during the actomyosin preparation.

Nature of inhibitor. The activity of the inhibitor was destroyed when it was digested with one-fiftieth of its weight of trypsin for 30min. at 25°, pH 9·0. Further indications of its protein nature were given by the complete loss of activity after heating solutions in 10mM-tris–hydrochloric acid buffer, pH 7·6, for 15min. at 60°. Activity was also lost after prolonged storage at 0°.

A feature of the IF preparations was the decreased solubility which resulted from the addition of low concentrations of magnesium chloride to solutions of IF in 10mM-tris–hydrochloric acid buffer, pH 7·6. At 10mM-magnesium chloride–10mM-tris–hydrochloric acid buffer, pH 7·6, between 25% and 35% of the protein of the preparation was precipitated but no further precipitation occurred on increasing

the magnesium chloride concentration to 50mM. Most of the inhibitor activity was present in the precipitate and this resulted in approximately a twofold increase in specific activity. In the decrease of its solubility by magnesium chloride the IF resembled actin (Barany, Biro & Molnar, 1954), but preparations of the latter protein that had been submitted to the extraction procedure used for the preparations of IF were not significantly precipitated by 10mM-magnesium chloride.

Neither myosin nor tropomyosin could be converted into IF by the normal preparative procedure. The IF preparations had no detectable ATPase activity, nor could significant amounts of tropomyosin be detected in them when viscometric assays (Hartshorne *et al.* 1966) for this protein were carried out.

The inhibitor preparations were very heterogeneous as judged by their ultracentrifugation behaviour and it has not yet been possible to determine the amount of IF present in the standard preparation.

Properties. The inhibitory factor was effective only on the Mg^{2+}-activated ATPase of actomyosin preparations, the enzymic activity of which was insensitive to EGTA. The Ca^{2+}-activated ATPase of these systems was only very slightly affected by the inhibitor. The factor was assayed with DAM (Schaub *et al.* 1967*a*), but it was also effective on synthetic actomyosin (4 parts by wt. of myosin to 1 of actin), actomyosin desensitized to EGTA by trypsin treatment (Ebashi, 1963) and acto-heavy-meromyosin (2·7–8·1 parts by wt. of heavy meromyosin to 1 of actin). The Mg^{2+}-activated ATPases of natural actomyosin, rabbit skeletal muscle granules, myosin and heavy meromyosin were not affected by the inhibitor. The ATPase activity of DAM in the absence of added magnesium chloride was also not affected.

Study of the time-course of the hydrolysis of ATP by DAM in the presence of inhibitor indicated that it was linear after 30sec., and although slower it was similar in form to that obtained in the absence of inhibitor (Fig. 1). The ADP and ATP concentrations after incubation in the presence and absence of inhibitor were what would be expected from the inorganic phosphate liberated.

Under identical ionic conditions ITP was split by DAM at a lower rate than ATP but its hydrolysis was not inhibited by the factor (Fig. 2).

Fig. 3 illustrates that under otherwise standard conditions the decrease in liberation of inorganic phosphate produced by a constant concentration of the IF was greater the higher the DAM concentration. At low DAM concentrations the inhibition flattened off with increasing inhibitor concentrations implying saturation of the system. This effect was much less apparent as the DAM concentration increased. In view of these effects, assays of IF were performed with a constant DAM concentration. In spite of these precautions different IF preparations varied considerably in specific activity. The most active preparations gave a 50% inhibition of the control DAM ATPase activity with 0·4–0·6mg. of the IF per assay.

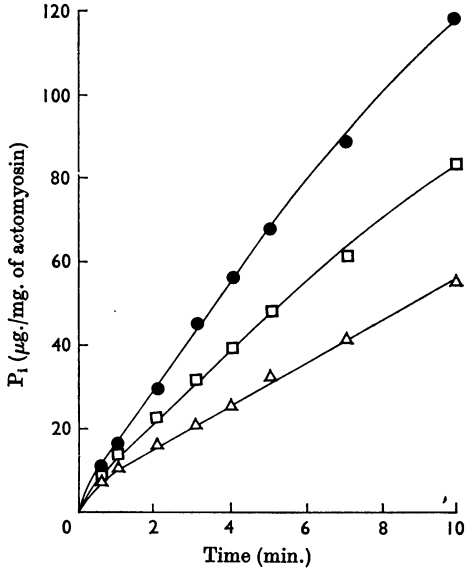

Fig. 1. Progress curve of the Mg^{2+}-activated ATPase of desensitized actomyosin in presence of inhibitor. Assay conditions: 2·5mM-$MgCl_2$–2·5mM-tris–ATP–25mM-tris–HCl buffer, pH 7·6; 0·31mg. of DAM/ml. ●, No IF; □, 0·31mg. of IF/ml.; △, 0·93mg. of IF/ml.

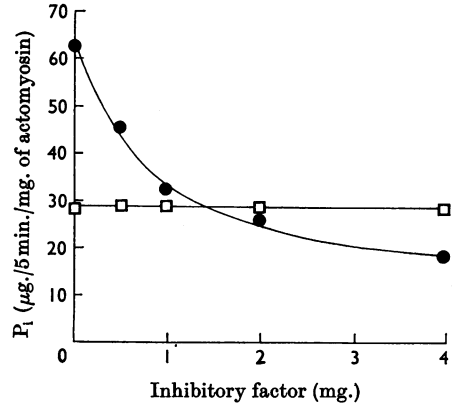

Fig. 2. Comparison of effect of IF with ITP and ATP as substrates. Assay conditions: 2·5mM-$MgCl_2$–2·5mM-tris–ATP (or ITP)–25mM-tris–HCl buffer, pH 7·6. IF as indicated in 2ml. assay volume. ●, ATP (0·64mg. of DAM per assay); □, ITP (1·28mg. of DAM per assay).

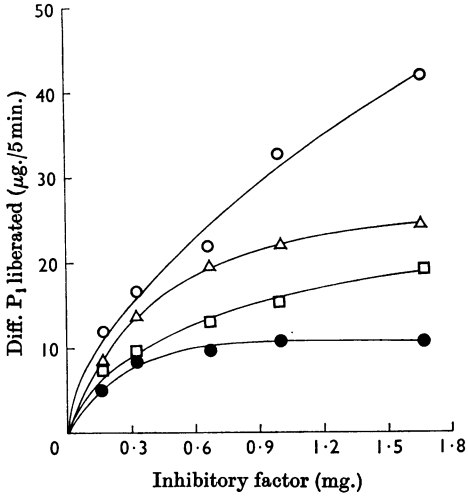

Fig. 3. Effect of different actomyosin concentrations on action of the IF. Assay conditions: 2·5mM-$MgCl_2$–2·5mM-tris–ATP–25mM-tris–HCl buffer, pH 7·6. IF as indicated in 2ml. assay volume. DAM per assay: ●, 0·21mg.; □, 0·42mg.; △, 0·63mg.; ○, 0·84mg. In the absence of IF the DAM concentrations used liberated 16·7, 31·6, 48·6 and 63·1 μg. of P_i in 5min. respectively.

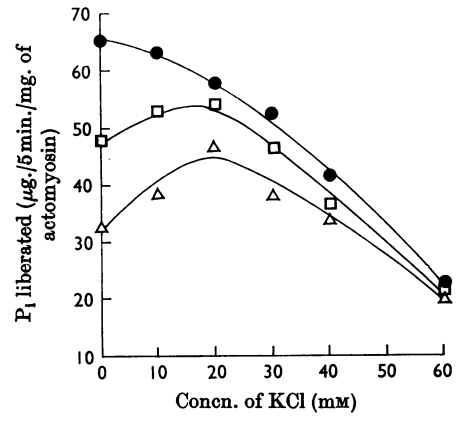

Fig. 4. Effect of increasing KCl concentration on the action of the IF. Assay conditions: 2·5mM-$MgCl_2$–2·5mM-tris–ATP–25mM-tris–HCl buffer, pH 7·6; 0·62mg. of DAM per assay. ●, No IF; □, 0·25mg. of IF per assay; △, 0·62mg. of IF per assay.

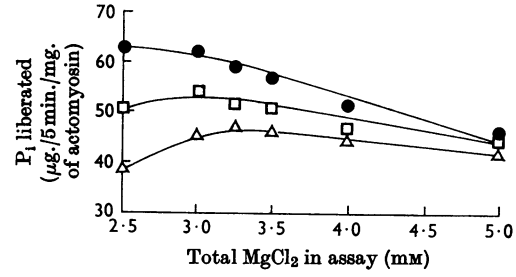

Fig. 5. Effect of increasing $MgCl_2$ concentration on the action of the IF. Magnesium chloride was added to basal assay medium containing 2·5mM-$MgCl_2$–2·5mM-tris–ATP–25mM-tris–HCl buffer, pH 7·6, and 0·64mg. of DAM per assay. Ionic strength was maintained at a constant value by the addition of compensating amounts of KCl. ●, No IF; □, 1·14mg. of IF per assay; △, 2·28mg. of IF per assay.

The extent of inhibition also depended on the ionic strength of the assay medium. It is shown in Fig. 4 that the inhibition decreased on increasing the potassium chloride concentration. Tris–hydrochloric acid buffer, pH 7·6, likewise reduced the effect of the IF. Precautions were therefore taken within any one series of assays to ensure a constant ionic strength.

Variation of pH in the range 7·6–9·0 caused no significant change in the degree of inhibition. Although the presence of Mg^{2+} was essential for inhibition, on increasing the magnesium chloride concentration above the 2·5mM normally used for assay the inhibitory effect was markedly decreased (Fig. 5). Calcium chloride was similarly effective in decreasing inhibition. This was a specific effect due to the bivalent cations as all assays were carried out at constant ionic strength.

Pretreatment of DAM with 1–2mM-calcium chloride and magnesium chloride before incubation also decreased the effect of the inhibitor (Fig. 6). This appeared also to be due to a direct effect of the bivalent cation on the DAM and not due to the small amounts of calcium chloride or magnesium chloride, which were added to the DAM and would be carried over into the assay tubes. Control experiments showed these additional amounts of bivalent cation were themselves ineffective when added to the assay medium before the untreated actomyosin. The treatment of DAM with potassium chloride (up to 15mM), calcium EGTA or magnesium EGTA, both at 2mM, did not decrease the inhibitory action of the factor. Pretreatment of DAM with 2mM-tris–ATP and 2mM-magnesium chloride was not so effective in decreasing the inhibitory effect of the factor as treatment with 2mM-magnesium chloride alone. This was possibly due to the competitive binding of the Mg^{2+} to the ATP.

Effect of other proteins. It has been reported (Schaub *et al.* 1967*a*) that a protein factor extracted from myofibrils at low ionic strength, the 'soluble fraction', contains the factor or factors responsible for restoring the properties of natural actomyosin to the desensitized preparation. As would be expected, the addition of the 'soluble fraction' to desensitized

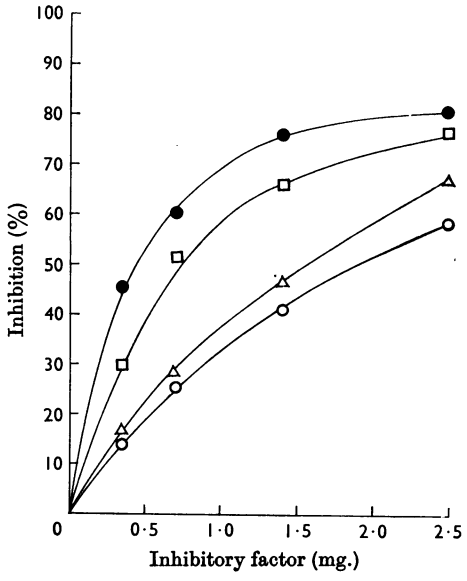

Fig. 6. Effect of pretreatment of DAM with $MgCl_2$ on the action of the IF. Assay conditions: 2·5 mM-$MgCl_2$–2·5 mM-tris–ATP–25 mM-tris–HCl buffer, pH 7·6; 0·62 mg. of DAM per assay; IF per assay as indicated. DAM was pretreated for 5 min. at 0° with $MgCl_2$ as indicated: ●, control; □, 0·5 mM-$MgCl_2$; △, 1·0 mM-$MgCl_2$; ○, 2·0 mM-$MgCl_2$.

actomyosin lowered the effectiveness of the inhibitor (Fig. 7). The DAM under these conditions resembled natural actomyosin, but the conversion was not complete as the inhibitor still had some effect on the ATPase.

The 'soluble fraction' contains tropomyosin (Perry & Corsi, 1958), which protein when prepared without the use of organic solvents (Ebashi, 1963) or in the presence of thiol compounds (Mueller, 1966) restores EGTA sensitivity to insensitive actomyosin preparations. The addition to 1 part of DAM of up to 2·5 parts of tropomyosin prepared by the Bailey (1948) method did not restore EGTA sensitivity nor did it modify the effect of the inhibitor on the Mg^{2+}-activated ATPase. Frequently, in the absence of EGTA, Bailey-type tropomyosin caused an activation of the Mg^{2+}-activated ATPase of DAM. This activating effect of tropomyosin did not prevent the inhibitor effect but merely increased the inorganic phosphate liberated both in the presence and absence of IF. With tropomyosin in large excess, i.e. about 5 parts by wt. of tropomyosin to IF, the inhibition was slightly diminished. The significance of this result was not clear but it would be expected if the tropomyosin possessed a slight amount of EGTA-sensitizing factor activity.

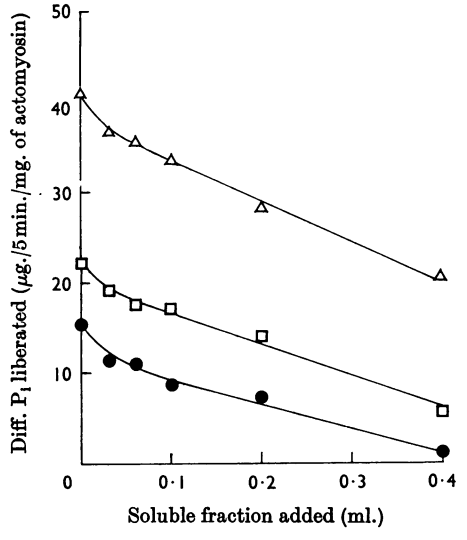

Fig. 7. Effect of 'soluble fraction' on the action of the IF. Assay conditions: 2·5 mM-$MgCl_2$–2·5 mM-tris–ATP–25 mM-tris–HCl buffer, pH 7·6; 0·64 mg. of DAM per assay. Amount of 'soluble fraction' ($E^{1\,cm}_{280}$ 3·3) and IF added as indicated per assay. Control values from which the differences in enzymic activity were calculated obtained with all additions except IF. ●, 0·31 mg. of IF; □, 0·62 mg. of IF; △, 1·53 mg. of IF.

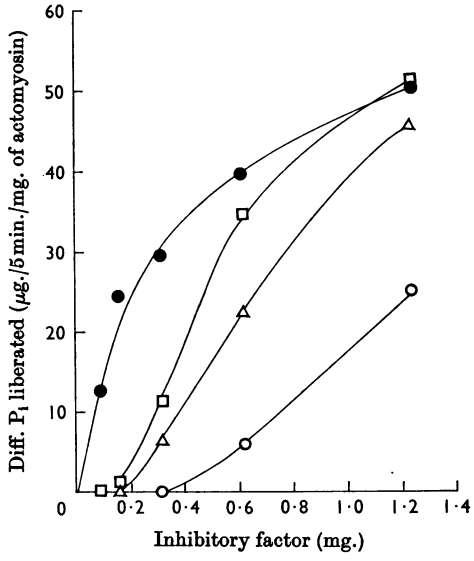

Fig. 8. Effect of actin on the action of the IF. Assay conditions: 2·5 mM-$MgCl_2$–2·5 mM-tris–ATP–25 mM-tris–HCl, pH 7·6; 0·64 mg. of DAM per assay. Amounts of IF added per assay as indicated. F-actin was added in 0·5 ml. of 10 mM-tris–HCl, pH 7·6, per assay. Control values from which the differences in enzymic activity were calculated were obtained with all additions except IF. ●, No actin added; □, 0·14 mg. of actin per assay; △, 0·28 mg. of actin per assay; ○, 0·70 mg. of actin per assay.

In the presence of EGTA, tropomyosin, which increased the Mg^{2+}-activated ATPase of DAM, had no effect whatsoever on the normal inhibition curve. After prolonged storage or freezing tropomyosin frequently lost the property of stimulating the Mg^{2+}-activated ATPase of DAM. Such stored preparations had no effect on the action of the IF.

On addition of increasing amounts of F-actin to a constant amount of DAM the inhibitory action of a given amount of IF was progressively decreased (Fig. 8). This effect could be explained either by the binding of IF to actin or competition between actin and the inhibitor for sites on the myosin.

DISCUSSION

The stability and properties of the IF indicate that it is protein in nature and since it could only be prepared from the washed myofibrillar fraction it is reasonable to suppose that in the muscle it is confined to the myofibrillar structure. Of the three major myofibrillar components it is most like actin in its salting-out characteristics with ammonium sulphate and its precipitation by low concentrations of magnesium chloride. Nevertheless when F- and G-actin were subjected to procedures simulating those used in the preparation of the IF they either had no effect or produced a stimulation of the Mg^{2+}-activated ATPase of DAM. Also, in contrast with actin (Barron, Eisenberg & Moos, 1966), the IF has no inhibitory action on the ATPase of myosin in the absence of added bivalent cations.

The non-identity with tropomyosin and myosin is apparent from the properties of the IF preparation. In addition absence of significant amounts of tropomyosin in the IF preparations is confirmed by the lack of effect of the IF on the Ca^{2+}-activated ATPase of DAM. Elsewhere it has been shown that low concentrations of tropomyosin (30–50 μg. per assay) will cause marked inhibition of the Ca^{2+}-activated ATPase of DAM (Schaub, Hartshorne & Perry, 1967*b*).

Direct comparison of the IF with other minor components of the myofibril reported in the literature is not easy, for often these components are not well characterized and in most cases have not yet been tested on desensitized actomyosin. Nevertheless it seems unlikely that it is identical with α-actinin (Ebashi & Ebashi, 1965) from its difference in behaviour on the superprecipitation of actomyosin. Likewise the effects of α- and β-actinin on the ATPase activity of actomyosin (Maruyama, 1966) bear little resemblance to those of the IF.

The relation of the IF to the EGTA-sensitizing factor is less clear, but the possibility exists that it is a modified form of this factor. On this hypothesis (Perry, 1967) the IF would represent a form of the EGTA-sensitizing factor which has been irreversibly changed so that its inhibitory action is no longer controlled by the Ca^{2+} concentration, with the result that it always functions as an inhibitor. It must be stated that as yet there is little evidence that the IF and EGTA-sensitizing factor are related in this way, for as judged by the conditions for extraction, the solubilities of the two factors are different. Also, unlike most preparations of the EGTA-sensitizing factor, the IF contains little or no tropomyosin, although the precise role of the latter protein in the EGTA-sensitizing factor system has yet to be determined. The fact that the IF can only be demonstrated in preparations of actomyosin that no longer contain the EGTA-sensitizing factor may, however, be significant and suggests that both factors are effective on similar regions of the actomyosin complex.

From the investigations described above it can be concluded that the effect is highly specific and that Mg^{2+} and actin are essential for its action. It may also be concluded from the experiments describing the treatment of actomyosin with Mg^{2+} or Ca^{2+} before addition to the normal medium and those in which the concentration of Mg^{2+} within the assay medium was altered, that these bivalent cations are bound to the actomyosin in such a way that the inhibitor or its complex with Mg^{2+} is ineffective. If the inhibitor does bind Mg^{2+} the complex is not particularly stable as the cation was readily removed by dialysis. From the shape of the curves illustrating the effect of actin it is possible the inhibitor competes with actin for the interaction at the enzymic site of myosin which is essential for the Mg^{2+}-activated ATPase.

At this stage the precise significance of the inhibitory factor in the contraction–relaxation cycle cannot be assessed. Although there is as yet no direct evidence of a role *in vivo*, the specificity of its effect with regard to myosin, actin and ATP implies such a function. The evidence suggests non-identity with all the known components of the myofibril, but the possibility exists, although it remains to be demonstrated, that it is part of an as yet ill-defined complex of factors concerned with the regulation *in vivo* of the myofibrillar ATPase and contractile response.

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