Phosphorylation of the Large Subunit of Myosin Phosphatase and Inhibition of Phosphatase Activity*

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The partially purified myosin-bound phosphatase had an associated protein kinase that phosphorylated the holoenzyme, primarily on the large (130-kDa) subunit. Phosphorylation of the 130-kDa subunit resulted in inhibition of phosphatase activity. The major site of phosphorylation was threonine 654 of the 130-kDa subunit or threonine 695 of the 133-kDa isoform. Phosphorylation of the large subunit did not dissociate the holoenzyme. Dephosphorylation of the large subunit was achieved by the holoenzyme, and addition of the catalytic subunit of the type 2A enzyme did not increase the rate of dephosphorylation. The associated kinase was inhibited by the type 2A enzyme did not increase the rate of dephosphorylation. The associated kinase was inhibited by chelerythrine, with half-maximal inhibition at approximately 5 μM (in 150 μM ATP). The associated kinase phosphorylated two synthetic peptides, one corresponding to the sequence flanking the phosphorylated threonine, i.e. 648–661 of the 130-kDa subunit, and the other to a known protein kinase C substrate, i.e. a modified sequence from the autoinhibitory region of ε protein kinase C. The associated kinase was activated by arachidonic and oleic acid and to a lesser extent by myristic acid. The protein kinase that phosphorylated the 130-kDa subunit and resulted in inhibition of myosin phosphatase activity was not identified.

Contraction of smooth muscle involves the phosphorylation of myosin by myosin light chain kinase. The Ca²⁺ dependence of this system derives from the activation of myosin light chain kinase by the Ca²⁺-calmodulin complex (1). Relaxation, following a reduction in the intracellular Ca²⁺ concentration, involves dephosphorylation of LC20 by the myosin light chain phosphatase.

In the simplest model a fixed relationship should exist among the Ca²⁺ concentration, myosin phosphorylation, and force. However, such was not observed in many studies carried out with intact or skinned smooth muscle preparations (2), and the need for an additional component was raised. This could involve independent mechanisms (3) or alteration of the Ca²⁺ dependence of phosphorylation (4). Use of Ca²⁺ indicators showed that the Ca²⁺ dependence of force could vary under different conditions. Usually higher force was achieved following agonist stimulation, compared with K⁺ depolarization (5–8). Force could also decrease at constant Ca²⁺ levels (9, 10). Subsequently it was shown that the changes in force reflected parallel changes in myosin phosphorylation, and thus the balance of myosin light chain kinase and phosphatase was altered (11–13).

One mechanism by which this can occur is by inhibition of phosphatase activity. This would increase myosin phosphorylation at a given Ca²⁺ concentration and lead to an increased Ca²⁺ sensitivity. Several laboratories made the observation that GTPγS increased the Ca²⁺ sensitivity of contraction, thus implicating a G protein-linked mechanism (8, 14, 15). Kitazawa et al. (12) suggested that this mechanism resulted in inhibition of phosphatase activity, and Kubota et al. (16) showed that in homogenates of tracheal muscle GTPγS inhibited the dephosphorylation of heavy meromyosin. The pathway that leads from receptor to G proteins to the ultimate inhibition of phosphatase is not established. Arachidonic acid has been suggested as a possible messenger (11). This compound dissociates the trimERIC structure of the phosphatase, leading to inhibition (11) and also is released in smooth muscle at concentrations and at time courses consistent with a physiological role (17). Masuo et al. (18) have suggested that the inhibition of phosphatase activity is linked to activation of PKC.¹

There are many reports of phosphatases that can dephosphorylate smooth muscle myosin (reviewed in Ref. 19), and clearly there is the possibility that more than one phosphatase is involved physiologically. In order to restrict the number of possibilities the assumption was made that the pertinent phosphatase should bind to myosin. Using myosin or actomyosin, as a source of the phosphatase, three laboratories obtained similar preparations; namely, the myosin phosphatase was composed of 3 subunits, 130, 38, and 20 kDa (20–22). The 38-kDa subunit is the PP1β isoform (also referred to as PP1γ (20)) of the catalytic subunit (21, 23) and the two other subunits are putative regulatory or target molecules. Because the trimeric holoenzyme is thought to bind to myosin (20–22) it is referred to as the myosin-bound phosphatase (MBP). An important objective is to determine the function of each subunit and its interactions within the holoenzyme. Amino sequences have been derived from cloned cDNAs of each subunit: the 38-kDa subunit (24); the gizzard 130-kDa subunit (21), the rat 110-kDa subunit (25), and the 20-kDa subunit (25). In gizzard, two isoforms of the large subunit exist, i.e. 130 and 133 kDa, differing by an insert in the central part of the molecule, residues

¹ The abbreviations used are: PKC, protein kinase C; LC20, 20,000-dalton myosin light chain; ³²P-LC20, LC20 phosphorylated using γ-³²P-labeled ATP; ³²P-myosin, phosphorylated myosin; PP1, protein phosphatase type 1; PP1c, catalytic subunit of PP1; PP1β, the δ isofom of PP1c; PP2A, protein phosphatase type 2A; MBP, myosin-bound phosphatase; PAGE, polyacrylamide gel electrophoresis; CAPS, 3-(cyclohexylamino)propanesulfonic acid; ATPγS, adenosine 5'-O-(thiotriphosphate); HPLC, high performance liquid chromatography.
512–552 (21). The 130-kDa subunit is the major isoform. Recently it was shown that treatment of α-toxin-permeabili-
zed portal vein with ATP resulted in increased Ca²⁺ sens-
sitivity of force output and reduced phosphorylation activity (26). This was achieved under conditions where LC20 thiophospho-
rylation by myosin light chain kinase was minimal. However, a number of high molecular weight proteins were thiphospho-
rylated, and among these was the large phosphatase subunit, equivalent to the gizzard 130-kDa subunit (26). Thus, the pos-
sibility was raised that phosphorylation of the 130-kDa subunit may be a regulatory mechanism for phosphatase activity. To investigate this further we examined the isolated gizzard phosphatase and found that a copurifying kinase phosphorylated the 130-kDa subunit and inhibited phosphorylated activity. These results are presented below.

**EXPERIMENTAL PROCEDURES**

Materials—Chemicals and suppliers were as follows: ATP-γS (Boe-
hringer Mannheim); [γ-32P]ATP and [γ-32P]ATP (DuPont NEN); chel-
erythrine chloride (Calbiochem); cAMP-dependent protein kinase inhib-
itor (PB140); ML9, H7, H8, calphostin C, arachidonic acid, oleic acid, myristic acid, phorbol-12-myristate-13-acetate, and L-
ophore (Sigma); microcystin-LR (LC Laboratories); and okadaic acid (Life Technologies, Inc.). The PKC peptide ε was kindly provided by Dr. M. P. Walsh (University of Calgary, Canada). The peptide 648–661 of the 130-kDa subunit (identical with 689–702 of the 130-kDa subunit) was synthesized by Macromolecular Resources (Colorado State University).

Protein Preparations—The smooth muscle MBP was purified from frozen turkey and fresh chicken gizzards (21). The preparation used for these studies was taken after the Mono S HR 5/5 column. Other proce-
dures were as follows: smooth muscle myosin from turkey gizzard (27); myosin light chains from gizzard myosin (28); 32P-labeled LC20 (29); myosin light chain kinase from frozen turkey gizzard (30); calmodulin from turkey gizzard; catalytic subunit of cAMP-dependent protein kinase from bovine heart (31); and the catalytic subunit of cAMP-dependent protein kinase from bovine serum albumin (32). The monoclonal antibody to the 130- and 133-kDa subunits was prepared as described earlier (33). Ascites fluid was isolated by conventional techniques. The monoclonal antibody was purified by DEAE Affi-Gel Blue and coupled to Affi-Gel 10 (Bio-Rad) following the manufacturer’s protocol. MBP, thiphosphory-
lated and nonphosphorylated (5 μg each), in 10 mM Tris-HCl (pH 7.5) was applied to the column, and after washing with 10 mM Tris-HCl (pH 7.5) the bound protein was eluted with 0.1 M glycine-HCl (pH 2.5). The eluted fractions were applied to SDS-PAGE and Western blot analysis using antibodies to the 58-kDa N-terminal fragment of the 130-kDa subunit and to PN1. The polyclonal antibody to PN1 (34) was kindly donated by Dr. L. A. Engvall (National Cancer Center Research Institute, Toky-

Phosphatase Assays—These were carried out at 30°C using [γ-
32P]ATP-γS (25) from rabbit skeletal muscle was treated dif-
ferently (28); 32P-labeled LC20 (29); ATP in the presence of ATP in solvent A. The 32P-
labeled LC20 at 5 μM and myosin (at 1 μM) as substrates (21). Assay con-
ditions were as follows: 50 mM Tris-HCl (pH 7.5), 0.1 mM EDTA, and 0.3 mM CoCl₂, unless otherwise indicated. The reactions were initiated by the addition of substrate and terminated by the addition of trichloro-
acetic acid and hydrolyzed in 6 N HCl for 3 h at 110°C. The hydrolysate was analyzed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5 (36). Positions of phosphoamino acids were established using phos-
phoethanolamine, and phosphotyrosine standards.

Other Procedures—Mapping of the [γ-32P]ATP label was collected, lyophilized, and reapplied to the C-18 column and eluted by a 5–20% acetone, 0.1% trifluoro-
acetic acid linear gradient. The major peak of radioactivity, at approximately 15% acetone, was collected and lyophilized, and its sequence was determined using a gas-phase sequencer (Applied Biosystems model 477A).

Identification of Phosphoamino Acids—The 32P-labeled 130-
and 20-kDa subunits were eluted from the polyvinylidene difluoride membrane and hydrolyzed in 6 N HCl for 3 h at 110°C. The hydrolysate was analyzed by two-dimensional thin-layer electrophoresis at pH 1.9 and 3.5 (36). Positions of phosphoamino acids were established using phos-
phoethanolamine, and phosphotyrosine standards.

**RESULTS**

Phosphorylation of MBP—The MBP holoenzyme was incubated with [γ-32P]ATP in solvent A. The 32P-
labeled MBP was applied to SDS-PAGE and transferred to polyvinyl-
diene difluoride membrane (Bio-Rad) in 10 mM CAPS-NaOH (pH 11.0) and 10% methanol (v/v). The area corresponding to the 130-kDa subunit was cut out and exposed for 24 h at 37°C to 1% α-chymotrypsin in 50 mM NH₄HCO₃-NaOH (pH 8.4) and 8% acetic acid. The solvent was lyophilized, dissolved in 0.1% trifluoroacetic acid, and applied to HPLC on a Spheri-5 RP-18 column (Brownlee Laboratories). The flow rate was 0.5 ml/min. Elution of the 32P-labeled peptides was achieved by a 0–30% acetonitrile, 0.1% trifluoroacetic acid gradient. The major peak (with respect to the 32P label) was collected, lyophilized, and reapplied to the C-18 column and eluted by a 5–20% acetone, 0.1% trifluoro-
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course progressed. Phosphorylation of the 38-kDa catalytic subunit was not found.

Brief digestion of MBP with α-chymotrypsin cleaved the 130-kDa subunit into a C-terminal fragment of 72 kDa and an N-terminal fragment of 58–60 kDa (33). Both fragments were phosphorylated (Fig. 1). Further digestion resulted in an accumulation of \(^{32}\)P-labeled lower molecular weight components.

The \(^{32}\)P-labeled 130- and 20-kDa subunits were eluted from gel slices, and their phosphoamino acid content was determined. As shown in Fig. 2 the 130-kDa subunit contained both phosphoserine and phosphothreonine. The 20-kDa subunit contained only phosphoserine (data not shown). Phosphotyrosine was not detected. The C-terminal and N-terminal α-chymotryptic fragments of the 130-kDa subunit also were analyzed. The 58-kDa N-terminal part of the molecule contained phosphoserine, and the C-terminal fragment contained phosphothreonine.

The time course and stoichiometry of phosphorylation for MBP and its subunits is shown in Fig. 3. For the holoenzyme, a relatively rapid phosphorylation to a level of 1 mol of phosphate/mol of MBP and subjected to proteolysis by α-chymotrypsin. The generated peptides were applied to HPLC, and the \(^{32}\)P content was determined for the eluted peptides (see “Experimental Procedures”). Four \(^{32}\)P-labeled peptides were detected, and the major peptide was eluted at approximately 17% acetonitrile (data not shown). The contribution of each peak to total radioactivity and the phosphoamino acid in each peak were determined (Table I). The major peak, peak II, was reapplied to HPLC and isolated, and its sequence was determined, as shown in Table I. The peptide matched the sequence 691–700 for the 133-kDa subunit, or 650–659 for the 130-kDa subunit. The phosphorylated T was at position 695 or 654 for the 133- and 130-kDa subunits.

Effect of Phosphorylation—MBP was phosphorylated for various times and assayed for phosphatase activity using \(^{32}\)P-myosin and \(^{32}\)P-LC20 (see “Experimental Procedures”). As the extent of phosphorylation increased, the phosphatase activity decreased (Fig. 4). The final extent of phosphorylation after 30 min of incubation was 1.5 mol of phosphate/mol of MBP.

The relationship between the stoichiometry of phosphorylation and the extent of inhibition was determined. MBP was phosphorylated to varying levels, and the corresponding phosphatase activities were assayed with \(^{32}\)P-myosin and \(^{32}\)P-LC20. In addition, the degree of phosphorylation for the 130-kDa subunit was estimated and related to phosphatase activity. As shown in Fig. 5A, the phosphatase activity was progressively inhibited as the extent of phosphorylation increased. Maximum inhibition was achieved at about 1 mol of phosphate/mol of 130 kDa subunit (Fig. 5B). Similar effects were observed with thio-phosphorylation, using \(^{[35]}\)S-ATP-S.

The endogenous kinase was removed by gel filtration of MBP (see “Experimental Procedures”), and the 130-kDa subunit was then thio-phosphorylated by the catalytic subunit of the cAMP-dependent protein kinase (10 \(\mu\)g/ml). Approximately 2 mol of thio-phosphate/mol 130-kDa subunit were incorporated, but this did not affect phosphatase activity. (Note that after gel filtration of MBP, incubation with ATP for 30 min resulted in less than 20% inhibition of phosphatase activity). Subsequent phosphorylation of the thio-phosphorylated 130-kDa subunit by a crude kinase fraction (obtained by gel filtration) inhibited phosphatase activity by 70%. These results suggest that the cAMP-dependent protein kinase did not phosphorylate the inhibitory site(s) on the 130-kDa subunit.

To determine if phosphorylation of MBP caused dissociation of the trimeric subunit structure the thio-phosphorylated (approximately 1.5 mol of thio-phosphate/mol of MBP) and non-phosphorylated MBP were applied to gel filtration. The elution sites of phosphorylation—MBP was phosphorylated to 1.5 mol of phosphate/mol of MBP and subjected to proteolysis by α-chymotrypsin. The generation of phosphorylated MBP and subjected to gel filtration, incubation with ATP for 30 min resulted in about 40%. Myelin basic protein (0.25 mg/ml) and histone III S (0.25 mg/ml), and α-casein (0.5 mg/ml) were not phosphorylated by the MBP-associated kinase.

**Fig. 1. Phosphorylation of MBP.** Lane 1, SDS-PAGE of MBP after chromatography on Mono S HR; lanes 2–9, autoradiograms from a time course of phosphorylation corresponding to 0, 2.5, 5, 10, 20, 30, 45, and 60 min, respectively; lanes 10–13, autoradiograms showing time course of digestion by α-chymotrypsin. MBP was phosphorylated for 30 min and digested at 25°C with α-chymotrypsin (1:1000 α-chymotrypsin:MBP (w/w)) for 0, 1, 2, and 3 min (lanes 10–13, respectively). The three arrows indicate the positions of the major phosphatase subunits at 130, 38, and 20 kDa. The major products of proteolysis (i.e. 72 and 60 kDa) are also indicated by the two arrows.
profiles for the two MBP samples were identical, and there was no indication of dissociation of the catalytic subunit (Fig. 6). The elution profile for the isolated catalytic subunit of PP1 also is shown in Fig. 6.

In addition, the thio-phosphorylated and nonphosphorylated samples were applied to a monoclonal antibody affinity column (see “Experimental Procedures”). The bound proteins were eluted at low pH and applied to SDS-PAGE. Western blots were carried out using the monoclonal antibody to the 130-kDa subunit and a polyclonal antibody to PP1. Both the thio-phosphorylated and nonphosphorylated MBP samples showed a similar subunit composition, again providing evidence that phosphorylation of MBP did not dissociate the holoenzyme.

Some kinetics of the inhibitory effect were determined. MBP was phosphorylated to 1.2–1.3 mol of phosphate/mol of 130-kDa subunit, and its activity was determined as a function of either 32P-LC20 or 32P-myosin. The constants derived from double-reciprocal plots are shown in Table II. Using 32P-LC20 as substrate the only effect of phosphorylation was a decrease in $V_{\text{max}}$. With 32P-myosin as substrate the effect of phosphorylation was more complex and involved a reduction of $V_{\text{max}}$ and an increase in $K_m$.

Dephosphorylation of MBP—The MBP was phosphorylated by its associated kinase and used as substrate for dephosphorylation assays. Dephosphorylation of MBP was catalyzed by component(s) present in the MBP preparation (Fig. 7). This, presumably, was due to either PP1β (i.e. the catalytic subunit of MBP) or a contaminant phosphatase. Okadaic acid was tested at concentrations that inhibit either PP2A or both PP2A and PP1. At 2 nM okadaic acid the dephosphorylation time curve was essentially the same as in the absence of okadaic acid. At higher concentrations of okadaic acid the phosphatase activity was inhibited (Fig. 7). Thus, if the phosphatase activity toward MBP is due to a contaminant enzyme it is probably a type 1 phosphatase. The most likely explanation is that the phospha-
tase activity reflects the presence of PP1α. Addition of the catalytic subunit of PP2A did not markedly increase the dephosphorylation rate. Other phosphatases were not tested.

The phosphorylated MBP was incubated for 60 min (conditions as in Fig. 7) and assayed for phosphatase activity with 32P-LC20 as substrate. Full recovery of light chain phosphatase activity occurred when MBP was dephosphorylated.

Properties of the MBP-associated Kinase—The major site on the 130-kDa subunit phosphorylated by the endogenous kinase is a potential site for either cAMP-dependent protein kinase or PKC. Since phosphorylation by the former kinase had no effect on MBP activity the possibility of a PKC-like enzyme was investigated. Fig. 8 shows that the kinase component of MBP was inhibited by chelerythrine. Half-maximal inhibition occurred at approximately 5 μM chelerythrine. An IC50 of 0.66 μM was reported previously (39) for PKC and histone II S at 10 μM ATP. ATP is a noncompetitive inhibitor for the effect of chelerythrine, and the assays reported in Fig. 8 were carried out at 150 μM ATP. However, two other PKC inhibitors, calphostin C (up to 30 μM at 150 μM ATP) and H7 (up to 200 μM at 150 μM ATP) had no effect on kinase activity.

Two synthetic peptides were tested as substrates for the MBP-associated kinase. One is a known PKC substrate, based on the autoinhibitory region of ε PKC. The second was a peptide incorporating the sequence around the phosphorylated Thr of the 130-kDa subunit, Arg648 to Asp661 of the 130-kDa sub-

TABLE II
Effect of phosphorylation of MBP

|                 | 32P-LC20 | 32P-Myosin |
|-----------------|----------|------------|
| Km              | 15.2 ± 1.8 | 16.0 ± 2.0 |
| Vmax            | 5.5 ± 0.5  | 4.3 ± 0.3  |

Phosphorylation and Inhibition of Myosin Phosphatase

Fig. 5. Phosphorylation of MBP and the 130-kDa subunit and its effect on activity of MBP. Two aliquots were withdrawn at each point; one was used for estimation of 32P incorporation into MBP (A) or the 130-kDa subunit (B), and the second was used for phosphatase assays (as in Fig. 7) using 32P-myosin (C), or, 32P-LC20 as substrate (△).

Fig. 6. Gel filtration of MBP and PP1c. Samples applied to the Hiprep Sephacryl S-300 16/60 column were as follows: thiophosphorylated MBP, 1.4 mol of thiophosphate/mol of MBP (●); nonphosphorylated MBP (○) and PP1c (△). The elution profiles were detected by phosphatase assays using 32P-LC20 as substrate. Arrows indicate (from left) void volume and elution positions of thyroglobulin (670 kDa), γ-globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa). The estimated masses from the elution positions were 240 kDa for MBP and 38 kDa for PP1c.

Fig. 7. Dephosphorylation of MBP. Time courses of dephosphorylation of MBP (initially 1.3–1.4 mol of phosphate/mol of MBP) are shown with the following: MBP alone (○); MBP plus 2 nM okadaic acid (△); MBP plus 1 μM okadaic acid (□); MBP plus 100-ng (0.5 μg/ml) catalytic subunit of PP2A (●).

Phosphorylation and Inhibition of Myosin Phosphatase

4737
unit, or 689–702 of the 133-kDa subunit (21). Both peptides were phosphorylated by the MBP-associated kinase, as shown in Fig. 9. The peptide from the 130-kDa subunit sequence was slightly more effective as a substrate, and its rate of phosphorylation was faster than the ePKC peptide. Addition of excess 130-kDa peptide to MBP blocked phosphorylation of the 130-kDa subunit. At 300 \text{mM} peptide the phosphorylation of MBP was reduced by 50%, and at 600 \text{mM} peptide it was reduced by 90%. As phosphorylation of MBP was reduced (by added peptide) the inhibition of MBP phosphatase activity also was reduced. At the higher levels of peptide (600 \text{mM}) no inhibition of MBP was observed.

The MBP-associated kinase phosphorylated the PKC sites on LC20. Incubation of MBP with 5 \text{mM} LC20 at 30° for 20 min, under the standard conditions of phosphorylation, phosphorylated approximately 0.3 mol of phosphate/mol of 130-kDa subunit. Other values were normalized to this (as 100%). Error bars indicate ± S.D. (n = 3).

**Fig. 8.** Inhibition of MBP-associated kinase by chelerythrine. Phosphorylation of the 130-kDa subunit was estimated after 30 min at 30 °C in 30 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.1 mM EGTA, 0.5 mM dithiothreitol, 150 \text{μM} \(\gamma^{32}\text{P}\)ATP, 1 \mu M microcystin-LR, 2 μg MBP, and varying concentrations of chelerythrine. Chelerythrine was dissolved in Me₂SO (3 mM stock solution), and all assays contained 4% Me₂SO. In the absence of chelerythrine the level of phosphorylation was 1.3 mol of phosphate/mol of 130-kDa subunit. Other values were normalized to this (as 100%). Error bars indicate ± S.D. (n = 3).

The MBP-associated kinase phosphorylated the PKC sites on LC20. Incubation of MBP with 5 μM LC20 at 30° for 20 min, under the standard conditions of phosphorylation, phosphorylated approximately 0.3 mol of phosphate/mol of LC20 at the various PKC sites (shown by peptide mapping). LC20 that was thiophosphorylated at S19 by myosin light chain kinase also was phosphorylated by MBP, to a similar stoichiometry.

The effect of various fatty acids on the activity of the MBP-associated kinase was tested. The results are shown in Fig. 10. Arachidonic acid and oleic acid activated the kinase, and these were effective over a range of 200–300 μM fatty acid. Activation by myristic acid was less efficient, and maximum activation occurred at about 1 μM. Phorbol 12-myristate 13-acetate (up to 200 μM) and phosphatidyl serine (to 100 μg/ml) had no effect on kinase activity, either alone or in combination.

**Fig. 9.** Phosphorylation of synthetic peptides by MBP-associated kinase. Assays with 130-kDa/133-kDa peptide (○) and with modified ePKC peptide (●) are shown. The amount of MBP used (0.5 μg) was chosen to give linear kinetics over this time course. Conditions were as follows: 30 mM Tris-HCl (pH 7.5), 1 mM MgCl₂, 0.5 mM dithiothreitol, 150 μM \(\gamma^{32}\text{P}\)ATP, 0.5 mg/ml bovine serum albumin, 1 μM microcystin-LR, and 100 μM peptide.

**Fig. 10.** Effect of fatty acids on the activity of the MBP-associated kinase. Phosphorylation of the 130-kDa subunit was estimated after 4 min with various concentrations of arachidonic acid (○), oleic acid (●), and myristic acid (■). Conditions were as in Fig. 9. Fatty acids were dissolved in Me₂SO, and all assays contained 2% Me₂SO.

**DISCUSSION**

The above results show that a kinase present in the MBP preparations phosphorylated the 130-kDa subunit and that phosphorylation inhibited phosphatase activity. The mechanism of inhibition is not known, and the interactions among the three subunits need to be documented before a reasonable model can be presented. Some general comments, however, can be made. With phosphorylated myosin as substrate, the phosphorylation of MBP caused a decrease in \(V_{\text{max}}\) and a slight increase in \(K_m\) to about 60 μM. Both factors may be involved, and the concentration of phosphorylated LC20 in vivo is expected to be between 10 and 60 μM. It is unlikely, however, that dissociation of the trimeric structure of the holoenzyme occurred as a consequence of phosphorylation since similar elution positions for phosphorylated and dephosphorylated MBP were obtained on gel filtration.

The putative inhibitory site is Thr⁶⁶⁴ (or Thr⁶⁶⁵ for the 133-
kDa subunit) in the C-terminal half of the molecule. Although this is the major phosphorylation site it cannot be concluded that it is the only inhibitory site. Other sites in this region of the molecule may also be inhibitory, and the contribution of phosphorylation at the minor sites has not been evaluated. Thus the assignment of Thr<sup>654</sup> as the inhibitory site is tentative. The corresponding residue in rat M110 is Thr<sup>641</sup>, and this is flanked by sequences identical to the chicken 130/133-kDa subunits (25). Phosphorylation of the rat MBP has not been investigated. The effects induced by phosphorylation are not known, and an obvious deficiency in this area is that the functions of different regions of the 130-kDa subunit have not been determined. Only a few properties have been proposed. It is suggested that the ankyrin repeat sequences in the N-terminal half may be involved in myosin binding, and this is based on characteristics of other proteins containing the ankyrin repeat. Certainly this is not conclusive, but it is known that the 58-kDa N-terminal fragment binds to myosin (21). This fragment also binds the 38-kDa catalytic subunit (23). Based on a linear model for the 130-kDa subunit it is difficult to imagine how phosphorylation at Thr<sup>654</sup> could affect distant interactions. Obviously, folding of the 130-kDa subunit is possible, and in addition the interactions of the 20-kDa subunit should be considered. These may involve C-terminal interactions since both the 20-kDa (rat and gizzard) and rat M110 subunits contain C-terminal leucine zipper sequences (25). The gizzard 130-kDa subunit does not contain this sequence. The C-terminal part of the molecule, however, appears to bind the 20-kDa subunit since the latter is not present with the 58-38-kDa complex (23). Although the 58-kDa subunit was phosphorylated by the endogenous kinase, albeit slowly, its phosphatase activity was not affected. Thus, the focus for the phosphorylation-dependent inhibition of MBP should be on interactions involving the C-terminal half of the 130-kDa subunit.

The kinase responsible for phosphorylation of the 130-kDa subunit is not identified. Based on the sequence flanking Thr<sup>654</sup>, two kinases are suggested, namely the cAMP-dependent protein kinase and PKC. It is unlikely that the former kinase is involved since the endogenous kinase was not affected by cAMP-dependent protein kinase inhibitors, nor did phosphorylation of the 130-kDa subunit by cAMP-dependent protein kinase influence phosphatase activity. The case for PKC also is not compelling. PKC-like characteristics include inhibition by chelerythrine, a reasonably specific inhibitor of PKC (39), and phosphorylation of known PKC sites, i.e. the PKC peptide and LC20. But the endogenous kinase was not activated by phorbol ester or phospholipids, and it was not inhibited by other PKC inhibitors. It is possible that the endogenous kinase was subject to proteolysis, and although this may explain the spontaneous activity in the MBP preparations, it would not account for all of the observed characteristics. Thus the identity of the endogenous kinase remains to be established. If this kinase is involved in the contraction cycle in smooth muscle then it is assumed that its activity would be regulated. One possibility is that the kinase is the last link of a signal cascade that results in inhibition of MBP and alteration of the Ca<sup>2+</sup>/calmodulin sensitivity of myosin phosphorylation.

If phosphorylation of MBP has a regulatory function then an effective dephosphorylation mechanism should exist, and this should occur within the time frame observed for physiological effects. From the available data it is difficult to assign an accurate time period for the recovery phase. The time required for restoration of full MBP activity, assuming this is linked to dephosphorylation of MBP, has not been determined, but intuitively a period of 1 or 2 min might be the upper limit. The in vitro dephosphorylation rate was slow, and 50% dephosphorylation required over 20 min. Obviously, the in vivo rate might be higher, due to slightly higher temperature, etc., but it should also be considered that another phosphatase is required for rapid dephosphorylation of MBP. If dephosphorylation by the PP1α isoform is not adequate, then it is important to determine which of the other phosphatases might be involved.

In summary, these results have shown that the large subunit of the trimeric MBP can be phosphorylated and that this results in inhibition of myosin phosphatase activity. Previously it was found that incubation of portal vein preparations with ATPγS caused inhibition of phosphatase activity and thiprophosphorylation of several proteins, including the phosphatase subunit (26). Thus the in vitro data support the more physiological experiments and suggest that inhibition of phosphatase via phosphorylation may play an important regulatory role in smooth muscle. The identity of the kinase involved is not known, but it has some characteristics of PKC. Priorities for future research involve characterization of the kinase and identification of the presumed link(s) between the agonist-induced membrane event and activation of the kinase at the level of the contractile apparatus.

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