Functional Interactions between Smooth Muscle Myosin Light Chain Kinase and Calmodulin†

Dean A. Malencik, Sonia R. Anderson,* Janice L. Bohnert, and Yechiel Shalitin‡

ABSTRACT: Calmodulin (CaM) binding by turkey gizzard myosin light chain kinase (MLCK) causes subtle changes in the fluorescence emission and polarization excitation spectra of the enzyme. Fluorescence experiments using 9-anthroylcholine (9AC), which competes with ATP in binding, demonstrate mutually stabilizing interactions between the CaM and ATP binding sites corresponding to $\Delta G = -0.6$ to -0.7 kcal/mol. Fluorescence titrations in the presence of 9AC or 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate] confirm the stoichiometry of 1 mol of CaM/MLCK. Phosphorylation of MLCK has no effect on either the protein fluorescence or the binding of ATP and 9AC. The dissociation constant for the MLCK–CaM complex is increased ~500-fold on phosphorylation. Values of K_d for the phosphorylated enzyme range from 0.5 to 1.1 μM in 0.2 N KCl, pH 7.3, 25 °C. We showed competition between MLCK and other CaM binding proteins and peptides by using both fluorescence and catalytic activity measurements. Competition for CaM occurs with ACTH, β-endorphin, substance P, glucagon, poly(L-arginine), myelin basic protein, troponin I, and histone H2A. Phosphorylation of the last three proteins by the adenosine cyclic 3′,5′-phosphate dependent protein kinase diminishes their ability to compete. Phosphorylation of MLCK by the protein kinase gives 0.95 ± 0.04 and 2.2 ± 0.4 mol of incorporated ^{32}P in the presence and absence of CaM, respectively. These stoichiometries agree with those recently reported [Conti, M. A., & Adelstein, R. S. (1981) *J. Biol. Chem. 256*, 3178].

Smooth muscle and the actin microfilaments of nonmuscle cells both lack troponin, the calcium binding protein characteristic of skeletal and cardiac muscle (Driska & Hartshorne, 1975; Sobieszek & Small, 1976; Hartshorne et al., 1977). Calcium control in these more generalized contractile systems involves instead *myosin light chain kinase* (MLCK),[1] an enzyme subject to dual regulation by calcium and cAMP (Aldestein et al., 1978, 1980; Conti & Adelstein, 1981). Smooth muscle myosin light chain kinase catalyzes phosphorylation of the 20000-dalton light chain of myosin, a modification necessary for cross-bridging and subsequent contraction in smooth muscle (Hartshorne & Siemankowski, 1981; Hartshorne & Persechini, 1980; Small & Sobieszek, 1980; Adelstein & Eisenberg, 1980). The activity of myosin light chain kinase is absolutely dependent on its binding of the calcium–calmodulin complex (Dabrowska et al., 1978; Dabrowska & Hartshorne, 1978; Hathway & Adelstein, 1979; Yerna et al., 1979; Blumenthal & Stull, 1980). cAMP exerts an antagonistic effect due to the decrease in affinity for calmodulin resulting when smooth muscle myosin light chain kinase itself is phosphorylated by the cAMP-dependent protein kinase (Adelstein et al., 1980; Conti & Adelstein, 1981). This effect may be due to direct modification of the calmodulin binding site since Malencik & Anderson (1982) have evidence that calmodulin and the cAMP-dependent protein kinase interact with similar recognition sequences. Myosin light chain kinase showing the dual effects of cAMP and calcium has been isolated from smooth muscle, including chicken and turkey gizzards (Dabrowska & Hartshorne, 1978; Adelstein & Klee, 1981), and from platelets (Hathaway & Adelstein, 1979; Hathaway et al., 1981). Calmodulin-dependent myosin light chain kinases of uncertain physiological function also occur in brain (Dabrowska & Hartshorne, 1978), skeletal muscle (Pires et al., 1974; Yazawa & Yagi, 1977; Nairn & Perry, 1979; Pires & Perry, 1977), and cardiac muscle (Wolf & Hofmann, 1980). Possible effects of phosphorylation on them have not been determined.

This paper deals with the mutual effects of calmodulin binding and phosphorylation on the structural and functional properties of turkey gizzard myosin light chain kinase. It includes direct measurement of the equilibrium constant for the binding of calmodulin by the phosphorylated enzyme, of ATP binding by both forms of the enzyme, and of the competition between myosin light chain kinase and other calmodulin binding proteins for calmodulin. Fluorescence binding measurements and activity measurements following the incorporation of ^{32}P into the isolated myosin light chains are used.

Materials and Methods

Materials. Binding experiments were carried out in the presence of 0.2 N KCl, 50 mM Mops, 1 mM dithiothreitol, and the indicated concentrations of $MgCl_2$ and $CaCl_2$, pH 7.3 at 25 °C. Glass-distilled water was used throughout.

Mixed bovine and porcine glucagon, human β-endorphin (met), porcine ACTH (93 IU/mg) and substance P were purchased from Sigma Chemical Co. All peptides were dissolved or suspended at 1 mg/mL in the buffer used for the experiments. The glucagon sample was titrated with 0.1 N HCl for solubilization and used immediately. All concen-

† From the Department of Biochemistry and Biophysics, Oregon State University, Corvallis, Oregon 97331. *Received February 5, 1982.* Supported by research grants from the Muscular Dystrophy Association and from the National Institutes of Health (AM 13912).

‡ Permanent address: Department of Biology, Technion-Israel Institute of Technology, Haifa, Israel.

[1] Abbreviations: Mops, 3-(*N*-morpholino)propanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; cAMP, adenosine cyclic 3′,5′-phosphate; CaM, calmodulin; TnI, troponin I; MLCK, smooth muscle myosin light chain kinase; dansyl, 5-(dimethylamino)-1-naphthalenesulfonyl; 9AC, 9-anthroylcholine; bis(ANS), 5,5′-bis[8-(phenylamino)-1-naphthalenesulfonate]; $NaDodSO_4$, sodium dodecyl sulfate; $\bar{A}$, anisotropy; K, dissociation constant; F_∞, fluorescence of totally bound ligand; F_0, fluorescence of unbound ligand; F, observed fluorescence; ϕ, fractional degree of saturation; $\bar{n}$, number of moles of ligand bound per mole of protein.

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trations are based on weight except for ACTH, which exhibits varying degrees of glycosylation. The concentrations of ACTH were determined by measurements of the absorbance at 280 nm and of the tryptophan fluorescence relative to that of free tryptophan in solutions containing 4.8 N guanidine hydrochloride. The results, based on 2 mol of tyrosine and 1 mol of tryptophan per mol of ACTH, showed that the preparation is 58–63% protein by weight. Poly(L-arginine), molecular weight range 60 000, ATP, and dithiothreitol were also supplied by Sigma. 9-Anthroylcholine bromide was obtained from Molecular Probes, Inc. We prepared bis(ANS) according to a modification of the original procedure described by Rosen & Weber (1969).

Porcine brain calmodulin, prepared according to Schreiber et al. (1981), was subjected to a final purification step by using affinity chromatography on a fluphenazine–Sepharose matrix (Charbonneau & Cormier, 1979). This additional purification removes tryptophan-containing impurities. The calmodulin concentrations are based on $E^{1\%}_{280nm} = 2.0$ and a molecular weight of 16 680 (Watterson et al., 1980). Dansylcalmodulin, containing an average of 1.7 mol of the dansyl moiety, was prepared by using 5-(dimethylamino)-1-naphthalenesulfonyl chloride as described by Malencik & Anderson (1982).

Turkey gizzard myosin light chain kinase was prepared essentially according to the procedure of Adelstein & Klee (1981), to which we added a final purification by using affinity chromatography on a support of α-casein–Sepharose 4B to remove contaminating proteases. The enzyme gave a single band corresponding to a molecular weight of 120 000 on $NaDodSO_4$ gel electrophoresis. Enzyme concentrations are based on that molecular weight and $E^{1\%}_{280nm} = 10.0$. The assays of enzymatic activity are described in the following section. Stock solutions of the enzyme (2–3 mg/mL) containing 50 mM Mops, 1 mM dithiothreitol, and 1 mM EDTA were stored at −70 °C. Most experiments were performed within 3 weeks of the completion of the preparations.

Turkey gizzard myosin was isolated according to Sobieszek & Bremel (1975). The 15 000- and 20 000-dalton myosin light chains were extracted from the purified myosin and resolved by the procedure described for the skeletal muscle myosin light chains (Blumenthal & Stull, 1980).

Troponin I was prepared from rabbit muscle by the method of Kerrick et al. (1980). Myelin basic protein and histone H2A were purchased from Calbiochem, and their purity was checked by $NaDodSO_4$ gel electrophoresis. The catalytic subunit of cAMP-dependent protein kinase was prepared from beef heart according to Peters et al. (1977).

Catalytic Activity Measurements. The phosphotransferase activity of myosin light chain kinase was assayed in a 70-μL volume of solution containing 50 mM Mops, 10 mM $Mg(CH_3CO_2)_2$, 0.2 mM $Ca(CH_3CO_2)_2$ (or 2 mM EGTA), 10 μM 20 000-dalton smooth muscle light chain, 0.1 mM [γ-^{32}P]ATP (New England Nuclear, ca. 0.3–0.7 Ci/mmol), and 1 mg/mL bovine serum albumin at pH 7.3 and 25 °C. The reaction was initiated by the addition of the enzyme, giving a 0.4 nM final concentration. Unless otherwise indicated, calmodulin was included with the enzyme and corresponded to a final concentration of 28 nM. Calmodulin effectors (ACTH, troponin I, etc.) were preincubated with calmodulin for 2 min before addition to the assay mixture. The reaction was terminated by spotting a 50-μL aliquot of Whatman GF/C filter paper (Corbin & Riemann, 1974) and immediately washing in a 10% trichloroacetic acid–2% sodium pyrophosphate solution in preparation for liquid scintillation counting. The assay was linear with respect to ^{32}P incorporation for 20 min. The reaction was usually stopped after 5 min. The specific activity of the enzyme in the above assay was 12 μmol of ^{32}P min^{-1} mg^{-1}.

Similar conditions were employed for phosphorylation of myosin light chain kinase by the cAMP-dependent protein kinase. The purified catalytic subunit of protein kinase was added to give 20 μg/mL in a solution containing 1 mg/mL myosin light chain kinase in 50 mM Mops, 4 mM $Mg(CH_3CO_2)_2$, 0.2 mM $Ca(CH_3CO_2)_2$, 0.2 mM [γ-^{32}P]ATP, 1 mM dithiothreitol, and 1 mg/mL bovine serum albumin, pH 7.3 and 25 °C. Two sets of experiments were done, in the presence and absence of 160 μM calmodulin. The phosphorylation was followed until a plateau was reached. The amount of ^{32}P incorporated at equilibrium was 0.95 ± 0.04 in the presence of calmodulin and 2.2 ± 0.4 in its absence. Omission of the catalytic subunit of protein kinase from this reaction shows that no autophosphorylation of myosin light chain kinase occurs.

Fluorescence Measurements. Measurements of fluorescence anisotropy and total intensity ($I_\parallel + 2I_\perp$) were obtained by using the SLM 4000 fluorescence polarization spectrophotometer. The excitation bandwidth was set at 1 nm for all experiments. Glass filters were placed between the sample and the photomultiplier tubes. Fluorescence polarization excitation spectra of the intrinsic protein fluorescence were obtained by using a Corning glass CSO-52 filter. The measurements on 9-anthroylcholine were obtained with 366-nm excitation and a Schott KV 389 filter, on bis(ANS) with 390-nm excitation and a Schott KV 418 filter, and on dansylcalmodulin with 340-nm excitation and a Schott KV 389 filter. The sample compartment was maintained at 25.0 ± 0.1 °C with a circulating water bath. All experiments were carried out with the SLM 4000 spectrophotometer except for the titrations given in Figure 2 and for the emission spectra, which were obtained by using the Hitachi Perkin-Elmer MPF 2-A fluorometer. All spectra are corrected for the wavelength dependence of the grating transmission and detector response. The details of the titrations and calculations are described by Anderson & Weber (1965) and by Anderson (1974). The intensities in Figure 2 were corrected to linear concentration response by comparison to fluorescence standards (9-anthroylcholine alone or quinine sulfate) of matching absorbancies.

Results

Effects of Calmodulin Binding and Phosphorylation on the Intrinsic Protein Fluorescence of Myosin Light Chain Kinase. Since the only fluorescent amino acid in calmodulin is tyrosine, the effects of calmodulin binding on the intrinsic tryptophan fluorescence of myosin light chain kinase are easy to determine. Excitation at 295 nm largely excludes the contribution of tyrosine to intrinsic protein fluorescence spectra (Teale, 1960; Weber, 1961). The binding of calmodulin by the unphosphorylated enzyme results in a shift in the fluorescence emission maximum from 331 to 327 nm and a 7% increase in quantum yield, suggesting subtle changes in the local environments of the tryptophan residues. Phosphorylation of myosin light chain kinase has no effect on its emission spectrum.

Fluorescence polarization excitation spectra of proteins reflect the relative rigidities of the tryptophanyl side chains, providing information not obtainable from emission spectra (Anderson & Weber, 1966). Figure 1A shows that the binding of calmodulin causes a uniform increase in the fluorescence anisotropy[2,3] of myosin light chain kinase reversed by the

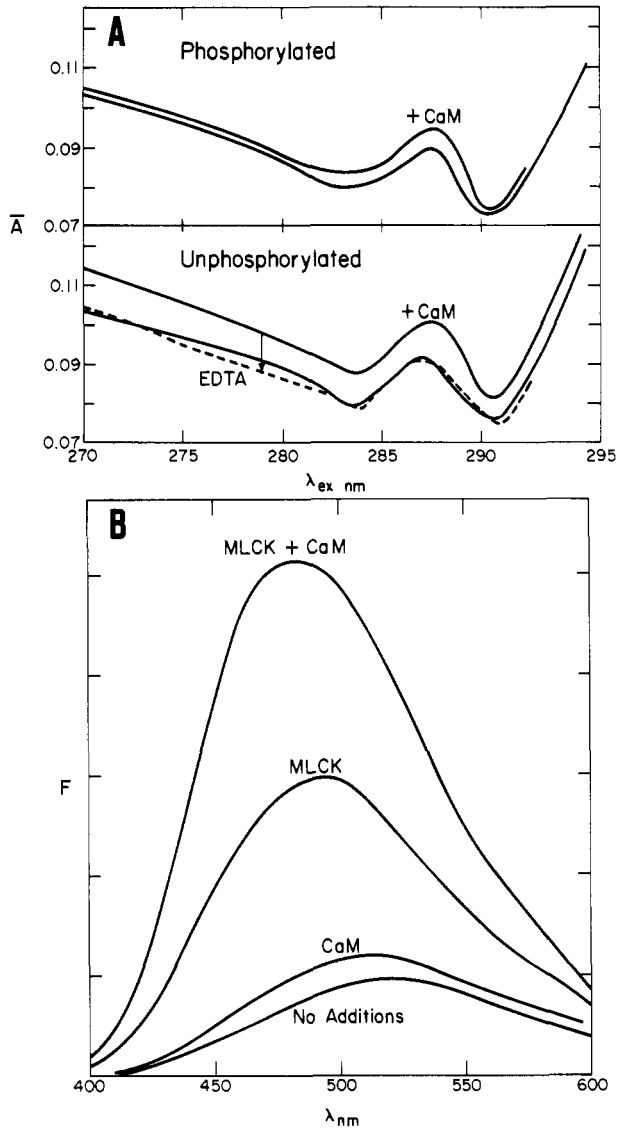

FIGURE 1: Fluorescence spectra. (A) Fluorescence polarization excitation spectra showing the binding of calmodulin by phosphorylated and unphosphorylated myosin light chain kinase. 1.25 μM calmodulin was added to 1.0 μM solutions of myosin light chain kinase containing 0.2 N KCl, 50 mM Mops, 1 mM dithiothreitol, and 0.85 mM $CaCl_2$, pH 7.3, 25 °C. The dashed line shows the spectrum recorded after the addition of 10 mM EDTA to the complex. The emitted light was observed through Corning glass filter CSO-52. (B) Fluorescence emission spectra of 7.0 μM 9-anthroylcholine with 0.5 μM calmodulin, with 0.5 μM myosin light chain kinase, with 0.5 μM calmodulin *plus* 0.5 μM myosin light chain kinase, and with no additions. The wavelength of the exciting light was 366 nm. Solution conditions are the same as in (A). The fluorescence intensity is in arbitrary units.

addition of excess EDTA. The overall increase in anisotropy is explained either by an actual increase in the average local rigidity of the tryptophan residues or by changes in individual quantum yields which increase the contribution of the more rigid residues to the observed anisotropy. The fluorescence

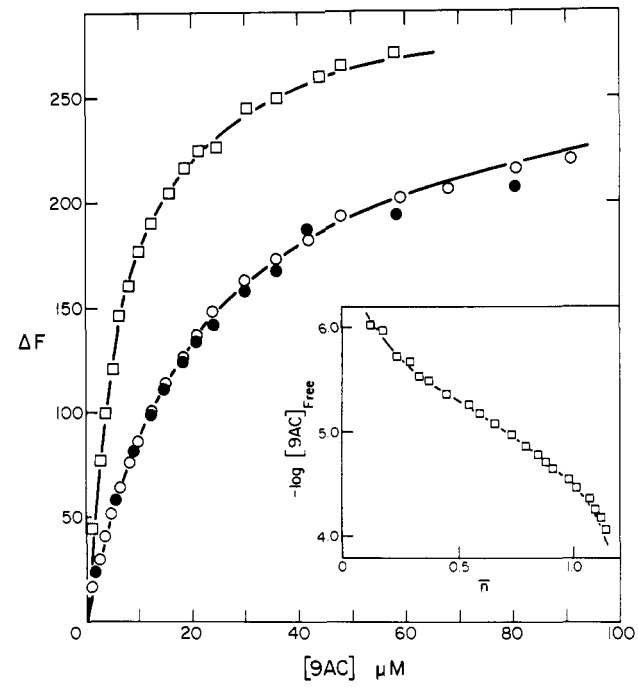

FIGURE 2: Fluorescence titration of myosin light chain kinase with 9-anthroylcholine: (□) 1.0 μM kinase + 1.0 μM calmodulin; (○) 1.0 μM kinase; (●) 1.0 μM phosphorylated kinase. The smooth curves were calculated for the binding of 1.2 mol of 9AC with dissociation constants of 20 μM (○, ●) and 6.4 μM (□). The inset contains the log plot of the concentrations of unbound 9AC vs. $\bar{n}$ in moles of 9AC bound per mole of kinase for a 2.0 μM solution of the enzyme–calmodulin complex. Excitation, 366 nm; emission, 460 nm. ΔF is the difference between the fluorescence intensities of 9AC obtained in the presence and absence of protein. Other conditions are given under Figure 1.

polarization spectrum of the phosphorylated myosin light chain kinase, not significantly different from that of the unphosphorylated enzyme, is considerably less affected by the addition of calmodulin, reflecting the reduced binding described in later sections.

Binding of 9-Anthroylcholine by Myosin Light Chain Kinase. The calcium–calmodulin complex binds four to six molecules of 9-anthroylcholine (9AC) with an average dissociation constant of 440 μM and a 24-fold enhancement of ligand fluorescence. Most of the bound 9AC dissociates when calmodulin binds troponin I, giving a stoichiometric fluorescence titration with an end point corresponding to 1 mol of troponin I/calmodulin (LaPorte et al., 1980). In experiments to determine whether similar titrations could be performed with calmodulin and myosin light chain kinase, we discovered that the enzyme binds 9AC with an affinity and fluorescence enhancement much larger than those obtained with calmodulin alone. In fact, the binding of 9AC to calmodulin makes minimal contribution to several of the experiments which follow.

Figure 1B shows the effects of calmodulin and myosin light chain kinase on the fluorescence emission spectrum of 9AC. The fluorescence enhancement obtained with the myosin light chain kinase–calmodulin complex exceeds the sum of the enhancements obtained with the two proteins alone, suggesting that either the fluorescence yield or the affinity of myosin light chain kinase for 9AC is increased in the complex. Addition of excess EDTA to the enzyme–calmodulin complex reverses this enhancement, giving the same fluorescence obtained with the enzyme and 9AC alone. To gain more information on these effects, we titrated both myosin light chain kinase and the kinase–calmodulin complex with varying concentrations

[2] $\bar{A} = (I_{\parallel} - I_{\perp})/(I_{\parallel} + 2I_{\perp})$, where $I_{\parallel}$ and $I_{\perp}$ are the intensities of the two linearly polarized components of the light emitted at right angles to the excitation direction. $I_{\parallel}$ vibrates in the direction of propagation of the exciting light, and $I_{\perp}$ vibrates normally to the plane corresponding to the directions of excitation and observation. In a mixture of fluorescent species, the average anisotropy equals the sum of the individual anisotropies weighed by the individual fractional contributions to the total fluorescence intensity (Weber, 1952): $\bar{A} = \sum f_i A_i$.

[3] The "blank" calmodulin fluorescence was negligible at all exciting wavelengths.

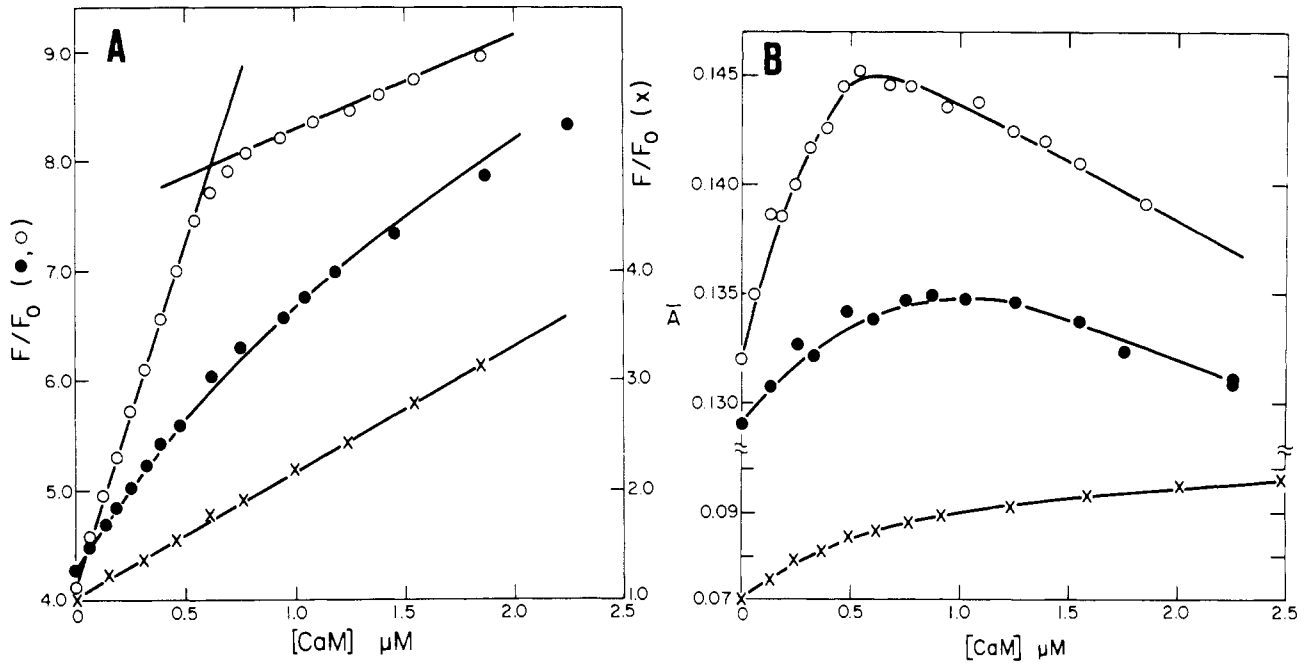

FIGURE 3: Fluorescence titration of 9-anthroylcholine with calmodulin in the presence of 0.5 μM myosin light chain kinase (○), 0.5 μM phosphorylated myosin light chain kinase (●), and with no additions (×). Panel A shows the relative fluorescence enhancements, where F_0 is the fluorescence of 9AC in the absence of protein. The smooth curve for the phosphorylated kinase (●) was calculated for the binding of 1 mol of calmodulin with a dissociation constant of 1.1 μM. Panel B shows the corresponding fluorescence anisotropy changes. The solutions contained 7.0 μM 9AC in addition to the constituents given under Figure 1. Excitation, 366 nm; emission, Schott KV 389 filter.

of 9AC (Figure 2). The convergence of the titration curves at high concentrations of 9AC, where the extrapolated values of ΔF agree within 11%, indicates that the total number of binding sites and the fluorescence yields are probably the same in the two cases. Extrapolation of the fluorescence of a 1.0 μM solution of 9AC to infinite concentration of the enzyme–calmodulin complex, using a plot of ΔF^{-1} vs. the reciprocal of the protein concentration, gives an enhancement factor (F_∞/F_0) of *260*. The fractions (f) of 9AC bound in the titrations are calculated from the relationship $f = (F_{obsd}/F_0 - 1)/(F_\infty/F_0 - 1)$.

Figure 2 (inset) shows the log plot of the calculated concentrations of unbound 9AC vs. $\bar{n}$, the number of moles of 9AC bound per mole of enzyme, for a 2.0 μM solution of the myosin light chain kinase–calmodulin complex. The smooth curve through the points was calculated for the binding of 1.2 mol of 9AC and a dissociation constant of *6.4 μM*. The dissociation constant for 9AC and the enzyme alone, either phosphorylated or unphosphorylated, is *20 μM*. *Thus, the increase in fluorescence observed upon the addition of calmodulin to the enzyme reflects increased binding of 9AC.*

Similar experiments performed in the presence of 0.3 mg/mL 20 000-dalton myosin light chain showed no additional effects on either the enzyme alone or the enzyme–calmodulin complex.

Calmodulin Binding by Myosin Light Chain Kinase. The binding of 9-anthroylcholine by myosin light chain kinase, showing complete insensitivity to phosphorylation and positive interaction with calmodulin binding, can be used to determine the stoichiometry and dissociation constants for the enzyme–calmodulin complex. The titrations in Figure 3A were carried out in the presence of 7.0 μM 9AC, a nonsaturating ligand concentration in excess of the protein concentrations. The enhanced binding of 9AC occurring upon the addition of varying amounts of calmodulin to the enzyme results in a linear increase in fluorescence intensity with a definite end point at 1.2 mol of calmodulin. Addition of excess calmodulin results in a slower rate of increase in fluorescence intensity nearly parallel to that obtained on the addition of calmodulin to 7.0 μM 9AC alone. This linear stoichiometric titration indicates virtually complete binding of calmodulin up to saturation. A similar experiment using the phosphorylated enzyme shows that the enzyme–calmodulin complex is extensively dissociated. By assuming that the fluorescence changes obtained on the complete binding of calmodulin by the phosphorylated enzyme are the same as those obtained with the unphosphorylated enzyme, we can calculate the fractional saturations. The smooth curve through the data points obtained with the phosphorylated enzyme corresponds to a dissociation constant of 1.1 μM for the enzyme–calmodulin complex.

Anisotropy[2] measurements obtained together with the above intensity measurements also reflect the extent of association between the enzyme and calmodulin (Figure 3B). The anisotropy observed with the unphosphorylated enzyme reaches a maximum at the stoichiometric ratio of 1.2 mol of calmodulin. The decrease in anisotropy seen at higher ratios reflects accumulation of the calmodulin–9AC complex, which has a lower anisotropy. The anisotropy obtained with the phosphorylated enzyme attains a lower maximum value, showing the presence of the unbound calmodulin–9AC complex throughout the titration.

Competition of Myosin Light Chain Kinase with Other Calmodulin Binding Proteins. The myosin light chains, ancestrally related to both calmodulin and troponin C (Barker et al., 1977), may bind some of the same proteins which bind calmodulin. Accordingly, the competition between myosin light chain kinase and calmodulin binding proteins may be difficult to detect in enzyme activity measurements where the intact light chains are used as substrate. We have applied a more direct method to detect competition based on the characteristic binding of 9-anthroylcholine by myosin light chain kinase. First, we showed that calmodulin binding proteins such as histone H2A and the myelin basic protein displace 9AC from calmodulin in the same way found by LaPorte et al. (1980) for troponin I. Next, we showed that these proteins have no effect on the binding of 9AC by the enzyme alone and

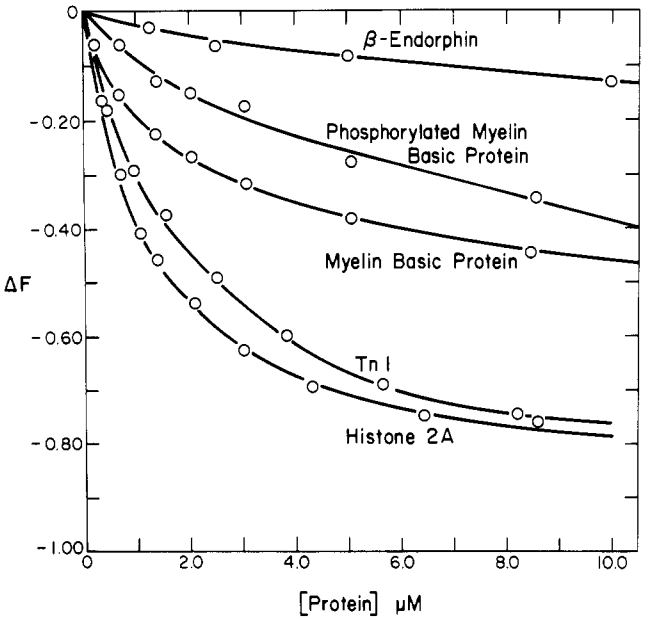

FIGURE 4: Dissociation of the myosin light chain kinase–calmodulin complex by other calmodulin binding proteins (troponin I, histone H2A, myelin basic protein, phosphorylated myelin basic protein, and β-endorphin). ΔF is the negative of the fraction of the difference between the fluorescence intensities of 0.5 μM myosin light chain kinase plus 0.5 μM calmodulin and of 0.5 μM myosin light chain kinase alone. The solutions contained 14.0 μM anthroylcholine in addition to the constituents given under Figure 1. See Figure 3 for other details.

that they do not bind 9AC themselves. Addition of one of these proteins to the calmodulin–myosin light chain kinase complex results in a net equilibrium such as the following shown for troponin I. Since the calmodulin concentration used

$$\text{MLCK·CaM} + \text{TnI} \rightleftarrows \text{MLCK} + \text{TnI·CaM}$$

is never in excess of the enzyme, the concentration of unbound calmodulin is negligible. Only two of the components in this equilibrium bind 9AC appreciably. Thus, the fluorescence obtained on the addition of troponin I, for example, should range between the values obtained with the enzyme–calmodulin complex and with the enzyme alone. Our experimental results are expressed as the negative of the fraction of the difference, ΔF, between these reference values.

The distribution of calmodulin between the two complexes and the ratio of the dissociation constants for the competing proteins are simple to determine. As illustrated for troponin I:

$$\frac{K_{\text{MLCK·CaM}}}{K_{\text{TnI·CaM}}} = \frac{\Delta F^2}{([\text{TnI}]/[\text{MLCK}]_{\text{total}})(1 - |\Delta F|)}$$

We have recently found that calmodulin binds small peptides such as ACTH, β-endorphin, glucagon, and substance P (Malencik & Anderson, 1982), following up the report of Weiss et al. (1980) that ACTH and β-endorphin inhibit the cyclic nucleotide phosphodiesterase. Figure 4 shows the competition resulting when β-endorphin, a representative peptide, and several known calmodulin binding proteins are added to solutions of the enzyme–calmodulin complex containing 14 μM 9AC. The ratios of dissociation constants calculated by applying the above equation to the data are $\sim 8 \times 10^{-4}$ for β-endorphin, $\sim 7 \times 10^{-3}$ for the phosphorylated myelin basic protein, $\sim 1.5 \times 10^{-2}$ for the unphosphorylated myelin basic protein, $\sim 4 \times 10^{-2}$ for troponin I, and $\sim 6 \times 10^{-2}$ for histone H2A. Dissociation constants are available for troponin I (60 nM) (LaPorte et al., 1981) and β-endorphin (2 μM) (Malencik & Anderson, 1982). If these values apply to the conditions of the present experiment, the dissociation constant for the enzyme–calmodulin complex must be 1.6–2.4 nM. Poly(L-arginine) dissociates the enzyme–calmodulin complex very efficiently, causing the fluorescence to fall somewhat below the base-line value. The additional displacement results from a smaller direct effect of poly(L-arginine) on the binding of 9AC by the enzyme alone.

Table I: Inhibition of Myosin Light Chain Kinase by Calmodulin Binding Proteins[a]

protein or peptide added	concn (μM)	% initial activity[b]
none	0	100
ACTH	22	22.5
β-endorphin	29	33
substance P	45	42
glucagon	39	70
histone H2A	6.0	18
phosphorylated H2A	6.0	35
troponin I	10.0	15
phosphorylated troponin I	10.0	45
myelin basic protein	10.0	12
phosphorylated myelin basic protein	10.0	39
poly(L-arginine)	20 μg/mL	67

[a] Assay conditions were the following: 0.4 nM MLCK, 28 nM CaM, 10 μM 20 000-dalton light chain, 0.1 mM ATP, 10 mM $Mg(CH_3COO)_2$, 0.2 mM $Ca(CH_3COO)_2$, 1.0 mM dithiothreitol, and 50 mM Mops containing 1 mg/mL bovine serum albumin at pH 7.3 (25 °C). [b] Reproducibility was generally ±5% of the measured activity.

Next we carried out inhibition experiments showing the effects of several calmodulin binding proteins and peptides on the catalytic activity of myosin light chain kinase. The results (Table I) should complement the preceding experiments, giving independent evidence for the competition with myosin light chain kinase and for effects of phosphorylation on calmodulin binding proteins. Comparison of the two sets of results may identify cases in which the 20 000-dalton myosin light chain interferes.

The order of effectiveness in inhibition by the four hormones and neurotransmitters (Table I) is consistent with the relative affinities obtained from direct binding measurements on calmodulin (Malencik & Anderson, 1982). The inhibition obtained at the reported concentrations of histone H2A, troponin I, or the myelin basic protein is generally less than that expected from the competition demonstrated in the presence of 9-anthroylcholine. Myelin basic protein is affected less than the other two proteins. Reduction of the concentrations to 1 μM or less diminishes the inhibition to a greater extent than expected from dilution alone (data not shown), indicating that the interference by the myosin light chain increases as the concentration of the added inhibitor becomes limiting. Phosphorylation attenuates the inhibitory properties of all three proteins.

Nucleotide Binding by Myosin Light Chain Kinases. The addition of ATP to solutions of myosin light chain kinase and 7.0 μM 9-anthroylcholine results in a decrease in observed fluorescence (Figure 5). The fluorescence enhancements can be reduced to 15% of the initial value with the enzyme–calmodulin complex and to 19% with the enzyme alone, suggesting actual displacement of 9AC by ATP.[4] Since the

[4] The addition of excess ATP reverses the quenching of the protein fluorescence which occurred on the binding of 9AC, indicating that most of the dye dissociates when ATP binds (data not shown). The residual enhancements of 15 and 19% seen at saturating ATP concentrations probably reflect unspecific binding of the dye to other sites.

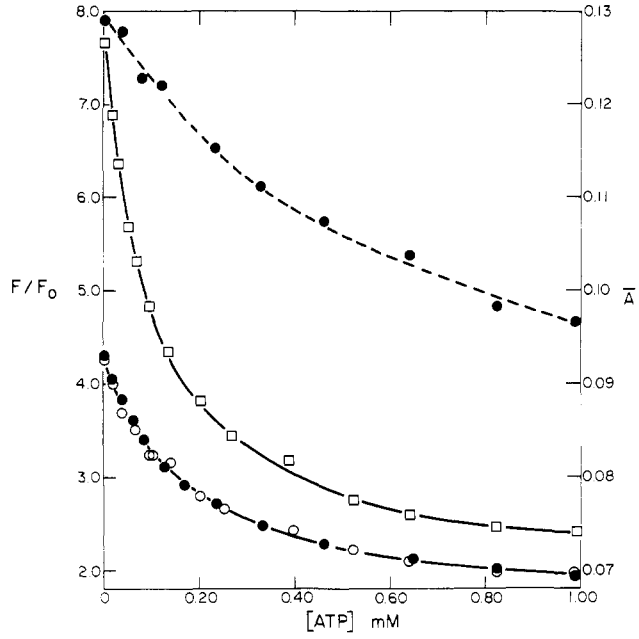

FIGURE 5: Effect of ATP concentration on 9-anthroylcholine binding by myosin light chain kinase. (□) 0.5 μM kinase + 0.5 μM calmodulin; (○) 0.5 μM kinase; (●) 0.5 μM phosphorylated kinase. The dashed line indicates the anisotropy changes obtained with the phosphorylated kinase. The solutions contained 7.0 μM 9-anthroylcholine and 2 mM $MgCl_2$ in addition to the constituents listed under Figure 1. See Figure 3 for other details.

concentrations of ATP greatly exceed that of the enzyme, the data can be analyzed according to the noncompetitive model of McClure & Edelman (1967). Plots of the changes in fluorescence ($I_0 - I$) vs. $(I_0 - I)/[ATP]$ are linear, yielding the apparent dissociation constants (K_{app}) for the enzyme–ATP complex. K_{app} is related to the true dissociation constant (K_d):

$$K_{app} = \frac{(P_0 + X_0)/K_2 + 1}{(P_0 + X_0)/K_3 + 1} K_d$$

P_0 and X_0 represent the total concentrations of enzyme and 9AC, respectively. K_2 and K_3 are the dissociation constants for the release of 9AC from the protein–9AC and the protein–9AC–ATP complexes, respectively. The values for K_2 were determined in the experiments shown in Figure 3. Since 9AC seems to be largely released on the addition of ATP,[4] we treat the reaction as competitive and ignore the denominator of the equation in order to approximate the values of K_d for ATP. The following tabulation summarizes the values of K_{app} and K_d obtained in the three cases.

	K_{app} (mM)	K_d (mM)
unphosphorylated MLCK	0.17	0.12
phosphorylated MLCK	0.17	0.12
unphosphorylated MLCK–CaM	0.093	0.043

Titrations using AMP and ADP also showed a fluorescence decrease. The values of K_{app} and K_d obtained with the unphosphorylated enzyme are tabulated below.

	K_{app} (mM)	K_d (mM)
ADP	0.46	0.32
AMP	1.1	0.8
GTP	∞	∞

Binding of 5,5'-Bis[8-(phenylamino)-1-naphthalenesulfonate] by Myosin Light Chain Kinase. The binding of bis(ANS) by myosin light chain kinase is interesting since it responds to phosphorylation of the enzyme as well as to ATP

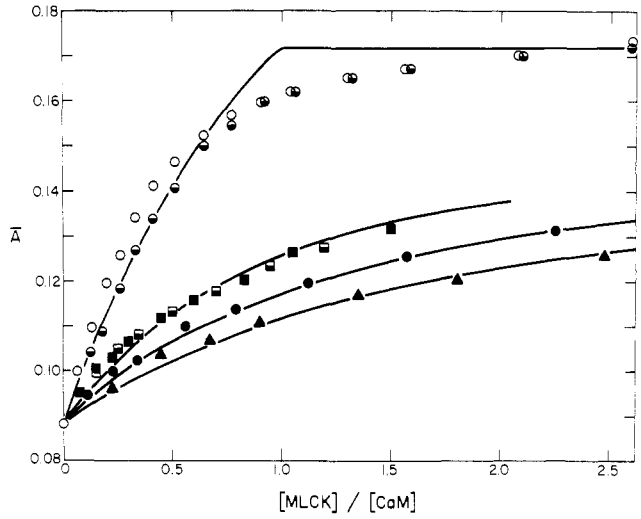

FIGURE 6: Fluorescence anisotropy changes on the binding of dansylcalmodulin by myosin light chain kinase. Titrations of 0.5 μM dansylcalmodulin with unphosphorylated kinase (○) and of 0.25 (▲), 0.50 (●), and 0.75 (■) μM dansylcalmodulin with phosphorylated kinase are shown. Mixing experiments using 0.25 μM dansylcalmodulin plus 0.25 μM unlabeled calmodulin (◒) and 0.38 μM dansylcalmodulin plus 0.38 μM unlabeled calmodulin (□) are also shown for the two forms of the enzyme. The smooth curves were calculated according to the text. Other conditions are given under Figure 1. Excitation, 340 nm; emission, Schott KV 389 filter.

and calmodulin binding. However, the binding of bis(ANS) seems to be considerably more heterogeneous than the binding of 9AC. The addition of ATP to solutions of the enzyme and 1.0 μM bis(ANS) causes the fluorescence of the bis(ANS) to decrease. Although the initial intensities of the phosphorylated and unphosphorylated enzymes differ by ~25%, the two titration curves with ATP are nearly parallel (data not illustrated but submitted to reviewers). Phosphorylation of the enzyme apparently causes either dissociation or quenching of bis(ANS) molecules which are not affected by ATP binding. Calmodulin binding has a complex effect, showing quenching or displacement of bis(ANS) together with a decline in sensitivity to ATP binding. Competition with ATP is difficult to detect when higher bis(ANS) concentrations are used, suggesting increased occupation of another class of binding sites by bis(ANS). The fluorescence anisotropy[2] of bis(ANS) was used to follow calmodulin binding by the enzyme. The results show a decrease in anisotropy, reflecting some change in the average environment of the remaining bound bis(ANS), and an end point of 1 mol of calmodulin (data not shown but examined by reviewers). Titration of the phosphorylated enzyme with calmodulin shows little detectable interaction, indicating either that there is none or that the bound bis(ANS) is insensitive to calmodulin binding.

Interaction of Dansylcalmodulin with Myosin Light Chain Kinase. Malencik & Anderson (1982) prepared a fluorescent conjugate of calmodulin with 5-(dimethylamino)-1-naphthalenesulfonyl chloride showing extraordinary sensitivity to both calcium and protein binding. They performed fluorescence titrations with the conjugate to demonstrate the binding of ACTH, β-endorphin, glucagon, and substance P by calmodulin. The dissociation constants for the complexes of ACTH and glucagon with dansylcalmodulin are close to those obtained with unmodified calmodulin when the changes in the intrinsic tryptophan fluorescence of the hormones are measured.

Figure 6 shows the anisotropy[2] changes occurring when dansylcalmodulin is titrated with myosin light chain kinase. The upper curve was calculated for the complete binding of

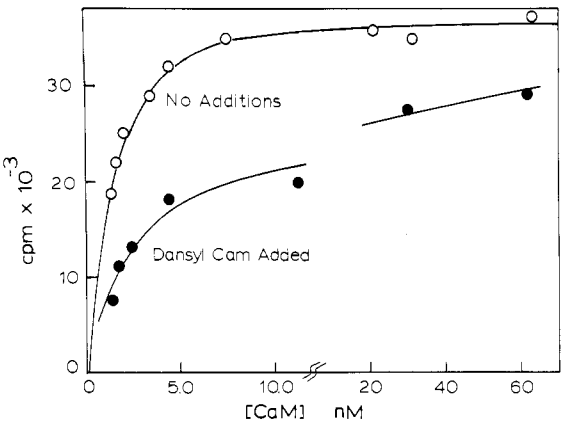

FIGURE 7: Dependence of the rate of phosphorylation of the 20 000-dalton myosin light chain on the native calmodulin concentration. Conditions: 0.4 nM myosin light chain kinase, 10 μM myosin light chain, 0.1 mM ATP, 10 mM $Mg(CH_3CO_2)_2$, 0.2 mM $Ca(CH_3CO_2)_2$, 1.0 mM dithiothreitol, 50 mM Mops, pH 7.3, 25 °C. (○) No other additions; (●) 0.5 nM dansylcalmodulin added.

1 mol of enzyme/calmodulin by using the fluorescence enhancement factor (1.7) and the anisotropies measured at the beginning (A_0) and the end of the titration (A_B). The equation relating the average anisotropy to these values and to the fractional degree of saturation of calmodulin with the enzyme (ϕ) derives directly from the principle of anisotropies (Weber, 1952).

$$\bar{A} = \frac{(F_\infty/F_0)\phi A_B + (1 - \phi)A_0}{(F_\infty/F_0)\phi + (1 - \phi)}$$

Deviations of the experimental points from the theoretical curve are modest. A mixing experiment, using a 1:1 mixture of dansylcalmodulin and native calmodulin, shows that the dansyl derivative still binds well in the presence of native calmodulin and limiting concentrations of myosin light chain kinase. The slightly lower anisotropies obtained with the mixture indicate some preference for unmodified calmodulin. The anisotropy changes are reversed on the addition of EDTA.

Repetition of the titration using the phosphorylated myosin light chain kinase shows extensive dissociation. The concentration dependence demonstrated with 0.25, 0.50, and 0.75 μM dansylcalmodulin shows that there is a dynamic equilibrium. The smooth curves were calculated for dissociation constants of 0.5, 0.6, and 0.4 μM, respectively. The best fit of the data was obtained with a value for A_B smaller than that observed with the unphosphorylated enzyme (0.15 vs. 0.172). This may reflect some difference between the complexes or possibly failure of a portion of the dansylcalmodulin to bind the phosphorylated enzyme.

Overall, myosin light chain kinase binds dansylcalmodulin well and shows the expected effects of calcium binding and phosphorylation in the fluorescence measurements. Consequently, we were surprised at the results when dansylcalmodulin was used in measurements of the catalytic activity of myosin light chain kinase. The maximum rate of incorporation of ^{32}P into the isolated myosin light chains was only 16% of that obtained with native calmodulin. Since saturating concentrations of dansylcalmodulin were being used, we suspected that a portion of the labeled calmodulin population is actually inhibitory. We carried out an experiment in which a fixed concentration (0.5 nM) of dansylcalmodulin was added to assay mixtures containing varying concentrations of native calmodulin. The results (Figure 7) show considerable inhibition reversed only at high concentrations of unmodified calmodulin. The concentration dependence of the rate of incorporation of ^{32}P into the myosin light chains corresponds to a K_m of 1.3 nM when none of the dansyl derivative is present.

Discussion

The binding of calmodulin by myosin light chain kinase causes subtle changes in the intrinsic fluorescence spectrum and the fluorescence polarization excitation spectrum of the enzyme, suggesting either the occurrence of small conformational changes affecting the tryptophan residues or direct involvement of tryptophan at the binding site. Johnson et al. (1981) found similar but larger changes occurring in the emission spectrum of skeletal muscle myosin light chain kinase which they used in a stoichiometric fluorescence titration of the enzyme with calmodulin. The binding of calmodulin by smooth muscle myosin light chain kinase enhances the binding of both ATP and 9-anthroylcholine, which seem to compete for a common binding site on the enzyme. The dissociation constants for ATP and 9-anthroylcholine are 120 and 20 μM, respectively, for the enzyme alone and 43 and 6.4 μM for the enzyme–calmodulin complex. This mutually stabilizing interaction between the calmodulin and ATP binding sites corresponds to a free energy of −0.6 to −0.7 kcal/mol.

The effects of phosphorylation on smooth muscle myosin light chain kinase seem to be even more localized. Phosphorylation has no effect on either the intrinsic fluorescence of the enzyme or the binding of ATP and 9-anthroylcholine. The phosphorylated enzyme is distinguished by a drastic decrease in affinity for calmodulin demonstrated in four different experiments. The dissociation constant for the complex of calmodulin with the phosphorylated enzyme ranges from 1.1 μM in the presence of 7.0 μM 9-anthroylcholine to ~0.5 μM when the dansyl derivative is used. In contrast, the dissociation constant for the unphosphorylated enzyme is almost beyond the range of direct determination. Corresponding K_m's from activity measurements by us (Figure 7) and by Adelstein & Klee (1981) are 1.3 and 1.0 nM, respectively. The dissociation constants which we have determined for the phosphorylated enzyme are at least 10-fold larger than the K_m reported by Conti & Adelstein (1981). Our phosphorylated myosin light chain kinase contains 2.2 ± 0.4 mol of phosphorus, the same amount present in the preparation of Conti and Adelstein. The difference between the K_m and the dissociation constant probably reflects the moderately stabilizing effect of MgATP, the ionic strengths used in the two experiments, and the sensitivity of the activity measurements to a trace of unphosphorylated enzyme. Conti and Adelstein used a 0.03 M tris(hydroxymethyl)aminomethane hydrochloride buffer while we used 0.05 M Mops plus 0.2 N KCl. Stabilization of the enzyme–calmodulin complex by the myosin light chains could also result in $K_m < K_d$ and has been suggested by Nairn & Perry (1979).

The phosphorylated myosin light chain kinase would have low rank in a hierarchy of calmodulin binding proteins. Its affinity for calmodulin is just severalfold larger than that found by Malencik & Anderson (1982) for specific small peptides. The concentrations of myosin light chain kinase and calmodulin in the gizzard are 1.2 (Adelstein & Klee, 1981) and 11 μM (Grand et al., 1979), respectively. In the absence of other calmodulin binding proteins, the phosphorylated enzyme would be nearly saturated in vivo. However, any decrease in the concentration of free calmodulin should result in an *immediate* decline in the activity of the phosphorylated, but not of the unphosphorylated, enzyme.

Competition experiments carried out in the presence of 9-anthroylcholine show that histone H2A and troponin I

compete very effectively with myosin light chain kinase in calmodulin binding. The partition coefficients show that the dissociation constants for the complexes of calmodulin with histone H2A and troponin I are just 16- and 25-fold larger, respectively, than that for the complex with myosin light chain kinase. These values suggest specific binding of these proteins by calmodulin, with competition for common binding sites. Inhibition of myosin light chain kinase by low concentrations of troponin I and histone H2A has been difficult to detect in activity measurements. Perry (1980) considered the possibility that troponin I and the enzyme occupy different binding sites on calmodulin. We believe that the failure of these proteins to inhibit the enzyme as well as expected reflects their binding by the substrate, the isolated 20000-dalton myosin light chain. Preliminary experiments by us show that both the 15000- and 20000-dalton smooth muscle light chains indeed bind troponin I.[5] The inhibition of myosin light chain kinase by calmodulin binding peptides—ACTH, β-endorphin, substance P, and glucagon—is consistent with competition between the peptides and the enzyme. The degree of inhibition is comparable to that found by Weiss et al. (1980) with the cyclic nucleotide phosphodiesterase and ACTH or β-endorphin.

Competition between myosin light chain kinase and any of these proteins or peptides is unlikely to occur in vivo, due either to compartmentalization or to their strong association with other proteins. Some regard the interaction between calmodulin and the histones or the myelin basic protein as entirely nonspecific (Itano et al., 1980). However, we note that nearly all the proteins which bind calmodulin are also substrates of the cAMP-dependent protein kinase. Malencik & Anderson (1982) found that the peptide hormones and neurotransmitters which bind calmodulin well ($K_d < 10$ μM) contain sequences similar to the recognition sequence for the cAMP-dependent protein kinase and to the phosphorylated sequences in several calmodulin binding proteins. We suggested that calmodulin and the cAMP-dependent protein kinase act on common recognition sequences in the target proteins. The similar specificities imply that proteins which are substrates of protein kinase tend to bind calmodulin, even though their functions may not be calmodulin dependent. The general effect of phosphorylation of the myelin basic protein, troponin I, or histone H2A on the competition with myosin light chain kinase (Figure 4, Table I) is consistent with modification of the calmodulin binding sites in these proteins. Experiments with a synthetic cAMP-dependent protein kinase substrate (Pro-Leu-Ser-Arg-Thr-Leu-Ser-Val-Ser-Ser-NH_2) which undergoes calcium-dependent binding by calmodulin support the hypothesis that calmodulin and protein kinase act on common recognition sequences (Anderson & Malencik, 1982). We believe that the binding of histone H2A by calmodulin involves both specific and nonspecific interactions. Parvalbumin, a protein descended from calmodulin which is considered to have lost any specific protein binding sites (Blum et al., 1977), binds histone H2A but not troponin I or the myelin basic protein.[5]

The localized effect of phosphorylation on myosin light chain kinase, consisting of a large change in calmodulin binding affinity, supports our suggestion that phosphorylation occurs near the calmodulin binding site. The fact that one of the phosphorylatable sites in myosin light chain kinase is blocked in the presence of excess calmodulin, as shown by Conti & Adelstein (1981) and verified by us (Table II), further supports our hypothesis. Cox & Edstrom (1981) have reported similar competition at the phosphorylation site in the β subunit of phosphorylase kinase. The proposed action of calmodulin and sequences in proteins subject to dual control allows for a level of interaction between the two second messengers, in which the binding of the calcium–calmodulin complex is regulated by phosphorylation and vice versa.

[5] D. A. Melancik and S. R. Anderson, unpublished results.

Table II: Summary of Properties of Turkey Gizzard Myosin Light Chain Kinase

	MLCK	phosphorylated MLCK	MCLK·CaM
K_{CaM}[a]	ND[c]	0.5–1.1 μM	NA[b]
$K_{m,CaM}$	1.3 nM	ND	NA
K_{ATP}	0.12 mM	0.12 mM	0.043 mM
K_{9AC}	20 μM	20 μM	6.4 μM
incorporable phosphate	2.2 ± 0.4	0	0.95 ± 0.04

[a] K designates dissociation constants. [b] NA, not applicable. [c] ND, not determined.

9-Anthroylcholine and bis(ANS) were both useful in discerning and describing the interactions of phosphorylated and unphosphorylated myosin light chain kinase with calmodulin. ATP and 9AC compete for a common binding site even though the affinity of the enzyme for 9AC is 6-fold higher than that for ATP. While the binding of bis(ANS) by the myosin light chain kinase is more heterogeneous than for 9AC, the addition of ATP causes a decline in the fluorescence of solutions of the enzyme and low concentrations of bis(ANS). Actual displacement of the dye by nucleotides has been shown in sedimentation experiments with lactate dehydrogenase·bis(ANS) adsorbates (Anderson, 1971; Lu & Anderson, 1973). The rate of phosphorylation of myosin light chain kinase by the cAMP-dependent protein kinase can be followed by using either bis(ANS) or 9AC. A suitable sampling pattern yielded linear rates with sensitivity comparable to that of the commonly employed ^{32}P technique. In addition, this procedure confirmed that no autophosphorylation of the myosin light chain kinase takes place (data not shown).

Activity measurements on myosin light chain kinase show that the dansylcalmodulin preparation contains an inhibitory component. The conjugate, containing an average of 1.7 mol of the dansyl moiety, is almost certain to be heterogeneously labeled since calmodulin contains a total of seven lysine residues (Cheung, 1980). However, the mixing experiments in Figure 6 show that the average affinity of the labeled population for the enzyme is not markedly different from that of unmodified calmodulin. Identification of the affected residues in the inhibitory species could provide information on the activation of myosin light chain kinase by calmodulin. We are presently working on the isolation and characterization of the inhibitor.

Fluorescent labeling of calmodulin without functional effects may be difficult. Walsh & Stevens (1977) showed that carbamoylation of lysine residues and carboxymethylation or oxidation of methionine residues interfere with the ability of calmodulin to activate the cyclic nucleotide phosphodiesterase. However, they did not check the products for actual binding ability or possible inhibitory properties. We have also prepared a derivative of calmodulin with dansylaziridine and found that it has a greatly reduced affinity for mysoin light chain kinase.[5] The spin-labeled calmodulin used by Hewgley & Puett (1980) gives a K_m which is 10 times larger than the K_m for native calmodulin in the cyclic nucleotide phosphodiesterase assay while the fluorescent conjugate prepared by LaPorte et al. (1981) by using *N*-[[(iodoacetyl)amino]ethyl]-5-naphthylamine-1-sulfonic acid gives a K_m which is 2.3 times larger.

Table II summarizes some of the principal information presented here.

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