Smooth muscle calcium sensitization reflects an inhibition of myosin light chain phosphatase (SMPP-1M) activity; however, the underlying mechanisms are not well understood. SMPP-1M activity can be modulated through phosphorylation of the myosin targeting subunit (MYPT1) by the endogenous myosin phosphatase-associated kinase, MYPT1 kinase (MacDonald, J. A., Borman, M. A., Murányi, A., Somlyo, A. V., Hartshorne, D. J., and Haystead, T. A.) (1). Recombinant chicken gizzard MYPT1 (M130) was phosphorylated in vitro by a recombinant MYPT1 kinase, and the sites of phosphorylation were identified as Thr695, Ser808, and Thr675. Introduction of recombinant MYPT1 kinase elicited a calcium-independent contraction in β-escin-permeabilized rabbit ileal smooth muscle. Using an antibody that specifically recognizes MYPT1 phosphorylated at Thr654 (M130 numbering), we determined that this calcium-independent contraction was correlated with an increase in MYPT1 phosphorylation. These results indicate that SMPP-1M phosphorylation by MYPT1 kinase is a mechanism of smooth muscle calcium sensitization.

Contractile activity in smooth muscle is primarily controlled by the level of free intracellular calcium ([Ca2+]i) and the level of phosphorylation of the 20-kDa myosin regulatory light chains (RLC). The phosphorylation level of myosin is mediated by the activity of two enzymes, the Ca2+-calmodulin-dependent myosin light chain kinase (MLCK) (2) and a Ca2+-independent myosin light chain phosphatase (SMPP-1M) (3).

Contractile stimuli that cause an increase in [Ca2+]i result in Ca2+ binding to calmodulin and activation of MLCK. MLCK catalyzes the phosphorylation of the regulatory myosin light chains at Ser19 (reviewed in Refs. 2, 4, and 5), resulting in cross-bridge cycling and force development (6). Relaxation follows a return of [Ca2+]i to resting levels, the subsequent inactivation of MLCK, and dephosphorylation of myosin catalyzed by SMPP-1M (3). Smooth muscle contraction may also occur in the absence of changes in [Ca2+]i, following agonist stimulation or by activation of G-proteins with GTPγS or AlF4-. This Ca2+ sensitization is thought to reflect an inhibition of SMPP-1M via a G-protein-linked mechanism (4, 7, 8). It is possible to elicit a Ca2+-independent contraction by completely inhibiting SMPP-1M activity with phosophatase inhibitors, e.g. microcystin (9–12). This contraction correlates with an increase in myosin regulatory light chain phosphorylation at Ser19 and Thr18 (12–14). A great deal of attention has recently been focused on identifying the kinase or kinases responsible for this myosin phosphorylation in the absence of changes in [Ca2+]i. The results of recent efforts have identified both zipper-interacting protein kinase (ZIPK) (14) and integrin-linked kinase (13) as kinases responsible for Ca2+-independent myosin phosphorylation and contraction in smooth muscle.

SMPP-1M is a heterotrimeric protein composed of a 37-kDa catalytic subunit (PP-1cβ), a 110–130-kDa regulatory myosin phosphatase targeting subunit (MYPT1), and a 20-kDa subunit of unknown function (10, 15, 16). The myosin phosphatase activity of SMPP-1M is believed to be regulated by phosphorylation of the MYPT1 subunit. Several phosphorylation sites have been identified on MYPT1, including an inhibitory site of phosphorylation by an endogenous kinase identified as Thr695 (in the chicken M133 isoform, equivalent to Thr654 in the chicken M130 isoform) (17).

The molecular mechanism through which activation of G-proteins inhibits SMPP-1M activity is not well established. Evidence suggests that the recently identified endogenous smooth muscle SMPP-1M-associated kinase (MYPT1 kinase) may be implicated in this pathway (1). In support of this, MYPT1 kinase and MYPT1 are colocalized in smooth muscle, and MYPT1 kinase is phosphorylated and activated in response to treatment with calcium-sensitizing agents (1). To elucidate the mechanism through which MYPT1 kinase acts, the following study was initiated. The following data show that introduction of a constitutively active recombinant MYPT1 kinase (rMYPT1) into β-escin-permeabilized smooth muscle provokes a Ca2+-independent contraction. We report that this Ca2+-independent contraction is associated with an inhibition of myosin phosphatase activity caused by phosphorylation at the Thr695 inhibition site.
MYPT1 Kinase Inhibition of Myosin Phosphatase

EXPERIMENTAL PROCEDURES

Chemicals—[γ-32P]ATP was obtained from ICN (Cost Mesa, CA). Cellulose TLC plates were from Fisher. β-Escin and carbocrohal were obtained from Sigma. A23187 and polyonal anti-ZIPK antibody were obtained from Calbiochem (San Diego, CA). Affinity purification anti-MYPT1 antibody was prepared by Quality Control Biochemicals (Hopkinton, MA). Phosphorylation state-specific MYPT1 antibody (pEM133) was prepared as described (18). Anti-rabbit antibody conjugated to horseradish peroxidase was purchased from Amersham Biosciences. The HPLC fraction containing the major peak of radioactivity was collected and subjected to nanoESI mass spectrometry on a QSTAR-Pulsar mass spectrometer. The HPLC fraction containing the major peak of radioactivity was collected and subjected to nanospray mass spectrometry on a QSTAR-Pulsar mass spectrometer.

Identification of a Calcium-independent Kinase That Phosphorylates Myosin Light Chains—To identify any additional Ca2+-independent kinase or kinases that phosphorylate myosin light chains, γ-linked ATP-Sepharose affinity chromatography was utilized. A substrate peptide sequence containing the Ser19 and Thr18 phosphorylation sites of RLC was synthesized. Kinase activity was isolated from the cytosolic fraction of rabbit muscle and utilized. A substrate peptide sequence containing the Ser19 and Thr18 phosphorylation sites of RLC was synthesized. Kinase activity was isolated from the cytosolic fraction of rabbit muscle, which was then subjected to sequential Edman degradation with a vapor phase amino acid sequencer (Applied Biosystems Procise 494) under conditions that liberated the characteristic m/z 78,997 corresponding to the phosphate ion. Positive mode time of flight was then used to measure the masses of peptide fragments produced by collision-induced dissociation (MS/MS) and, ultimately, to predict the sequence of the unknown phosphopeptide.

RESULTS

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The remainder of the phosphosite characterization was performed using differential proteolysis and cleaved radioactive protein (CPR) analysis. The tryptic phosphopeptide samples were immobilized on immobilized metal-ion affinity membrane (Millipore) following the manufacturer's instructions. The phosphorylated residues within phosphopeptides were located by determining the cycles in which [32P] was released when the samples were subjected to sequential Edman degradation with a vapor phase amino acid sequencer (Applied Biosystems Procise 494) under conditions that optimize recovery of [32P] (22). The CPR analysis program (23) was used to assign a phosphorylation site to the [32P] released in a specific cycle. The CPR analysis program is available at fasta.bioch.virginia.edu/erpc.

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Expression and Characterization of MYPT1 Kinase—MYPT1 kinase was originally identified as a kinase that phosphorylates MYPT1 (1); subsequently this same kinase was shown to phosphorylate a RLC substrate peptide (present data). The phosphorylation of RLC by HeLa ZIPK has been previously demonstrated (24). To determine the cellular function of MYPT1 kinase, recombinant MYPT1 kinase (rMYPT1K) was expressed in Escherichia coli, and its enzymatic properties were examined in vitro. We have analyzed the specificity of rMYPT1K toward two known protein substrates, RLC and MYPT1, by comparing enzyme affinities. Michaelis constants (Km) were determined for RLC protein and C130, a C-terminal fragment of chicken gizzard M130 that contains the known inhibitory phosphorylation sites. Km values were 53 ± 6 μM (n = 4) for the RLC protein and 3 ± 0.9 μM (n = 4) for the C130 protein. Catalytic constants (kcat) were also measured for RLC and C130 and were 1.9 × 10−2 s−1 ± 9.6 × 10−4 (n = 4) and 5.0 × 10−2 s−1 ± 1.8 × 10−2 (n = 4), respectively. These results suggest that MYPT1 may be a preferred substrate in vivo.

In Situ Phosphorylation of MYPT1 by rMYPT1K—Because the in vitro kinetics results for rMYPT1K against the two putative protein substrates suggested that MYPT1 was a better substrate, we investigated the in situ phosphorylation of MYPT1 by rMYPT1K in permeabilized smooth muscle. The effect of 10 μM rMYPT1K on the extent of MYPT1 phosphorylation was determined in calcium-free conditions (G10). As shown in Fig. 2A, incubation of rabbit ileal strips with rMYPT1K provoked a Ca2+-independent contraction equivalent to 37.5% ± 2.0% (n = 7) of the maximal calcium (CaG) contraction. To determine whether the contractile effect of rMYPT1K was associated with phosphorylation of MYPT1, rabbit ileal strips were mounted in a bubble chamber and permeabilized with β-escin. Following maximal calcium contraction, the muscles were washed in calcium-free solution containing 10 mM EGTA and then incubated for 20 min in the same solution in the absence or presence of 10 μM rMYPT1K. After 20 min, the strips were rapidly frozen, and MYPT1 phosphorylation at Thr695 was measured (see “Experimental Procedures”). Fig. 2 (B and C) shows that incubation of muscle strips...
with 10 μM rMYPT1K causes a 5-fold increase in the level of Thr\(^{655}\) phosphorylation over control muscle strips (5.0 ± 0.8 in rMYPT1K-treated strips \((n = 9)\); 1.0 ± 0.2 in control strips \((n = 12)\).

**Phosphorylation of MYPT1 in Vitro by Native MYPT1 Kinase and Recombinant GST-rMYPT1K**—Initial studies using native MYPT1 kinase suggested phosphorylation of multiple sites on M133. Both phosphoserine and phosphothreonine residues were detected by phosphoamino acid analysis (Fig. 3, inset). To identify the individual phosphorylation sites, we used recombinant MYPT1 proteins phosphorylated by GST-rMYPT1K. As shown in Table I, GST-rMYPT1K phosphorylated multiple sites of M130 in vitro. The reverse phase chromatography profile showed a major peak of \({}^{32}P\) in fraction 40 of the recombinant wild-type C130 and rM133\(^{7865A}\) protein digests. This peak was absent in the chromatography profile of the recombinant C130\(^{7865A}\) protein digest. Three additional peaks (I, II, and IV) of \(^{32}P\) were observed in the reverse phase chromatography profile.

We used mass spectrometry to identify the major phosphorylation site (peak III) in the C-terminal fragment of the wild-type rM130 protein (Fig. 4). Phosphopeptides in fraction 40 were treated with trypsin overnight and subjected to nanospray mass spectrometry. A precursor ion scan in the time of flight negative mode identified parent ions with \(^{32}P\)-labeled fractions of the protein digests were subjected to mass spectrometry analysis. The HPLC fraction (fraction 40) containing the major phosphorylation site was digested with trypsin and analyzed on a QSTAR/Pulsar mass spectrometer. A, negative mode time of flight precursor ion scan displaying \(m/z\) of peptides liberating the 79.997 corresponding to the phosphate ion. The \(m/z\) values of 927.4 and 618.8 measured in the positive mode time of flight spectra were assigned to doubly and triply charged phosphorylated peptides; the \(m/z\) values of 887.9 and 591.8 were assigned to doubly and triply charged unphosphorylated peptides. These peptide ions were subjected to collision-induced dissociation to produce MS/MS spectra. The doubly and triply charged peptides were identified by the \(b\) and \(y\) series ions in the MS/MS spectra as \(STQGVTLTDLQEAEK\) and \(RSTQGVTLTDLQEAEK\), respectively. The phosphorylation of the Thr\(^{654}\) site within these peptides was confirmed by mass spectrometry of the 929.8 and 619.8 \(^{32}P\) phospho-species.

The additional minor sites of phosphorylation were identified by determining the cycles in which \(^{32}P\) was released when \(^{32}P\)-labeled fractions of the protein digests were subjected to sequential Edman degradation under conditions that optimized recovery of \(^{32}P\). Solid phase Edman sequencing of peak I and CRP analysis of MYPT1 (Fig. 5) revealed that Thr\(^{675}\) was the only residue that could yield the release of \(^{32}P\) in the eighth cycle following digestion with endoproteinase Lys-C and in the fourth cycle following digestion with endoproteinase Arg-C. From a similar analysis of peak II, we have identified Ser\(^{808}\) which corresponds to Ser\(^{849}\) in Rat3 MBS and Ser\(^{808}\) in chicken M130, as a minor phosphorylation site. In addition, phosphorylation of the peptide in peak II was reduced but not eliminated in the rM133\(^{7865A}\) protein digest; this is also consistent with the phosphorylation of Thr\(^{850}\) on the wild-type M130 protein. A phosphoamino acid analysis of peak II phosphopeptides from wild-type M130 protein confirmed the presence of both Ser(P) and Thr(P) (data not shown). The phosphorylation of Ser\(^{854}\) was reported to be specific to Rho-associated protein kinase (ROK), and Ser\(^{854}\) phosphorylation has been used in previous studies to identify ROK-specific phosphorylation of MBS in vivo (25).

**DISCUSSION**

Substrate specificity in signal transduction is often conferred to a common catalytic subunit through a unique targeting subunit (reviewed in Ref. 26). The targeting subunits themselves may be targets of signaling pathways that can modulate the activity of the enzyme toward its specific substrate. The smooth muscle myosin phosphatase targeting subunit (MYPT1) is regulated by multiple signaling pathways. In the

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**TABLE I**

| Peptide | Amino acid sequence | Phosphorylated residue | Phosphate content |
|---------|---------------------|------------------------|------------------|
| I | \(^{644}\)TIGRGRSTTREQNEETK \(^{685}\) | Thr\(^{675}\) | 7.5 |
| II | \(^{803}\)RSTGSGVFQDSDENEREQTGDSTEDGSK \(^{835}\) | Ser\(^{854}\) | 7.5 |
| III | \(^{644}\)ARKRQGQISRRTQGVTLTDLQEAEK \(^{667}\) | Thr\(^{850}\) | 76.2 |
| IV | ND | ND | 12.1 |

*a* Residue numbers correspond to chicken M130 MYPT1.  
*b* The phosphorylated residue in each peptide is underlined.  
*c* The values were determined from the radioactivity in the HPLC analysis of wild-type M130 as shown in Fig. 3.  
*d* ND, not determined.
current study, we have provided evidence for a mechanism regulating the activity of MYPT1. There is substantial evidence that smooth muscle Ca\textsuperscript{2+}/H\textsuperscript{11001} sensitization reflects an inhibition of myosin phosphatase activity (reviewed in Refs. 3 and 4). However, the mechanism of SMPP-1M inhibition remains unclear. Several theories have now been described: inhibition by CPI-17, a 17-kDa phosphatase inhibitor and protein kinase C substrate protein that becomes a potent inhibitor of SMPP-1M following phosphorylation by protein kinase C (27, 28); dissociation of the SMPP-1M holoenzyme through arachidonic acid interaction (29); and phosphorylation of MYPT1 by an endogenous kinase (1, 17) or by ROK (30).

Previous studies reported that phosphorylation of MYPT1 at Thr\textsuperscript{695} by an endogenous kinase caused inhibition of SMPP-1M activity (17). We subsequently identified this endogenous kinase that copurifies with the holoenzyme of myosin phosphatase as MYPT1 kinase (1), which shows significant sequence homology to the previously described ZIPK (31). The data presented above indicate that MYPT1 kinase phosphorylates MYPT1 in situ in rabbit ileal smooth muscle and that this MYPT1 phosphorylation is correlated with a Ca\textsuperscript{2+}-independent contraction. Our data suggest that MYPT1 is indeed a target of smooth muscle MYPT1 kinase contributing to calcium sensitization.

We previously reported that MYPT1 kinase phosphorylates the inhibitory Thr\textsuperscript{695} site of MYPT1 (1). In the present study, we report the presence of three additional MYPT1 phosphorylation sites, two of which we have identified. Ser\textsuperscript{854} in Rat3 MBS was previously reported to be a phosphorylation site specific to ROK and was proposed to be an effective indicator of ROK activation \textit{in vivo} (25). Phosphorylation of Ser\textsuperscript{849}, equivalent to Ser\textsuperscript{854} in Rat3 MBS, did not have an inhibitory effect on SMPP-1M activity (18). In the present study, we have identified Ser\textsuperscript{808}, which corresponds to Ser\textsuperscript{854} in Rat3 MBS and Ser\textsuperscript{849} in chicken M133, as a minor MYPT1 kinase phosphorylation site. Our data indicate that Ser\textsuperscript{854} phosphorylation is not unique to ROK and suggest a renewed examination of the role of ROK in SMPP-1M regulation. We also identify Thr\textsuperscript{675} as a minor MYPT1 phosphorylation site. The role of this newly identified phosphorylation site in the regulation of SMPP-1M remains unknown.

Native MYPT1 kinase was recovered from the cytosolic fraction of bladder smooth muscle following homogenization in the absence of any detergent. Native MYPT1 kinase was previously isolated from a Triton-solubilized fraction consisting of myofilament and cytoskeletal components (1), suggesting the presence of two distinct subcellular fractions of MYPT1 kinase in smooth muscle. Presumably, it is the myofilament-associated pool of MYPT1 kinase that would associate with and phosphorylate myosin phosphatase \textit{in vivo}. An additional substrate for MYPT1 kinase that may be important in the regulation of smooth muscle contraction is myosin. It was previously reported that a full-length smooth muscle ZIPK phosphorylated myosin at both Ser\textsuperscript{19} and Thr\textsuperscript{18} (14). We have also observed
that rMYPT1K phosphorylates RLC and whole myosin on its regulatory light chains in vitro (data not shown). The change in calcium sensitivity associated with rMYPT1K treatment may therefore reflect two distinct phosphorylation mechanisms, i.e. MYPT1 and myosin. This possibility will require further investigation. Current procedures for investigating the contribution of MYPT1 phosphorylation in the calcium sensitization of force development involve the indiscriminate use of MLCK inhibitors (i.e. ML-9). It should be noted that this commonly used MLCK inhibitor also inhibits the activity of MYPT1 kinase in vitro at levels below those required for full MLCK inactivation (IC50 for MYPT1 kinase: ~150 µM ML-9). Researchers who use MLCK inhibitors to investigate GTPyS- or ATPγS-induced force enhancement should be aware that they may be inadvertently influencing different signaling pathways.

It is unclear why the phosphorylation results obtained with native MYPT1 kinase and rMYPT1K in our studies (this paper and Ref. 1) are different from those recently reported by Niiro (32), that ZIPK (and native MYPT1 kinase) only phosphorylates the M133 isoform of MYPT1 (i.e. Thr695 site) and not the shorter, spliced-out M130 isoform (i.e. Thr654 site). The expression of MYPT1 isoforms is both developmentally regulated and tissue-specific (3), thereby raising the possibility that MYPT1 isoforms could modulate the magnitude of agonist-induced Ca2+ sensitization (32). Although we have not completed a comprehensive examination of the phosphorylation kinetics for the M130/M133 isoforms, our preliminary studies (Fig. 3) have shown that both the M130 and M133 isoforms are phosphorylated by MYPT1 kinase.

We have now identified three substrates for the smooth muscle MYPT1 kinase that could be involved in regulating calcium sensitization: MYPT1 (Ref. 1 and present data), myosin (present data), and the protein kinase C-activated peptide inhibitor (CPI-17) (33). We have established the sites of phosphorylation of MYPT1, which include the known inhibitory site (Thr654), a site previously thought to be unique to ROK (Ser608), and a newly identified phosphorylation site whose role in SMPP-1M regulation has not been established (Thr675). We have also demonstrated that a constitutively active fragment of MYPT1 kinase induces contraction in β-escin permeabilized ideal smooth muscle and that this Ca2+ sensitization is correlated with an increase in phosphorylation of SMPP-1M at Thr654. If this occurs in vivo, it would likely be under conditions where MLCK activity is reduced, i.e. at low calcium concentrations. This would clearly necessitate a signal other than calcium to activate MYPT1 kinase.

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