Calcium-Independent Myosin Light Chain Kinase of Smooth Muscle. Preparation by Limited Chymotryptic Digestion of the Calcium Ion Dependent Enzyme, Purification, and Characterization†

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ABSTRACT: Limited α-chymotryptic digestion of Ca^{2+}-, calmodulin-dependent myosin light chain kinase partially purified from smooth muscle (turkey gizzard) yielded a Ca^{2+}-independent form of the enzyme. Digestion to yield the Ca^{2+}-independent kinase required the enzyme complexed with Ca^{2+}–calmodulin; when digestion was performed on the apoenzyme, i.e., in the absence of Ca^{2+}, the dependence of kinase activity on Ca^{2+} was retained. The Ca^{2+}-independent kinase was purified by ion-exchange chromatography and shown to have an apparent molecular weight of ~80 000. The specific activity of the freshly prepared enzyme was 6.5 ± 0.2 μmol of P_i incorporated min^{-1} mg^{-1} in the presence of Ca^{2+} and 8.3 ± 0.3 μmol min^{-1} mg^{-1} in the absence of Ca^{2+}, using the isolated light chains of gizzard myosin as the substrate. The Ca^{2+}-independent enzyme also phosphorylated the 20 000-dalton light chains of purified myosin and crude actomyosin from turkey gizzard. The K_m of the Ca^{2+}-independent kinase for Mg^{2+}–ATP (54 μM) was not significantly different from that of the native, Ca^{2+}-dependent enzyme (68 μM). These observations indicate maintenance of the integrity of the active site after digestion with α-chymotrypsin. It is suggested that the loss of Ca^{2+} sensitivity of the kinase after limited proteolysis is due to loss of the calmodulin-binding site from the 80 000-dalton fragment. The two sites of phosphorylation by the cyclic AMP dependent protein kinase were also removed by the chymotryptic hydrolysis.

The most widely accepted theory for the regulation of smooth muscle actomyosin involves the phosphorylation of the 20 000-dalton light chains of myosin (Adelstein & Eisenberg, 1980; Hartshorne & Siemankowski, 1981). Evidence has been accumulated in support of the phosphorylation theory from a variety of experimental approaches. These include the following: the use of adenosine 5′-*O*-(3-thiotriphosphate) (ATPγS),[1] which generates a stable thiophosphorylated state and a resultant loss of Ca^{2+} sensitivity in gizzard actomyosin and in skinned smooth muscle fibers; a positive correlation between the phosphorylation of myosin and either the ATPase activities of several in vitro actomyosin systems or tension development in various smooth muscle fiber preparations; and, finally, the use of calmodulin antagonists such as the phenothiazines, which inhibit both myosin phosphorylation and ATPase activity of gizzard actomyosin and tension development in muscle strips [for bibliography, see Hartshorne & Mrwa (1982)].

Despite this impressive array of supporting data, the phosphorylation theory is not accepted universally as the only regulatory mechanism in smooth muscle. The dissention ranges from those who believe that phosphorylation is not involved as a regulatory mechanism and propose instead a system based on the thin filaments termed leiotonin [Mikawa et al., 1978; Nonomura & Ebashi, 1980; see also Murray & England (1980)] to those who propose dual regulatory systems (Chacko et al., 1977; Marston et al., 1980; Walters & Marston, 1981; Persechini et al., 1981).

An objective of ours is to define more clearly the role of myosin phosphorylation and to establish whether or not other Ca^{2+}-dependent mechanisms might constitute dominant features of the regulatory process in smooth muscle. One approach to this problem was suggested by the earlier studies of Hartshorne et al. (1977), who found that limited proteolysis of a crude preparation of MLCK rendered it Ca^{2+} independent. The rationale developed that if the MLCK could be isolated in a Ca^{2+}-independent form then the effects of myosin phosphorylation could be assessed in the absence of Ca^{2+}. This, therefore, would provide a means of evaluating the significance of phosphorylation relative to other Ca^{2+}-dependent processes that might also exist. In this presentation, we describe the initial phases of this program, namely, the isolation and some properties of the Ca^{2+}-independent MLCK from turkey gizzard smooth muscle.

Materials and Methods

[γ-^{32}P]ATP was obtained from New England Nuclear. α-Chymotrypsin and lima bean trypsin inhibitor were purchased from Millipore Corp. (Freehold, NJ). The following proteins were prepared by methods described earlier: myosin (Persechini et al., 1981) and mixed myosin light chains (Perrie & Perry, 1970) from turkey gizzard, calmodulin from bovine testes (Autric et al., 1980), catalytic subunit of bovine cardiac cAMP-dependent protein kinase type II [preparation (Demaille et al., 1977) and storage (Peters et al., 1977)].

Protein concentrations were determined by the biuret method (Itzhaki & Gill, 1964) or the dye-binding assay as described by Spector (1978).

Electrophoresis was performed on 7.5–20% polyacrylamide gradient slab gels in the presence of 0.1% $NaDodSO_4$ at 30 mA by using the discontinuous buffer system of Laemmli (1970). Coomassie blue stained and destained gels were scanned at 550 nm by using a Zeiss Spektralphotometer PM6 attached to a Spectra-Physics SP 4050 printer/plotter, an SP 4020 data interface, and an SP 4000 central processor.

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[1] Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); cAMP, adenosine cyclic 3′,5′-phosphate; DEAE, diethylaminoethyl; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; LBTI, lima bean trypsin inhibitor; MLCK, myosin light chain kinase; $NaDodSO_4$, sodium dodecyl sulfate.

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Calmodulin was coupled to cyanogen bromide activated Sepharose 4B (Sigma Chemical Co., St. Louis, MO) essentially by the method of March et al. (1974). The resin was equilibrated with 15 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 20 μM $CaCl_2$, and 0.02% sodium azide and stored at 4 °C in a plastic bottle until used. The amount of bound calmodulin was determined to be 0.9 mg/mL of packed resin.

MLCK Assays. Phosphate (^{32}P) incorporation into the 20 000-dalton light chain of gizzard myosin was measured as described by Mrwa & Hartshorne (1980).

ATPase Activity Measurements. Mg^{2+}–ATPase activities of smooth muscle actomyosin (3 mg/mL) were measured in 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 60 mM KCl, 0.75 mM [γ-^{32}P]ATP (~1000 cpm/nmol), and either 0.1 mM $CaCl_2$ or 1 mM EGTA, in the presence and absence of the Ca^{2+}-independent MLCK (16 nM), as previously described (Ferenczi et al., 1978).

Preparation and Purification of Ca^{2+}-Independent Myosin Light Chain Kinase. All procedures were carried out at 4 °C unless otherwise noted. Frozen turkey gizzards (200 g) were minced and washed with overhead stirring for 15 min in 5 volumes of 15 mM Tris-HCl (pH 7.5), 60 mM KCl, 1 mM $MgCl_2$, 10 μM $CaCl_2$, 0.5 mM dithiothreitol, and 0.5% (v/v) Triton X-100 (buffer A) and centrifuged at 16000*g* for 5 min. The pellet was washed as described above in 5 volumes of buffer A from which Triton X-100 was omitted. The final pellet was suspended in 3 volumes of 0.3 M KCl, 0.15 M potassium phosphate (pH 6.5), 10 mM sodium pyrophosphate, 1 mM $MgCl_2$, and 0.5 mM dithiothreitol (buffer B) with overhead stirring for 3 h. The extract was centrifuged at 16000*g* for 10 min. The pellet was suspended in 3 volumes of buffer B for 1 h with overhead stirring and centrifuged as before. The two supernatants were combined, filtered through glass wool, and dialyzed vs. two changes (10 L each) of 15 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, and 0.5 mM dithiothreitol (buffer C).

First Ion-Exchange Chromatography.[2] The dialyzed extract was centrifuged at 16000*g* for 60 min to pellet the actomyosin. The supernatant was filtered through glass wool and loaded on a column (4.5 × 30 cm) of DEAE-Sephacel previously equilibrated with buffer C. Proteins were eluted with a linear gradient made from 600 mL each of buffer C and buffer C containing 0.5 M KCl. Fractions (6 mL) were collected at a flow rate of 50–60 mL/h. Aliquots (50 μL) of selected fractions (based on the A_{280} elution profile) were assayed for MLCK activity in the presence and absence of Ca^{2+} as described by Mrwa & Hartshorne (1980). Fractions containing Ca^{2+}-dependent MLCK were pooled.

Chymotryptic Digestion. The pool of Ca^{2+}-dependent MLCK was diluted to a protein concentration of 0.5 mg/mL with 15 mM Tris-HCl (pH 7.5) to lower the ionic strength below that of buffer D (see below). α-Chymotrypsin was added at a protease:total protein ratio of 1:500. Digestion was allowed to proceed at 25 °C for 5 min and was terminated by addition of lima bean trypsin inhibitor at a weight ratio to α-chymotrypsin of 10 to 1.

Second Ion-Exchange Chromatography. The chymotryptic digest was loaded immediately on a column (2.5 × 90 cm) of DEAE-Sephacel previously equilibrated with 15 mM Tris-HCl (pH 7.5), 0.1 M KCl, 1 mM $MgCl_2$, 10 μM $CaCl_2$, and 0.5 mM dithiothreitol (buffer D). Proteins were eluted with a linear gradient made from 350 mL each of buffer D and buffer D containing 0.4 M KCl. Fractions (5 mL) were collected at a flow rate of 30–40 mL/h. Aliquots (20 μL) of selected fractions were assayed for MLCK activity in the presence and absence of Ca^{2+} as before and their protein contents examined by 0.1% $NaDodSO_4$ and 7.5–20% polyacrylamide gradient slab gel electrophoresis. Fractions containing Ca^{2+}-independent MLCK were pooled and stored in 1-mL aliquots at −20 °C until use.

[2] A figure of this chromatogram was submitted to the reviewers. Copies are available on request.

Table I: Effect of α-Chymotrypsin on the Ca^{2+} Dependence of Smooth Muscle Myosin Light Chain Kinase[a]

digestion mixture		MLCK activity[b] +Ca^{2+}	MLCK activity[b] +EGTA
+Ca^{2+}:	α-chymotrypsin/LBTI[c]	101	106
	LBTI/α-chymotrypsin[d]	100	14
	LBTI[e]	89	11
	no addition[f]	100	8
+EGTA:	α-chymotrypsin/LBTI[c]	85	3
	LBTI/α-chymotrypsin[d]	100	2
	LBTI[e]	102	2
	no addition[f]	86	2

[a] Ca^{2+}-dependent MLCK, purified through the first DEAE-Sephacel column, was digested with α-chymotrypsin in the presence of calmodulin (1.8 μM) and either 0.1 mM $CaCl_2$ (+Ca^{2+}) or 1 mM EGTA (+EGTA) as described under Materials and Methods. Digestion was terminated with lima bean trypsin inhibitor (inhibitor:protease = 10:1 by weight), and MLCK activity was measured immediately (at a concentration of 80 nM) in the presence and absence of Ca^{2+} as described previously (Mrwa & Hartshorne, 1980) with a 10-min incubation time. The higher level of phosphorylation compared with that in Figure 3 is due to the higher level of MLCK used in the assay. [b] Expressed as a percentage of the P_i incorporation into myosin (0.85 mol of P_i/mol of 20 000-dalton light chain) catalyzed by untreated kinase under identical conditions. [c] Digestion terminated after 5 min at 25 °C with LBTI. [d] LBTI added to reaction mixture before α-chymotrypsin. [e] Kinase incubated with LBTI in the absence of α-chymotrypsin. [f] Kinase incubated with no further addition.

Results

It was shown earlier by Hartshorne et al. (1977) that the Ca^{2+} sensitivity of partially purified smooth muscle (chicken gizzard) MLCK was lost by digestion with α-chymotrypsin with retention of enzymatic activity. Our strategy, therefore, in an attempt to obtain an active but Ca^{2+}-insensitive smooth muscle MLCK involved an initial partial purification of the native Ca^{2+}-dependent enzyme, followed by chymotryptic digestion and purification of the Ca^{2+}-independent enzyme. The partial purification of Ca^{2+}-dependent MLCK, described in detail under Materials and Methods, was similar to that described earlier by Walsh et al. (1980) through ion-exchange chromatography, with the exception that Ca^{2+} ions were included throughout so that MLCK was complexed with calmodulin. The effect of α-chymotryptic digestion of this enzyme preparation on the Ca^{2+} dependence of the MLCK is shown in Table I. Proteolysis in the presence of Ca^{2+}–calmodulin induced loss of Ca^{2+} sensitivity, while the appropriate controls retained Ca^{2+} dependence. If digestion was carried out in the absence of Ca^{2+}, no loss of Ca^{2+} sensitivity was observed (Table I). Under these latter conditions, the kinase was digested to a 95 000-dalton fragment (data not shown).

The Ca^{2+}-independent MLCK was then purified by a further ion-exchange chromatograph (Figure 1). In most purifications performed, a peak of Ca^{2+}-dependent MLCK eluted at 0.2 M KCl. This represents undigested kinase, as the native kinase eluted at 0.2 M KCl from the first column. A peak of Ca^{2+}-independent kinase eluted immediately after the Ca^{2+}-dependent enzyme (Figure 1). Aliquots (20 μL) of selected fractions from this column were subjected to Na-

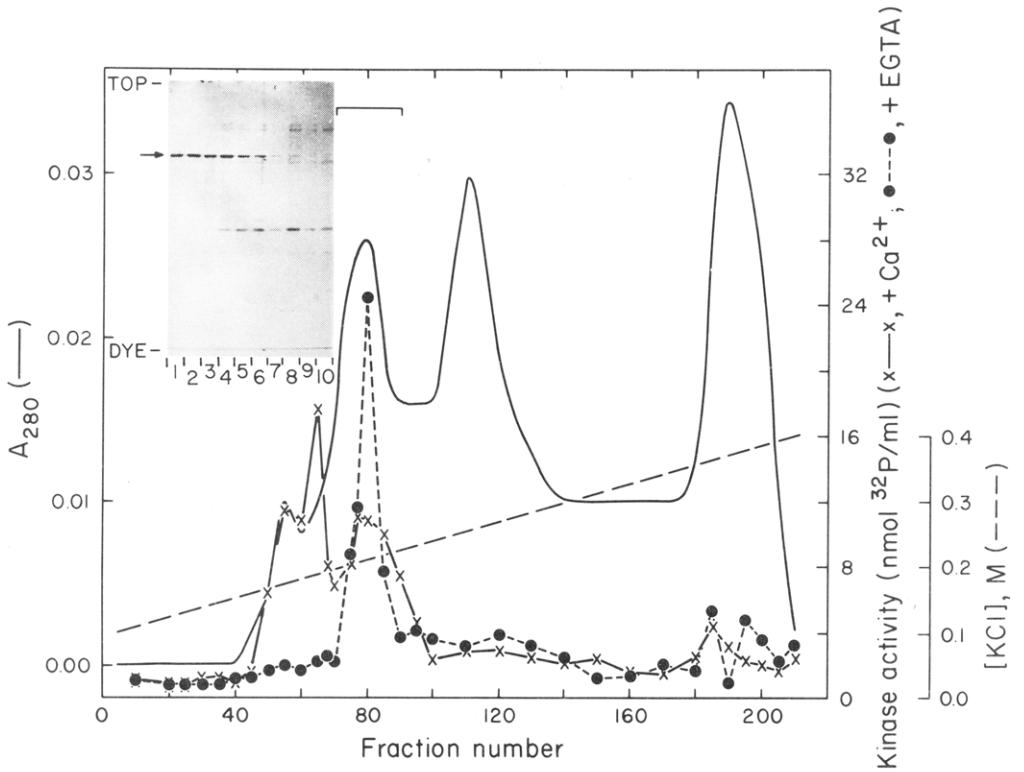

FIGURE 1: Ion-exchange chromatography of the chymotryptic digest of MLCK. The digest, prepared as described under Materials and Methods, was loaded on a column (2.5 × 90 cm) of DEAE-Sephacel equilibrated with buffer D and eluted with a linear 0.1–0.4 M KCl gradient (---). Protein was monitored by A_{280} (—). MLCK activity was assayed in the presence of 0.1 mM $CaCl_2$ (×) or 1 mM EGTA (●). Fractions indicated by the bar were pooled and stored in 1-mL aliquots at −20 °C. Selected fractions were electrophoresed on a 0.1% $NaDodSO_4$ and 7.5–20% polyacrylamide gradient slab gel. These are shown as insets in the figure. Numbers 1–10 under the gel lanes refer to fractions 75–84, respectively. The arrow indicates the position of the protein band which corresponds with Ca^{2+}-independent MLCK activity. The kinase activities are not specific activities but represent P_i incorporation following a 5-min incubation for a given volume (10 μL) of each fraction.

$DodSO_4$–polyacrylamide gradient slab gel electrophoresis (Figure 1, inset). Ca^{2+}-independent MLCK activity corresponded with the protein indicated by the arrow since kinase activity was detected only in those fractions which contained this band. This is particularly evident in fractions 75–77 (gel slots 1–3). This conclusion was confirmed by limited α-chymotryptic digestion of the purified 130 000-dalton gizzard kinase followed by separation of the fragments on a column of DEAE-Sephacel (data not shown).

Characterization of Ca^{2+}-Independent Myosin Light Chain Kinase. Purity and M_r Determination. The purity of the kinase preparation is shown by the densitometric gel scan in Figure 2. A major and a minor band are apparent. It is clear from Figure 1 that the higher M_r band represents the Ca^{2+}-independent MLCK. The M_r of this kinase was determined to be 78 500 as shown in the inset of Figure 2. The contaminant had an M_r of 32 000.

Ca^{2+} Independence. The Ca^{2+} independence of this kinase is shown clearly by the time course of phosphate incorporation into isolated myosin light chains (Figure 3). From these data, and other preparations, the specific activity of the Ca^{2+}-independent kinase was determined to be 6.5 ± 0.2 μmol of P_i incorporated min^{-1} mg^{-1} in the presence of Ca^{2+} and 8.3 ± 0.3 μmol min^{-1} mg^{-1} in the absence of Ca^{2+}. When stored frozen at −20 °C, the activity of the Ca^{2+}-independent enzyme was found to diminish slowly over a period of several weeks. The enzyme activity was more stable following lyophilization in 5% (w/v) sucrose and storage at −20 °C. Lyophilization under these conditions had no effect on enzymatic activity. The enzyme was also stable upon dialysis.

It was clearly of importance to determine whether or not the Ca^{2+}-independent kinase phosphorylated the 20 000-dalton

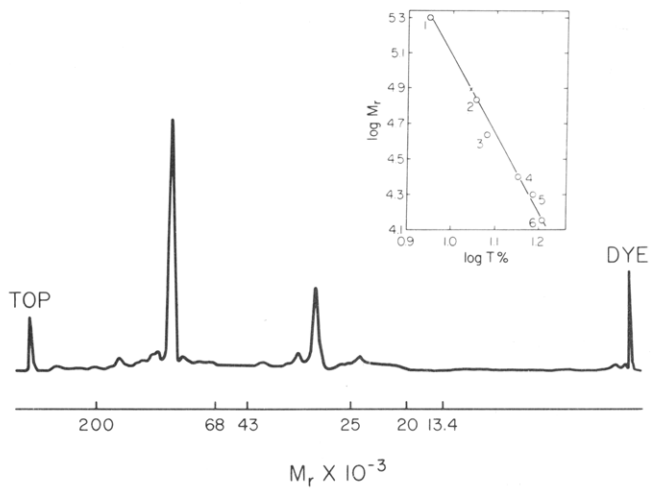

FIGURE 2: Purity and M_r determination of the Ca^{2+}-independent MLCK. An aliquot (10 μg) of the Ca^{2+}-independent kinase was electrophoresed on a 7.5–20% polyacrylamide gradient slab gel in the presence of 0.1% $NaDodSO_4$. The stained and destained gel was scanned as described under Materials and Methods. The inset shows the M_r determination as described by Lambin (1978). The following M_r standards were used in the determination: 1 = myosin heavy chain, 2 = bovine serum albumin, 3 = ovalbumin, 4 = chymotrypsinogen, 5 = 20 000-dalton light chain of myosin, 6 = cytochrome *c*, x = Ca^{2+}-independent MLCK.

light chain in purified myosin and partially purified actomyosin. The data in Figure 3 show that the kinase does indeed phosphorylate purified myosin, and similar results were obtained with actomyosin. The sites of phosphorylation in each case were specific for the 20 000-dalton light chains of myosin as revealed by autoradiography (data not shown). In agree-

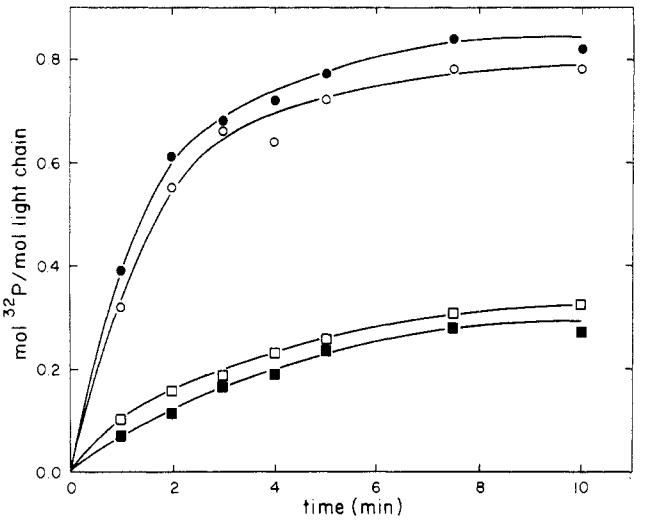

FIGURE 3: Time course of phosphorylation of myosin and isolated light chains by the Ca^{2+}-independent MLCK. Smooth muscle mixed light chains (22.5 μg/mL; ○, ●) or whole myosin (1 mg/mL; □, ■) was incubated at 25 °C in 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (~3000 cpm/nmol), calmodulin (0.2 μM), and Ca^{2+}-independent MLCK (16 nM), in the presence of 0.1 mM $CaCl_2$ (○, □) or 1 mM EGTA (●, ■). The extent of phosphorylation was determined on aliquots [0.48 (○, ●) or 0.225 mL (□, ■)] withdrawn at the indicated times.

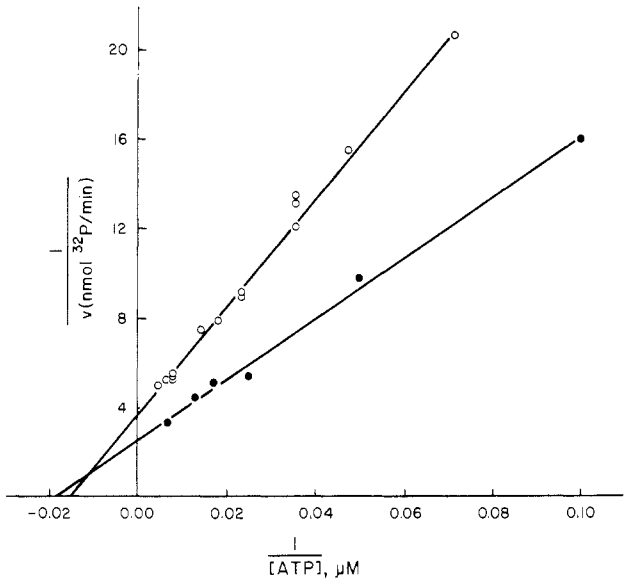

FIGURE 4: Comparison of the K_m values for Mg^{2+}–ATP of Ca^{2+}-dependent and Ca^{2+}-independent MLCKs. Initial velocity measurements were made by using the Ca^{2+}-independent MLCK (10 nM, ●) and Ca^{2+}-dependent MLCK (0.7 nM, ○) at various concentrations of ATP as shown. Other conditions were identical: 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 60 mM KCl, 3.2 μM 20 000-dalton light chain of myosin, and 2.4 μM calmodulin. The specific radioactivity of [γ-^{32}P]ATP was ~4000 cpm/nmol. For each point, a time course of light chain phosphorylation was measured by withdrawing aliquots of the reaction mixtures at the following times: 12, 27, 45, 63, 84, and 105 s.

ment with earlier studies on the Ca^{2+}-dependent form of the enzyme (Mrwa & Hartshorne, 1980; Adelstein & Klee, 1981), the rate of phosphorylation of whole myosin was significantly less than that of the isolated light chains (Figure 3).

K_m for Mg^{2+}–ATP. Figure 4 shows that the K_m for Mg^{2+}–ATP is not significantly affected by chymotryptic digestion; the K_m of the Ca^{2+}-independent MLCK was determined to be 54 μM, compared with 68 μM for the native Ca^{2+}-dependent enzyme.

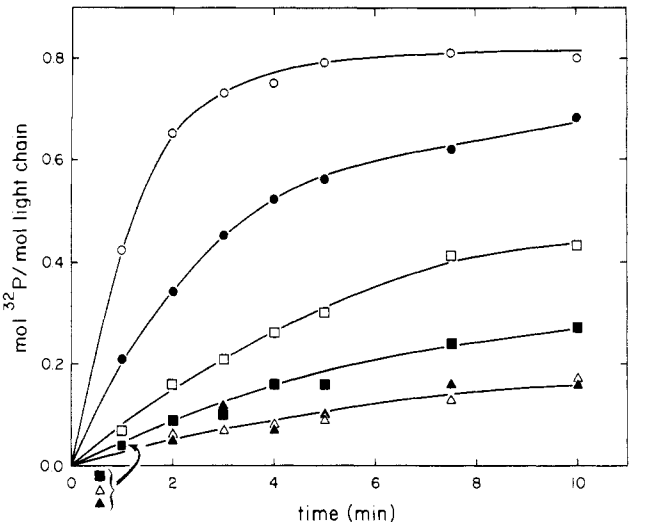

FIGURE 5: Effect of ionic strength on the activity of Ca^{2+}-independent MLCK. Ca^{2+}-independent MLCK (1.2 nM) was incubated at 25 °C in 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 1 mM [γ-^{32}P]ATP (~3000 cpm/nmol), 0.35 μM calmodulin, 1 mM EGTA, 1.6 μM 20 000-dalton light chain of myosin, and varying concentrations of KCl: (○) 0; (●) 0.1; (□) 0.2; (■) 0.3; (△) 0.4; (▲) 0.5 M. The extent of phosphorylation was determined in aliquots (0.5 mL) withdrawn at the indicated times.

Effect of Ionic Strength. It was of interest to determine the effect of ionic strength on the activity of Ca^{2+}-independent MLCK since later studies with skinned smooth muscle fibers would require a bathing medium at an ionic strength of 0.15 (Cassidy et al., 1979). Furthermore, if the kinase were to retain its activity at high ionic strength, it would be possible to conduct in vitro experiments with soluble myosin as a substrate. The results depicted in Figure 5 show that the activity of the kinase is quite markedly affected by increasing ionic strength. However, at the ionic strength required for the skinned fiber experiments, considerable activity was retained. Above 0.3 M KCl, the kinase retained little activity, indicating that it may not be suitable for use in in vitro systems where myosin is soluble. The effect of ionic strength on the activity of the native enzyme was less dramatic but was not studied in detail owing to the complication that multiple equilibria (Ca^{2+}–calmodulin, calmodulin–MLCK, and MLCK–myosin) are involved.

Loss of Calmodulin-Binding Site. For determination of whether or not the Ca^{2+}-independent kinase had retained the calmodulin-binding site, the chymotryptic digest of the pool from the first DEAE-Sephacel column was further purified by gel filtration and applied to the calmodulin affinity column in the presence of Ca^{2+}. As shown in Figure 6, the bulk of the kinase activity either was not bound to the column or was bound nonspecifically and was eluted with 0.2 M NaCl in the presence of Ca^{2+}. Both peaks contained the M_r ~80 000 kinase as shown by $NaDodSO_4$–polyacrylamide gradient gel electrophoresis. These observations suggest that loss of Ca^{2+} dependence by limited chymotryptic digestion is a result of the removal of the calmodulin-binding site.

Loss of Phosphorylation Sites. It is established that the Ca^{2+}-dependent MLCK of smooth muscle is phosphorylated by the cAMP-dependent protein kinase (Adelstein et al., 1978; Conti & Adelstein, 1981), and two phosphorylation sites have been identified. Bound calmodulin blocked phosphorylation at one of these sites (Conti & Adelstein, 1981). The chymotryptic digest of Ca^{2+}-, calmodulin-dependent MLCK was treated with the purified catalytic subunit of cAMP-dependent protein kinase. Maximal phosphate incorporation of 2

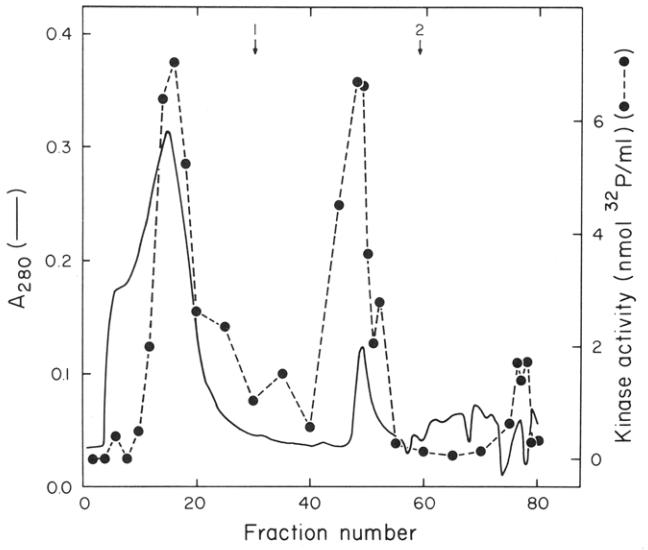

FIGURE 6: Affinity chromatography of Ca^{2+}-independent MLCK. An aliquot of the chymotryptic digest (see Materials and Methods) was lyophilized in 5% (w/v) sucrose. The lyophilized material was desalted and further purified by gel filtration as follows: the sample was dissolved in 5 mL of deionized water and loaded on a column (2.5 × 90 cm) of Sephacryl S-200 previously equilibrated with 15 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 10 μM $CaCl_2$, and 0.5 mM dithiothreitol (buffer E). The column was developed with the same buffer. Ca^{2+}-independent MLCK-containing fractions were pooled (no Ca^{2+}-dependent MLCK activity was detected in this pool) and loaded on a column (1 × 19 cm) of calmodulin–Sepharose 4B prepared as described under Materials and Methods and equilibrated with buffer E. Fractions (1 mL) were collected at a flow rate of 12 mL/h and monitored for A_{280} (—) and MLCK activity in the presence of 1 mM EGTA (●). Eluting buffers were changed as indicated by the arrows: 1 = buffer E containing 0.2 M NaCl; 2 = buffer E containing 0.2 M NaCl and 2 mM EGTA instead of $CaCl_2$.

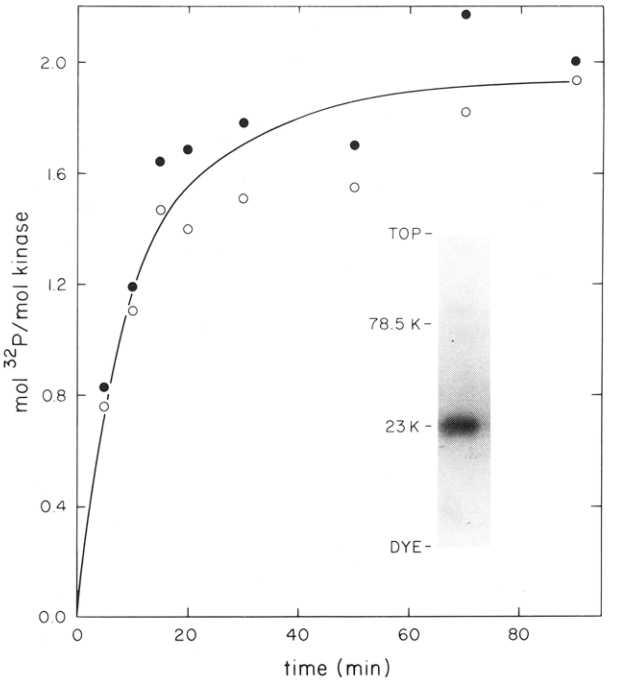

FIGURE 7: Phosphorylation of the α-chymotryptic digest of MLCK by the catalytic subunit of cAMP-dependent protein kinase. The α-chymotryptic digest (0.5 mg/mL) was incubated at 25 °C in 25 mM Tris-HCl (pH 7.5), 4 mM $MgCl_2$, 0.1 mM $CaCl_2$, 1 mM [γ-^{32}P]ATP (~5000 cpm/nmol), and purified catalytic subunit of cAMP-dependent protein kinase (at a weight ratio to total protein of 1 to 100) in the presence (○) and absence (●) of 10 μM calmodulin. The extent of phosphorylation was determined on aliquots (0.25 mL) withdrawn at the indicated times. Samples from which the protein kinase catalytic subunit was omitted exhibited no phosphate incorporation. An aliquot of the reaction mixture (–calmodulin) was withdrawn at time 80 min for 0.1% $NaDodSO_4$ and 7.5–20% polyacrylamide gradient slab gel electrophoresis and autoradiography (inset).

mol/mol of kinase was observed (Figure 7) whether or not calmodulin was present. Phosphorylated peptides were visualized after $NaDodSO_4$–polyacrylamide gradient slab gel electrophoresis and autoradiography. As shown in the inset of Figure 7, the only labeled peptide had an M_r of 23 000; no ^{32}P was incorporated into the 80 000-dalton Ca^{2+}-independent kinase. This observation has been confirmed by α-chymotryptic digestion of pure native MLCK phosphorylated at both sites by the catalytic subunit of cAMP-dependent protein kinase: all the radioactive label was recovered in the 23 000-dalton fragment of MLCK.

Effect of Ca^{2+}-Independent MLCK on the Ca^{2+} Sensitivity of Smooth Muscle Actomyosin ATPase. The potential of the Ca^{2+}-independent MLCK as a tool to investigate the regulatory mechanism of the contractile apparatus of smooth muscle was tested by assaying its ability to activate the Mg^{2+}–ATPase activity of a crude gizzard actomyosin preparation (Figure 8). In the absence of Ca^{2+}, and in the absence of added MLCK, an approximate steady-state rate (see legend to Figure 8) of 1.4 nmol of P_i min^{-1} (mg of actomyosin)$^{-1}$ was obtained. This was increased to 5.8 nmol of P_i min^{-1} (mg of actomyosin)$^{-1}$, or 23.2 nmol of P_i min^{-1} (mg of myosin)$^{-1}$ (see legend to Figure 8), by the addition of Ca^{2+}. A similar rate, 5.5 nmol of P_i min^{-1} (mg of actomyosin)$^{-1}$, or 22.0 nmol of P_i min^{-1} (mg of myosin)$^{-1}$, was obtained when the Ca^{2+}-independent MLCK was added in the absence of Ca^{2+}. As shown by autoradiography, the only detectable sites of phosphorylation by the Ca^{2+}-independent MLCK in the absence of Ca^{2+} were the 20 000-dalton light chains of myosin. In the presence of Ca^{2+}, the ATPase activity was unaffected by the addition of the Ca^{2+}-independent MLCK. These preliminary experiments indicate that significant activation of Mg^{2+}–ATPase activity can be achieved by phosphorylation of myosin in the absence of Ca^{2+}.

Discussion

Smooth muscle myosin light chain kinase is regulated in vitro by Ca^{2+}–calmodulin (Dabrowska et al., 1978) and by cAMP-dependent phosphorylation. Conti & Adelstein (1981) have shown that MLCK contains two sites of phosphorylation by the catalytic subunit of cAMP-dependent protein kinase. In the presence of bound calmodulin, only one of these sites is phosphorylated, and this has no observable effect on kinase activity. In the absence of bound calmodulin, on the other hand, both sites are phosphorylated, and the result is an approximately 20-fold decrease in the affinity of the kinase for calmodulin. The two sites of phosphorylation were found in a 22 000-dalton fragment released by limited tryptic digestion of phosphorylated MLCK, indicating that both sites are located near one terminus of the 130 000-dalton kinase molecule (Adelstein et al., 1978). Subsequent work by Walsh et al. (1980) showed that the MLCK molecule could be cleaved into its three domains by limited tryptic digestion under somewhat different conditions than those used by Adelstein et al. (1978): one fragment (M_r 22 000) contained the sites of phosphorylation by cAMP-dependent protein kinase; a second fragment (M_r 59 000) contained at least part of the active site, since this peptide exhibited a low level of kinase activity which was Ca^{2+} independent; and a third fragment (M_r 50 000) retained the calmodulin-binding site since it was retained on an affinity column of calmodulin–Sepharose in the presence of Ca^{2+} and was eluted with EGTA (Walsh et al., 1980). These obser-

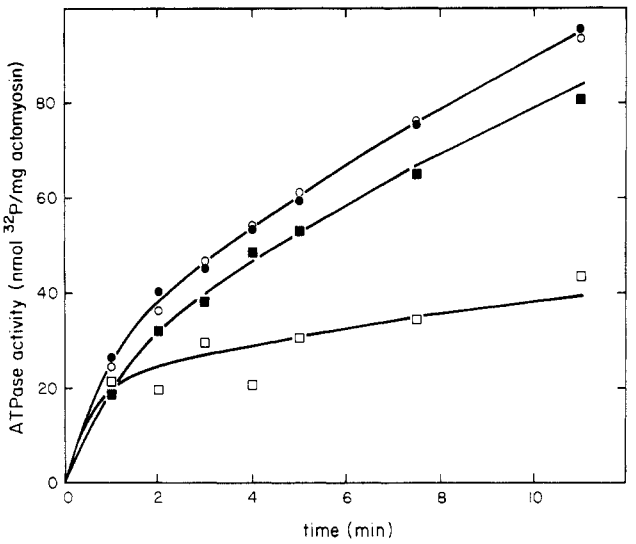

FIGURE 8: Effect of Ca^{2+}-independent MLCK on the Ca^{2+} sensitivity of smooth muscle actomyosin Mg^{2+}-ATPase activity. Actomyosin prepared during the MLCK preparation (see Materials and Methods) was resuspended in a minimum volume of 10 mM Tris-HCl (pH 7.5), 0.2 mM dithiothreitol, and 50 mM KCl and dialyzed against 2 × 4 L of the same buffer. The actomyosin was incubated (at a final concentration of 3 mg/mL) in 25 mM Tris-HCl (pH 7.5), 60 mM KCl, 4 mM $MgCl_2$, 0.8 mM [γ-^{32}P]ATP (~1000 cpm/nmol), and either 0.1 mM $CaCl_2$ (○, ●) or 1 mM EGTA (□, ■), in the presence (●, ■) and absence (○, □) of Ca^{2+}-independent MLCK (16 nM). Aliquots (0.5 mL) of reaction mixtures were withdrawn at the indicated times for measurement of ATPase activity as described by Ferenczi et al. (1978). Steady-state rates of ATPase activity were measured from the approximately linear portions of the curves following the initial rate. The actomyosin preparation contained 25% myosin as determined by densitometric scanning of a 0.1% $NaDodSO_4$ and 7.5–20% polyacrylamide gradient slab gel of the preparation (30 μg of protein).

vations indicated that the phosphorylation sites and the calmodulin-binding site are located in different parts of the molecule. One can visualize the calmodulin-binding site as completely accessible to Ca^{2+}–calmodulin in the nonphosphorylated kinase. Phosphorylation of both sites on the kinase could then cause this terminal region of the molecule to swing over and mask, at least partially, the calmodulin-binding site, thereby decreasing the affinity of the kinase for calmodulin. The major advantage of the limited α-chymotryptic hydrolysis reported here is that it yielded a kinase which retained full enzymatic activity but was no longer regulated by either Ca^{2+}–calmodulin or cAMP-dependent phosphorylation. Similar to limited tryptic digestion, α-chymotrypsin released a small peptide (M_r 23 000) which contained both phosphorylation sites. It is important to note that the 80 000-dalton Ca^{2+}-independent MLCK was produced only when digestion with α-chymotrypsin was performed on the calcium–calmodulin–kinase complex. MLCK appears to be more resistant to proteolysis by α-chymotrypsin in the calmodulin-free form. We are pursuing similar limited digestion studies with a number of proteases with a view to defining more clearly the domains of the MLCK molecule.

Aside from providing considerable structural information on the domains of MLCK, we felt that the Ca^{2+}-independent MLCK would be a useful tool for probing the function of myosin phosphorylation in the regulation of smooth muscle contractile activity. Hartshorne et al. (1977) had previously observed that treatment of partially purified chicken gizzard MLCK with a limiting amount of α-chymotrypsin in the presence of bound calmodulin resulted in loss of the Ca^{2+} dependence of the kinase but maintenance of enzymatic activity. We confirm this observation here with turkey gizzard MLCK and further purify the 80 000-dalton Ca^{2+}-independent MLCK by ion-exchange chromatography. The pattern of digestion observed in the absence of bound calmodulin was quite different. In this case, a 95 000-dalton peptide was generated, and full Ca^{2+} dependence was retained.

The 80 000-dalton Ca^{2+}-independent MLCK was shown to have a high specific activity (6.5 ± 0.2 μmol of P_i incorporated min^{-1} mg^{-1} in the presence of Ca^{2+} and 8.3 ± 0.3 μmol min^{-1} mg^{-1} in the absence of Ca^{2+}), which is close to that of the native, Ca^{2+}–calmodulin-dependent enzyme [5–30 μmol min^{-1} mg^{-1} (Dabrowska et al., 1977; Mrwa & Hartshorne, 1980; Adelstein & Klee, 1981)]. The K_m for Mg^{2+}–ATP was similar for both Ca^{2+}-dependent and -independent MLCKs. These facts suggest maintenance of the integrity of the active site after α-chymotryptic digestion. Our aim of obtaining an active Ca^{2+}-independent MLCK was thus achieved.

Further characterization of the 80 000-dalton kinase fragment showed that it had lost both the calmodulin-binding site and the sites of phosphorylation by cAMP-dependent protein kinase. This form of the enzyme is not, therefore, regulated either by Ca^{2+}–calmodulin or by cAMP-dependent phosphorylation. Its potential use to probe the regulatory mechanism in smooth muscle is encouraged by the results shown in Figure 8. Although of a preliminary nature, it is demonstrated that the phosphorylation of myosin in the absence of Ca^{2+} resulted in an activation of the actin-activated ATPase activity. Since significant activation occurred, it might be argued that phosphorylation plays a major role in the activation of the contractile apparatus in smooth muscle and it is unlikely that other Ca^{2+}-dependent mechanisms are essential for activation. This suggestion must be verified in future studies, and it is hoped that the application of the Ca^{2+}-independent MLCK to actomyosins or skinned fibers will provide the means of achieving this objective. These studies will be reported at a later date. The Ca^{2+}-independent kinase might also be useful for comparative studies on the regulatory mechanism in muscles from a variety of different species, e.g., in invertebrate muscles.

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Fluorescently Labeled Myosin Subfragment 1: Identification of the Kinetic Step Associated with the Adenosine 5′-Triphosphate Induced Fluorescence Decrease†

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ABSTRACT: Marsh and Lowey [Marsh, D. J., & Lowey, S. (1980) *Biochemistry 19*, 774–784] introduced a fluorescence label, 5-(iodoacetamido)fluorescein (IAF), into the alkali 1 light chain of myosin subfragment 1 (S-1) without perturbing the K^+-, Ca^{2+}-, or actin-activated ATPase activities of the labeled S-1. They also found that the addition of MgATP to the labeled S-1 led to a 6–10% decrease in the fluorescence of the bound IAF label. In the present study, we investigated the pre-steady-state kinetics of the fluorescence decrease of the IAF label caused by the binding of MgATP to the labeled S-1. Our results show that most of the decrease in IAF fluorescence is caused by the ATP-hydrolysis step. This implies that a conformational change in the S-1 molecule associated with ATP hydrolysis has led to a change in the microenvironment of the IAF fluorophore bound to the alkali 1 light chain.

The interaction of ATP with skeletal muscle myosin is accompanied by a significant increase in tryptophan fluorescence (Werber et al., 1972). Recently it has become apparent that most of this fluorescence enhancement is due to the ATP-hydrolysis step rather than the binding of ATP (Johnson & Taylor, 1978; Chock et al., 1979). The ATP hydrolysis step follows the binding of ATP as shown in the kinetic model for the myosin ATPase activity:

$$M + ATP \underset{}{\overset{K_1}{\rightleftharpoons}} M\cdot ATP \overset{K_2}{\rightleftharpoons} M^*\cdot ATP \overset{K_3}{\rightleftharpoons} M^{**}\cdot ADP\cdot P_i \overset{K_4}{\rightleftharpoons} M + ADP + P_i$$

where M is myosin, P_i is phosphate, and the stars indicate conformational states of myosin corresponding to different degrees of tryptophan fluorescence enhancement. The rate-limiting step in this model is the very slow conformational change which allows release of P_i (step 4), while the first-order step which causes the fluorescence enhancement (step 3) occurs quite rapidly and is responsible for the initial phosphate burst (Bagshaw & Trentham, 1974; Bagshaw et al., 1974).

Recently, Marsh & Lowey (1980) have been able to introduce a fluorescent label into the alkali 1 light chain of myosin subfragement 1 (S-1) without perturbing the K^+-, Ca^{2+}-, or actin-activated ATPase activities of the enzyme. These authors also found that the addition of MgATP to the labeled subfragment 1 causes a 6–10% decrease in the fluorescence of the label.

In the present study we investigated the pre-steady-state kinetics of the extrinsic fluorescence decrease which occurs when ATP interacts with subfragment 1 labeled on the alkali 1 light chain with 5-(iodoacetamido)fluorescein (IAF). In particular, we wished to establish which step in the kinetic cycle was responsible for the decrease in IAF fluorescence. Our results show that, as with the increase in intrinsic tryptophan fluorescence, much of the decrease in IAF fluorescence is caused by the ATP-hydrolysis step. It would appear that the conformational change associated with ATP hydrolysis has an effect on the microenvironment of the IAF fluorophore bound to the alkali 1 light chain.

Materials and Methods

Protein Preparations. Actin was prepared and its concentration determined as previously described (Fraser et al., 1975). The preparation and characterization of myosin subfragment

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