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Smooth muscle myosin as a calmodulin binding protein. Affinity increase on filament assembly

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Summary

Smooth muscle myosin is normally copurified with myosin light chain kinase (MLCKase) and calmodulin (CM). We have now established the binding affinities and stoichiometries of these two components with respect to monomeric and filamentous myosin. The relative amounts of CM and MLCKase in fresh synthetic myosin filaments were approximately stoichiometrical but for both in a molar ratio to myosin of about 1 to 30 or less. A 10^7 dilution of filaments did not result in any significant decrease in the amount of endogenous MLCKase and CM except in the absence of Ca^{2+} when the CM content was reduced around five-fold.

Binding assays were performed with myosin depleted of CM and MLCKase by passage over melittin- and CM-affinity columns, arranged in tandem. For binding to myosin preassembled into filaments three classes of CM binding sites could be demonstrated. (1) A high affinity binding characterized by a dissociation constant of 20–30 nM and a rather low binding stoichiometry of below 1 to 500. (2) An intermediate affinity, characterized by a dissociation constant of 1.2 μM and 1 to 100 binding stoichiometry. (3) A low affinity with a $K_d > 10\mu$M and with an approximate 1 to 1 binding ratio relative to myosin. If CM was made available during filament assembly the high affinity binding predominated, with a stoichiometry in the presence of Ca^{2+} of about 1 to 50. The binding affinity but not the stoichiometry was reduced several fold by the removal of Ca, excluding a non-specific trapping of CM within the filament architecture. Collectively, these data demonstrate an independent and specific association of MLCKase and CM with myosin, that is strengthened by filament assembly.

Introduction

In many stimulus-induced cellular responses such as muscle contraction or cell motility Ca^{2+} plays a triggering role. Its target is commonly an intermediate reactant in the form of a Ca-binding protein. In skeletal muscle Ca^{2+} acts on the troponin–tropomyosin system where its binding to troponin C releases the inhibition of the actin–myosin interaction (see El-Saleh *et al.*, 1986; Zot and Potter, 1987). For many other cellular processes the functional intermediate is calmodulin (CM), a unique and highly conserved regulatory protein (Klee and Vanaman, 1982; Johnson and Mills, 1986).

In vertebrate smooth muscle it is CM and not troponin C that is involved in signal transmission. In the presence of Ca^{2+}, CM binds to myosin light chain kinase (MLCKase) which in turn activates myosin by phosphorylation of its regulatory light chain (see Kamm and Stull, 1985; Marston, 1982) The activity of MLCKase is fully CM-dependent and only phosphorylated myosin can cyclically interact with F-actin to produce contraction (Small and Sobieszek, 1980). The system is therefore myosin-linked although more recent studies suggest that CM may also be involved in smooth muscle in actin-dependent modulation of the actin–myosin interaction via its action on another CM-binding protein, caldesmon (Sobue *et al.*, 1982; Smith *et al.*, 1987). The exact role of caldesmon as well as the interrelationship between the two calmodulin-regulated systems is at present poorly understood.

In this respect, studies on the localization of CM in smooth muscle, in general, or on its binding to the major contractile proteins are of particular relevance. But so far only rather limited information is available and the conclusions are controversial. There are reports that CM may bind to myosin (Sobieszek, 1985b) but not to actin (Howe *et al.*, 1980) filaments. The binding of MLCKase to both thick and thin filaments has already been demonstrated (Dabrowska *et al.*, 1982; Sellers and Pato, 1984; Sobieszek, 1985a) but values for its affinity for myosin are in dispute. Furthermore, this latter binding appears to be independent of CM and Ca^{2+} although CM weakens the binding of MLCKase to myosin (Sellers and Pato, 1984). In the present studies the binding of CM to myosin was characterized in some detail. As is shown, the binding of this regulatory protein to myosin shows an interesting dependence on the polymeric state of the myosin molecule.

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Materials and methods

Enzyme preparations

Although turkey gizzard myosin was mainly used in this study, some experiments were performed with chicken gizzard or pig stomach myosin. The myosin was purified from freshly extracted actomyosin as described previously (Sobieszek, 1985a; Small and Sobieszek, 1983) using a myofibril-like preparation as starting material (Sobieszek and Bremel, 1975). Proteolytic activity commonly present in pig stomach myosin was removed by passing the myosin fraction (dissolved 45–60% ammonium sulphate pellet) through a 3.2 cm × 30 cm Fractogel hydrophobic column (TSK Butyl-650; Merk, Darmstadt, FRG). After precipitation as filaments and resuspension to a concentration of 20–40 mg ml^{-1}, 3 ml aliquots of the purified myosin were frozen in liquid nitrogen and stored at −70°C until use.

Myosin light chain kinase (MLCKase) was also extracted from the myofibrils with a buffer containing 25 mM $MgCl_2$ (Sobieszek and Small, 1977). It was purified as described in detail previously (Sobieszek and Barylko, 1984). Smooth muscle calmodulin was purified from 60% ammonium sulphate supernantants of the first wash of muscle mince (see Sobieszek and Bremel, 1975) and from the crude actomyosin that remained after myosin precipitation. The calmodulin precipitated from these supernantants at pH 4.2 was then purified by chromatography on Phenyl Sepharose 4B-Cl and DEAE sepharose 6B-CL (both of Pharmacia, Uppsala, Sweden) followed by gel filtration chromatography on AcA54 (LKB, France).

Binding experiments

For the measurement of CM binding to filamentous myosin two types of binding experiments were employed. In the first, increasing concentrations of ^{125}I-CM were added to a fixed amount of filamentous myosin resuspended in a BIS-TRIS buffer (containing in mM: KCl, 40; $MgCl_2$, 2; DTT, 0.5; imidazole, 10 and BIS-TRIS, 10 at pH 6.6). The assay volume was 0.6 ml with a myosin concentration between 15 and 25 mg ml^{-1}. After 15 min incubation at 25 °C the filaments were pelleted by 30 min centrifugation at 19,000 rpm. The concentration of free and bound CM was then determined from the amount of radioactivity present in supernantants and pellets respectively. A correction was made for the volume of supernantant 'trapped' in the pellet using a calibration curve made by pelleting known amounts of myosin in the presence of D_2O. The binding data were then presented in the form of Scatchard plots (1949).

In the second type of binding experiments ^{125}I-CM was made available during formation of myosin filaments. In this case it was added to myosin dissolved in 0.56 M KCl solution, also containing 2 mM $MgCl_2$, 0.5 mM DTT and 10 mM imidazole at pH 6.8. The filaments were then formed by slow dialysis as described earlier (Sobieszek, 1977a). After final dialysis against BIS-TRIS buffer at pH 6.6 (see above) 0.5ml aliquots of the ^{125}I-CM-filamentous myosin mixture were diluted (with the same buffer) to the required ^{125}I-CM concentration. The free and bound CM was then determined as in the first type of binding experiment.

Affinity columns and other procedures

Melittin and mastoparan were purchased from Sigma (St. Louis, MO). These as well as calmodulin were covalently linked onto CNBr-activated sepharose 4B (Pharmacia, Uppsala, Sweden), as recommended by the producer. For cost reasons larger size (2.2 × 7cm) affinity columns were prepared only from melittin and calmodulin. The columns were connected in tandem and dissolved myosin, or myosin fractions, were applied in the presence of $CaCl_2$ (0.1–0.5 mM) at a flow rate of 20 to 40 ml h^{-1}. After extensive washing with the 0.3 M KCl AA buffer (see below) the columns were eluted separately. Elution was carried out with the same buffer, in which $CaCl_2$ was replaced by 2 mM EGTA followed by a solution of 5 M urea, 10 mM EDTA at pH 10.7. Attempts to elute all CM and other proteins bound to the affinity columns under non-denaturating conditions (including 15% glycerol) were not successful.

Phosphorylation assays were carried out in a medium containing 60 mM KCl, 2 mM $MgCl_2$ 0.5 mM DTT and 10 mM imidazole at pH 7.2 (AA solution) to which $CaCl_2$ (0.1 mM) was added. The reaction was initiated by addition of γ^{32}P ATP(Mg) to a final concentration of 1.5–2.5 mM and terminated by addition of 50 µl (final volume 175 µl) of saturated guanidine hydrochloride solution. For samples analysed additionally by urea-glycerol gel electrophoresis the reactions were terminated by addition of 102 mg of solid urea (Sobieszek and Jertschin, 1986a), aliquots of 150 µl of the terminated mixture were spotted onto 2 × 4cm Whatman (Maidstone, England) 3 MM chromatographic paper and, after four TCA, two ethanol and two water washes, the paper pieces were counted, in water, in a scintillation counter.

Sodium dodecyl sulphate gel electrophoresis was carried out essentially according to Laemmli (1970) with some modification (Sobieszek and Jertschin, 1986b), and urea-glycerol-acrylamide gel electrophoresis was performed according to a method given in detail elsewhere (Sobieszek and Jertschin, 1986a). For analysis of CM containing samples 2 mM EGTA were added to the samples and 0.2 mM EGTA was included in the stacking gel and the running buffer. This resulted in good separation of CM from the myosin light chains but reduced slightly the separation between the light chains. It should be noted, however, that variable amounts of CM may be lost from acrylamide gels during fixation and staining. These losses which often resulted in 'hollow' CM bands, limited the detection of CM to amounts above 5 to 10 pmoles.

Protein concentration of myosin was measured using the Biuret method (Gornall *et al.*, 1948) calibrated with a Sigma (St. Louis, MO) protein standard. MLCKase and CM concentration was measured by absorption at 278 nm using extinction coefficients (0.1%) of respectively 1.1 and 0.18 (Adelstein and Klee, 1981).

Results

Determination of endogenous CM and MLCKase

Fresh smooth muscle myosin preparations are normally copurified with endogenous myosin light chain kinase (MLCKase) and calmodulin (CM). These endogenous contaminants could not be detected on normally-loaded electrophoresis gels of myosin (Fig. 1B, E) but their presence was evident from the high phosphorylation rates observed (see Fig. 3A). They could be demonstrated by electrophoresis using excessive sample loadings of appropriately enriched CM or MLCKase fractions (e.g. Fig. 5,

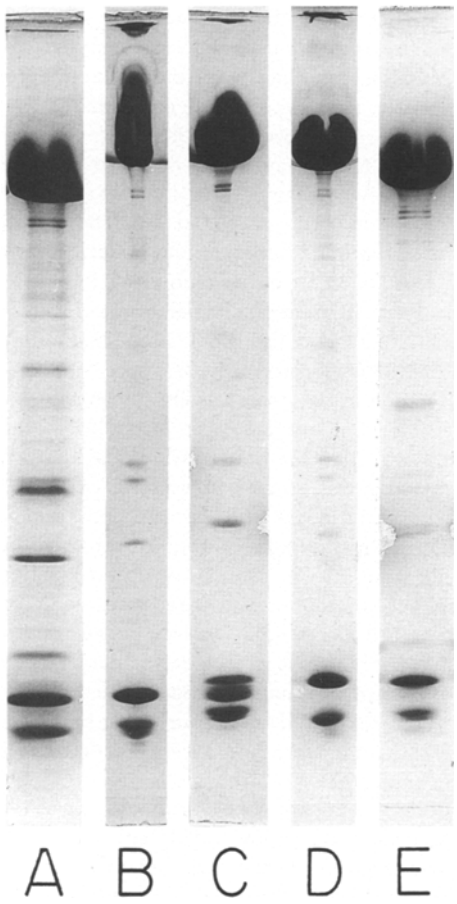

Fig. 1. (A) Turkey gizzard (TG) myosin fraction, containing endogenous CM, MLCKase and contaminating tropomyosin. Typical preparation used for melittin- and CM-affinity chromatography. The major bands (from the top) correspond to myosin heavy chain, tropomyosin (doublet) phosphorylatable (20kDa) and 17kD light chains of myosin. (B) Purified TG myosin containing relatively high amounts of endogenous CM and MLCKase. (C) Pig stomach (PS) myosin prepared exactly as for gizzard myosin (see Materials and methods). Note slight proteolytic degradation of the 20 kD light chain; this coincided with a major loss of endogenous CM and MLCKase (see also legend to Fig 1E). (D) 'Rinsed' TG myosin. Resuspended filamentous myosin, corresponding to gel B was diluted 2–3 fold, pelleted and resuspended to remove trace impurities. (E) PS myosin purified as in C except for an additional pass through a hydrophobic affinity column (see Materials and methods), which removed the proteolytic activity present. This myosin retained equivalent amounts of endogenous CM and MLCKase as its gizzard counterpart.

Cross & Sobieszek, 1985). With excessive sample loading CM could also be detected by using urea-glycerol gel electrophoresis (Fig. 2E-G).

Significantly, the molar amounts of MLCKase and CM were approximately the same. This was demonstrated by measurements of myosin phosphorylation rates with and without addition of CM and kinase. As shown in Fig. 3A addition of high amounts of MLCKase or CM did not produce any significant increase of the existing (endogenous) phosphorylation rates. When added together, CM and kinase produced an elevation of the phosphorylation rates and this amplification was used to estimate the endogenous amounts of CM–MLCKase complex (Fig. 3A). To facilitate more accurate measurements of the differences in phosphorylation rates assays were performed on ice (e.g. at 0–2 °C).

Aged myosin preparations had generally lower endogenous phosphorylation rates which correlated with a time dependent proteolytic degradation of the MLCKase. The degraded kinase was less tightly associated with filamentous myosin and normally remained in the supernantant after filament sedimentation. For such myosin preparations the phosphorylation rates increased two- to threefold, after the addition of the MLCKase alone, indicating that the myosin had lost 50–70% of its endogenous kinase but not CM.

Using this type of assay the molar ratio of CM to myosin could be estimated and was found to be rather variable depending on the type of myosin preparation. This ratio was as high as 1 to 20 for myosin extracted from myofibril-like preparations (Sobieszek and Bremel, 1975) and purified by ammonium sulphate fractionation followed by sedimentation of assemble filaments (Sobieszek, 1985a). When myofibrils were used that were preextracted for MLCKase, the CM–MLCKase content of the purified myosin was reduced about 10 times. However, even these latter amounts were sufficient to induce full phosphorylation of myosin at its first site within the first 20–40 s after addition of Ca^{2+} and ATP (Mg). Longer incubations resulted in a partial phosphorylation of the second threonine site (Fig. 2C; Cole *et al.*, 1985; Ikebe and Hartshorne, 1985) but this never exceeded 50% even for myosin extracted directly from myofibrils. Estimations of the endogenous CM content were also made by comparison of the intensity of CM bands obtained from myosin preparations with known amounts of purified CM on urea-glycerol gels (Fig. 2E-G). By this means the endogenous CM to myosin molar ratio was measured as around 1 to 30. More accurate estimates of the CM to myosin ratio were obtained using mastoparan affinity columns to separate endogenous CM from myosin. This column acted in very much the same way as the melittin affinity one described below, except that the bound CM could conveniently be eluted with an EGTA-containing buffer. These estimations confirmed the validity of the indirect kinetic estimations.

Lack of CM removal by dilution

As already indicated (see Materials and methods) CM extracted with actomyosin was specifically bound to myosin. The amount in the myosin fraction, prior to the first sedimentation of myosin filaments was essentially retained during the subsequent purification of myosin. Figure 4 shows that the removal of this endogenous CM

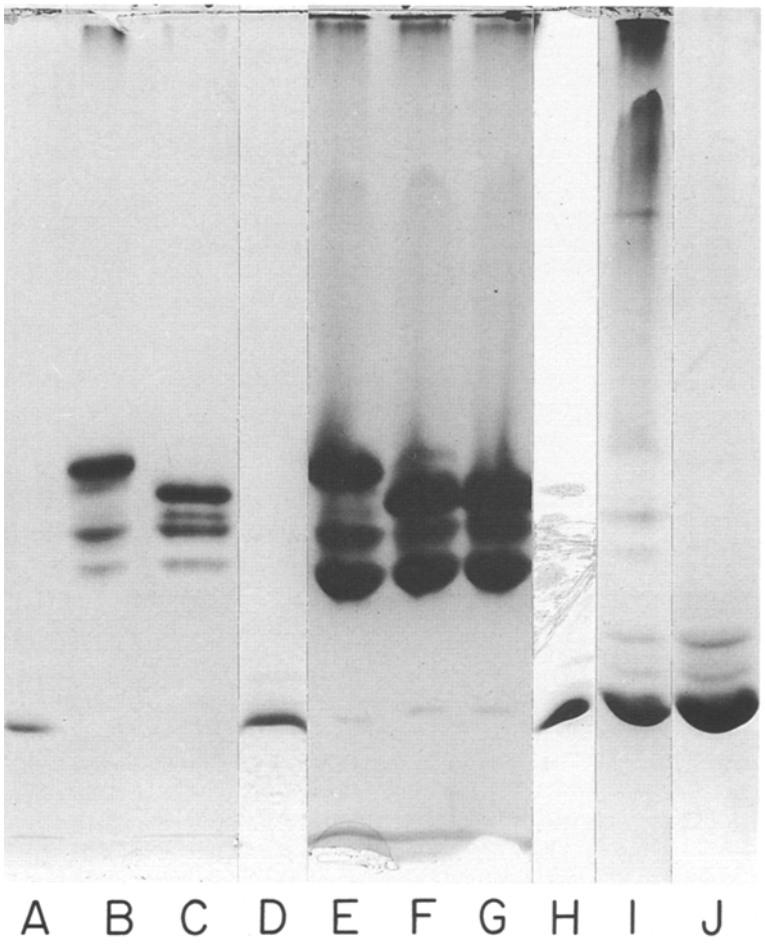

Fig. 2. Urea-glycerol electrophoresis of TG myosin and CM samples. (A) CM removed from purified TG myosin by mastoparan-affinity column and elution with Ca-free buffer as used in the estimation of the CM to myosin ratio. (B) unphosphorylated TG myosin. The upper most band corresponds to unphosphorylated 20 kD light chain and the lower bands to the two differently charged 17 kD light chain species. (C) phosphorylated TG myosin. Note a shift in position of the 20 kD light chain due to phosphorylation and the appearance of an additional lower band that corresponds to this light chain phosphorylated at its second site. (D) purified TG calmodulin. (E), (F), (G) detection of CM in purified TG myosin. (E) unphosphorylated myosin, (F) and (G) represent the same preparation after respectively 10 and 20s incubation with Ca^{2+} and (Mg) ATP. (H) and (I) CM removed from TM-myosin fraction by melittin-affinity column. (H) elution with Ca-free buffer and (I) subsequent elution with urea/EDTA buffer. (J) purified $PS^{125}I$-CM used in the binding experiments.

by dilution and sedimentation of myosin filaments was ineffective. By this means a twofold reduction of the CM content, in the presence of 0.1 mM $CaCl_2$, required a millionfold dilution carried out in six consecutive dilutions (Fig. 4A). The initial CM concentration was in the range of 1–2μM, therefore the apparent affinity of CM for filamentous myosin was in the nanomolar range. Since the MLCKase apoenzyme was also bound to filamentous myosin with the same high affinity (Fig. 4A), the question arose as to whether or not it was complexed with CM under these conditions. Similar experiments carried out in the presence of EGTA indicated, however, that this was not so. Thus a similar 10^7 fold dilution of the myosin in the absence of Ca resulted only in a fourfold reduction of the CM and MLCKase content (Fig. 4B). Since in solution, CM and MLCKase are not complexed in the absence of Ca^{2+} these data indicate an independent binding of these two components to filamentous myosin.

Removal of endogenous CM and MLCKase

As we have shown (Cross and Sobieszek, 1985) endogenous MLCKase can be separated from myosin by gel filtration under myosin folding conditions. Measurements of the control phosphorylation rates of such myosin, as described above, indicated at the same time that endogenous CM was likewise lost from folded myosin. In view of its limited solubility, only small amounts of folded myosin could however be subjected to gel filtration making purification of MLCKase- and CM-free myosin impractical by this means. A more efficient removal of the

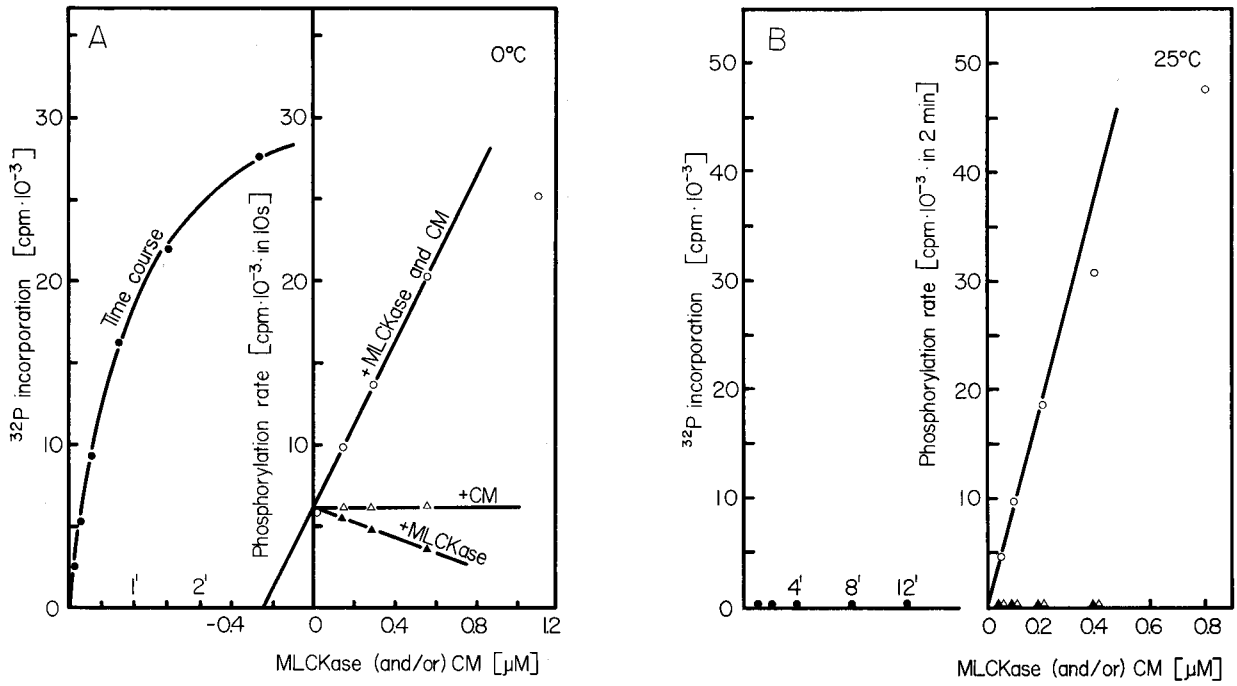

Fig. 3. Determination of endogenous CM and MLCKase content of myosin. The rates of ^{32}P incorporation of myosin were measured in the presence of increasing amounts of added CM (open triangles), MLCKase (closed triangles) or both (open circles). The relative amounts of endogenous CM and MLCKase were given respectively by the maximal rates in the presence of added MLCKase and CM. The absolute amount of endogenous MLCKase was then estimated from the linear range of the rate increase in the presence of added known amounts of MLCKase saturated with CM. Its value was given by the intercept of the linear range line on the negative side of the abscissa. Time course of ^{32}P incorporation for myosin with no added CM and MLCKase was also measured (closed circles). Myosin purified by filament precipitation (A) contained very high levels of CM and MLCKase. The assays were therefore carried out on ice (0 °C) with an assay time of 10 s. In the case of CM- and MLCKase-free myosin (B) all the phosphorylation rates (except those with added CM/MLCKase) were low and could readily be measured with an assay time of 2 min at 25 °C. Myosin concentration was 32 and 40 μM in (A) and (B) respectively.

endogenous components was achieved by application of myosin, dissolved in a buffer of moderate ionic strength, on melittin- and CM-affinity columns arranged in tandem. In the presence of Ca^{2+}, melittin, which has a very high affinity for CM (Anderson and Malencik, 1986), effectively removes CM from myosin (Fig. 5A, F, G). CM-free myosin may then be passed through a second CM-affinity column to remove the MLCKase (Fig. 5C, I). Care was taken not to exceed the capacity of the affinity columns, especially the melittin column which rapidly became saturated with calmodulin. When the capacity of this column was lower relative to that of the CM-affinity column the resulting myosin was free of MLCKase but not of CM. When myosin was applied onto the melittin-affinity column alone the myosin was almost completely depleted of CM but not of MLCKase. This indicated that even in the presence of Ca, interaction of CM with MLCKase associated with dissolved myosin was relatively weak (see Discussion). The presence or absence of CM or MLCKase on the subsequently precipitated myosin was assayed as described above. Phosphorylation rates of CM- and MLCKase-free myosin were negligible even at 25 °C (Fig. 3B).

After extensive washing, the two affinity columns were separately eluted to recover CM and MLCKase. Elution of most of the MLCKase from the CM affinity column required Ca-free buffer (Fig. 5C, I) while for a similar elution of CM from the melittin affinity column the addition of 10–15% glycerol was necessary (Fig. 5A). Elution of the other components bound to these columns, under native condition was, despite many attempts, not possible and they could be recovered only in 5 M urea, 10 mM EDTA buffer (pH 10.6). As shown in Fig. 5, the melittin columns retained a number of proteins some of which could be identified on the basis of their mobility on SDS-gels. In contrast, relatively few proteins were eluted by the urea buffer from CM-affinity columns. A major component with a polypeptide chain weight of 120 k_D was eluted with CM (Fig. 5E, J). This protein, which may be tenaciously bound to myosin could not be investigated further since attempts to obtain it in a native form after the removal of urea were unsuccessful. From its very high affinity for myosin and size this protein may represent a native form of CM-dependent myosin light chain phosphatase (see Sellers and Pato, 1984; Sobieszek and Barylko, 1984).

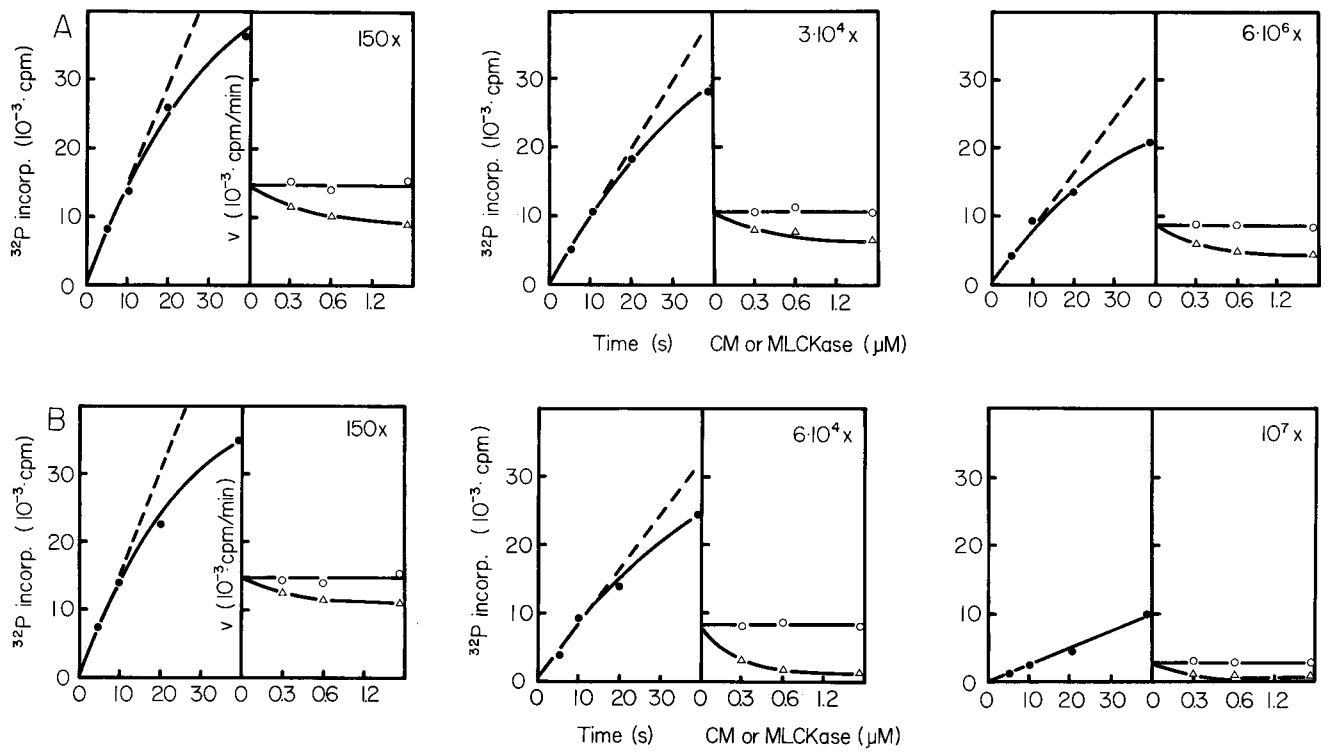

Fig. 4. Data showing that endogenous CM and MLCKase could not be removed from assembled myosin filaments by their repetitive dilution and precipitation in presence (A; 0.1 mM) as well as in the absence (B; 0.5 mM EGTA) of $CaCl_2$. The relative amounts of CM and MLCKase were determined and indicated as in Fig. 5A (assay time 10 s) except that the assays in the presence of added CM and MLCKase are not shown. The myosin chosen for this experiment had stoichiometrical amounts of CM and MLCKase and their ratio remained the same throughout. The amount of CM and MLCKase relative to myosin was proportional to the initial phosphorylation rate (broken line) or to the incorporation level at increasing CM concentration (open triangles). Reduction of the ^{32}P incorporation level at increasing concentrations of added MLCKase (closed triangles) resulted from the formation of unproductive (CM-free) complexes of myosin with the kinase (Sobieszek, 1985b). Every second assay is shown for a series of six consecutive dilutions, indicated in the upper right corner and precipitations involved 20 s centrifugation at 2000 rpm.

Binding of CM to myosin filaments

Having obtained a CM- and MLCKase-free myosin (Fig. 3B) it was possible to characterize the CM binding properties of smooth muscle myosin. In the first, simpler type of binding experiments (see Materials and methods) binding to preformed filaments was measured over a wide range of CM concentrations. As shown in Fig. 6 the binding in the presence of Ca^{2+} could be characterized by three (or possibly two, see Discussion) classes of binding sites. The first, with the highest affinity, had an apparent dissociation constant of about 30 nM and a rather low stoichiometry (CM to monomeric myosin) of about 1 to 500. The second class, of intermediate affinity, could be characterized by a dissociation constant of about 1.2μM and a higher stoichiometry in the range of 1 to 50–100. For the third class a binding stoichiometry in the range of one CM per myosin was obtained, but with the dissociation constant well above 10 μM.

The endogenously bound CM was copurified with myosin in stoichiometric amounts corresponding approximately to the occupation of the intermediate affinity sites characterized by binding studies but was bound with a 100–1000 fold higher affinity, in the nanomolar range. This implied that the sites available for CM during the filament assembly process were not exactly equivalent to the ones already existing on assembled filaments. Accordingly when CM was present during filament formation, its binding to myosin was found to be different (Fig 7). In this case only high affinity binding was observed with very well defined stoichiometry for each assembly experiment. This stoichiometry was approximately the same as observed for freshly purified filamentous myosin. The binding in the absence of Ca^{2+} was also measured and was generally 20 or more fold weaker (Fig. 7).

Binding of CM to pig stomach myosin

The turkey gizzard and pig stomach muscle myosins used in the present study were indistinguishable with respect to their endogenous CM and MLCKase content, provided the porcine myosin was subjected to a purification step on a hydrophobic affinity column. This column removed proteolytic activity present in the myosin fraction. With-

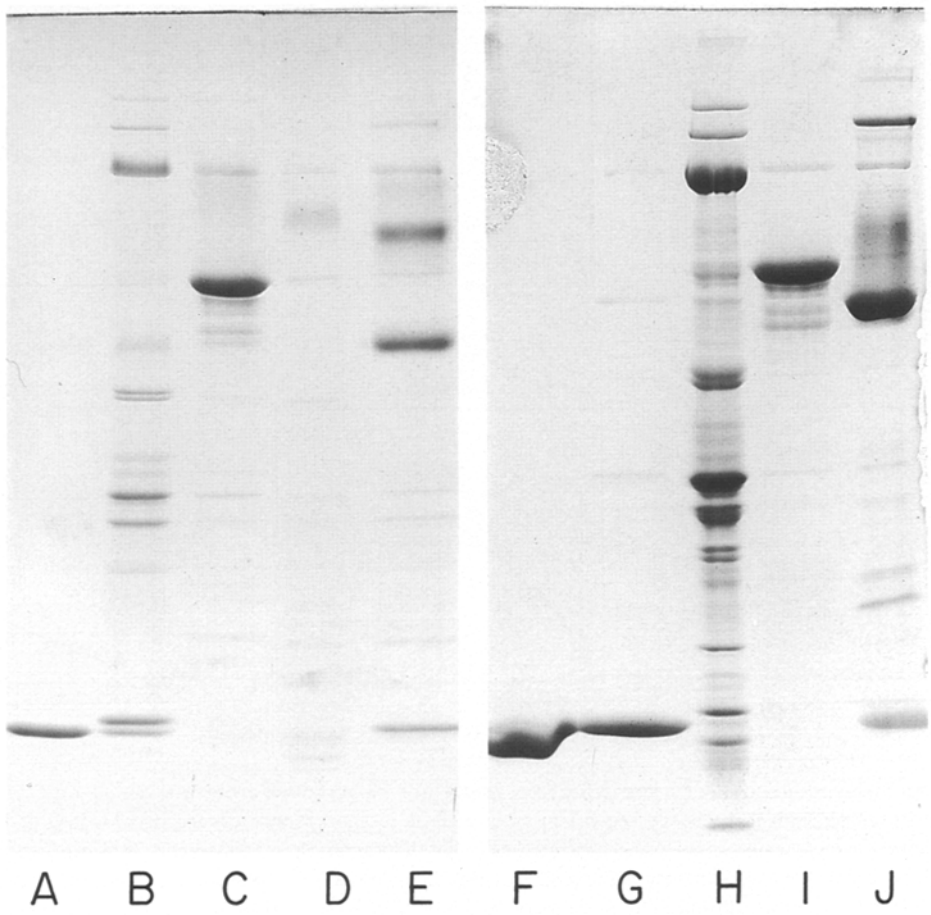

Fig. 5. SDS-gel electrophoresis of samples obtained by affinity chromatography of purified TG myosin (A-E) and myosin fraction (F-J). (A) eluate of melittin-affinity column obtained with Ca-free buffer containing 10% glycerol. Note that only CM was eluted and it was confirmed by CM-assays measurements. (B) subsequent elution of the same column (as A) with urea/EDTA buffer. (C) eluate of CM-affinity column with Ca-free buffer. Activity measurements showed that the major band corresponded to MLCKase. (D) eluate of column as in (C) with the same buffer but containing 15% glycerol. (E) subsequent eluate of column as above (D) with urea/EDTA buffer. The major band corresponds to a 120 kD protein. (F) eluate of mastoparan-affinity column with Ca-free buffer. (G) eluate of melittin-affinity column with Ca-free buffer. (H) eluate of column as in (G) but with urea/EDTA buffer. (I) eluate of CM-affinity column with Ca-free buffer. (J) subsequent eluate of the column in (I) with urea/EDTA buffer.

out this treatment, purified pig stomach myosin showed slight degradation of its regulatory, 20 k_D light chain (Fig. 1C). The apparent polypeptide weight of this light chain was reduced only by a few residues. Interestingly the CM binding properties of such myosin were modified. Its affinity for CM was reduced so that after 100 fold dilution, the collected filaments had a very low CM and MLCKase content. For CM removal it was not necessary to subject this myosin to affinity chromatography. Correspondingly pig stomach myosin with slight degradation of its 20 k_D light chain exhibited only low affinity CM binding sites. Thus the few residues lost during the purification appear to be directly involved in CM binding and are most likely located in the neck region of the molecule in close proximity to the light chain (Vibert and Cohen, 1988).

Discussion

Numerous biochemical studies have established that CM plays a key role in the regulation of contraction in smooth muscle. Estimations of calmodulin content in smooth muscle tissue indicate that it is an abundant protein, occurring in amounts up to 40mg per 100g of wet muscle (Grand and Perry, 1980), and a corresponding concentration of around 25 μM. How calmodulin is segregated within the cell, between the contractile and other components is, however, unknown. Of the contractile proteins, actin, at a concentration of around 700 μM (Cohen and Murphy, 1978) is the most abundant. Actin, however, does not bind CM as demonstrated for skeletal muscle F-actin by Howe and co-workers (1980) and confirmed for the smooth muscle F-actin by Sobue and co-workers

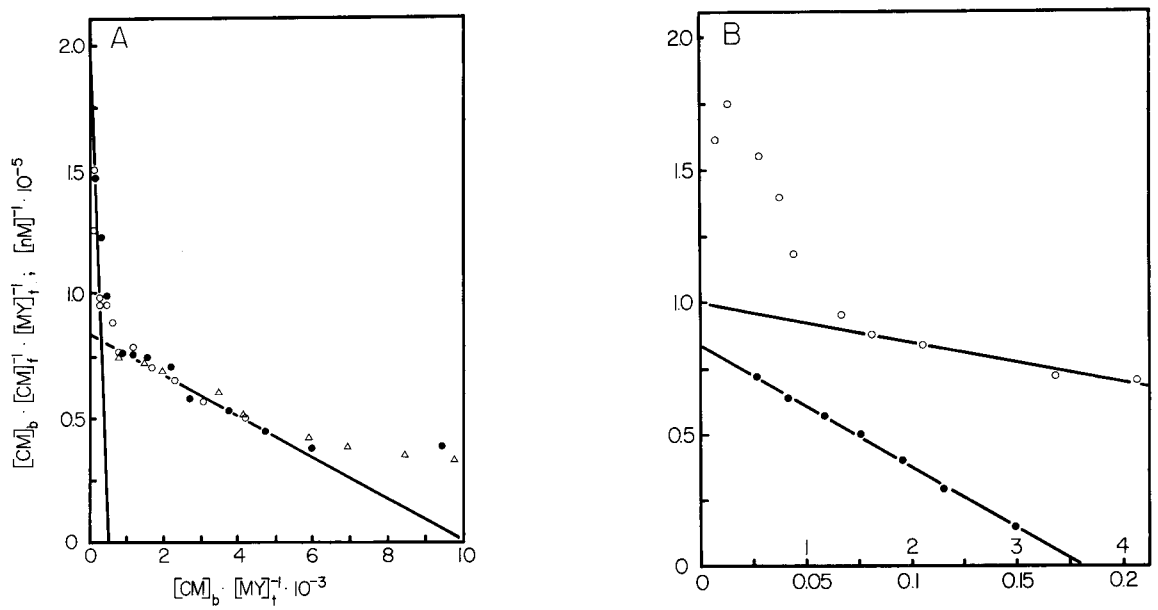

Fig. 6. Scatchard plots of CM-binding to preassembled myosin filaments. In (A) the binding was carried out in the presence of Ca and for a wide range (0.1–11 μM) of CM concentration. The three different symbols corresponded to three separate experiments. The two lines drawn correspond to the high and intermediate affinity binding (B). Binding in the presence (0.1 mM open circles) and absence (2 mM EGTA, closed circles) of Ca in a parallel experiment.

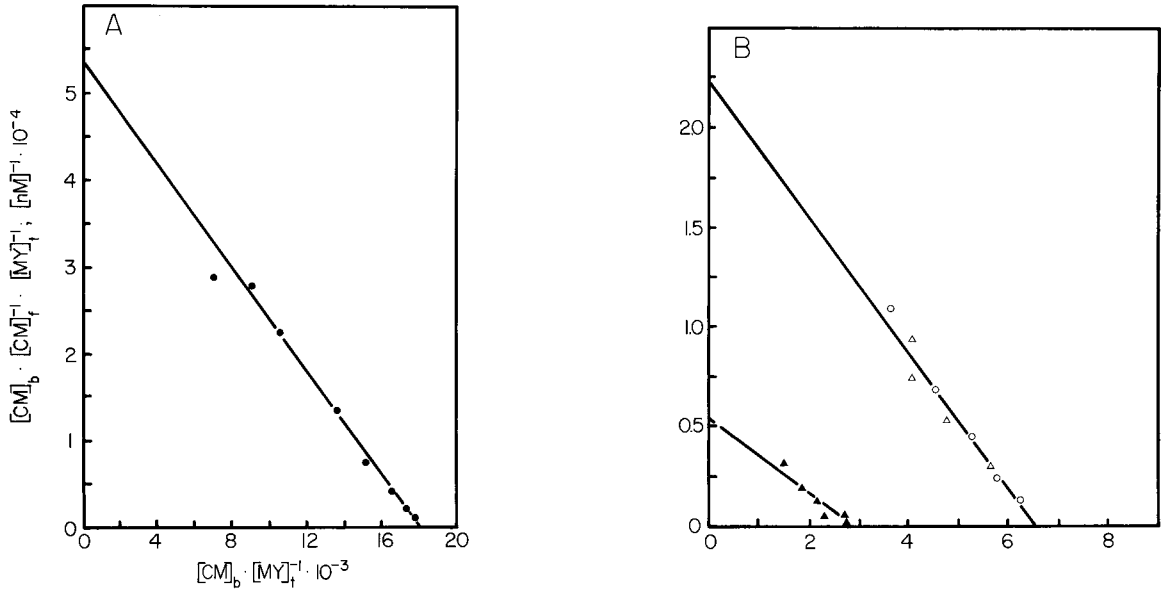

Fig. 7. Scatchard plots of CM-binding to myosin filaments with CM present during the filament assembly. In this case only high affinity binding was observed. In (A) apparent dissociation constant was 33 nM and binding stoichiometry 1 to 55 (CM to myosin). In (B) removal of Ca by EGTA resulted in a 20 fold reduction of the binding affinity but in only a twofold reduction in the binding stoichiometry.

(1972) and in the present study. It has been shown that CM association with smooth muscle thin filaments involves another protein, caldesmon, which binds both actin and CM (Sobue *et al.*, 1982). According to these authors caldesmon occurs in sufficient amounts to account for as much as 70% of the total CM; however, since the affinity of CM for caldesmon is relatively low (around 1 μm; Smith *et al.*, 1987) this figure may be grossly overestimated. Caldesmon appears to be bound to F-actin only in the absence of CM or when its interaction with CM is inhibited by Ca^{2+} removal. Although variations of this scheme have also been proposed (Smith *et al.*, 1987), it was shown more recently that caldesmon also has a considerable affinity for smooth muscle myosin (Ikebe and

Reardon, 1988), so that the question of whether CM is localized on the thin or the thick filaments remains unsettled.

The present demonstration that filamentous and not monomeric myosin bound CM is significant in the context of the myosin phosphorylation theory of regulation in smooth muscle (see Small and Sobieszek, 1980; Marston. 1982; Kamm and Stull, 1985). CM is here required for the activation of MLCKase which phosphorylates myosin so that cyclic interaction between actin and myosin can take place. In contrast to other regulatory enzymes such as phosphodiesterase (see Cox *et al.*, 1981) or phosphorylase (see Burger *et al.*, 1983) MLCKase has no basal activity on its own (Sobieszek and Barylko, 1984; Malencik and Anderson, 1986) as would be expected of an all or none response to Ca^{2+} during muscle stimulation. Despite its relatively high content of between 1 and 4 μM (Adelstein and Klee, 1981; Sobue *et al.*, 1982) there is at least three times less MLCKase in smooth muscle than CM which would suggest that around 30% of total CM is associated with the thick, myosin containing, filaments. Accordingly we found that the binding of CM to filamentous myosin was also substoichiometrical. Significantly, when both CM and MLCKase were present during filament assembly they were bound at approximately the same molar ratios to myosin. However, this ratio was rather variable but filamentous myosin preparations contained as much as one CM per 20 myosin molecules. In view of the substantial losses of CM and not MLCKase (Sobieszek and Barylko, 1984) during the preparation of myofibrils the latter ratio compares realistically with the total content of these components.

The binding of CM and MLCKase to filamentous myosin was not interdependent and in general took place also in the absence of Ca^{2+}. We therefore suggest that CM as well as the kinase are localized on the thick myosin containing filament. However the present results cannot exclude the possibility that another component present in small amounts in myosin is necessary for the binding of one or both of these components. A CM-binding protein would be required for binding of at least CM to myosin. Whether or not it could be the 120 k_D, high affinity CM binding protein present in urea eluates of the CM-affinity column, is not known at present. We may, however, exclude other CM-binding proteins since such proteins would be bound and identified during the CM-affinity chromatography steps used for myosin purification.

The demonstrated nanomolar affinity of CM for filamentous myosin is in the same range as that of MLCKase for myosin filaments K_d = 30 nM: (Sobieszek, 1985a). In other reports the measured K_d was in the range of 1 to 2 μM (Sellers and Pato, 1984), a difference that may be readily explained from the different methods used in the binding experiments. Both methods were tested for binding of CM to filamentous myosin in the present studies. On this basis we conclude that indirect measurements of the unbound fraction remaining in the supernantants, as made by Sellers and Pato (1984), obscure substoichiometrical binding of high affinity when lower affinity binding sites are also present.

In view of the nanomolar affinity of MLCKase for CM (Adelstein and Klee, 1981; Malencik and Anderson, 1986) it could be argued that, at least in the presence of Ca^{2+}, binding of CM or MLCKase alone could be sufficient to retain their respective counterpart on the myosin filament. But as we have shown, CM and MLCKase bind equally well to myosin in the absence of Ca^{2+} and cannot be removed from myosin by simple dilution. Only by a dilution of 10^6 fold can removal be effected, indicating a nanomolar affinity range of binding similar to that in the presence of Ca^{2+}. CM and MLCKase are therefore firmly incorporated into the filament structure but their relationship and possible interaction while on the myosin filament remain to be established. A direct effect of endogenous MLCKase on the binding of CM to myosin was not investigated. However, in agreement with Sellers and Pato (1984), it was noted that at least the low affinity binding of the kinase was not enhanced, but reduced by the additional presence of CM. Thus it may be concluded that the affinity of the CM–MLCKase complex for myosin might be somewhat lower than their separate affinities. In view of the apparently independent binding of CM and MLCKase to myosin we presume that, in the presence of Ca^{2+}, binding of ATP to myosin or to the kinase and associated conformational changes are a prerequisite for the formation of the active CM–MLCKase complex. The decrease in the affinity associated with subsequent myosin phosphorylation observed earlier (Sellers and Pato, 1984; Sobieszek, 1985a). is assumed to trigger the release of CM–MLCKase complex so that it can be transmitted to adjacent heads along the myosin filament.

The appearance of the high affinity binding sites during filament formation that are absent on monomeric myosin suggests that CM binding to myosin may play a role in filament assembly. The substoichiometrical binding in addition points to its role in the initiation of filament assembly rather than in filament elongation. As shown previously phosphorylation of myosin in the presence of CM and MLCKase could initiate the assembly of short filaments under ionic conditions very close to physiological (Scholey *et al.*, 1980). Our own preliminary observations indicate that very long synthetic filaments, such as published in previous electron microscope studies (Sobieszek, 1977), have a much lower CM content. Since removal of CM could be accompanied by the depletion of other minor myosin impurities, it is unclear at present whether there is any relationship between the CM to myosin ratios and the length of the filaments assembled *in vitro*. Studies now in progress, on the effect of CM on filament assembly, should shed more light on this interesting possibility.

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