The 204-kDa Smooth Muscle Myosin Heavy Chain Is Phosphorylated in Intact Cells by Casein Kinase II on a Serine near the Carboxyl Terminus*

Christine A. Kelley and Robert S. Adelstein
From the Laboratory of Molecular Cardiology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

The heavy chain of smooth muscle myosin was found to be phosphorylated following immunoprecipitation from cultured bovine aortic smooth muscle cells. Of a variety of serine/threonine kinases assayed, only casein kinase II and calcium/calmodulin-dependent protein kinase II phosphorylated the smooth muscle myosin heavy chain to a significant extent in vitro. Two-dimensional maps of tryptic peptides derived from heavy chains phosphorylated in cultured cells revealed one major and one minor phosphopeptide. Identical tryptic peptide maps were obtained from heavy chains phosphorylated in vitro with casein kinase II but not with calcium/calmodulin-dependent protein kinase II. Of note, the 204-kDa smooth muscle myosin heavy chain but not the 200-kDa heavy chain isoform was phosphorylated by casein kinase II. Partial sequence of the tryptic phosphopeptides generated following phosphorylation by casein kinase II yielded Val-Ile-phosphorylated by casein kinase II. Partial sequence for rabbit uterine smooth muscle myosin (Nagai, R., Kuro-o, M., Babij, P., and Periasamy, M. (1989) J. Biol. Chem. 264, 9734–9737), we have localized the phosphorylated serine residue to the nonhelical tail of the 204-kDa isoform of the smooth muscle myosin heavy chain. The ability of the 204-kDa isoform, but not the 200-kDa isoform, to serve as a substrate for casein kinase II suggests that these two isoforms can be regulated differentially.

Myosin is a contractile protein involved in a variety of motile events including muscle contraction (1), cell locomotion (2), and cell division (3, 4). The conventional myosin molecule is composed of two heavy chain subunits of approximately 200 kDa each, which form a globular amino-terminal head region, and a rod-like coiled coil carboxyl-terminal tail. The globular head region is noncovalently associated with two pairs of light chains of 17 and 21 kDa.

Reversible phosphorylation of the heavy chain and/or light chain subunits of myosin occurs throughout nature. The nonmuscle and smooth muscle myosins of some invertebrates have been shown to be regulated by heavy chain phosphorylation. Phosphorylation of the heavy chains of Dictyostelium and Acanthamoeba myosin inhibits the actin-activated Mg-ATPase activity and affects its ability to form stable bipolar filaments (5–8). The myosin heavy chains from a molluscan smooth muscle can also be phosphorylated, and this modification may control the transition from a long lived state called "catch" to the relaxed state (9).

Vertebrate nonmuscle and smooth muscle myosin are regulated by phosphorylation of the 20-kDa light chains (LC20).1 Myosin light chain kinase phosphorylation of LC20 in vitro allows the myosin Mg-ATPase to be activated by actin and favors myosin polymerization into filaments (1, 10, 11). Studies performed in situ suggest that LC20 phosphorylation is required for the initiation of contraction (1, 10, 11). The heavy chains of vertebrate nonmuscle (12–20) and smooth muscle myosin (21) can also be phosphorylated although the biological effects of these phosphorylations are not yet known.

The identification of myosin heavy chain kinases in Acanthamoeba and Dictyostelium and myosin light chain kinases in vertebrate nonmuscle and smooth muscle cells and the localization of their phosphorylation sites have been essential to understanding the effects of phosphorylation on the regulation of myosin activity (1, 22). Likewise, this information would help to elucidate the role of heavy chain phosphorylation in vertebrate nonmuscle and smooth muscle myosin function. Several heavy chain kinases and in some cases the sites of phosphorylation have been identified for vertebrate nonmuscle myosins. A calcium/calmodulin-dependent kinase has been shown to phosphorylate both intestinal brush-border epithelial cell myosin heavy chains (18) as well as the heavy chains of brain myosin (17). Casein kinase II has also been shown to phosphorylate brain myosin heavy chains (14), and recently the site of phosphorylation in brain myosin was identified within an amino acid sequence in the tail region of the molecule (23). The heavy chains of human platelet and rat basophil myosin have been shown to be phosphorylated by protein kinase C in vitro (19) and in intact cells (19, 20), and the site of phosphorylation was localized to a serine residue in the carboxyl-terminal region of the heavy chain (24). To date, the myosin heavy chain kinase(s) catalyzing phosphorylation of the myosin heavy chain in vertebrate smooth muscle cells has not been identified, nor have the sites of heavy chain phosphorylation been determined.

Two isoforms of myosin heavy chains have been identified in smooth muscle cells which differ in their amino acid sequence in the carboxyl-terminal tail regions (25). These

*The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1The abbreviations used are: LC20, 20 kDa myosin light chain; CaM kinase II, calcium/calmodulin-dependent protein kinase II; MOPS, 3(1-N-morpholinol)propanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitriilo)]tetraacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; MHC, and MHCs, 204- and 200-kDa smooth muscle myosin heavy chains, respectively; HPLC, high performance liquid chromatography.

17876
isoforms (204 and 200 kDa) are generated from a single gene through alternative mRNA splicing. The functional significance of these two isoforms is not yet known although their expression appears to be developmentally regulated in the vascular system (26). In this report, we provide the first direct evidence that casein kinase II, a kinase believed to play a role in regulating cellular growth (27), catalyzes the phosphorylation of smooth muscle myosin heavy chains in intact vascular cells. Specifically, casein kinase II phosphorylates the 204-kDa myosin heavy chain but not the 200-kDa heavy chain on a single serine residue in the carboxyl-terminal tail region.

**EXPERIMENTAL PROCEDURES**

**Purification of Myosin** — Smooth muscle myosin was purified from the medial layer of bovine aorta. The tissue was homogenized in wash buffer consisting of 20 mM MOPS (pH 7.0), 60 mM KCl, 4 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride (Sigma), and 50 µg/ml leupeptin (Boehringer Mannheim), sedimented at 38,000 × g for 15 min and the pellet resuspended in wash buffer plus 5 mM ATP (Sigma) for 20 min. Centrifugation was repeated, and the supernatant was made 20 mM with respect to MgSO4, 10 mM with ATP, and 0.5 M with respect to NaCl. A 0-40% ammonium sulfate fractionation was followed by a 40-60% fractionation of the supernatant in precipitation of myosin. The 40-60% ammonium sulfate fraction was dissolved in buffer containing 10 mM MOPS, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 0.1 mM phenylmethylsulfonyl fluoride, 50 µg/ml leupeptin, 1 mM dithiothreitol, and 3 mM NaN3 and dialyzed against the same buffer. The dialyzed was sedimented as described above, resuspended in the same buffer, and applied to a Sepharose CL-4B gel filtration column (5 × 90 cm, Pharmacia LKB Biotechnology Inc.) equilibrated with a buffer consisting of 10 mM MOPS (pH 7.0), 0.5 mM NaCl, 0.1 mM EDTA, and 3 mM NaN3. Immediately before applying the sample to the column, it was made 1 mM with respect to MgCl2 and ATP, and 0.5 M with respect to NaCl. The fractions containing myosin were pooled, dialyzed against the dialysis buffer described above, and stored at 0 °C on nitrogen.

**Phosphorylation and Dephosphorylation of Myosin in Vitro** — Purified serine/threonine kinases were used to phosphorylate myosin in vitro. The catalytic subunit of cAMP-dependent protein kinase and cGMP-dependent protein kinase (purified from bovine heart (28) and bovine lung (29), respectively) were a gift of Dr. M. Elzinga (New York State Institute for Basic Research). Protein kinase C from rat brain (31) was a gift of Dr. J. Sellers (NIH). Protein kinase C from bovine aortic smooth muscle was a gift of Dr. A. Nairn (Rockefeller University). Casein kinase II from bovine aortic smooth muscle was a gift of Dr. J. DiSalvo (University of Minnesota). Bovine brain casein kinase II (purified as described (4)) was a gift of Dr. N. Murakami and Dr. M. Elzeng (New York State Institute for Basic Research). Protein kinase C from rat brain (31) was a gift of Dr. J. Sellers (NIH).

Phosphorylation of myosin by the catalytic subunit of cAMP-dependent protein kinase, cGMP-dependent protein kinase, and casein kinase II was carried out for 30 min at 25 °C with 6.4 µm myosin, 50 µm Tris-HCl (pH 7.5), 40 mM NaCl, 7.5 mM MgCl2, 0.2 mM EGTA, and 0.5 mM ATP ([γ-32P]ATP, 192 Ci/mmol, Du Pont-New England Nuclear). The same conditions were used for protein kinase C and CaM kinase II with the addition of 0.5 mM CaCl2. Calmodulin, purified from bovine brain (32), was also included with CaM kinase II at a concentration of 10 µM, and 25 µg/ml phosphatidyserine (Sigma) and 1 µg/ml 1,3-diolein (Sigma) were added to the protein kinase C assays. The approximate concentrations of kinases used were 50-70 µM.

Dephosphorylation of myosin was carried out for 24 h at 0 °C with bacterial alkaline phosphatase (E. coli, calf, BAPF, Worthington) in 15 mM Tris-HCl (pH 8.0), 10 mM MgCl2, and 0.5 M KCl at a phosphatase:myosin ratio of 1.7 (mg/mg). The phosphatase was subsequently separated from myosin by gel filtration on Sepharose 4B (2.5 × 90 cm). Untreated myosin was prepared in the same manner except that no phosphatase was added.

**Stoichiometry of Myosin Phosphorylation** — The stoichiometry of phosphorylation of myosin heavy and light chains in vitro was determined by spotting aliquots of the phosphorylation reaction mixture onto Whatman No. 3 filter paper discs that were immediately immersed in 10% trichloroacetic acid and 5% sodium pyrophosphate on ice and then washed as described by Corbin and Reimann (33). The total [32P]phosphate content was calculated from the radioscopic activity of [γ-32P]ATP. Additional aliquots were withdrawn at the same time and added to Laemmli sample buffer and boiled. These samples were electrophoresed in SDS-polyacrylamide gels (12.5%), transferred to nitrocellulose and the phosphorylated heavy and light chains were detected by phosphorimaging, cut out of the gel, and the radioactivity was measured by scintillation counting. The proportion of the total [32P]phosphate in the heavy and light chains was then calculated.

**Antibodies** — Anti smooth muscle myosin antibodies were produced in rabbits using purified bovine aortic smooth muscle myosin. The IgG fraction of serum was prepared as a 50% ammonium sulfate fractionation. Antipeptide antibodies were prepared in the same manner. No cross-reactivity of these antibodies was detected by immunoblotting of purified smooth muscle and platelet myosin at the dilutions used.

**Cell Culture and Immunoprecipitation** — Bovine aortic smooth muscle cells were derived from medial explant outgrowths of bovine aortas according to the method of Ross (34) and grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. Bovine retinal pericytes were isolated and cultured as described previously (35). All experiments were performed with first passage cells grown in 35-mm tissue culture dishes upon reaching confluence (basal conditions). Cells were labeled metabolically with [3H]thymidine (0.2-0.5 µCi/ml, Du Pont-New England Nuclear) in phosphate-free Dulbecco's modified Eagle's medium for 4 h. The labeling medium was removed, and the cells were quickly frozen in liquid nitrogen. Preparation of cell extracts and immunoprecipitation of smooth muscle myosin were carried out as described previously (21).

The immunoprecipitated myosin was boiled in Laemmli sample buffer, and the subunits were separated in SDS-polyacrylamide gels (12.5% or 5%). The labeled proteins were identified by autoradiography of dried gels.

**SDS-Polyacrylamide Gel Electrophoresis** — Gel electrophoresis was performed in either SDS-polyacrylamide gels (12.5%) with 0.13% cetylpyridinium bromide or SDS-polyacrylamide gels (5%) with 0.065% cetylpyridinium bromide, using the buffer system of Laemmli (36). Quantitation of protein and radioactivity in the gels, unless otherwise noted, were by scanning densitometry using a Pharmacia LKB Biotechnology Inc. Ultrascan XL laser densitometer. In experiments in which [32P]-labeled myosin heavy chain peptides were prepared for purification and sequencing, gels were not stained, and phosphorylated heavy chains were localized by autoradiography of SDS-polyacrylamide gels (5%).

**Peptide Mapping** — Tryptic digestion of myosin heavy chains and light chains in the polyacrylamide gels and two-dimensional mapping of the tryptic phosphopeptides were carried out as described previously (90). Phosphorylated peptides were localized by autoradiography.

**Phosphoamino Acid Analysis** — Radioactive phosphopeptides from the two-dimensional mapping plates were hydrolyzed in 6 N HCl for 3 h at 106 °C. The acid-hydrolyzed peptides were electrophoresed at 1000 V for 3 h in acetic acid/formic acid/water, 78:25:897, pH 1.9. The [32P]-labeled phosphoamino acids were identified by autoradiography, and their migration was compared with that of standards of phosphoserine, phosphothreonine, and phosphoserine (Sigma) stained with ninhydrin.

**Purification of Tryptic Phosphopeptides** — Bovine aortic smooth muscle myosin (2.5 mg) was phosphorylated by casein kinase II as described above. Heavy chains were separated from light chains by a cDNase II phase in SDS-polyacrylamide gels (6%) and digested with trypsin as described for the two-dimensional peptide mapping experiments (20). The tryptic digests were lyophilized, dissolved in 0.1 M acetic acid, and applied to an Fe3+-iminodiacetic acid-Sepharose affinity chromatography column (1 × 5 cm, Pharmacia) (37). Unphosphorylated peptides were eluted from the column by sequential washing in 0.1 M acetic acid, 0.1 M acetic acid/NaOH, pH 5.0, and 0.7 M sodium acetate. The phosphorylated peptide bands were subsequently eluted with 1% ammonium acetate adjusted to pH 8.3 with NH3. The column was run at a flow rate of 0.5 ml/min, and 1.0-ml fractions were collected. The fractions were analyzed for peptides by A280 and radioactivity by Cerenkov counting. Radioactive fractions were pooled, lyophilized, and resuspended in 0.1% trifluoroacetic acid in water. The sample was spotted onto a C18 phase HPLC column (Waters Associates) in a mobile phase of 10% acetonitrile (Fisher) and 0.1% trifluoroacetic acid (Fisher) in water. Peptides were eluted with a linear gradient of 10-0% (v/v) acetonitrile, 0.1% trifluoroacetic acid in water developed over 60 min at 1.0 ml/min. One-min fractions were collected, and radioactive peptides were identified by autoradiography of dried gels.
were identified by Cerenkov counting. The major radioactive peaks were pooled separately.

**Amino Acid Analysis and Sequence Determination**—Amino acid analysis of the isolated phosphopeptide fractions was carried out using a Hewlett-Packard Amino-Quant system after hydrolysis of aliquots by gaseous HCl; sequences were determined in an Applied Biosystems 470A instrument equipped with an on-line 120A HPLC.

**RESULTS**

**Phosphorylation of Smooth Muscle Myosin Heavy Chains in Cultured Bovine Aortic Cells**—Using polyclonal antibodies prepared against bovine aortic smooth muscle myosin, we immunoprecipitated myosin from first passage cultures of bovine aortic smooth muscle cells labeled metabolically with [32P]orthophosphate. Following SDS-PAGE (12.5% gel) and autoradiography, we observed that 32P had been incorporated into both the myosin heavy chain and the 20-kDa light chain (Fig. 1B, lane 1A), in agreement with a previous report using rat aorta (21). Closer analysis of the myosin heavy chain immunoprecipitate on SDS-polyacrylamide gels (5%) revealed one major and one minor myosin heavy chain band by Coomassie Blue staining (Fig. 1A, lane 2). The major band was identified as smooth muscle myosin by immunoblotting using antibodies specific for smooth muscle and nonmuscle myosin (results not shown). We further identified the major band as the higher molecular weight isofrom of the smooth muscle myosin heavy chain (MHC), based on its mobility in SDS-polyacrylamide gels (5%) compared with purified aortic smooth muscle myosin (see below, Fig. 4, lane 2). The autoradiogram of the SDS-polyacrylamide gel (5%) of myosin heavy chains from cultured aortic cells (Fig. 1B, lane 2A) showed incorporation of 32P into the smooth muscle MHC.

The minor band just below the smooth muscle MHC band, seen on Coomassie Blue-stained gels and autoradiography (Fig. 1A, lane 2, and 1B, lane 2A, respectively), was identified as a nonmuscle myosin heavy chain by immunoblotting (results not shown). This nonmuscle myosin heavy chain was not present in immunoprecipitates if ATP was included in the immunoprecipitation buffer, indicating that it is co-precipitated and not immunoprecipitated.

**Phosphorylation of Smooth Muscle Myosin Heavy Chains in Vitro**—Having established that the smooth muscle myosin heavy chain was phosphorylated in cultured bovine aortic smooth muscle cells, we next examined the ability of a variety of purified kinases to catalyze the phosphorylation of bovine aortic smooth muscle myosin in vitro. Myosin was incubated with kinase either after treatment with bacterial alkaline phosphatase or without prior phosphatase treatment (Table I, dephosphorylated and untreated myosin, respectively). Of the several enzymes tested, only protein kinase C, CaM kinase II, and casein kinase II were able to phosphorylate the heavy chains of untreated myosin (Table I). cAMP- and cGMP-dependent protein kinase did not catalyze the phosphorylation of myosin heavy chains or light chains. When myosin was treated with bacterial alkaline phosphatase prior to kinase addition, a 3-fold increase in phosphorylation of the heavy chain by casein kinase II to 0.6 mol/mol and a 2-fold increase in phosphorylation by CaM kinase II to 0.6 mol/mol was observed. The stoichiometry of phosphorylation with protein kinase C did not increase with dephosphorylated myosin as the substrate. These results suggest that myosin is already phosphorylated by casein kinase II or CaM kinase II in intact tissue or that it becomes phosphorylated during the purification process.

Phosphorylation of myosin by casein kinase II was specific for the heavy chain whereas CaM kinase II and protein kinase C also phosphorylated the LC20 subunit (Table I). The protein kinase C phosphorylation sites on the 20-kDa light chains have been identified as serine 1 and 2 and threonine 9 (38, 39). Two-dimensional tryptic peptide mapping revealed that CaM kinase II phosphorylates LC20 on serine 19, one of the sites phosphorylated by myosin light chain kinase.

**Identification of the Smooth Muscle Myosin Heavy Chain Kinase in Cultured Cells**—We next determined whether the region of the smooth muscle myosin heavy chain that was phosphorylated in cultured cells corresponded to that phosphorylated in vitro by either protein kinase C, CaM kinase II, or casein kinase II. Two-dimensional tryptic peptide maps of myosin heavy chains phosphorylated either in cultured cells or in vitro by protein kinase C, CaM kinase II, or casein kinase II were compared. One major and one minor phosphopeptide were generated following tryptic digestion of 32P-labeled smooth muscle myosin heavy chains immunoprecipitated from cultured aortic smooth muscle cells (Fig. 2, panel 1, A and B, respectively). The same two phosphopeptides were

---

**TABLE I**

| Kinase          | Untreated myosin | Dephosphorylated myosin |
|-----------------|------------------|-------------------------|
|                 | Heavy chain      | Light chain             |
| Casein kinase II| 0.2              | 0                       |
| CaM kinase II   | 0.3              | 0.6                     |
| Protein kinase C| 0.2              | 1.0                     |

---

2 C. A. Kelley, unpublished results.
Myosin Heavy Chain Phosphorylation

Cultured Cells In Vitro, Casein Kinase II Co-migration

Fig. 2. Autoradiograms of two-dimensional tryptic phosphopeptide maps of myosin heavy chains. The 204-kDa heavy chains of smooth muscle myosin immunoprecipitated from cultured cells labeled with \[^32P\]orthophosphate or purified myosin phosphorylated with casein kinase II \textit{in vitro} were separated by SDS-PAGE (5% gel) and digested with trypsin as described under “Experimental Procedures.” The resulting tryptic peptides were separated by two-dimensional thin layer electrophoresis and chromatography. The phosphopeptides were located by autoradiography. Panel 1, phosphopeptides of heavy chains from intact cells; panel 2, phosphopeptides of heavy chains phosphorylated \textit{in vitro} with casein kinase II; panel 3, co-mapping of 1 and 2 above. The origin is marked with an X. The letters indicate the common phosphopeptides. The directions of electrophoresis and chromatography are marked by the arrows.

Identified on two-dimensional maps of tryptic digests from smooth muscle myosin heavy chains phosphorylated with casein kinase II \textit{in vitro} (Fig. 2, panel 2). We confirmed that the peptides were the same by co-mapping the tryptic digests of myosin heavy chains phosphorylated with casein kinase II \textit{in vitro} and those phosphorylated in cultured cells (Fig. 2, panel 3). Of note, two-dimensional tryptic phosphopeptide maps of \[^32P\]-labeled smooth muscle myosin heavy chains immunoprecipitated from cultured bovine retinal pericytes were identical to those in Fig. 2 (results not shown). These results suggest that casein kinase II phosphorylation of smooth muscle myosin heavy chains is not limited to bovine aortic smooth muscle cells.

Two-dimensional tryptic phosphopeptide maps of myosin heavy chains phosphorylated with protein kinase C revealed numerous (>10) phosphopeptides (results not shown). Because the stoichiometry of phosphorylation of both untreated and dephosphorylated myosin was low, and the phosphate was distributed among several sites not phosphorylated in intact cells, we conclude that protein kinase C is not responsible for the myosin heavy chain phosphorylation observed in cultured cells. The peptide maps of myosin heavy chains phosphorylated with CaM kinase II contained one major and three minor phosphopeptides. The pattern, however, was completely different from that obtained with heavy chains from cultured cells (results not shown).

Phosphoamino Acid Analysis of Myosin Heavy Chains—The phosphoamino acid content of the common peptides from myosin heavy chains phosphorylated in cultured cells or \textit{in vitro} with casein kinase II was determined. Fig. 3 shows the results from the \textit{in vitro} phosphorylation with casein kinase II and establishes that both peptides contain phosphoserine with no detectable amounts of either phosphothreonine or phosphotyrosine. Identical results (not shown) were obtained with phosphopeptides from myosin heavy chains labeled in cultured cells.

Localization of the Casein Kinase II Phosphorylation Site to the MHC, Isoform—In order to determine whether both isoforms of the myosin heavy chain were phosphorylated by casein kinase II, purified bovine aortic smooth muscle myosin was phosphorylated by casein kinase II and analyzed by SDS-PAGE (5% gel). The results confirmed that the bovine aortic smooth muscle myosin heavy chain consists of two isoforms (204 and 200 kDa, designated MHC, and MHC\textsubscript{2}, respectively; Fig. 4A, lane 2). Both bands were identified as smooth muscle.
myosin by immunobLOTS using anti-smooth muscle myosin antibodies and by lack of cross-reactivity with antiplatelet myosin antibodies. By densitometry, we estimated that the relative abundance of the isoforms was equal although in cultured cells only MHC1 is present (see Fig. 1). The distribution of 32P in the two isoforms is shown in Fig. 4B, lane 2A. Densitometry of these bands showed that of the total 32P incorporated into heavy chains, greater than 85% was incorporated into MHC1.

Identification of the Casein Kinase II Phosphorylation Site—Tryptic peptides of smooth muscle myosin heavy chains phosphorylated in vitro with casein kinase II were separated by Fe3+-iminodiacetic-Sepharose affinity column chromatography (Fig. 5). The tryptic digest was applied to the column in 0.1 M acetic acid, pH 2.0, unphosphorylated peptides were eluted by stepwise washing at acidic pH, and phosphorylated peptides were eluted at pH 8.3. Fractions 44–54 (77% of the total radioactivity applied) were pooled and applied to reverse phase HPLC. Four radioactive peaks were eluted (Fig. 6, A, B, C, and D) with an overall 32P recovery of 86%. Pooled fractions from the peaks were analyzed for amino acid composition and amino acid sequence. Peaks A and D had no absorption at 220 nm, contained no interpretable amino acid sequence, and therefore were not examined further. Peaks B and C, which were combined, gave the results presented in Table II. The sequence through the first 12 steps was Val-Ile-Glu-Glu-Ala-Asp-Gly-Ser-Glu-Glu-Glu-Val. The amino acid composition of this fraction was consistent with this sequence plus the amino acids Asp-Ala-Arg.

To determine the exact location of this phosphopeptide in the smooth muscle MHC1, we compared the sequence with the published carboxyl-terminal sequence of the MHC1 and MHC2 isoforms of rabbit uterine smooth muscle myosin (25). We recognized our peptide sequence within a predicted tryptic peptide of rabbit MHC1, as shown between the vertical arrows in Fig. 7. This sequence is not present in rabbit MHC2. We demonstrated above (Fig. 3) that casein kinase II phosphorylates a serine residue in the smooth muscle MHC1. We demonstrated above (Fig. 3) that casein kinase II phosphorylates a serine residue in the smooth muscle MHC1. We demonstrated above (Fig. 3) that casein kinase II phosphorylates a serine residue in the smooth muscle MHC1. We demonstrated above (Fig. 3) that casein kinase II phosphorylates a serine residue in the smooth muscle MHC1. Since the tryptic phosphopeptide we isolated as well as the corresponding tryptic peptide shown in Fig. 7 contain a single serine residue, we conclude that this is the location of the serine residue phosphorylated by casein kinase II in the MHC1 isoform of bovine aortic smooth muscle myosin.

DISCUSSION

In the present study we found that casein kinase II, CaM kinase II, and protein kinase C catalyzed the phosphorylation of smooth muscle myosin heavy chains in vitro although the stoichiometry of phosphorylation with protein kinase C was...
further that the MHCi isoform contains 43 amino acids after represent smooth muscle myosin heavy chain isoforms that Nagai et al. (25) presented evidence that the two bands that the site of casein kinase II phosphorylation on the MHC, in the carboxyl-terminal tail region. In this report we demonstrated that MHCI, and could occur in response to increased cytosolic calcium. Several investigators have reported two smooth muscle myosin heavy chain phosphorylations in cultured smooth muscle cells which were identical to tryptic maps of myosin heavy chains phosphorylated with casein kinase II in vitro and were different from those phosphorylated by CaM kinase II and protein kinase C in vitro. We conclude, therefore, that casein kinase II is probably solely responsible for the basal phosphorylation of smooth muscle myosin heavy chains in cultured cells. We cannot, of course, exclude the possibility that another kinase with substrate specificity similar to casein kinase II catalyzes the observed phosphorylation.

Although basal phosphorylation of smooth muscle myosin heavy chains in cultured cells appears to be due to casein kinase II activity, treatment of smooth muscle cells with various agonists may result in stimulation of other kinases that phosphorylate myosin heavy chains. Kamm et al. (40) reported that myosin heavy chains in cultured tracheal smooth muscle cells were phosphorylated under basal conditions and that the phosphorylation increased after treatment of the cells with a calcium ionophore. Our in vitro results suggest that heavy chain phosphorylation by CaM kinase II could occur in response to increased cytosolic calcium.

Several investigators have reported two smooth muscle myosin heavy chain bands on low percentage polyacrylamide gels, referred to here as MHCi and MHC2 (41-43). Recently, Nagai et al. (25) presented evidence that the bands are generated by alternative mRNA splicing. They showed that the nonmuscle myosin heavy chain peptide phosphorylated by casein kinase II and the amino acid sequence surrounding the site phosphorylated by casein kinase II in vitro and were different from those phosphorylated by CaM kinase II and protein kinase C in vitro. We conclude, therefore, that casein kinase II is probably solely responsible for the basal phosphorylation of smooth muscle myosin heavy chains in cultured cells. We cannot, of course, exclude the possibility that another kinase with substrate specificity similar to casein kinase II catalyzes the observed phosphorylation.

Although basal phosphorylation of smooth muscle myosin heavy chains in cultured cells appears to be due to casein kinase II activity, treatment of smooth muscle cells with various agonists may result in stimulation of other kinases which phosphorylate myosin heavy chains. Kamm et al. (40) reported that myosin heavy chains in cultured tracheal smooth muscle cells were phosphorylated under basal conditions and that the phosphorylation increased after treatment of the cells with a calcium ionophore. Our in vitro results suggest that heavy chain phosphorylation by CaM kinase II could occur in response to increased cytosolic calcium.

Several investigators have reported two smooth muscle myosin heavy chain bands on low percentage polyacrylamide gels, referred to here as MHCi and MHC2 (41-43). Recently, Nagai et al. (25) presented evidence that the bands are generated by alternative mRNA splicing. They showed that the MHC2 isoform contains 43 amino acids after the point of divergence from MHCi in the carboxyl-terminal tail region. In this report we demonstrated that MHCi, and not MHC2, was phosphorylated by casein kinase II, suggesting that the site of casein kinase II phosphorylation on the MHC2 isoform was located carboxyl-terminal to its divergence from MHCi. We purified and sequenced the smooth muscle myosin heavy chain peptide phosphorylated by casein kinase II and obtained the sequence Val-Ile-Glu-Asn-Ala-Asp-Gly-Ser*, Glu-Glu-Glu-Val, where Ser* denotes the phosphorylated residue. Sequences containing serine or threonine located within clusters of acidic residues and a critical acidic residue at each tryptic peptide, VIENAD SEEEVDP, in MHCt of bovine aortic smooth muscle is identical with an expected tryptic peptide, delineated by the arrows, in the carboxyl-terminal tail of rabbit uterine smooth muscle MHCt (25). This region is absent in rabbit uterine MHCt. Casein kinase II phosphorylated the serine marked with an asterisk in the bovine aortic sequence. The amino acid sequence and casein kinase II phosphorylation sites of this tryptic peptide are also highly conserved in rat aortic MHCt and are absent from rat aortic MHC2 (46). The casein kinase II phosphorylation site and surrounding consensus sequence required for phosphorylation (boxed bold letters) are also conserved among nonmuscle myosins. The human macrophage myosin sequence is from Ref. 47. The chicken epithelial myosin sequence is from Ref. 48. The bovine brain myosin sequence is from Ref. 23.

We looked for conservation of the casein kinase II phosphorylation site in smooth muscle and nonmuscle myosins. Fig. 7 compares the carboxyl-terminal amino acid sequence of the smooth muscle myosin heavy chains for rabbit uterine MHCt and MHC2 (25), rat aortic MHCt and MHC2 (46), and the nonmuscle myosin heavy chains of human macrophage myosin (47), chicken intestinal epithelial cell myosin (48), and the amino acid sequence surrounding the site phosphorylated by casein kinase II in bovine brain myosin (23). The 6 amino acids shown in enlarged print within the box are exceptional in being highly conserved among smooth muscle and nonmuscle myosins. The work reported herein as well as previous work reported by others (23) indicate that the serine residues, shown within the box, in both the bovine aortic smooth muscle MHCt and the brain myosin heavy chain are substrates for casein kinase II. The boxed acidic amino acids carboxyl-terminal to the phosphorylated serines constitute a conserved consensus sequence for casein kinase II phosphorylation. We suspect that the analogous serines in the rat aortic smooth muscle MHCt, the human macrophage myosin, and the chicken intestinal epithelial cell myosin will also serve as substrates for this enzyme. It is likely that additional smooth muscle MHCt and nonmuscle myosin heavy chain isoforms from a variety of species and tissues will contain this conserved casein kinase II phosphorylation site. However, it is noteworthy that in the embryonic chicken gizzard amino acid sequence (not shown), the phosphorylatable serine is replaced by a glycine (49).

The functional consequences of heavy chain phosphorylation by casein kinase II are not yet known. The conservation...
of amino acid sequence in an otherwise divergent region suggests that casein kinase II phosphorylation is important in myosin function and that smooth muscle MHC1, and not MHC2 implies that these isoforms are regulated differently. This area of sequence similarity is within a region of myosin which is required for assembly into filaments (50-53), and phosphorylation of some myosin heavy chain phosphorylation is high in cultured (see Fig. 1) (41, 42). Previous work has demonstrated that muscle myosin may be regulated similarly. Moreover, the myosin isoforms in this region has been shown to interfere with filament formation (6, 8). There is to date no evidence in myosin function and that smooth muscle MHCl and non-

MHC phosphorylation by casein kinase II may be important in smooth muscle cell proliferation. Casein kinase II phosphorylates a number of substrates that are critical in regulating cellular proliferation (44, 45), and its activity may

Acknowledgments—We wish to thank Dr. Marshall Elzinga for helpful advice, Dr. Peter McPhie for assistance in the HPLC purification, Dr. Donald Bottaro for critical reading of the manuscript, and Catherine Magruder for assistance in the typing of the manuscript.

REFERENCES
1. Hartshorne, D. J. (1987) in Physiology of the Gastrointestinal Tract (Johnson, L. R., ed) Vol. 2, pp. 423-442, Raven Press, New York
2. Clarke, M., and Spudich, J. A. (1977) Annu. Rev. Biochem. 46, 797-822
3. Fujinaga, R., and Pollard, T. D. (1970) J. Cell Biol. 47, 729-7296
4. Fukui, Y., De Lozanne, A., and Spudich, J. A. (1990) J. Biol. Chem. 265, 264-268
5. Gauthier, P. G., and Bukiejko, U. (1987) J. Biol. Chem. 262, 1065-1072
6. Kuczmarski, E. R., and Spudich, J. A. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 920-9206
7. Conti, M. A., Elzinga, M., McPhie, P., Sellers, J. R., and Adelstein, R. S. (1990) J. Biol. Chem. 265, 3950-3955
8. Corbin, J. D., and Reimann, E. M. (1975) Methods Enzymol. 38, 287-290
9. Klee, C. B. (1977) Biochemistry 16, 1017-1024
10. Laemmli, U. K. (1970) Nature 227, 680-685
11. Karm, J. P., Swanljung-Collins, H., and Collins, J. H. (1987) J. Biol. Chem. 262, 1099-1102
12. Kawamoto, S., Bengur, A. R., Sellers, J. R., and Adelstein, R. S. (1989) J. Biol. Chem. 264, 2258-2265
13. Kamm, K. E., Hsu, L.-C., Kubota, Y., and Stull, J. T. (1989) J. Biol. Chem. 264, 21223-21229
14. Kamm, K. E., and Stull, J. T. (1989) Annu. Rev. Physiol. 51, 299-313
15. Feichheimer, M., and Cebra, J. J. (1982) J. Cell Biol. 93, 264-270
16. Bengur, A. R., Robinson, E. A., Appella, E., and Sellers, J. R. (1987) J. Biol. Chem. 262, 7613-7617
17. Kamm, K. E., and Stull, J. T. (1989) Annu. Rev. Physiol. 51, 299-313
18. Rieker, J. P., Swanljung-Collins, H., and Collins, J. H. (1987) J. Biol. Chem. 262, 9136-9140
19. Waterston, R. H. (1985) J. Mol. Biol. 183, 543-551
20. Carroll, D., and Marshak, D. R. (1989) J. Biol. Chem. 264, 21223-21229
21. Kamm, K. E., Hsu, L.-C., Kubota, Y., and Stull, J. T. (1989) J. Biol. Chem. 264, 21223-21229
22. Rieker, J. P., Swanljung-Collins, H., and Collins, J. H. (1987) J. Biol. Chem. 262, 9136-9140
23. Bengur, A. R., Robinson, E. A., Appella, E., and Sellers, J. R. (1987) J. Biol. Chem. 262, 7613-7617
24. Kamm, K. E., and Stull, J. T. (1989) Annu. Rev. Physiol. 51, 299-313
25. Feichheimer, M., and Cebra, J. J. (1982) J. Cell Biol. 93, 264-270
26. Bengur, A. R., Robinson, E. A., Appella, E., and Sellers, J. R. (1987) J. Biol. Chem. 262, 7613-7617
27. Kamm, K. E., Hsu, L.-C., Kubota, Y., and Stull, J. T. (1989) J. Biol. Chem. 264, 21223-21229
28. Rieker, J. P., Swanljung-Collins, H., and Collins, J. H. (1987) J. Biol. Chem. 262, 9136-9140
29. Bengur, A. R., Robinson, E. A., Appella, E., and Sellers, J. R. (1987) J. Biol. Chem. 262, 7613-7617
30. McCuinness, T. L., Lai, Y., and Greengard, P. (1985) J. Biol. Chem. 260, 367-378
31. Huang, K.-P., Ghan, K.-F. J., Singh, T. J., Nakabayashi, H., and Huang, F. L. (1986) J. Biol. Chem. 261, 12134-12140
The 204-kDa smooth muscle myosin heavy chain is phosphorylated in intact cells by casein kinase II on a serine near the carboxyl terminus.

C A Kelley and R S Adelstein

J. Biol. Chem. 1990, 265:17876-17882.

Access the most updated version of this article at http://www.jbc.org/content/265/29/17876

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC’s e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/265/29/17876.full.html#ref-list-1