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Biochemistry

Human platelet myosin light chain kinase requires the calcium-binding protein calmodulin for activity

(calcium-dependent regulator/phosphorylation of nonmuscle contractile protein/affinity chromatography)

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ABSTRACT **In an actomyosin fraction isolated from human platelets, phosphorylation of the 20,000-dalton light chain of myosin is stimulated by calcium and the calcium-binding protein calmodulin. The enzyme catalyzing this phosphorylation has been isolated by using calmodulin-affinity chromatography. Platelet myosin light chain kinase activity was monitored throughout the isolation procedures by using the 20,000-dalton smooth muscle myosin light chain purified from turkey gizzards as substrate. The partially purified myosin kinase requires both calcium and calmodulin for activity and has a specific activity of 3.1 μmol of phosphate transferred to the 20,000-dalton light chain per mg of kinase per min under optimal assay conditions. K_m values determined for ATP and myosin light chains are 121 μM and 18 μM, respectively. Of several substrates surveyed as phosphate acceptors (α-casein, histone II-A, phosphorylase b, protamine, histone V-S, and phosvitin), only the 20,000-dalton myosin light chain is phosphorylated at a significant rate. These results suggest that platelet myosin light chain kinase is a calcium-dependent enzyme and that the requirement for calcium is mediated by the calcium-binding protein calmodulin.**

Despite their nonmuscle origin, platelets possess an abundance of the contractile proteins actin and myosin; the combined content has been estimated as 10–30% of the total cellular protein (1, 2). Although platelet actin may serve a structural role, the function of actomyosin is related to its unique capacity to generate force and thereby to create tension or to shorten. This feature has been implicated as an important property involved in mediation of cell secretion, shape change, and clot retraction (1).

The regulation of actin–myosin interaction in platelets was found earlier to involve phosphorylation–dephosphorylation of the 20,000-dalton light chain of myosin (3). Phosphorylation of this subunit is necessary for actin-induced activation of myosin ATPase activity. Moreover, the myosins isolated from smooth muscle (4–6), skeletal muscle prefusion myoblasts (7), and rabbit alveolar macrophages (J. Trotter and R. S. Adelstein, unpublished data) have been shown to require phosphorylation for actin-induced activation of myosin ATPase activity.

Striated and smooth muscle actomyosins are regulated by calcium. In skeletal and cardiac muscles, the proteins troponin and tropomyosin bind stoichiometrically to actin and, in the absence of calcium, inhibit actin-induced activation of myosin ATPase activity. The binding of calcium to one component of troponin, troponin C, removes this inhibition (8, 9). In contrast, a principal site of calcium control in smooth muscle is the myosin light chain kinase which catalyzes phosphorylation of the 20,000-dalton light chain of myosin (4–6). Recently, the calcium dependence of chicken gizzard myosin kinase has been shown to be mediated by a 16,500-dalton calcium-binding subunit (10, 11). In the absence of this component the kinase is inactive. Furthermore, this calcium-binding protein has been shown to be identical to the calcium-dependent regulator* of cyclic AMP phosphodiesterase (11).

Although a growing body of evidence suggests that nonmuscle myosin, such as that isolated from platelets, is regulated by light chain phosphorylation, the nature or existence of calcium control mechanisms has not been clarified. In an earlier study, platelet myosin kinase was found to be active in the absence of calcium (12). Recently, we isolated, from human platelets, an actomyosin fraction in which phosphorylation of the 20,000-dalton myosin light chain is dependent upon calcium. Furthermore, this phosphorylation requires not only calcium but also the calcium-binding protein calmodulin. In this report we describe a method for the isolation of a calcium- and calmodulin-dependent myosin light chain kinase from human platelets.

MATERIALS AND METHODS

Preparation of Platelet Actomyosin. All isolation procedures were performed at 4°C. Human platelets were collected by sedimentation of fresh (<24 hr old) platelet concentrates (54 units) at 9000 × *g* for 10 min. The more rapidly sedimenting erythrocyte pellet was removed from the bottom of the centrifuge tube by aspiration. The platelet pellet (25–35 g) was resuspended in 2 vol of 2.5 mM dithiothreitol/150 mM NaCl/3 mM citrate buffer, pH 6.8, and sedimented again. At the end of each of two such washes, any remaining erythrocytes were removed by aspiration. The final platelet pellet was suspended in 2–3 vol of 15 mM Tris·HCl, pH 7.5/0.5 M KCl/2.5 mM dithiothreitol/5 mM Na_2EDTA. With continuous stirring, the platelet suspension was equilibrated with nitrogen [1500 psi (10.4 megapascals)] for 20 min in a Parr bomb. Disruption was effected by opening the release valve and permitting platelets to flow slowly into an erlenmeyer flask at atmospheric pressure. The extract was then sedimented at 28,000 × *g* for 30 min. The supernatant fraction (80–100 ml) was collected and dialyzed overnight against 20 vol of 20 mM Tris·HCl, pH 7.5/2.5 mM dithiothreitol/5 mM EDTA. The pellet obtained by sedimentation at 10,000 × *g* for 20 min was the source of crude actomyosin.

Preparation of Platelet Myosin Light Chain Kinase. The platelet myosin light chain kinase characterized in this report was prepared from 25–35 g of platelets that were washed as described above. The final, washed pellet was resuspended in 100 ml of 20 mM Tris·HCl, pH 7.5/2.5 mM dithiothreitol/10 mM EDTA/1 mM ethylene glycol bis(β-aminoethyl ether)-

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Abbreviations: $NaDodSO_4$, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid.

* This protein is also known as CDR. Other names appearing in the literature include "modulator protein" and "phosphodiesterase activator." The preferred term "calmodulin" is used throughout the text of this paper.

N,N'-tetraacetic acid (EGTA)/phenylmethylsulfonyl fluoride at 75 mg/liter (75 mg in 5 ml of dimethyl sulfoxide added to 1 liter of buffer)/soybean trypsin inhibitor at 0.1 mg/ml. After disruption of whole platelets by nitrogen decompression (see above), the extract was sedimented at 28,000 × *g* for 30 min and the supernatant was collected for subsequent purification, as described below.

Kinase Assay. Unless specified otherwise, all kinase assays were performed at room temperature (23–25°C) in a final volume of 0.2 ml containing 20 mM Tris·HCl, pH 7.5, 0.2 mM [γ-^{32}P]ATP (10^5 dpm/nmol), 20 μM smooth muscle 20,000-dalton myosin light chain, 10 mM $MgCl_2$, and either 0.1 mM $CaCl_2$ or 1 mM EGTA. The final concentration of calmodulin, unless stated otherwise, was 0.1 μM. Assays were terminated by the addition of 50 μl of 50% trichloroacetic acid/10% sodium pyrophosphate, and ^{32}P incorporation into substrate myosin light chains was determined by Millipore filtration of samples followed by liquid scintillation counting of the Millipore filters (4).

Gel Electrophoresis. Specific phosphorylation of 20,000-dalton platelet myosin light chains was confirmed by electrophoresis of 150 μg of actomyosin in cylindrical 7.5% polyacrylamide/1% sodium dodecyl sulfate ($NaDodSO_4$) gels with the Fairbanks buffer system (13). The 20,000-dalton light chain was identified after staining with Coomassie blue; slices containing light chains were assayed for ^{32}P incorporation. The subunit molecular weight of the platelet myosin light chain kinase was determined by electrophoresis in 1% $NaDodSO_4$ slab gels containing a 5–10% gradient of polyacrylamide. For this procedure, the Laemmli buffer system was used (14). The Fairbanks buffer system, excluding $NaDodSO_4$, was used for electrophoresis under nondenaturing conditions. Samples of platelet myosin kinase purified through affinity chromatography (0.2 mg/ml) were concentrated 2-fold by dialysis against 100 vol of 20 mM Tris·HCl, pH 7.5/100 mM KCl/5 mM EDTA/30% (wt/vol) glycerol. Approximately 75 μl of the concentrated material was applied to cylindrical 3.5% acrylamide gels (5 × 80 mm). After electrophoresis for 6 hr (4°C) at 2 mA per gel, the gels were sliced into 2-mm segments and eluted overnight into 100 μl of 20 mM Tris·HCl, pH 7.5/100 mM KCl/5 mM EDTA/10% glycerol/0.02 mg of β-lactoglobulin per ml. Kinase activity was determined by assaying 10-μl aliquots in an incubation mixture consisting of 20 mM Tris·HCl (pH 7.5), 10 mM $MgCl_2$, 40 μM 20,000-dalton smooth muscle myosin light chain, 0.1 mM $CaCl_2$, 0.1 μM calmodulin, and 50 μM [^{32}P]ATP (10^6 dpm/nmol) for 10 min at room temperature. For subunit protein composition and molecular weight determinations, 50-μl aliquots of the samples were applied to $NaDodSO_4$ gels as described above.

Other Methods. Porcine brain calmodulin was prepared by the method of Klee (15). The calmodulin coupled to Sepharose 4B was prepared as described (16). Protein was determined by the method of Lowry *et al.* (17). The 20,000-dalton smooth muscle myosin light chain fraction was isolated from turkey gizzards by a method similar to that reported by Perrie and Perry (18). The light chain fraction was freed of contaminating calmodulin by ion exchange chromatography on DEAE-Sephacel (15).

Materials. Casein, phosvitin, phosphorylase b, histone, and protamine were purchased from Sigma. [γ-^{32}P]ATP was obtained from New England Nuclear.

RESULTS

Endogenous Phosphorylation of Platelet Actomyosin. An actomyosin-rich fraction containing endogenous myosin light chain kinase was prepared from platelets to study phosphorylation of the 20,000-dalton platelet myosin light chain. The time-dependent incorporation of ^{32}P into the platelet myosin light chains was determined as described in Fig. 1. In the absence of calcium (1 mM EGTA), a slow rate of phosphate incorporation was observed. Addition of calcium to a final concentration of 0.1 mM resulted in a 2-fold increase in the rate of phosphorylation. This effect of calcium was significantly enhanced by addition of calmodulin to a concentration of 1 μM. This enhancement suggested that the effect of calcium might be due to the presence of subsaturating levels of calmodulin in the actomyosin. For this reason, actomyosin fractions were analyzed for calmodulin content by using activation of brain phosphodiesterase as the method of detection (15). In these fractions, calmodulin was found in the concentration range 1–10 nM.

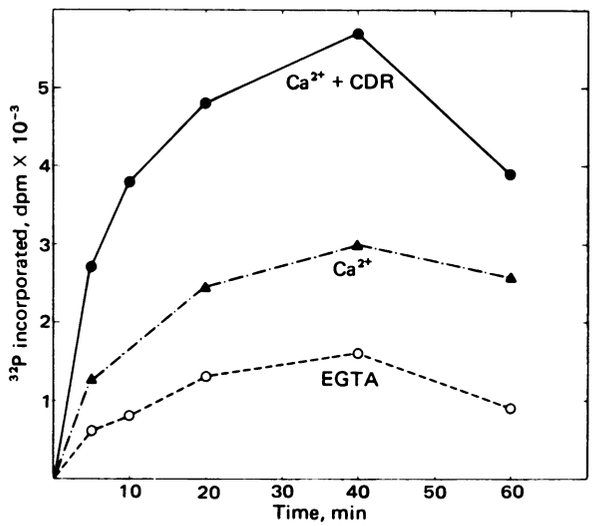

FIG. 1. Endogenous phosphorylation of platelet actomyosin. Each time point represents 200 μg of crude platelet actomyosin incubated in 20 mM Tris·HCl, pH 7.5/0.1 mM $CaCl_2$/125 mM KCl/1 μM calmodulin (CDR)/10 mM $MgCl_2$/1 mM [γ-^{32}P]ATP (10^4 dpm/nmol) in a final volume of 0.2 ml. The incubations were terminated by the addition of 1 ml of 6 M guanidine HCl. Samples were dialyzed against water, applied to 1% $NaDodSO_4$/7.5% polyacrylamide gels, and stained with Coomassie blue as described (4). ^{32}P incorporation into the 20,000-dalton myosin light chain was determined by liquid scintillation counting of gel slices.

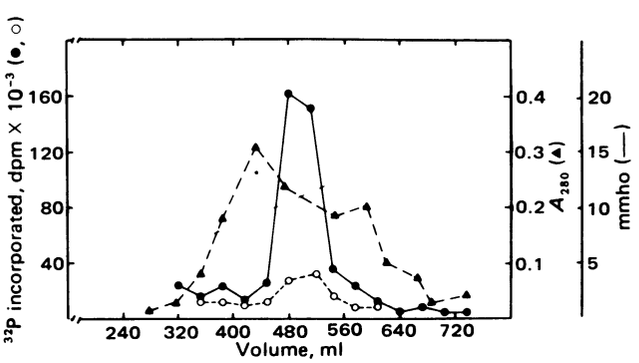

FIG. 2. Ion-exchange chromatography of platelet myosin kinase. Platelet extract (90 ml; 400 mg) was applied to a 2 × 30 cm DEAE-Sephacel column that had been equilibrated with 20 mM Tris·HCl, pH 7.5/10 mM EDTA/1 mM EGTA/2.5 mM dithiothreitol/20 mM KCl. Kinase was eluted from the column with a 1200-ml linear KCl gradient (20–500 mM KCl) at a flow rate of 60 ml/hr; 10-ml fractions were collected. Kinase activity was determined in the presence of either 0.1 mM $CaCl_2$ and 0.1 μM calmodulin (●—●) or 1 mM EGTA (○---○).

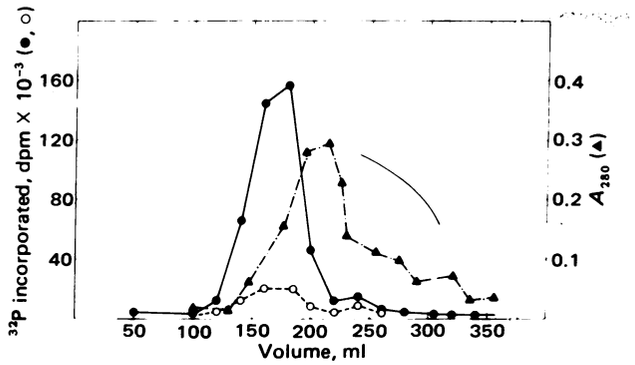

FIG. 3. Gel filtration of platelet myosin kinase. The active fractions from the DEAE-Sephacel column were pooled, concentrated to 25 ml (28 mg), and applied to a 2.5 × 90 cm Sephacryl S200 column that had been equilibrated with 0.5 M KCl/20 mM Tris·HCl, pH 7.5/10 mM EDTA/1 mM EGTA. Fractions (5 ml) were collected at a flow rate of 60 ml/hr. Kinase activity was measured either in the presence of 0.1 mM $CaCl_2$ and 0.1 μM calmodulin (●—●) or 1 mM EGTA (○---○).

At 60 min a decline in myosin phosphorylation was observed and is most likely due to the presence of phosphatase(s) and the complete hydrolysis of ATP. A similar but more rapid pattern of phosphorylation–dephosphorylation has been reported to occur with smooth muscle actomyosin (4).

Purification of Calmodulin-Dependent Platelet Myosin Kinase. In order to isolate the enzyme catalyzing the calcium- and calmodulin-dependent phosphorylation of the 20,000-dalton light chain of platelet myosin, a low ionic strength extract of platelets was applied to a 2 × 30 cm DEAE-Sephacel column. Kinase activity, determined in the presence of 0.1 μM calmodulin, 0.1 mM $CaCl_2$, and 20 μM 20,000-dalton smooth muscle myosin light chains, was eluted as a single peak with a linear gradient of KCl (Fig. 2). When the active fractions were assayed in the presence of 1 mM EGTA, phosphorylation was inhibited by more than 90%.

The active fractions obtained by ion exchange chromatography were pooled (100 ml) and concentrated 4-fold by ultrafiltration (Amicon, PM-10 Diaflo filter). This pool was then

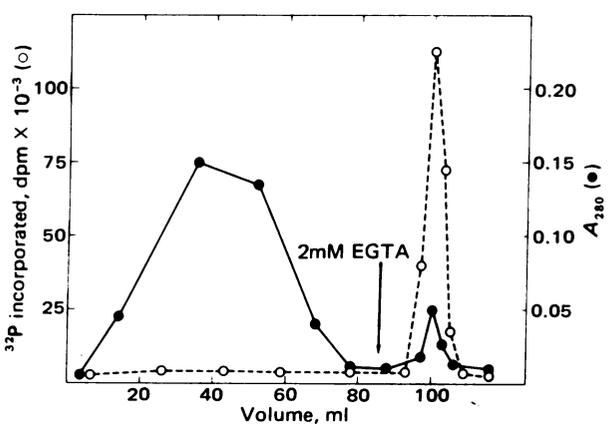

FIG. 4. Calmodulin-affinity chromatography of platelet myosin kinase. Approximately 10.5 mg of protein (70 ml) containing the myosin kinase activity was obtained from the Sephacryl S200 column step and applied to a 1.5 × 10 cm Sepharose 4B-calmodulin column containing 1.5 mg of calmodulin per ml of gel. The column was then washed sequentially with 40 mM Tris·HCl, pH 7.5/50 mM KCl/0.2 mM $CaCl_2$/3 mM $MgCl_2$/0.5 mM dithiothreitol, the same buffer containing 200 mM KCl, and finally with the same buffer containing 200 mM KCl, 1 mM $MgCl_2$, and 2 mM EGTA. In each case the volume of the wash was sufficient to decrease the A_{280} units in the eluant to sufficient baseline level.

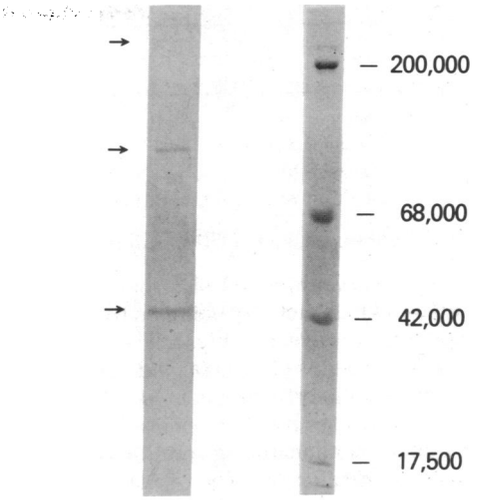

FIG. 5. NaDodSO$_4$/polyacrylamide gel electrophoresis pattern of platelet myosin light chain kinase. Approximately 2 μg of sample obtained from the calmodulin-affinity chromatography step was applied to a 1% NaDodSO$_4$/5–10% polyacrylamide gel. The sample is shown at the left. Protein standards, shown at the right, were skeletal muscle myosin heavy chain (200,000), bovine serum albumin (68,000), skeletal muscle actin (42,000), and β-lactoglobulin (17,500).

further purified by gel filtration as shown in Fig. 3. Only one peak of enzyme activity could be identified by this procedure. Furthermore, the requirement for calcium after gel filtration was verified by the inhibition of kinase activity in the presence of 1 mM EGTA.

After gel filtration, the active fractions were pooled and dialyzed overnight against 40 mM Tris·HCl, pH 7.3/1 mM dithiothreitol/40 mM KCl/3 mM $MgCl_2$. After addition of $CaCl_2$ to a final concentration of 0.8 mM, the dialyzed sample was applied to a column of calmodulin coupled to Sepharose. Most of the UV-absorbing material passed unimpeded through the column whereas the kinase activity was quantitatively adsorbed and eluted as a sharp peak with a buffer containing 2 mM EGTA (Fig. 4).

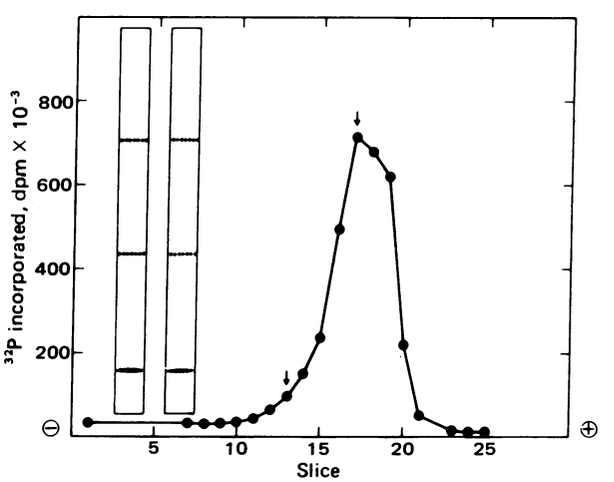

FIG. 6. Electrophoresis of platelet myosin light chain kinase under nondenaturing conditions. At the right is a profile of myosin kinase activity eluted from 2-mm slices of a nondenaturing gel. Diagrams of two lanes from a 1% NaDodSO$_4$/5–10% polyacrylamide gel, which represent aliquots from slices 13 and 17, respectively, are shown at the left. The vertical arrows point to slices 13 and 17 and indicate the peak intensity of Coomassie blue staining for the 45,000- and 105,000-dalton components, respectively. The prominent lower band in both lanes is β-lactoglobulin.

Table 1. Purification summary

Step	Protein, mg	Activity*	Recovery, %	Purification, fold
Extract	400	0.018	—	—
DEAE-Sephacel	28	0.150	58	8.3
Sephacryl S200	10.5	0.243	35	13.5
Calmodulin-affinity chromatography	0.3	3.10	13	172

* Shown as μmol of ^{32}P transferred per mg per min.

A $NaDodSO_4$/polyacrylamide gel electrophoresis pattern of the peak of myosin kinase activity obtained by calmodulin-affinity chromatography is shown in Fig. 5. Two major protein bands with molecular weights of 105,000 and 45,000 could be identified, as well as a minor component at 230,000. These proteins have been separated by electrophoresis in 3.5% polyacrylamide gels under nondenaturing conditions.

Fig. 6 summarizes these results. The two lanes from a $NaDodSO_4$ gradient slab gel shown at the left correspond to the peaks of staining for the 45,000- and 105,000-dalton components. The peak of myosin light chain kinase activity correlated with the 105,000-dalton subunit (slice 17) which contains some of the 45,000-dalton protein as well. On the other hand, the peak staining intensity of the 45,000-dalton band (slice 13) was associated with negligible catalytic activity. These results suggest that the 105,000-dalton component is the active myosin kinase but do not exclude the possibility that the 45,000-dalton protein may be a regulatory subunit of the enzyme.

The purification scheme is shown in Table 1. A yield of approximately 13% was obtained with an overall purification of 170-fold.

Characterization of Platelet Myosin Light Chain Kinase. The calmodulin dependence of the kinase purified by calmodulin-affinity chromatography was verified by titrating the enzyme with the calcium-binding protein (Table 2). At very low concentrations of calmodulin (0.027 nM) the kinase was inactive, even in the presence of 0.1 mM $CaCl_2$. Calmodulin concentrations of 27 nM or greater were sufficient to activate the platelet myosin kinase fully, but only in the presence of calcium. An accurate estimation of the stoichiometry between calmodulin and myosin kinase is not possible from this experiment because of the presence of two additional calmodulin-binding proteins which constitute 50% of the protein present (Fig. 5).

Table 3 summarizes other properties of the platelet myosin kinase purified by calmodulin-affinity chromatography. The specific activity of the partially purified platelet enzyme measured under optimal conditions is comparable to the value determined for the purified smooth muscle myosin light chain kinase (unpublished data) and corresponds to a turnover of 310 min^{-1}.

The substrate specificity of the platelet myosin light chain kinase was determined with the following substrates: phosvitin (25 μM), α-casein (20 μM), histone II-A (25 μM), histone V-S (25 μM), phosphorylase b (3 μM), and smooth muscle myosin light chain (20 μM). Under conditions identical to those used for smooth muscle myosin light chain phosphorylation, only the light chains were phosphorylated at a significant rate.

Table 2. Calmodulin requirement of platelet myosin light chain kinase

Calmodulin, nM	Myosin kinase activity*
0.027	0.0001
0.27	0.18
2.7	0.73
27	2.61
270	2.61

* Approximately 0.3 μg of the partially purified platelet kinase was added to start the assay. Incubations were terminated at 2 min. Other conditions were as described under *Materials and Methods*. Results are shown as μmol ^{32}P transferred per mg per min.

DISCUSSION

Phosphorylation of the 20,000-dalton light chain of platelet myosin has been observed to occur in response to stimuli that result in aggregation or secretion (19–21). These observations have implied not only that the contractile proteins actin and myosin are involved in platelet function but also that myosin phosphorylation (and hence interaction of myosin with actin) requires some activating factor. The isolation of a calmodulin- and calcium-dependent myosin light chain kinase and the identification of a calmodulin-like protein in platelets (22) suggest that endogenous platelet myosin phosphorylation is modulated by changes in intracellular calcium levels.

The myosin light chain kinase described in this report differs from the enzyme first isolated from human platelets in its requirement for calcium and calmodulin for activity (12). In addition, the estimated molecular weight of 105,000 is greater than the value, 78,000, obtained for the calcium-independent species previously isolated (12). It is possible that limited proteolysis of the native enzyme may account for the observed differences in molecular weight and calcium dependence. In favor of such a mechanism is the finding that the calmodulin-dependent cyclic AMP phosphodiesterase from bovine brain can be converted to an active, calmodulin- and calcium-independent form by proteolytic degradation (23). Our present procedure, which yields a calcium-dependent myosin kinase, differs from the procedure that yielded a calcium-independent species (12) in three major ways: (*i*) only fresh, unfrozen platelets were used, (*ii*) nitrogen decompression rather than 1-butanol was used for platelet lysis, and (*iii*) 10 mM EDTA and several protease inhibitors were included throughout the early preparative stages. Although all of these steps were designed to limit proteolysis, the exact relationship between the calcium-dependent and -independent platelet myosin light chain kinases must await further detailed studies on the enzyme structure.

On the other hand, a calcium-independent myosin light chain kinase has been isolated from aortic smooth muscle cells grown in culture whereas uncultured aortic smooth muscle yields a calcium-dependent enzyme (24). Furthermore, al-

Table 3. Properties of platelet myosin light chain kinase

K_m, ATP	121 μM
K_m light chain	18 μM
Specific activity	3.1 μmol/mg per min

The K_m value for ATP was determined at a light chain concentration of 50 μM; that for light chains was determined at an ATP concentration of 400 μM. In all instances, 0.1 μM calmodulin and 0.1 mM $CaCl_2$ were present. The assay was started by the addition of 0.3 μg of kinase and terminated at 2 min.

though a calcium-independent myosin kinase from cultured skeletal muscle prefusion myoblasts has been characterized, similar isolation techniques have yielded a calcium-dependent enzyme from a more differentiated cell form grown in culture (unpublished data). Whether these discrepancies reflect differences in proteolytic degradation that may occur in the isolation procedures, differences in myosin light chain kinase regulation in the various tissues, or more than one isoenzyme for the myosin light chain kinase remains to be clarified.

In addition to the myosin light chain kinases from smooth (10, 11) and skeletal muscles (25–27), calmodulin also regulates other calcium-modulated processes (28) including bovine brain phosphodiesterase (29), brain adenylate cyclase (30), erythrocyte membrane Ca,Mg-ATPase (31–33), and skeletal muscle phosphorylase kinase (34). Moreover, recent reports identify a calmodulin-dependent phosphorylation of neuronal membrane fractions (35) and a role for calmodulin in tubulin depolymerization (36). In view of these findings, it seems likely that additional calmodulin-regulated systems will be discovered. Recently, we partially purified a calmodulin-dependent myosin light chain kinase from bovine brain (unpublished data). Collectively, these results suggest that calmodulin regulation of myosin kinases is not restricted to muscle and may provide a model for the calcium regulation of other nonmuscle myosins that require light chain phosphorylation for actin-induced activation of myosin ATPase activity.

Note Added in Proof. Two recent reports describe the presence of a calmodulin-dependent myosin light chain kinase in platelets and brain (37) and in BHK-21 cells (38).

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