J. Biochem. 119, 783-790 (1996)

Two Phosphorylations Specific to the Tail Region of the 204-kDa Heavy Chain Isoform of Porcine Aorta Smooth Muscle Myosin[1]

Yasuhiro Fukui and Fumi Morita[2]

Division of Chemistry, Graduate School of Science; Hokkaido University, Sapporo, Hokkaido 060

Received for publication, November 15, 1995

In a porcine aorta extract, we observed two protein kinase activities which specifically phosphorylate the 204-kDa heavy chain isoform of aorta myosin in the absence of conventional kinase activators. We referred to these two protein kinases, eluted at 0.15 and 0.2 M KCl from a DEAE-column, as myosin kinases I (MKI) and II (MKII), respectively. The phosphorylation site for MKI was determined using a purified phosphopeptide derived from porcine aorta myosin phosphorylated with MKI. By comparison with the deduced amino acid sequence for smooth muscle myosins, the site corresponded to a Ser located at 3 amino acids upstream from a Pro, the putative end of the α-helical segment of the 204-kDa heavy chain tail. A homologous Ser is only present in smooth muscle myosins, *i.e.* not in nonmuscle myosins. MKI was purified 130-fold, but not separated from a kinase activity phosphorylating Ser1 or Ser2 in the 20-kDa regulatory light chain of aorta myosin. In contrast, MKII was purified to near homogeneity. MKII phosphorylated the porcine aorta myosin heavy chain at a Ser 19 amino acids downstream from the MKI site. The amino acid sequence around the Ser shared a consensus sequence of the phosphorylation site for casein kinase II and was homologous to that reported for bovine aorta myosin [Kelley, C.A. and Adelstein, R.S. (1990) *J. Biol. Chem.* 265, 17876-17882]. MKII was identified as a multifunctional protein kinase, casein kinase II.

Key words: aorta smooth muscle, casein kinase II, myosin heavy chain, phosphorylation, protein kinase.

Vertebrate smooth muscle myosin is regulated through phosphorylation of Ser19 in the 20-kDa regulatory light chain (LC20). Actin-activated myosin ATPase activity increases with the phosphorylation, which results in initiation of smooth muscle contraction. The phosphorylation of LC20 is catalyzed by myosin light chain kinase (MLCK), which is activated by Ca^{2+}-calmodulin when the intracellular Ca^{2+} concentration increases. The phosphorylation of Ser19 in myosin LC20 is thus the most important signal transduced in smooth muscles (*1, 2*).

In vertebrate smooth muscle and nonmuscle myosins, heavy chain subunits are phosphorylated *in vivo* and *in vitro* (*3-11*), in addition to LC20. Myosin heavy chains of bovine brain (*6, 7*) and bovine aorta smooth muscle (*3, 4*) were shown to be phosphorylated by casein kinase II. The site phosphorylated by casein kinase II is a Ser present in the nonhelical tailpiece of the myosin heavy chain (*4, 7*), and the amino acid sequences around the phosphorylatable Ser are highly conserved among vertebrate smooth muscle and nonmuscle myosin heavy chains (*4, 7, 8, 12-16*). Nonmuscle myosin heavy chains also contain another site phosphorylatable by protein kinase C 30 amino acids upstream from the casein kinase II site (*8-10*). A protein kinase C site is not present in smooth muscle myosins. The heavy chains of nonmuscle myosins of lower eukaryotes such as *Acanthamoeba* (*17, 18*) and *Dictyostelium* (*19, 20*) are phosphorylated by specific protein kinases, and the phosphorylation inhibits the ATPase activity and filament assembly of myosins. Therefore, the phosphorylation of vertebrate myosin heavy chain tails may also be of physiological significance, although their roles remain obscure. There has been no report of the direct detection or characterization of endogenous myosin heavy chain kinase in vertebrate smooth muscles.

We studied the myosin heavy chain kinase in porcine aorta smooth muscle and found the presence of two kinases independent of various conventional kinase activators. We referred to these two kinases as myosin kinases I (MKI) and II (MKII), and identified their phosphorylation sites on the aorta myosin heavy chain after their purification. We report, for the first time, that the MKI site is a Ser located near the end of the helical region of the 204-kDa heavy chain tail in smooth muscle myosins. MKII was purified to near homogeneity and identified as casein kinase II.

MATERIALS AND METHODS

Materials—[γ-^{32}P]ATP was purchased from NEN.

[1] This work was supported in part by a Special Grant-in-Aid for the Promotion of Education and Science in Hokkaido University, and by a Grant-in-Aid for General Scientific Research from the Ministry of Education, Science and Culture. A part of this work was performed at the Research Center for Molecular Genetics of Hokkaido University.

[2] To whom correspondence should be addressed.

Abbreviations: LC20, 20-kDa myosin light chain; MLCK, myosin light chain kinase; PMSF, phenylmethylsulphonyl fluoride; PTH, phenylthiohydantoin; TPCK-trypsin, L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone-treated trypsin.

Phosphatidylserine was obtained from Funakoshi. Egg phosvitin, calf thymus histone (Type IIA), salmon protamine, rabbit muscle phosphorylase *b*, α-casein (dephosphorylated), and TPCK-trypsin were from Sigma. Lysyl-endopeptidase was from Wako Pure Chemical. Polyclonal anti-casein kinase II antibodies against a peptide corresponding to residues 70-91 of the α-subunit, and polyclonal anti-human cdc2 kinase antibodies against a peptide corresponding to residues 42-57 containing the PSTIR sequence were from Seikagaku-Kogyo.

Proteins—All purification procedures were carried out at 4°C. Acid phosphatase was prepared from potatoes by ammonium sulfate fractionation and DEAE Toyopearl 650S (Tosoh) column chromatography. One unit of acid phosphatase was defined as the amount of enzyme necessary to release 1 μmol of P_i/min at 25°C with *p*-nitrophenylphosphate as a substrate.

Myosin was prepared from porcine aorta media smooth muscle as described previously (*21*). The heavy chain of the purified myosin usually contained 1.3 to 4.1 mol of alkali-labile phosphate per mol of myosin, depending on the preparation. The phosphate covalently bound to myosin (5 mg/ml) was dephosphorylated by incubation with acid phosphatase (1,200 U per nmol of phosphate in myosin) in a solution comprising 30 mM MOPS-KOH (pH 7.0), 0.3 M KCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol, at 25°C for 2 h. The dephosphorylated myosin was dissolved in 0.25 M KCl, 2 mM EDTA, 2 mM EGTA, 20 mM Tris-HCl (pH 7.5), and 0.2% 2-mercaptoethanol, and then passed through a phosphocellulose column (Whatman, P11) equilibrated with the same solution to remove acid phosphatase and contaminating heavy chain kinase activities (*6*).

Chicken gizzard calmodulin, MLCK, and porcine aorta LC20 were purified as described previously (*22, 23*). The aorta LC20 was phosphorylated for 10 min at 25°C with 10 μg/ml gizzard MLCK in a solution of 10 μg/ml calmodulin, 0.25 M KCl, 67 mM NaCl, 4.4 mM NaP_i, 5 mM $MgCl_2$, 0.1 mM $CaCl_2$, and 0.2 mM [γ-^{32}P]ATP (78 dpm/pmol), and then passed through a small Sephadex G25 gel filtration column.

Quantitative Analysis of Alkali-Labile Phosphate in Myosin—Myosin (approximately 5 mg/ml) suspended in 120 μl of water was mixed with 24 μl of 6 M NaOH and then boiled for 12 min to release alkali-labile phosphate (*24*). The sample was neutralized with 24 μl of 6 M HCl. The concentration of free phosphate was determined by the method of Chifflet *et al.* (*25*). As a control, the concentration of free phosphate released by 6 M HCl and 6 M NaOH without heat treatment was determined.

Kinase Assay—Unless otherwise indicated, MKI was assayed at 25°C in a solution comprising 0.15 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 20 mM Tris-HCl (pH 7.5), and 0.5 mM [γ-^{32}P]ATP (220-890 dpm/pmol), and MKII was assayed in a solution of 0.2 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 0.5 mM [γ-^{32}P]ATP (220-890 dpm/pmol), at 25°C (standard assay conditions). The reaction was started by the addition of [γ-^{32}P]ATP, and 3 mg/ml myosin was usually reacted during 1-2 min with less than 0.3 U/ml of the kinase fraction (1 U = 1 nmol/min at 25°C, with myosin heavy chain as the substrate). The reaction was terminated by the addition of the SDS-PAGE sample buffer, and then the sample was subjected to SDS-PAGE after boiling. The gel was stained with Coomassie Brilliant Blue. Radioactivity in the myosin heavy and light chain bands was quantitatively determined with an image analyzer (Fujix BAS2000). In some assays during the purification of the kinases, radioactivity in myosin heavy chain bands sliced from the gel was directly determined by Cerenkov counting.

Purification of MKI and MKII—All procedures were performed at 4°C. Freshly prepared porcine aorta mediae were minced and then homogenized 5 times with a Polytron for 15-30 s in three volumes of a solution comprising 0.6 M KCl, 20 mM MOPS-KOH (pH 7.0), 2 mM EGTA, 1 mM benzamidine, 0.2 mM PMSF, and 0.1% 2-mercaptoethanol, and then further stirred for 15 min. The homogenate was centrifuged at 4,700 × *g* for 30 min, and the resulting supernatant was passed through four layers of cheesecloth. The filtrate was fractionated with ammonium sulfate, between 30 and 60% saturation, and then the precipitate was homogenized in a small volume of water. The suspension was dialyzed overnight against buffer A [20 mM Tris-HCl (pH 8.0), 1 mM EGTA, 1 mM benzamidine, 0.1% 2-mercaptoethanol, and 0.1 mM PMSF], and then centrifuged at 13,900 × *g* for 1 h. The supernatant was loaded on a DEAE-Toyopearl 650S column equilibrated with buffer A. The MKI and MKII activities were eluted at 0.15 and 0.2 M KCl, respectively, by applying a linear KCl gradient.

MKI, eluted at 0.15 M KCl, was brought to 60% saturation with ammonium sulfate, and then the precipitate was collected and dialyzed against 20 mM Tris-HCl (pH 7.5), 1 mM EGTA, and 0.1% 2-mercaptoethanol. The dialyzed sample was loaded on a Affigel blue column (BioRad, 100-200 mesh) equilibrated with buffer B [20 mM Tris-HCl (pH 7.5), 1 mM EGTA, 0.1% 2-mercaptoethanol, 0.1 mM PMSF, and 1 mM benzamidine], and then developed with a linear KCl gradient. The active fractions eluted at 0.45 M KCl were loaded on a Sephacryl S300 column equilibrated with buffer B plus 0.5 M KCl. The active fractions were pooled and then dialyzed against buffer A plus 10% glycerol, and finally concentrated on a small DEAE-Toyopearl 650S column (1-2 ml) equilibrated with buffer A plus 10% glycerol. The proteins were eluted with buffer A plus 0.5 M KCl, and stored at −20°C after dialysis against 20 mM KCl, 20 mM Tris-HCl (pH 7.5), 50% glycerol, 1 mM benzamidine, 0.1% 2-mercaptoethanol, and 0.1 mM PMSF.

MKII fractions eluted at 0.2 M KCl from the first DEAE-Toyopearl 650S column were equilibrated with buffer A plus 0.1 M KCl, precipitated by 60% saturation with ammonium sulfate, and then dialyzed against buffer C [20 mM MOPS-KOH (pH 7.0), 1 mM EGTA, 1 mM benzamidine, 0.1 mM PMSF, and 0.1% 2-mercaptoethanol]. After ultracentrifugation at 125,000 × *g* for 2 h, the supernatant was loaded on a CM-Toyopearl 650S column (Tosoh) equilibrated with buffer C plus 0.1 M KCl, and then developed with a linear gradient of 0.1-0.4 M KCl. The active fractions eluted at around 0.2 M KCl were concentrated and then applied to a Sephacryl S300 column in buffer A plus 0.5 M KCl. The pooled active fractions were diluted to 0.3 M KCl with buffer B and the pH was adjusted to 7.5 with 6 M HCl. The sample was then loaded on a heparin-agarose ALD column (GIBCO BRL) equilibrated with buffer B plus 0.3 M KCl, and then developed with a linear gradient of 0.3-0.8 M KCl. The active fractions free from contaminants were collected and concentrated on a

small DEAE-Toyopearl 650S column equilibrated with buffer A plus 20 mM KCl and 10% glycerol. The sample was stored at −20°C after dialysis against 20 mM Tris-HCl (pH 8.0), 30 mM KCl, 50% glycerol, 1 mM benzamidine, 0.1 mM PMSF, and 0.1% 2-mercaptoethanol.

Purification of Tryptic Phosphopeptides of Myosin—Myosin (6.5 mg) was phosphorylated under the standard kinase assay conditions, using [γ-^{32}P]ATP, at 25°C with either 0.14 U of MKI for 1 h or 0.48 U of MKII for 15 min. The MKI used was the fraction purified omitting the Affigel blue step. The kinase reactions were terminated by adding urea to saturation, and myosin subunits were separated on a Sepharose CL6B column (Pharmacia) equilibrated with 8 M urea and 20 mM Tris-HCl (pH 8.0). The fractions containing the myosin heavy chain or LC20 were exhaustively dialyzed against 50 mM ammonium bicarbonate. The heavy chain fraction was digested at 30°C for 24 h with TPCK-trypsin (1 : 50 w/w). The LC20 fraction phosphorylated with MKI was digested with lysylendopeptidase (1 : 50 w/w) at 30°C for 12 h. Each sample was lyophilized, and phosphopeptides were isolated using an Fe-immobilized chelating affinity column (Pharmacia) (*4, 7, 8*) and HPLC with a C18 reverse phase column (ODS-80Ts; Tosoh). The fractions containing radioactivity were further purified by rechromatography. The LC20 phosphopeptide obtained on the chelating column was not retained on the HPLC column and thus passed through a Sephadex G10 column.

Two-Dimensional Phosphopeptide Mapping of LC20—LC20 phosphorylated with MKI was separated from the heavy chain on the Sepharose CL6B column described above, and then dialyzed against 20 mM ammonium bicarbonate, lyophilized, and then dissolved in a small volume of 20 mM ammonium bicarbonate. The sample (180 μg) was then digested at 30°C with TPCK-trypsin (1 : 9 w/w). The same amount of TPCK-trypsin was added four times, every 2 h, after the digestion was started, and the incubation was continued for 14 h. The reaction mixture was lyophilized and purified by repeated dissolution (in water)-lyophilization twice. The lyophilized sample was then dissolved in 5 μl of the electrophoresis buffer (acetic acid : formic acid : water = 9 : 3 : 88) (*26*), and subjected to electrophoresis at 950 V for 30 min (5°C) after spotting onto a cellulose thin-layer plate (10 × 10 cm; Funakoshi). The plate was dried, and then ascending chromatography, as the second dimension, was carried out with a solution of 1-buthanol : pyridine : acetic acid : water = 195 : 150 : 30 : 120. The LC20 (90 μg) phosphorylated with MLCK was treated in the same manner. Spots of phosphopeptides were detected with Fuji X-ray RX film or BAS2000.

Other Methods—The amino acid compositions and sequences of the peptides were determined as described previously (*27*) without performic acid oxidation of Cys. Phosphoamino acid analysis of the purified tryptic phosphopeptide from the myosin heavy chain was performed by partial hydrolysis of the peptide in 6 M HCl at 110°C for 3 h. Electrophoresis of the partially hydrolyzed products was carried out at 950 V for 150-180 min on a thin-layer cellulose plate in electrophoresis buffer. The phosphoamino acid standards were stained with cadmium-ninhydrin, and radioactivity in the samples was monitored with X-ray film and BAS2000. SDS-PAGE was performed as in Ref. *28* and the gel was stained with Coomassie Brilliant Blue or silver. Immunoblot analysis was performed by transferring proteins in the gel to 0.1 μm nitrocellulose membranes (Schleicher & Schuell) for 80 min at 30 mA. After blocking with gelatin, the membranes were incubated with antibodies for 2 h. The immunoreactive bands were stained with alkaline phosphatase conjugate substrate kit (Bio-Rad). The concentration of myosin was determined from the absorbance at 280 nm using the absorption coefficient of $A^{1\%}_{280} = 0.48$. The concentrations of phosvitin, casein, histone, protamine, and phosphorylase *b* were determined by the biuret method (*29*), and other proteins were determined by the Coomassie Brilliant Blue method (*30*) using a Bio-Rad Protein Assay kit.

RESULTS

Survey of Myosin Heavy Chain Kinase Activities—Using porcine aorta media extract, we searched for an endogenous myosin heavy chain kinase activities. Two myosin kinases activated by calcium and calmodulin, and two other kinases exhibiting activities in the absence of conventional kinase activators were found in porcine aorta smooth muscle. We first focused on characterization of the latter two kinases.

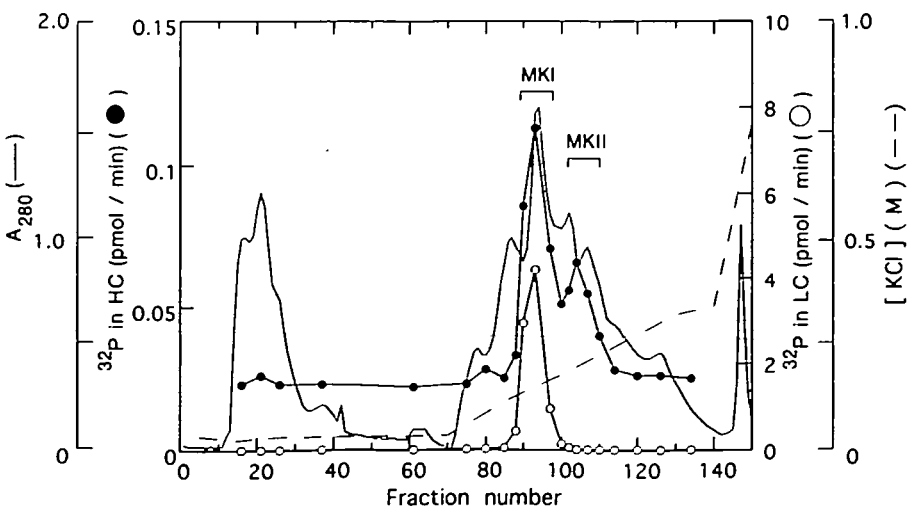

Fig. 1. **Elution profile of myosin heavy chain kinase activity in a porcine aorta extract from a DEAE-Toyopearl 650S column.** The 30-60% saturated ammonium sulfate fraction of a porcine aorta extract was applied to a DEAE-Toyopearl 650S column equilibrated with 20 mM Tris-HCl (pH 8.5), 1 mM EGTA, 1 mM benzamidine, 0.1% 2-mercaptoethanol, 0.1 mM PMSF, and 20 mM KCl. Kinase activity was measured as the incorporation of radioactivity into myosin (3 mg/ml) with 6 μl of dialyzed eluted fractions and 0.5 mM [γ-^{32}P]ATP (670 dpm/pmol) in a 12 μl reaction mixture comprising 0.2 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, and 20 mM Tris-HCl (pH 8.0). (●) kinase activity toward the myosin heavy chain; (○) kinase activity toward myosin LC20.

As shown in Fig. 1, DEAE Toyopearl 650S column chromatography of the porcine aorta extract gave two peaks of heavy chain kinase activities detected in the presence of EGTA. The first heavy chain kinase peak, eluted at 0.15 M KCl, also contained myosin LC20 kinase activity, while the second peak, at 0.2 M KCl, showed only heavy chain kinase activity. We referred to these two kinases as MKI and MKII, respectively, and attempted to characterize them.

MKI—This kinase activity was further purified by two steps of column chromatography. MKI was not ultimately purified to homogeneity, but the heavy and light chain kinase activities were purified 130 and 140 times, respectively (Table I). Both kinase activities were co-eluted from the Affigel blue column at 0.45 M KCl and from the Sephacryl S300 gel filtration column at a K_{av} value of 0.50, corresponding to a molecular mass of 40 kDa. These results suggest that an identical protein kinase catalyzes the phosphorylation of both the heavy chain and LC20 of aorta myosin. MKI phosphorylated protamine and α-casein in addition to the myosin heavy and light chains (Table II). The kinase activity was not inhibited by heparin with either myosin or phosvitin as the substrate (data not shown).

The phosphorylation site on the myosin heavy chain for MKI was determined using porcine aorta myosin phosphorylated with MKI and [γ-^{32}P]ATP. The tryptic phosphopeptide derived from the heavy chain gave a single radioactive peak on reverse phase HPLC. The peak fractions collected were subjected to protein sequencing. Two major PTH-amino acids were recovered at each cycle of Edman degradation, indicating the presence of two peptide components in the sample. Since one of the two PTH-amino acids recovered in each cycle appeared in the subsequent cycle, the two peptides were shown to be the same fragment, but having a one residue difference in the N-terminal end. As shown in Table III, the yield of PTH-amino acids of the longer peptide was significantly low at cycle 6, suggesting phosphorylation of the corresponding residue. Phosphoamino acid analysis of the purified phosphopeptide showed a radioactive spot of phosphoserine but not one of phosphothreonine (data not shown). These results indicated that the site on the myosin heavy chain phosphorylated by MKI was in the segment having the amino acid sequence of Arg-Gly-Asn-Glu-Thr-Ser*-Phe-Val-Pro, where Ser* was the phosphoacceptor. This sequence is analogous to partial sequences of the reported 204-kDa heavy chain isoforms of smooth muscle myosins (*15, 16, 31*), and the C-terminal Pro of the peptide corresponds to that breaking the α-helical segment of the 204-kDa heavy chain (Fig. 5).

The phosphorylation site of LC20 for MKI was determined from the tryptic phosphopeptide map of LC20 derived from aorta myosin phosphorylated with MKI (Fig. 2A). The two major peptides phosphorylated by MKI hardly migrated in the chromatographic dimension, but one of the two peptides migrated in the electrophoretic dimension faster than the peptide derived from porcine aorta LC20 phosphorylated with MLCK (Fig. 2B). These degrees of migration suggest that the two MKI phosphopeptides (Fig. 2A) are amino-terminal fragments of LC20 in which Ser1 or Ser2, or both are phosphorylated (*9, 26*). Additionally, we observed only a single component for the lysylendopeptidase phosphopeptide of LC20 derived from aorta myosin phosphorylated with MKI. The amino acid composition of the lysylendopeptidase phosphopeptide was Ser : Lys = 2.1 : 1, consistent with that of the amino-terminal fragment of LC20, Ac-Ser-Ser-Lys (*32*). All these results suggest that the phosphorylation site on aorta LC20 for MKI is either Ser1 or Ser2, or both of them.

MKII—MKII was purified approximately 1,100-fold from porcine aorta smooth muscle. Figure 3A shows the silver-stained SDS-PAGE pattern of the purified product.

TABLE II. **Substrate specificity of MKI.** The kinase assay was carried out with less than 0.2 U/ml of MKI under the standard assay conditions. In the case of substrates other than myosin, the assay was performed in the absence of KCl. The reaction was terminated after incubation for 1-2 min at 25°C, and phosphate incorporated into substrates was determined by the method of Corbin and Reimann (*46*). Kinase activity toward protamine was determined using P81 phosphocellulose paper (*47*). The values for myosin are cited from Table I.

Substrate	Concentration of substrate (μM)	MKI activity (nmol/min/mg)
Myosin heavy chain	6.3	1.3
Myosin LC20	6.3	28
Histone IIA	10	0
Protamine	10	2.9
Phosphorylase *b*	10	0.7
α-Casein	10	1.4
Phosvitin	10	0.6

TABLE III. **Results of amino acid sequence analysis of a heavy chain phosphopeptide derived from porcine aorta myosin phosphorylated with MKI.**

Cycle No.	PTH-amino acid	Yield (pmol)
1	Arg	10.1
2	Gly	27.1
3	Asn	27.6
4	Glu	26.7
5	Thr	17.8
6	—	—
7	Phe	20.9
8	Val	16.9
9	Pro	6.73

TABLE I. **Summary of purification of MKI from porcine aorta smooth muscle.**

Purification step	Protein (mg)	Heavy chain		Light chain	
		Total activity (nmol/min)	Specific activity (nmol/min/mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)
Extract	19,000	190	0.010	3,800	0.20
30-60% SASFn[a]	7,000	50	0.0072	590	0.084
DEAE-Toyopearl	620	27	0.044	870	1.4
Affigel blue	18	6.8	0.38	140	7.9
Sephacryl S300	0.9	1.2	1.3	25	28

[a]Fraction precipitated between 30 and 60% ammonium sulfate saturation.

43-, 40-, and 26-kDa polypeptides comprized the MKII. The molecular mass of MKII under nondenaturing conditions was estimated to be 160-kDa on Sephacryl S300 column chromatography. MKII favored acidic proteins such as casein and phosvitin as substrates, and was inhibited strongly by heparin with myosin as the substrate (data not shown). Maximum kinase activity was observed in the presence of 0.2 M KCl and 5 mM $MgCl_2$ at pH 8.0.

The phosphorylation site of the aorta myosin heavy chain for MKII was determined. Tryptic phosphopeptides derived from myosin phosphorylated with MKII gave two radioactive peaks on HPLC on a reverse phase column. Amino acid sequence analysis revealed that both radioactive components contained peptides having identical amino acid sequences, Val-Ile-Glu-Asn-Ala-Asp-Gly-Ser*-Asp-Glu-Glu-Met-Asp-Ala-Arg, where Ser* was estimated to be phosphorylated because of the significantly low yield. Phosphorylation of this Ser was confirmed by phosphoamino acid analysis of the purified phosphopeptides. This amino acid sequence is analogous to that of bovine aorta myosin containing the phosphorylation site for casein kinase II reported by Kelley and Adelstein (*4*). We then performed immunoblot analysis of the purified MKII with anti-casein kinase II antibodies (Fig. 3B). Both the 43-kDa main and 40-kDa minor components of MKII cross-reacted with the antibodies, indicating that MKII is the well-characterized ubiquitous protein kinase, casein kinase II. The silver-stained band corresponding to a high molecular mass in Fig. 3B is due to a contaminant because the MKII used in this experiment was not the best purified fraction.

Localization of Endogenous Phosphates on the Porcine Aorta Myosin Heavy Chain—The heavy chain derived from purified porcine aorta smooth muscle myosin usually contained up to 4.1 mol covalently bound phosphate per mole of myosin. The degree of phosphorylation with MKI,

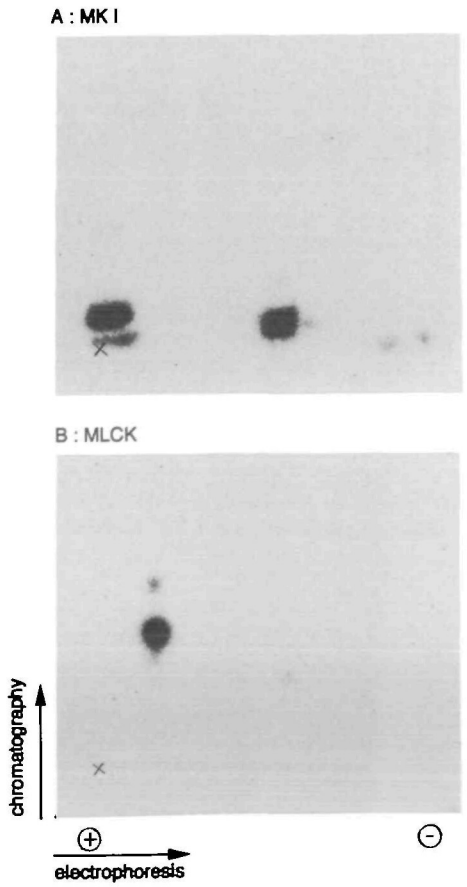

Fig. 2. **Two-dimensional tryptic phosphopeptide mapping of LC20 from porcine aorta myosin phosphorylated with MKI.** (A) Mapping of LC20 derived from myosin phosphorylated with MKI; (B) mapping of LC20 phosphorylated with MLCK. The origin is denoted by ×.

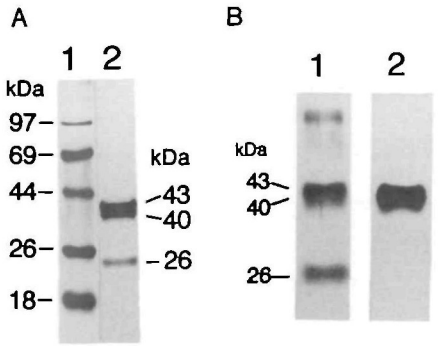

Fig. 3. **SDS-PAGE and immunoblot analysis of MKII.** (A) SDS-PAGE pattern of the purified MKII stained with silver. Lane 1, molecular mass standards, phosphorylase (97 kDa), bovine serum albumin (69 kDa), ovalbumin (44 kDa), chymotrypsinogen (26 kDa), and β-lactoglobulin (18 kDa); lane 2, MKII. (B) Immunoblot analysis of MKII with anti-casein kinase II antibodies. MKII (0.5 μg) was subjected to SDS-PAGE, transferred to a nitrocellulose sheet, and then cross-reacted with anti-casein kinase II antibodies which recognize the α and α′-subunits (43 and 40 kDa), but not the β-subunit (26 kDa). Lane 1, silver staining; lane 2, immunostaining.

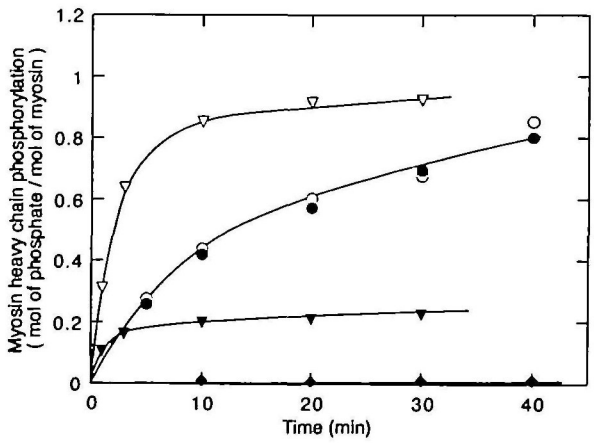

Fig. 4. **Effect of acid phosphatase treatment of myosin on myosin heavy chain phosphorylation by MKI or MKII/casein kinase II.** The time courses indicate the phosphate incorporation into myosin after incubation of the myosin with each kinase under the standard assay conditions. Open symbols, myosin treated with phosphatase; closed symbols, myosin untreated with phosphatase. Phosphate incorporation was measured after incubation with MKI (circles), MKII (triangles), or without kinase (rhomboids). Myosin untreated with phosphatase was prepared by passage through a phosphocellulose column to eliminate contaminating heavy chain kinases. This myosin contained 1.5 mol of covalently bound phosphate/mol of myosin.

or with MKII/casein kinase II was examined before and after dephosphorylation of the endogenous phosphate by treatment with acid phosphatase. The phosphate incorporation into the dephosphorylated myosin increased with MKII/casein kinase II, but not with MKI (Fig. 4). These results indicate that a part of the phosphate covalently bound to porcine aorta myosin was localized at the MKII/casein kinase II site, not at the MKI site.

DISCUSSION

We have surveyed myosin heavy chain kinase activities in a porcine aorta smooth muscle extract and found the presence of two kinases independent of conventional kinase activators. One kinase, MKII, which only had heavy chain kinase activity, *i.e.* no LC20 kinase activity, was purified to near homogeneity, and identified as casein kinase II based on both the molecular and subunit masses, enzymatic properties, substrate specificity, amino acid sequence around the phosphorylation site (*33-35*), and results of immunoblot analysis (Fig. 3). This result is consistent with the report of Kelley and Adelstein (*4*) that the tryptic phosphopeptide map of the myosin heavy chain derived from bovine aorta myosin phosphorylated in cultured aortic cells was identical to that of the heavy chain derived from the myosin phosphorylated with casein kinase II *in vitro*. The results suggested that casein kinase II catalyzes the phosphorylation of the myosin heavy chain in intact aortic cells. We presented here direct evidence that the endogenous heavy chain kinase phosphorylating the nonhelical tailpiece of the 204-kDa myosin heavy chain is actually casein kinase II in aorta smooth muscle.

Nonmuscle myosin heavy chains from bovine (*7*) and chicken brains (*14*), human macrophages (*12*), and chicken intestinal epithelial cells (*13*) also contain a Ser homologous to that phosphorylatable by casein kinase II (Fig. 5). The conservation of the phosphorylation site among vertebrate nonmuscle and smooth muscle myosins seems to indicate that the phosphorylation of such a characteristic site may be involved in an indispensable function. The role of this phosphorylation, however, remains to be determined, though the carboxyl terminal tailpiece region of the myosin heavy chain is important for filament formation in smooth muscle and nonmuscle myosins (*36-38*).

The other endogenous kinase, MKI, phosphorylated a Ser residue at 19 amino acids upstream from the MKII/casein kinase II site based on the deduced amino acid sequence for smooth muscle myosins (Fig. 5). Interestingly, the Ser phosphorylatable by MKI is near a Pro which corresponds to the putative site breaking the α-helix and starting the nonhelical tailpiece region of the 204-kDa isoform. A corresponding Ser exists in the 204-kDa heavy chain of rat aorta and rabbit uterus myosins, but not in their 200-kDa isoforms (Fig. 5). Chicken gizzard myosin also contains a corresponding Ser, although it does not contain a Ser phosphorylatable with casein kinase II, which is substituted by Gly (*31*) (Fig. 5). It should be noted that the homologous Ser phosphorylatable by MKI is only present in the heavy chain of smooth muscle myosins, *i.e.* not in that of nonmuscle myosins (Fig. 5). As shown in Fig. 4, this Ser phosphorylatable by MKI was not phosphorylated in the purified porcine aorta myosin, suggesting that its phosphorylation occurs transiently *in vivo*. Its phosphorylation may be induced by an unknown particular signal, or there may be a strong endogenous phosphatase specific to this phosphorylation site. Of note is that the total heavy chain kinase activity of MKI was more than twice that of MKII/casein kinase II in the aorta smooth muscle extract (Fig. 1). All these results suggest that the phosphorylation of the 204-kDa myosin heavy chain by MKI may play a significant role in the smooth muscle function. This should be clarified by future studies.

In contrast to MKII/casein kinase II, MKI also phosphorylated LC20, at the N-terminal Ser1 or Ser2 (Fig. 2). These sites on LC20 are also phosphorylated by protein kinase C (*39*) or cyclin-p34^{cdc2} kinase (*40*). We could not observe, however, the activation of MKI by Ca^{2+} and phosphatidylserine plus diolein, or the cross-reactivity of MKI with the polyclonal anti-p34^{cdc2} kinase antibodies. The molecular mass of the catalytic subunit of cAMP-dependent protein kinase, 40-kDa, is similar to that of MKI. However, the aorta myosin heavy and light chains were reported not to be phosphorylated by cAMP-dependent protein kinase (*4*). MKI, thus, is unlikely to be the catalytic subunit of cAMP-dependent protein kinase. The proximity of the Ser phosphorylatable with MKI to the Pro at the helix end of the porcine aorta myosin heavy chain (Fig. 5) suggests that MKI may be a kind of glycogen synthase kinase-3 (GSK-3) (*41*). The phosphorylation of glycogen synthase (*42*) and inhibitor-2 (*43*) of type 1 protein phosphatase by GSK-3 is synergistically activated on the phosphorylation of the casein kinase II site, which is also

	1	2
Porcine aorta	RGNE-T**S**FV<u>P</u>	VIEN-ADG**S**DEEMDAR
Bovine aorta		VIEN-ADGSEEEVDAR
Rat aorta (204)	LKSKLRRGNE-ASFVPSRRAGGRRVIEN-TDGSEEEMDARDSDFNGTKASE*	
Rabbit uterus (204)	LKSKLRRGNE-TSFVPTRRSGGRRVIEN-ADGSEEEVDARDADFNGTKSSE*	
Chicken gizzard	LKSKLRRGNEPVSFAPPRRSGGRRVIENATDGGEEEIDGRDGDFNG-KASE*	
Chicken epithelium (NM-A)	LKSKLRRGD-----LPFVVT-RRLVRKGTGECSDEEVDGKAEAGDA-KATE*	
Chicken brain (NM-B)	LKNRLRRGGPITFSSSRSGRRQLHIEGASLELSDDDAESKGSDVNEAQPTPAE*	
Rat aorta (200)	LKSKLRGPPPQETSQ*	
Rabbit uterus (200)	LKSKLRGPPPQETSQ*	

Fig. 5. **Homology of the amino acid sequences of MKI and MKII/casein kinase II phosphorylation sites on the myosin heavy chain.** The amino acid sequences of two tryptic phosphopeptides, 1 and 2, derived from porcine aorta myosin phosphorylated with MKI and MKII/casein kinase II, respectively, were compared with the published sequences of myosin heavy chains. The outlined letter represents the phosphorylated Ser residue. The underlined proline corresponds to the putative carboxy-terminus of the α-helical rod. (204) 204-kDa heavy chain of smooth muscle myosin; (200) 200-kDa heavy chain of smooth muscle myosin. NM-A and NM-B, nonmuscle myosin heavy chains of the A-form and B-form, respectively, according to the classification of Kawamoto and Adelstein (*48*). Amino acid sequences were obtained for bovine aorta [Kelley and Adelstein (*4*)]; rat aorta [Babij and Periasamy (*15*)], rabbit uterus [Nagai *et al.* (*16*)]; chicken gizzard [Yanagisawa *et al.* (*31*)]; chicken epithelium [Shohet *et al.* (*13*)]; and chicken brain [Takahashi *et al.* (*14*)]. The carboxy-terminal ends of the myosin heavy chains are denoted by *.

present in both proteins. The MKI site on the myosin heavy chain was separated by only 19 amino acids from the casein kinase II site (Fig. 5). The presence of these two phosphorylation sites on a myosin heavy chain also suggests that MKI may potentially be a type of GSK-3. We do not, however, have any convincing evidence to support this assumption at present. Identification of MKI remains to be performed.

The actin-activated Mg-ATPase activity of gizzard heavy meromyosin was reported to decrease by more than 50% on the phosphorylation of LC20 by protein kinase C. This large decrease in the ATPase activity appears due to the phosphorylation of Thr9 rather than that of Ser1 or Ser2 (*39, 44, 45*). Furthermore, it was reported that the phosphorylation of protein kinase C sites in LC20 was not detected during the contraction-relaxation cycle of bovine tracheal smooth muscle (*26*). Therefore, the phosphorylation of Ser1 or Ser2 of LC20 by MKI may not directly be correlated with the contraction-relaxation cycle. The specific activity of the obtained MKI is more than ten times higher for myosin LC20 than for the myosin heavy chain (Tables I and II). It may be possible *in vivo*, however, that an unknown regulator for MKI or an organized structure of thick and thin filaments surrounded by many other cytosolic protein components regulates the MKI activity differently toward the myosin heavy chain and LC20. Clarification of the localization of MKI in aorta cells is necessary.

REFERENCES

1. Kamm, K.E. and Stull, J.T. (1985) The function of myosin and myosin light chain kinase phosphorylation in smooth muscle. *Annu. Rev. Pharmacol. Toxicol.* **25**, 593-620
2. Sellers, J.R. and Adelstein, R.S. (1987) Regulation of contractile activity. *The Enzymes* **18**, 381-418
3. Kawamoto, S. and Adelstein, R.S. (1988) The heavy chain of smooth muscle myosin is phosphorylated in aorta cells. *J. Biol. Chem.* **263**, 1099-1102
4. Kelley, C.A. and Adelstein, R.S. (1990) The 204-kDa smooth muscle myosin heavy chain is phosphorylated in intact cells by casein kinase II on a serine near the carboxyl terminus. *J. Biol. Chem.* **265**, 17876-17882
5. Kamm, K.E., Hsu, L., Kubota, Y., and Stull, J.T. (1989) Phosphorylation of smooth muscle myosin heavy and light chains. *J. Biol. Chem.* **264**, 21223-21229
6. Murakami, N., Matsumura, S., and Kumon, A. (1984) Purification and identification of myosin heavy chain kinase from bovine brain. *J. Biochem.* **95**, 651-660
7. Murakami, N., Healy-Louie, G., and Elzinga, M. (1990) Amino acid sequence around the serine phosphorylated by casein kinase II in brain myosin heavy chain. *J. Biol. Chem.* **265**, 1041-1047
8. Conti, M.A., Sellers, J.R., Adelstein, R.S., and Elzinga, M. (1991) Identification of the serine residue phosphorylated by protein kinase C in vertebrate nonmuscle myosin heavy chains. *Biochemistry* **30**, 966-970
9. Kawamoto, S., Bengur, A.R., Sellers, J.R., and Adelstein, R.S. (1989) *In situ* phosphorylation of human platelet myosin heavy and light chains by protein kinase C. *J. Biol. Chem.* **264**, 2258-2265
10. Murakami, N., Elzinga, M., Singh, S.S., and Chauhan, V.P.S. (1994) Direct binding of myosin II to phospholipid vesicles *via* tail regions and phosphorylation of the heavy chains by protein kinase C. *J. Biol. Chem.* **269**, 16082-16090
11. Kelley, C.A., Oberman, F., Yisraeli, J.K., and Adelstein, R.S. (1995) A *Xenopus* nonmuscle myosin heavy chain isoform is phosphorylated by cyclin-p34^{cdc2} kinase during miosis. *J. Biol. Chem.* **270**, 1395-1401
12. Saez, C.G., Myers, J.C., Shows, T.B., and Leinwand, L.A. (1990) Human nonmuscle myosin heavy chain mRNA: Generation of diversity through alternative polyadenylylation. *Proc. Natl. Acad. Sci. USA* **87**, 1164-1168
13. Shohet, R.V., Conti, M.A., Kawamoto, S., Preston, Y.A., Brill, D.A., and Adelstein, R.S. (1989) Cloning of the cDNA encoding the myosin heavy chain of a vertebrate cellular myosin. *Proc. Natl. Acad. Sci. USA* **86**, 7726-7730
14. Takahashi, M., Kawamoto, S., and Adelstein, R.S. (1992) Evidence for inserted sequences in the head region of nonmuscle myosin specific to the nervous system. *J. Biol. Chem.* **267**, 17864-17871
15. Babij, P. and Periasamy, M. (1989) Myosin heavy chain isoform diversity in smooth muscle is produced by differential RNA processing. *J. Mol. Biol.* **210**, 673-679
16. Nagai, R., Kuro-o, M., Babij, P., and Periasamy, M. (1989) Identification of two types of smooth muscle myosin heavy chain isoforms by cDNA cloning and immunoblot analysis. *J. Biol. Chem.* **264**, 9734-9737
17. Cote, G.P., Collins, J.H., and Korn, E.D. (1981) Identification of three phosphorylation sites on each heavy chain of *Acanthamoeba* myosin II. *J. Biol. Chem.* **256**, 12811-12816
18. Kuznicki, J., Albanesi, J.P., Cote, G.P., and Korn, E.D. (1983) Supramolecular regulation of the actin-activated ATPase activity of filaments of *Acanthamoeba* myosin II. *J. Biol. Chem.* **258**, 6011-6014
19. Cote, G.P. and Bukiejko, U. (1987) Purification and characterization of a myosin heavy chain kinase from *Dictyostelium discoideum*. *J. Biol. Chem.* **262**, 1065-1072
20. Ravid, S. and Spudich, J.A. (1989) Myosin heavy chain kinase from developed *Dictyostelium* cells. *J. Biol. Chem.* **264**, 15144-15150
21. Hasegawa, Y., Tanahashi, K., and Morita, F. (1990) Regulatory mechanism by the phosphorylation of 20-kDa light chain of porcine aorta smooth muscle myosin. *J. Biochem.* **108**, 909-913
22. Yoshida, M., Minowa, O., and Yagi, K. (1983) Divalent cation binding to wheat germ calmodulin. *J. Biochem.* **94**, 1925-1933
23. Hasegawa, Y., Ueno, H., Horie, K., and Morita, F. (1988) Two isoforms of 17-kDa essential light chain of aorta media smooth muscle myosin. *J. Biochem.* **103**, 15-18
24. Weller, M. (1979) *Protein Phosphorylation*, pp. 452-456, Pion Limited, London
25. Chifflet, S., Torriglia, A., Chiesa, R., and Tolosa, S. (1988) A method for the determination of inorganic phosphate in the presence of labile organic phosphate and high concentrations of protein: Application to lens ATPases. *Anal. Biochem.* **168**, 1-4
26. Colburn, J.C., Michnoff, C.H., Hsu, L., Slaughter, C.A., Kamm, K.E., and Stull, J.T. (1988) Sites phosphorylated in myosin light chain in contracting smooth muscle. *J. Biol. Chem.* **263**, 19166-19173
27. Hasegawa, Y., Ueda, Y., Watanabe, M., and Morita, F. (1992) Studies of amino acid sequences of two isoforms of 17-kDa essential light chain of smooth muscle myosin from porcine aorta media. *J. Biochem.* **111**, 798-803
28. Porzio, M.A. and Pearson, A.M. (1977) Improved resolution of myofibrillar proteins with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta* **490**, 27-34
29. Gornall, A.G., Bardawill, C.J., and David, M.M. (1949) Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177**, 751-766
30. Bradford, M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254
31. Yanagisawa, M., Hamada, Y., Katsuragawa, Y., Imamura, M., Mikawa, T., and Masaki, T. (1987) Complete primary structure of vertebrate smooth muscle myosin heavy chain deduced from its complementary DNA sequence. *J. Mol. Biol.* **198**, 143-157
32. Watanabe, M., Hasagawa, Y., Katoh, T., and Morita, F. (1992) Amino acid sequence of the 20-kDa regulatory light chain of porcine aorta smooth muscle myosin. *J. Biochem.* **112**, 431-432
33. Pinna, L.A. (1990) Casein kinase 2: An 'eminence grise' in cellular regulation? *Biochim. Biophys. Acta* **1054**, 267-284
34. Hathaway, G.M. and Traugh, J.A. (1983) Casein kinase II in

Methods in Enzymology (Corbin, J.D. and Hardman, J.G., eds.) Vol. 99, pp. 317-331, Academic Press, New York
35. Mitev, V., Pauloin, A., and Houdebine, Louis-M. (1994) Purification and characterization of two casein kinase type II isozymes from bovine brain gray matter. *J. Neurochem.* **63**, 717-726
36. Hodge, T.P., Cross, R., and Kendrick-Jones, J. (1992) Role of the COOH-terminal nonhelical tailpiece in the assembly of a vertebrate nonmuscle myosin rod. *J. Cell Biol.* **118**, 1085-1095
37. Tan, J.L., Ravid, S., and Spudich, J.A. (1992) Control of nonmuscle myosins by phosphorylation. *Annu. Rev. Biochem.* **61**, 721-759
38. Ikebe, M., Hewett, T.E., Martin, A.F., Chen, M., and Hartshorne, D.J. (1991) Cleavage of a smooth muscle myosin heavy chain near its C-terminus by α-chymotrypsin. *J. Biol. Chem.* **266**, 7030-7036
39. Bengur, A.R., Robinson, E.A., Appella, E., and Sellers, J.R. (1987) Sequence of the sites phosphorylated by protein kinase C in the smooth muscle myosin light chain. *J. Biol. Chem.* **262**, 7613-7617
40. Satterwhite, L.L., Lohka, M.J., Wilson, K.L., Scherson, T.Y., Cisek, L.J., Corden, J.L., and Pollard, T.D. (1992) Phosphorylation of myosin II regulatory light chain by cyclin-p34^{cdc2}: A mechanism for the timing of cytokinesis. *J. Cell Biol.* **118**, 595-605
41. Hemmings, B.A. and Cohen, P. (1983) Glycogen synthase kinase-3 from rabbit skeletal muscle in *Methods in Enzymology* (Corbin, J.D. and Hardman, J.G., eds.) Vol. 99, pp. 337-345, Academic Press, New York
42. Picton, C., Woodgett, J., Hemmings, B., and Cohen, P. (1982) Multisite phosphorylation of glycogen synthase from rabbit skeletal muscle. *FEBS Lett.* **150**, 191-196
43. DePaoli-Roach, A.A. (1984) Synergistic phosphorylation and activation of ATP-Mg-dependent phosphoprotein phosphatase by F_A/GSK-3 and casein kinase II. *J. Biol. Chem.* **259**, 12144-12152
44. Ikebe, M., Hartshorne, D.J., and Elzinga, M. (1987) Phosphorylation of the 20,000-dalton light chain of smooth muscle myosin by the calcium-activated, phospholipid-dependent protein kinase. *J. Biol. Chem.* **262**, 9569-9573
45. Nishikawa, M., Sellers, J.R., Adelstein, R.S., and Hidaka, H. (1984) Protein kinase C modulates *in vitro* phosphorylation of the smooth muscle heavy meromyosin by myosin light chain kinase. *J. Biol. Chem.* **259**, 8808-8814
46. Corbin, J.D. and Reimann, E.M. (1974) Assay of cyclic AMP-dependent protein kinases in *Methods in Enzymology* (Hardman, J.G. and O'Malley, B.W., eds.) Vol. 38, pp. 287-290, Academic Press, New York
47. Roskoski, R., Jr. (1983) Assays of protein kinase in *Methods in Enzymology* (Corbin, J.D. and Hardman, J.G., eds.) Vol. 99, pp. 3-6, Academic Press, New York
48. Kawamoto, S. and Adelstein, R.S. (1991) Chicken nonmuscle myosin heavy chains: Differential expression of two mRNAs and evidence for two different polypeptides. *J. Cell Biol.* **112**, 915-924