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# A Comparative Study of the Myosin Light Chain Kinases from Myoblast and Muscle Sources. Studies on the Kinases from Proliferative Rat Myoblasts in Culture, Rat Thigh muscle, and Rabbit Skeletal Muscle

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# A Comparative Study of the Myosin Light Chain Kinases from Myoblast and Muscle Sources

## STUDIES ON THE KINASES FROM PROLIFERATIVE RAT MYOBLASTS IN CULTURE, RAT THIGH MUSCLE, AND RABBIT SKELETAL MUSCLE*

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**Myosin light chain kinases have been isolated from rat thigh and rabbit skeletal muscle and cultured rat myoblasts. From these preparations, two types of kinases can be distinguished: calcium-dependent and calcium-independent. Both types of kinases can phosphorylate isolated P-light chains of myosin from several sources (skeletal muscle, cardiac muscle, and platelet). Data are shown which support the phosphorylation of the same site on the non-muscle P-light chains by both types of kinases. The rates of these reactions are, however, different for the two types of kinases. Kinetic analysis of the myoblast kinase shows differing affinities for various P-light chains (non-muscle > cardiac > skeletal). In the proliferative rat myoblast, phosphorylation of myosin is a prerequisite for actin activation of the myosin ATPase activity.**

Myosin, thought to be the protein responsible for the transduction of chemical energy (ATP) to force generation in muscle contraction, can be phosphorylated (1). The protein kinase which catalyzes the transfer of the γ-phosphate of ATP to myosin has been described in a number of muscle and non-muscle contractile systems (2-7). The covalent addition of a phosphate group to the myosin molecule is localized to one of the low molecular weight light chains, comprising the myosin hexamer, hence the name of the enzyme, myosin light chain kinase. The specific light chains of myosin that are phosphorylated are those with apparent molecular weights between 18,000 to 20,000 on SDS[1]-polyacrylamide gel electrophoresis, the so-called P-light chains (8). There are 2 mol of P-light chains/mol of native myosin. Since each P-light chain can be phosphorylated maximally to 1 mol of phosphate/mol (9), the native myosin molecule can be phosphorylated to 2 mol of phosphate/mol of myosin. Isolated P-light chains incorporate 1 mol of phosphate.

The myosin light chain kinase was first described in rabbit white skeletal muscle (1). This protein has been subsequently purified to homogeneity and some of its properties have been described (7). The analogous enzyme from a non-muscle source, human blood platelets, has also been studied (4).

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[1] The abbreviations used are: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid.

This paper deals with a comparison of myosin light chain kinases from muscle and non-muscle sources. The following enzymes of each type were isolated: skeletal kinases from rat thigh and rabbit back and leg muscles; and nonmuscle kinase from proliferative rat myoblasts in culture. The same purification procedure was used for each system and therefore, results of the purification will be shown only for one system. The enzymes will then be compared as to their: 1) requirement for calcium ions for activity and 2) ability to phosphorylate P-light chains of different myosins. The affinity of the proliferative rat myoblast myosin light chain kinase for P-light chains of various myosins will be described, as well as the function of the myosin phosphorylation in this system. Some preliminary results on the function of phosphorylation in proliferative rat myoblasts have been published (10).

### MATERIALS AND METHODS

*Cells and Tissues*—Back and leg muscles were excised from New Zealand white male rabbits, thigh muscles from adult Osborne Mendel rats; and cardiac muscle from National Institutes of Health foxhounds with the great vessels and pericardium removed. Yaffee L5 proliferative rat myoblasts were cloned by Dr. W. Uhlendorf. These L5 A10 cells were generously provided by Drs. W. Uhlendorf and G. Cantoni (Laboratory of General and Comparative Biochemistry, National Institute of Mental Health, Bethesda, Md.). The cells and tissues, as well as human blood platelets, were stored frozen at −20°C.

*Myosin and Myosin Light Chain Kinase Purification*—Cells or tissues were thawed at room temperature and extracted in 3 to 5 volumes (1 volume equal to the sample weight) of Buffer I (0.5 M KCl, 0.015 M Tris-HCl (pH 7.5), 2.5 mM dithiothreitol, 1 mM EDTA) with 10 mM $Na_4P_2O_7$ and an additional 7.5 mM dithiothreitol. The temperature was maintained at 4°C and deionized water was used throughout. The samples were homogenized in a VirTis homogenizer (model 23) for 3 min in 1-min pulses with 2 min cooling between each pulse at medium speed. This high ionic strength extract was stirred for 30 to 60 min. The pH was checked and maintained at 7.5. This slurry was centrifuged at 50,000 × *g* for 15 to 20 min. The supernatant was dialyzed overnight against 50 mM KCl, 10 mM Tris-HCl (pH 7.5), 2.5 mM dithiothreitol, 0.1 mM EDTA. Myosin and the myosin light chain kinase then were further precipitated by lowering the pH to 6.3 by addition of 0.4 M acetic acid and allowed to stand for 1 h at 4°C. This precipitate was collected by centrifugation at 50,000 × *g* for 15 min and then suspended in Buffer I at about 5 to 10 mg/ml, making sure that the pH was adjusted to 7.5. This suspension was made 10 mM in $MgCl_2$ and $Na_2ATP$ immediately prior to fractionation by addition of saturated $(NH_4)_2SO_4$ (Mann ultrapure) which contained 10 mM EDTA at pH 8.2. Two fractions, 0 to 30% and 30 to 55% saturation were collected by centrifugation. The 0 to 30% fraction contained primarily actin, whereas the 30 to 55% fraction contained some actin, the bulk of the myosin, and the myosin light chain kinase. The 30 to 55% fraction was resuspended in Buffer I at about 2 to 8 mg/ml and was dialyzed overnight against Buffer I to remove the $(NH_4)_2SO_4$. Immediately prior to gel chromatography on Sepharose 4B or CL-4B, this fraction was made 10 mM in $MgCl_2$ and $Na_2ATP$.

Then it was applied to a column (either 1.5 × 90, 2.5 × 90, or 5 × 90 cm), equilibrated, and eluted with Buffer I, which effected the separation of the actomyosin, myosin, and the myosin light chain kinase (see Fig. 2).

*Myosin Light Chain Purification*—Myosin light chains were prepared by the method of Perrie and Perry (11) from rabbit skeletal, human platelet, and canine cardiac actomyosin (35 to 55% saturation $(NH_4)_2SO_4$ fraction as above). The supernatant which contained the light chains was dialyzed exhaustively against 20 mM $NH_4HCO_3$, 1 mM dithiothreitol (pH 7.5) and lyophilized. When used in the phosphorylation assays, the light chains were solubilized in 10 mM KCl, 10 mM Tris-HCl or imidazole/HCl (pH 7.5), and 1 mM dithiothreitol (see Fig. 3).

*Actin Purification*—Actin was purified from back and leg muscles from New Zealand white male rabbits by the method of Spudich and Watt (12).

*Column Analyses for Myosin and Myosin Light Chain Kinase*—Myosin was detected by measuring the ($K^+$-EDTA)-activated ATPase activity (see below) of 0.1 ml of each column fraction. Myosin light chain kinase activity was determined by incubation of 0.1 ml of each column fraction with 0.005 to 0.014 mM human platelet, rabbit skeletal, or canine cardiac myosin light chains, 0.2 to 0.5 mM $Na_2ATP$, 5 to 12.5 mM $MgCl_2$, 0.125 M KCl, and sufficient $Na_4[\gamma\text{-}^{32}P]ATP$ (10 to 30 Ci/mmol) to give 50,000 to 100,000 dpm in 5 μl of the final mixture. These fractions were then analyzed by the Millipore filtration method (see below).

*Phosphorylation Analyses*—Two methods for analyzing the extent of phosphorylation were employed: either filtration through Millipore filters or electrophoresis on SDS-polyacrylamide gels. Phosphate incorporations to be analyzed by the filtration method were terminated by addition of cold 50% trichloroacetic acid, 10% $Na_4P_2O_7$ to a final concentration of 10 and 2%, respectively. These samples were heated then to 90–95°C for 20 to 30 min, cooled on ice, and filtered through the center of a Millipore HAWP 02500 0.45-μm filter by application with a Pasteur pipette. Each filter was washed exhaustively with cold 5% trichloroacetic acid, 1% $Na_4P_2O_7$. The radioactivity remaining on the filters was presumably bound to protein. The filters were counted in a Searle Mark III liquid scintillation spectrometer using ACS (Aqueous counting scintillant, Amersham Corp.) as the scintillation mixture.

Phosphate incorporations to be analyzed by SDS-polyacrylamide gel electrophoresis were terminated either by addition of 7 M guanidine/HCl to a final concentration of 3.5 M or by addition of 5% SDS, 1% $Na_4P_2O_7$ to a final concentration of 1 and 0.05%, respectively. These samples were dialyzed for 8 to 12 h against 0.3, 0.2, 0.1 M NaCl and against water at room temperature to remove the excess ATP and KCl. 1% SDS-10% polyacrylamide gel electrophoresis was carried out as described below with the exception that diallyltartardiamide (DATD/Bio-Rad Laboratories) was substituted for bisacrylamide in the same molar ratio to the acrylamide according to the method of Anker (13). The gels were then run, stained in 30% $CH_3OH$, 8.4% $CH_3COOH$, 0.03% Coomassie brilliant blue R, destained in the same solution without the dye, scanned at 584 nm, and cut into 2-mm slices, and each slice was dissolved in 1 ml of an aqueous 2% periodic acid solution at room temperature and counted in the ACS scintillant.

*Myosin P-Light Chain Phosphorylation*—Myosin can be phosphorylated by the endogenous myosin light chain kinase either prior to or after gel filtration chromatography on Sepharose 4B. For endogenous phosphorylation prior to column chromatography the 30 to 55% $(NH_4)_2SO_4$ fraction of actomyosin was dialyzed against Buffer I overnight to remove the $(NH_4)_2SO_4$ and then incubated as follows: 0.2 M KCl, 30 mM Tris-HCl (pH 7.5), 12.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2 mM $CaCl_2$ or 2 mM EGTA (ethylene glycol bis(β-aminoethyl ether)*N,N'*-tetraacetic acid), and 0.1 to 0.5 mM $Na_2ATP$ with sufficient $Na_4[\gamma\text{-}^{32}P]ATP$ (10 to 30 Ci/mmol) so as to give 200,000 to 400,000 dpm in 5 μl of the final mixture.

After column chromatography, purified myosin (0.05 to 0.15 mg/ml) can be phosphorylated by the partially purified myosin light chain kinase by incubation in the following mixture: 0.125 to 0.3 M KCl, 30 mM Tris-HCl, or imidazole/HCl (pH 7.4), 5 to 12.5 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.2 mM $CaCl_2$ or 2 mM EGTA, and 0.1 to 1.0 mM $Na_2ATP$ with sufficient $Na_4[\gamma\text{-}^{32}P]ATP$ (10 to 30 Ci/mmol) so as to give 40,000 to 200,000 dpm in 5 μl of the final reaction mixture (0.4 ml). The kinase fractions from a Sepharose 4B column were pooled and 0.05 to 0.2 ml were used in this assay.

Isolated light chains of various myosins were phosphorylated in the same incubation mixture as was the column-purified myosin with the exception that the KCl concentration varied from 0.02 to 0.25 M, 0.125 M being used most frequently.

*ATPase Assays*—High ionic strength ($K^+$-EDTA)- and $Ca^{2+}$-activated myosin ATPases were measured at 37°C in the following medium: 0.5 M KCl, 20 mM Tris-HCl or imidazole/HCl (pH 7.4), 2 mM $Na_2ATP$, and either 2 mM EDTA or 10 mM $CaCl_2$ in a final volume of 1.5 ml. The myosin concentration varied from 0.04 to 0.07 mg/ml. The low ionic strength $Mg^{2+}$- and actin-activated $Mg^{2+}$ myosin ATPases were measured at 37°C in the following medium: 17 mM KCl, 10 mM Tris-HCl or imidazole/HCl (pH 7.4), 1.4 mM $MgCl_2$, 1 mM $Na_2ATP$, and either 0.1 mM $CaCl_2$ or 1 mM EGTA. The actin concentration was 10-fold greater (0.4 to 1.0 mg/ml) than the myosin concentration (0.04 to 0.1 mg/ml). Inorganic phosphate liberation was measured by the method of Martin and Doty (14) as modified by Pollard and Korn (15). Protein concentrations were estimated by the method of Lowry *et al.* (16), using crystalline bovine serum albumin as the standard.

*Gel Electrophoresis*—Analytical SDS-polyacrylamide gel electrophoresis was performed according to the method of Fairbanks *et al.* (17) in 1% SDS. Samples were dialyzed against water at room temperature, lyophilized, and then solubilized in 10 mM Tris-HCl (pH 8.0), 1% SDS, 50 mM dithiothreitol, and boiled for 2 to 3 min.

## RESULTS

*Myosin Light Chain Kinase Detection*—Endogenous myosin light chain kinase activity can be demonstrated in the actomyosin fraction of proliferative myoblast myosin by incubation of the 30 to 55% $(NH_4)_2SO_4$ fraction with $Mg[\gamma\text{-}^{32}P]ATP$. A time course of such an incubation demonstrated that the proliferative myoblast myosin in this fraction is essentially completely phosphorylated (0.8 mol of phosphate/mol of P-light chain) within 10 min (see Fig. 1). Maximal incorporation of phosphate would be 2 mol of phosphate/mol of native myosin. Fig. 1 further demonstrates that this myosin light chain kinase is not dependent on trace calcium concentrations for its activity, since in the presence of the calcium chelator EGTA, which at the concentration used (2 mM), lowers the free calcium ion concentration to below $10^{-8}$ M, the myosin P-light chain is phosphorylated to roughly the same extent within the same amount of time. By

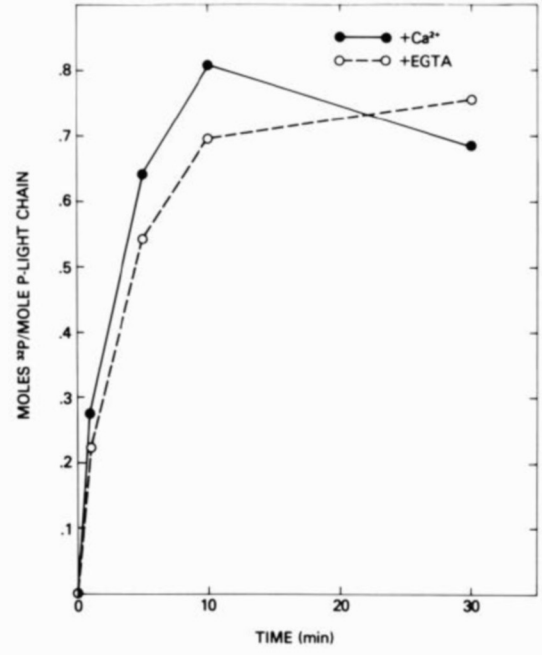


FIG. 1. Time course of $^{32}P$ incorporation into proliferative myoblast myosin P-light chain by the endogenous myosin light chain kinase in the presence of calcium ions (0.2 mM) or EGTA (2.0 mM). The 30 to 55% saturation $(NH_4)_2SO_4$ fraction of actomyosin was incubated as described under "Materials and Methods." 1% SDS, 10% polyacrylamide gels were run in duplicate at each time point. The P-light chain was cut out, solubilized, and counted.

inspection, it is apparent that the time courses of phosphate incorporation with calcium and with EGTA present are not significantly different from one another.

*Protein Purification*—Myosin and the endogenous myosin light chain kinase co-purify in the purification scheme shown in Fig. 2 up until the molecular sieve chromatography step. Upon gel filtration on Sepharose 4B, the myosin elutes at $K_{av}$ 0.17, whereas the myosin light chain kinase activity elutes nearer the salt boundary at $K_{av}$ 0.58. This chromatographic separation effects the complete separation of the myosin and the myosin light chain kinase and also purifies the myosin since few other proteins have a molecular weight of 460,000. An SDS-polyacrylamide gel of myosin purified in this manner is shown in Fig. 3.

The elution profiles of a gel filtration column from rat thigh muscle are shown in Fig. 4. Myosin is detected by determination of the ($K^+$-EDTA)-ATPase activity of each column fraction. The myosin light chain kinase elution profile is determined by incubation of each column fraction with isolated myosin light chains and [$\gamma$-$^{32}$P]ATP, and then analyzing the reaction products for incorporation of $^{32}$P into the light chains by the filtration method (see "Materials and Methods"). By this chromatographic technique, these two proteins are well separated. Furthermore, no kinase activity is associated with the myosin peak.

*Kinase Phosphorylation of Various Substrates and Calcium Requirement*—Having demonstrated endogenous myosin light chain kinase activity in the actomyosin fraction (Fig. 1), it was of interest to determine whether the separated myosin and myosin light chain kinase could be added back together to achieve phosphorylation of the P-light chain. A nonphosphorylated fraction of proliferative rat myoblast actomyosin was chromatographed on Sepharose 4B and elution profiles similar to those of Fig. 4 were obtained. An aliquot of the proliferative myoblast myosin light chain kinase fraction was then incubated with some of the pooled column-purified myosin from the same column under the phosphorylation conditions. The controls for this experiment, namely incubation of the myosin alone and of the myosin light chain kinase fraction alone under the phosphorylation conditions, demonstrated no autophosphorylation in these two fractions (Fig. 5, *A* and *B*). When these same two fractions were combined (Fig. 5*C*) the myosin P-light chain was phosphorylated by the myosin light chain kinase to 0.8 mol of $^{32}$P/mol of the P-light chain. Moreover, this non-muscle myosin light chain kinase

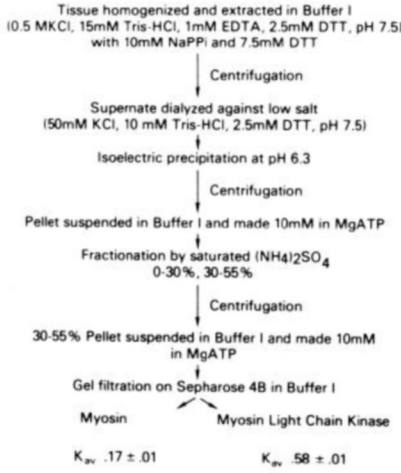


FIG. 2. Purification scheme used for the isolation of myosin and myosin light chain kinase from each source described in this report. The partition coefficients ($K_{av}$) are expressed as the mean ± standard deviation, $n = 8$. *DTT*, dithiothreitol.

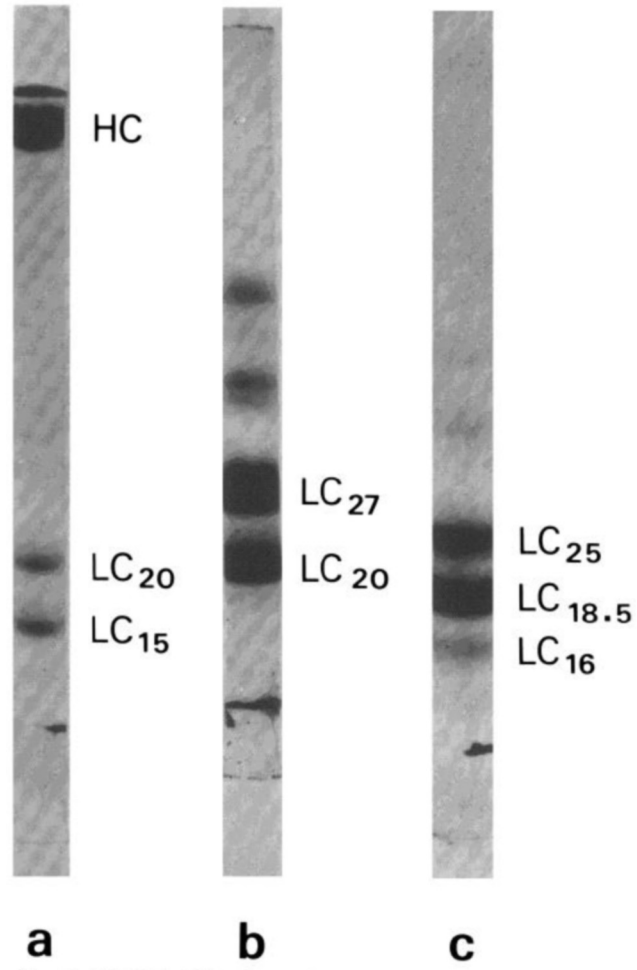


FIG. 3. 1% SDS-10% polyacrylamide gels of myosin and isolated myosin light chains. Electrophoresis was carried out from *top* to *bottom*. *a*, proliferative rat myoblast myosin after Sepharose 4B column chromatography showing the heavy chain of 200,000 daltons (*HC*) and the 20,000- and 15,000-dalton light chains ($LC_{20}$ and $LC_{15}$); *b*, isolated canine cardiac myosin light chains of 27,000 and 20,000 daltons ($LC_{27}$ and $LC_{20}$); *c*, isolated rabbit skeletal muscle myosin light chains of 25,000, 18,500, and 16,000 daltons ($LC_{25}$, $LC_{18.5}$, and $LC_{16}$). The gels of the isolated light chains show small amounts of contamination by other proteins (actin and tropomyosin). The P-light chains are as follows: *a* and *b*, 20,000-dalton light chain; *c*, 18,500-dalton light chain.

will phosphorylate isolated non-muscle myosin light chains from another source. Human platelet myosin light chains were incubated with the kinase from the rat myoblasts and with Mg[$\gamma$-$^{32}$P]ATP (Fig. 5*D*). The amount of phosphate incorporated per mol of the platelet P-light chain was 0.7 mol.

To determine whether the non-muscle myosin light chain kinase can phosphorylate isolated muscle myosin light chains, the proliferative rat myoblast myosin light chain kinase was incubated with isolated canine cardiac myosin light chains. Both in the presence of EGTA and in the presence of calcium the non-muscle myosin light chain kinase incorporated 0.8 mol of phosphate/mol of the cardiac P-light chain (Fig. 6). Therefore, the proliferative rat myoblast myosin light chain kinase can phosphorylate cardiac muscle myosin light chains equally well whether or not free calcium ions are present at greater than $10^{-8}$ M.

To extend these observations further and to contrast this non-muscle kinase with a skeletal muscle myosin light chain kinase, a series of myosin light chains were incubated with either the myosin light chain kinase isolated from proliferative

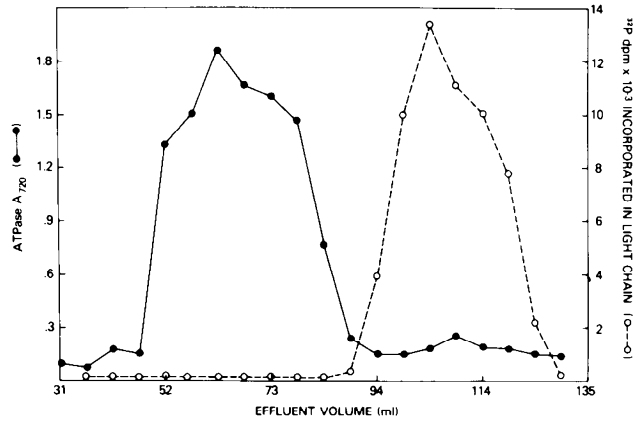


FIG. 4. The elution profile from Sepharose CL-4B chromatography of rat thigh muscle actomyosin (30 to 55% $(NH_4)_2SO_4$ fraction). A 4.3-ml sample was applied to a column (1.5 × 90 cm) equilibrated and eluted with Buffer I (see "Materials and Methods") at 17 ml/h. Fractions of 2.6 ml were collected, of which 0.1 ml was used for the ($K^+$-EDTA)-ATPase assay ($A_{720}$) (●) and 0.1 ml was used in the kinase detection assay (○) utilizing canine cardiac myosin light chains. The void volume and salt boundary of this column were at 36 and 134 ml, respectively.

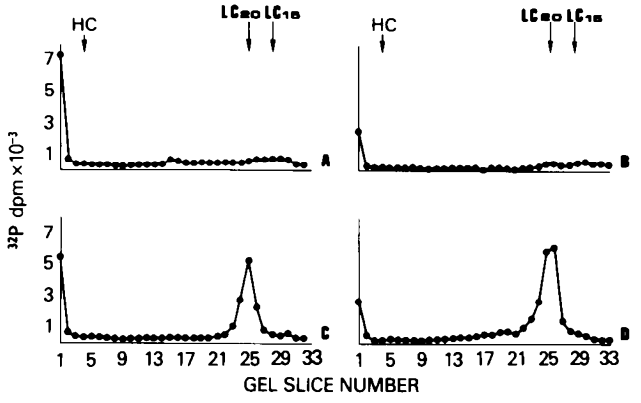


FIG. 5. Profiles of $^{32}$P radioactivity eluted from 1% SDS-10% polyacrylamide gels of column-purified proliferative rat myoblast myosin, myosin light chain kinase, and human blood platelet myosin light chains. Each of these fractions was incubated for 45 min under the identical phosphorylation conditions (see "Materials and Methods"), run on the gels, and scanned at 584 nm; the entire gel was sliced, and each slice was counted for $^{32}$P radioactivity. *A*, column-purified proliferative rat myoblast myosin light chain kinase showing no $^{32}$P incorporation; *B*, column-purified proliferative rat myoblast myosin showing no $^{32}$P incorporation into either heavy chain (*HC*) or the 20,000- and 15,000-dalton light chains ($LC_{20}$ and $LC_{15}$, respectively); *C*, the combination of the kinase shown in *Panel A* and the myosin in *Panel B* showing $^{32}$P incorporation into the P-light chain ($LC_{20}$) equivalent to 0.76 mol of $P_i$/mol of P-light chain; and *D*, the combination of the kinase (*Panel A*) with human blood platelet myosin light chains showing $^{32}$P incorporation into the P-light chain ($LC_{20}$) equivalent to 0.74 mol of $P_i$/mol of P-light chain. Electrophoresis was from *left* to *right*.

rat myoblasts, or the skeletal muscle one, isolated from rat thigh muscle (this fraction is the pooled peak shown in Fig. 4). As can be clearly seen in Table I, both types of myosin light chain kinases will phosphorylate non-muscle and muscle P-light chains to the same extent. Further, the data in this table show the difference in the calcium dependency of the two types of kinases: the skeletal muscle kinase requires trace calcium ions for its activity, whereas the myoblast kinase does not. No significant differences in the phosphate incorporations of the P-light chains by the non-muscle kinase with and without calcium were detected. The muscle kinase, however was almost completely inhibited by the low free calcium concentrations. This observation eliminates the possibility that the light chains are conferring the calcium dependency to the kinases.

*Same Site Phosphorylation*—The fact that some myosin light chain kinases require calcium ions for activity and that other ones do not allows for a direct determination of whether the same site on the P-light chain is being phosphorylated by both types of kinases. In this experiment, rabbit skeletal muscle myosin light chain kinase is used as the calcium-dependent kinase and human platelet myosin light chain

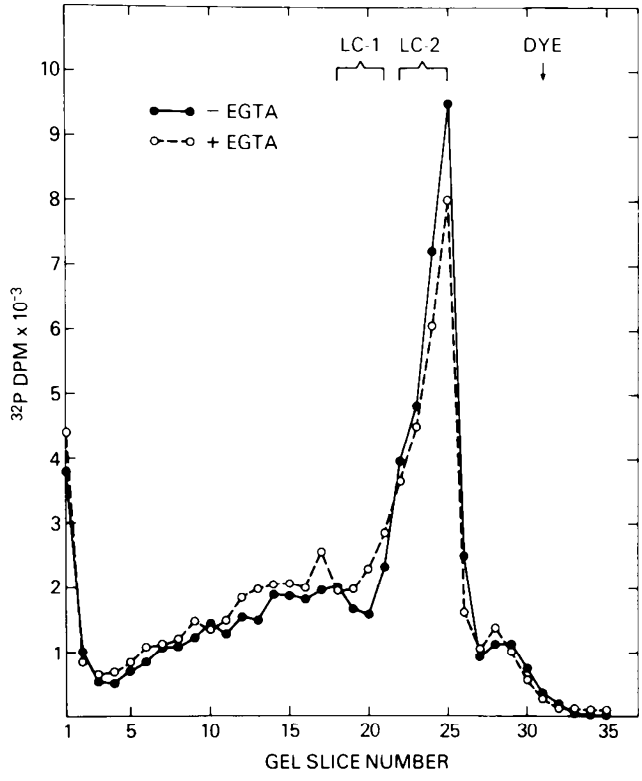


FIG. 6. Incorporation of $^{32}$P into isolated canine cardiac myosin light chains (*LC-2*) by the column-purified proliferative rat myoblast myosin light chain kinase in the presence of calcium ions (0.2 mM) and in the presence of EGTA (2.0 mM). Identical amounts of the kinase and the light chains were incubated under the phosphorylation conditions (see "Materials and Methods") and run on 1% SDS-10% polyacrylamide gels. The gels were scanned and cut, and the $^{32}$P eluted from each slice was counted. When corrections for protein loading differences were made, the P-light chains incorporated equivalent amounts of $^{32}$P in the presence of calcium and in the presence of EGTA (0.83 and 0.77 mol of $P_i$/mol of P-light chain, respectively). Electrophoresis was from *left* to *right*.

TABLE I

*Isolated myosin light chains and column-purified myosin phosphorylation by partially purified myosin light chain kinases from proliferative rat myoblasts and rat thigh muscle in the presence of either calcium ions or EGTA*

Both kinases were purified as described in Fig. 2 and added back to the light chains or myosin under the phosphorylation conditions and analyzed on SDS-polyacrylamide gels (see "Materials and Methods").

| Kinase | Substrate | Moles $^{32}$P Incorporated Per Mole P-Light Chain | |
|---|---|---|---|
| | | $+Ca^{2+}$ | $-Ca^{2+}$ |
| Proliferative Rat Myoblast | Myoblast Myosin | 0.75 | 0.81 |
| | Platelet Myosin Light Chains | 0.74 | 0.72 |
| | Cardiac Myosin Light Chains | 0.83 | 0.77 |
| | Skeletal Myosin Light Chains | 0.91 | 0.86 |
| Rat Skeletal Muscle | Platelet Myosin Light Chains | 0.84 | 0.01 |
| | Cardiac Myosin Light Chains | 0.87 | 0.02 |
| | Skeletal Myosin Light Chains | 0.93 | 0.01 |

$+Ca^{2+}$ = 200μM $CaCl_2$ $Ca^{2+}$ = 2mM EGTA

kinase as the calcium-independent kinase. The calcium requirement of the muscle kinase phosphorylation of the human platelet myosin P-light chain is shown in the lower portion of Fig. 7. In the presence of calcium ($+Ca^{2+}$), the muscle kinase incorporates 0.95 mol of phosphate/mol of the platelet P-light chain, whereas in the presence of EGTA ($-Ca^{2+}$), only 0.02 mol are incorporated. In the upper left hand portion of this figure is shown that platelet light chains, incubated first with nonradioactively labeled ATP without the kinase and then incubated with radioactively labeled [$\gamma$-$^{32}$P]ATP with EGTA ($-Ca^{2+}$) with the platelet kinase, will incorporate 0.98 mol of phosphate/mol of the P-light chain. The platelet kinase functions in the presence of EGTA, whereas the muscle kinase does not. Further, this portion of the experiment demonstrates that no kinase exists in the light chain fraction and that the platelet kinase functions under the conditions of this experiment. If the same site on the P-light chain is phosphorylated by both kinases, then preincubation with the muscle kinase in the presence of calcium with nonradioactively labeled ATP should preclude any additional phosphorylation by the platelet kinase after the muscle kinase is inhibited by chelation of the free calcium to below $10^{-8}$ M.

Indeed, this is what was found. Platelet light chains were incubated with the muscle kinase in the presence of calcium and nonradioactively labeled ATP (*right portion* of Fig. 7). The reaction was allowed to run long enough to phosphorylate

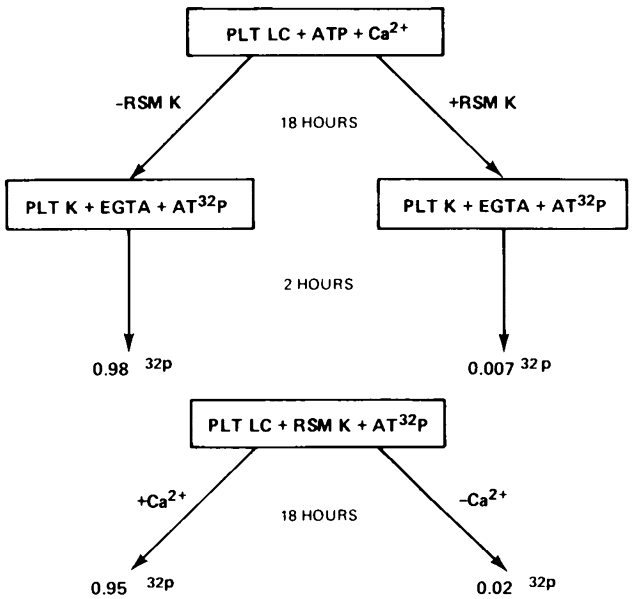


FIG. 7. Diagram summarizing the evidence for the phosphorylation of the same site on the platelet P-light chain by both the human platelet and rabbit skeletal muscle myosin light chain kinases. The lower portion demonstrates that the rabbit skeletal muscle kinase (*RSM K*) will phosphorylate platelet light chains (*PLT LC*) in a calcium-dependent manner. The calcium concentration ($+Ca^{2+}$) was 0.2 mM and the EGTA concentration ($-Ca^{2+}$) was 2.0 mM. In the *upper left-hand portion* of this diagram, platelet light chains are incubated with ATP. EGTA is added, sufficient to lower the free calcium ion concentration to below $10^{-8}$ M. The platelet myosin light chain kinase (*PLT K*) is added with Mg[$\gamma$-$^{32}$P]ATP. The platelet light chains are then maximally phosphorylated (0.98 mol of $P_i$/mol of P-light chain). In the *upper right-hand portion* of this diagram, platelet light chains are phosphorylated by the *RSM K* (as in the *lower portion* of this diagram, $+Ca^{2+}$), but with nonradioactive ATP. Then EGTA is added to inhibit this kinase (see *lower portion*, $-Ca^{2+}$). The platelet kinase and Mg[$\gamma$-$^{32}$P]ATP are incubated with these light chains. No significant incorporation is seen in the P-light chains, indicating that the phosphorylatable site on the P-light chains is already phosphorylated. Each step of these experiments was analyzed on SDS-polyacrylamide gels (see "Materials and Methods").

the light chains maximally. Then the muscle kinase was inhibited by addition of 2 mM EGTA. Mg[$\gamma$-$^{32}$P]ATP and the platelet kinase were added and allowed to incubate sufficiently long to ensure that significant phosphorylation could occur if the P-light chain acceptor were available. As is seen in the right hand portion of Fig. 7, no significant incorporation of $^{32}$P was observed in the P-light chains (less than 1%, 0.007 mol of phosphate/mol of P-light chain). These results indicate that both kinases are phosphorylating the same site on the platelet P-light chain.

*Substrate Specificity and Phosphorylation Kinetics*—It has been demonstrated that myoblast and muscle myosin light chain kinases will phosphorylate the P-light chain to the same extent (Table I), even though the muscle kinases are dependent on trace calcium ions for their activity. The questions now arise as to whether the rates of phosphate incorporation are similar and as to whether the kinases demonstrate a specificity for particular light chains. To answer the first of these questions, time courses of incorporation into three types of light chains, non-muscle, cardiac muscle, and skeletal muscle, by both a non-muscle kinase (proliferative rat myoblast) and a muscle kinase (rabbit skeletal) were run concurrently under identical conditions. In both cases, the rates of phosphate incorporation varied markedly (Fig. 8). The non-muscle kinase phosphorylated the non-muscle (platelet) light chains most quickly. A slower rate was found for the cardiac light chains, and the slowest for the skeletal (rabbit) light chains. The converse was true for the muscle kinase (rabbit skeletal). The skeletal light chains were phosphorylated most quickly, the cardiac light chains at an intermediate rate, and the non-muscle light chains the least quickly. These experiments demonstrate that both kinases incorporate phosphate into different light chains at different rates. The kinases seem to have a specificity for the P-light chain of the myosin to which they are most homologous.

To characterize some kinetic parameters of the myoblast kinase, the extent of phosphate incorporation at a fixed time into varying concentrations of myosin P-light chains from different sources was examined. Non-muscle, cardiac, and skeletal muscle myosin light chains were incubated in duplicate at five different concentrations varying from 6 to 100 $\mu$M for 7½ min with a constant amount of proliferative rat myo-

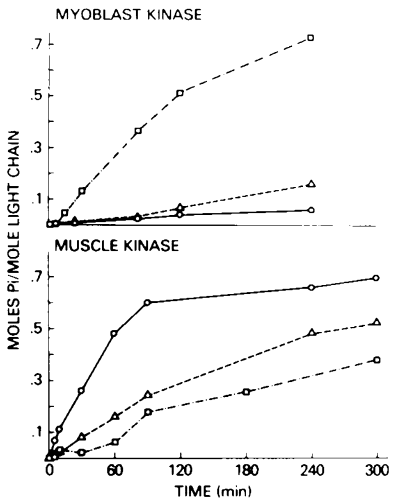


FIG. 8. Time courses of $^{32}$P incorporation into human platelet (□), canine cardiac (△), and rabbit skeletal (○) myosin P-light chains by the proliferative rat myoblast and rabbit skeletal muscle myosin light chain kinases. In each case, the experiments were run concurrently, under the identical conditions with pooled fractions of kinases from Sepharose 4B columns. The amount of $^{32}$P incorporation was analyzed on SDS-polyacrylamide gels (see "Materials and Methods").

blast myosin light chain kinase. This time was chosen so that less than 10% of the light chains in any of the samples would be phosphorylated. The rates of incorporation within this time period were linear. The reactions were terminated by SDS denaturation and the fractions were analyzed by SDS-polyacrylamide gel electrophoresis. The velocities of phosphate incorporation were then calculated and analyzed graphically on a double reciprocal plot of 1/velocity of phosphate incorporation *versus* 1/P-light chain concentration (Fig. 9). The results indicate that the apparent affinity of the kinase for different P-light chains does vary, although the maximal velocity of the reaction is identical. The value for $V_{max}$ is 0.077 μmol/min/mg (0.2 μM/min) for all three P-light chain acceptors (Table II). However, the extrapolated $K_m$ values vary by a factor of 15; the smallest $K_m$ of 2 μM for the non-muscle platelet light chains and the largest of 35 μM for the rabbit skeletal muscle myosin P-light chains.

*Function of Myosin Phosphorylation*—The non-muscle myosin light chain kinases elicit a functional change in the non-muscle myosins upon phosphorylation (for the data on the platelet, see Ref. 19). In proliferative rat myoblast myosin (Table III), phosphorylation of the P-light chains is a prerequisite for the activation of the myosin low ionic strength ATPase activity by actin (0.017 M KCl). Unphosphorylated myoblast myosin, whether or not calcium ions are present, cannot be activated by actin, even though the actin concentration is 10-fold greater on a weight basis (milligrams per mg)

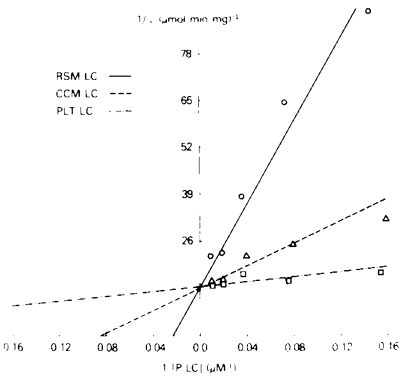


FIG. 9. Double reciprocal plot of the velocity of phosphate incorporation by the proliferative rat myoblast myosin light chain kinase *versus* the P-light chain (*LC*) concentration of human platelet (*PLT*), canine cardiac (*CCM*), and rabbit skeletal muscle (*RSM*) myosin. All these points were from duplicate experiments analyzed on SDS-polyacrylamide gels (see "Materials and Methods"). The phosphorylation reactions were carried out for 7½ min in 1-ml volumes with varying P-light chain concentrations (6 to 100 μM). The final protein concentration of the pooled kinase fraction was 2.6 μg/ml. Phosphate incorporation was linear under these conditions and in all the samples less than 10% of the P-light chains were phosphorylated. The data were analyzed according to the method of Cleland (18). The ATP concentration was 0.5 mM, which was saturating.

TABLE II

*The $K_m$ and $V_{max}$ of the proliferative rat myoblast myosin light chain kinase for isolated myosin light chains*

These values were determined from the double reciprocal plot shown in Fig. 9. The protein concentration of the kinase fraction was 2.6 μg/ml.

| Light Chain Source | Human Platelet | Canine Cardiac | Rabbit Skeletal |
|---|---|---|---|
| Km (μM) | 2 | 11 | 35 |
| Vmax (μM/min) | 0.2 | 0.2 | 0.2 |

TABLE III

*ATPase activities of phosphorylated and unphosphorylated proliferative rat myoblast myosin*

Actomyosin fractions (30 to 55% $(NH_4)_2SO_4$) from the myoblasts were divided in half: one-half was phosphorylated, the other half was not. Both halves were then purified by gel filtration and the myosin showed electrophoretic patterns as in Fig. 3*a*. The ATPase activity data are from 10 determinations from six different preparations (see "Materials and Methods" for assay conditions).

| Actin-Activated (0.017 M KCl) | - Actin | | + Actin | |
|---|---|---|---|---|
| | $Ca^{2+}$ | EGTA | $Ca^{2+}$ | EGTA |
| Phosphorylated | .015±.003 | .018±.003 | .182±.006 | .179±.005 |
| Unphosphorylated | .013±.002 | .020±.003 | .019±.003 | .020±.003 |

| Myosin Alone (0.5 M KCl) | $K_2EDTA$ | $Ca^{2+}$ |
|---|---|---|
| Phosphorylated | 1.45±.01 | .217±.026 |
| Unphosphorylated | 1.42±.06 | .216±.043 |

Specific Activities are expressed as μmol Pi/mg myosin/min at 37° C.

than the myosin. Phosphorylated myosin ATPase activities measured at low ionic strength in the absence of actin are identical with the unphosphorylated values. In the presence of actin, however, a 9- to 14-fold increase in phosphorylated myosin ATPase activities is observed both in the presence and the absence of calcium ions. This dramatic increase in the actin-activated myosin ATPase activity is observed only in the phosphorylated myosin. Therefore, phosphorylation is a prerequisite for actin activation of proliferative rat myoblast myosin. Note that the high ionic strength (0.5 M KCl) myosin ATPase activities are not affected by phosphorylation.

## DISCUSSION

Protein kinases which phosphorylate the P-light chain of myosin (ATP:myosin P-light chain phosphotransferase) have been partially purified from several sources. These myosin light chain kinases have been characterized and some of their properties have been compared. The purification methods used (see Fig. 2) will isolate myosin and the myosin light chain kinase separately from widely divergent sources; for example, murine astrocytic neuroglial cells in culture (5), human blood platelets (20), vas deferens smooth muscle (21), proliferative rat myoblasts, rat thigh muscle, and rabbit skeletal muscle. Using the identical isolation scheme for all the kinases, the overall conclusion of the experiments reported here is that two types of myosin light chain kinase can be distinguished, those which depend on trace calcium ions for their activities (muscle) and those which are independent of trace calcium ions (myoblast).

Although the calcium dependence of these two types of kinase is different, several similarities do exist between them. Both types of kinase can phosphorylate any P-light chain with which they are challenged (see Table I), albeit at different rates of phosphate incorporation (Fig. 8). Data have been presented which suggest that the calcium-independent and calcium-dependent kinases phosphorylate the same site on the P-light chain isolated from human platelet myosin (Fig. 7).

Dissimilar between the two types of myosin light chain kinases is the calcium dependence. This property seems to be

an intrinsic attribute of the kinase itself and not of the phosphate acceptors even though the P-light chains have been reported to bind calcium (22, 23). Using the identical preparations of myosin light chains, differing results with the two classes of kinases were clearly demonstrated (see Table I). It is concluded, therefore, that the light chains do not confer calcium dependency to the muscle kinase and independence to the non-muscle kinase.

The P-light chains of skeletal, cardiac, and non-muscle myosin are recognized differently by the muscle and myoblast myosin light chain kinases. A specificity for the myosin light chains from the most similar source is evident in Fig. 8. These results tend to indicate differences in the kinases' P-light chain recognition site. Data which further support this conclusion, for the non-muscle rat proliferative myoblast kinase, are shown in Fig. 9 and Table II. If the $K_m$ values obtained from the double reciprocal plot reflect the apparent affinities of the P-light chains for the kinase, then these affinities vary by more than 1 order of magnitude. The value obtained for the $K_m$ of the P-light chain of human platelet myosin, a non-muscle myosin, with the partially purified non-muscle kinase is 2 $\mu$M. Using the purified rabbit skeletal muscle myosin light chain kinase and P-light chains, Pires and Perry (7) have reported values in the 100 to 200 $\mu$M range. This implies that the non-muscle kinase has a higher affinity for the non-muscle P-light chain, than the skeletal kinase has for the skeletal P-light chains. However, the non-muscle kinase $K_m$ for rabbit skeletal muscle myosin P-light chains is 35 $\mu$M. This value is roughly comparable to the value reported for the skeletal kinase with the same substrate. These differences could be accounted for by the preparative methods or the skeletal P-light chains or could be intrinsic to the two different kinases.

The $K_m$ values reported for the myoblast kinase are all within the realm of possibility in the *in vivo* biological system. If the $K_m$ values for the P-light chains can be translated into values for native myosin, then the range of myosin concentrations for the enzyme to operate at the $K_m$ for the substrate within the cell would be 0.9 mg/ml for the non-muscle myosin and 1.6 mg/ml for the skeletal myosin. It has been estimated that the myosin concentration of human blood platelets is 1.5 mg/ml (24). Given that the cell is not a sac of homogeneous composition and that myosin is thought to be organized into supramolecular arrays, these concentrations of myosin within the cell are well within the possible range.

The consequences of phosphorylation of non-muscle myosin by non-muscle myosin light chain kinase are readily apparent from Table III. Functionally, the kinase represents a regulatory system which is capable of controlling the interaction of actin with myosin. As myosin P-light chain phosphatases have been described (25) or detected (26–28) in several systems, it is probable that the interaction of the myosin light chain kinase with the myosin light chain phosphatase and myosin serves as a regulatory system for the actin activation of the myosin low ionic strength ATPase. The extension of this argument to the regulation of contraction and relaxation in non-muscle cells by phosphorylation-dephosphorylation cycles can be made, although no direct evidence for such regulation has been reported.

The unregulated nature of the myoblast kinase (calcium independence) as isolated in this study can be a consequence of numerous factors. Firstly, the kinase could be truly unregulated and the steady state of myosin phosphorylation within the cell could be regulated by the phosphatase. Secondly, the non-muscle kinase could be regulated by other proteins or cofactors which are not isolated with it. Thirdly, the ever present problem of proteolysis could have rendered the non-muscle kinase calcium independent. Several points argue against this third possibility: (*a*) myosin, which is very sensitive to proteolysis, is isolated almost completely intact (see Fig. 4, and Ref. 10); (*b*) using the identical isolation procedure, the kinases from rat thigh and rabbit skeletal muscle are completely calcium-dependent; and (*c*) during *in vitro* development, the non-muscle proliferative rat myoblasts can fuse and form sarcomere-containing myotubes in which the bulk of the myosin light chain kinase is calcium-dependent (29). These arguments tend to rule out a major effect of proteolysis on the calcium dependence or independence of the myoblast light chain kinase as isolated in this report.

The most likely reasons for the calcium independence of the myoblast kinase are that it is truly unregulated or that other proteins confer calcium dependence on the kinase, or both. Some evidence as to the interaction of another protein(s) with the myosin light chain kinase from smooth and skeletal muscle has been reported (30–32), but these studies are too preliminary so as to allow conclusions to be drawn and extended to the myoblast myosin light chain kinases.

Phosphorylation of non-muscle and smooth muscle myosin by myosin light chain kinases appears to be a general phenomenon with functional ramifications (10, 19, 21, 30). Kinases have also been reported in skeletal muscle and cardiac muscle which phosphorylate their respective myosins. In these two cases, however, no functional change has been reported upon phosphorylation. It is probable that in non-muscle and smooth muscle cells one of the primary regulatory pathways for the actin-myosin interaction is through phosphorylation of the myosin P-light chain by the endogenous myosin light chain kinase.

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*Note Added in Proof*—Recent work has shown that depending on the method of preparation, the platelet kinase can be isolated in a calcium-dependent or a calcium-independent form. Similar to the smooth muscle (30) and skeletal muscle (31) kinases, a $M_r$ = 16,500 calcium-binding protein is required for activity of the calcium-dependent platelet kinase.[2]

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