THE JOURNAL OF BIOLOGICAL CHEMISTRY Vol. 279, No. 33, Issue of August 13, pp. 34496–34504, 2004

Printed in U.S.A.

Smooth Muscle Phosphatase Is Regulated *in Vivo* by Exclusion of Phosphorylation of Threonine 696 of MYPT1 by Phosphorylation of Serine 695 in Response to Cyclic Nucleotides*

Received for publication, May 27, 2004
Published, JBC Papers in Press, June 11, 2004, DOI 10.1074/jbc.M405957200

Anne A. Wooldridge‡, Justin A. MacDonald§¶, Ferenc Erdodi‖, Chaoyu Ma‡, Meredith A. Borman§, David J. Hartshorne, and Timothy A. J. Haystead‡ ‡‡**

*From the ‡Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710, the §Department of Biochemistry and Molecular Biology, Smooth Muscle Research Group, University of Calgary, Calgary T2N 4N1, Canada, the ‖Department of Medical Chemistry, Medical and Health Science Center, University of Debrecen, Debrecen, Hungary, and **Muscle Biology Group, University of Arizona, Tucson, Arizona 85721*

Regulation of smooth muscle myosin phosphatase (SMPP-1M) is thought to be a primary mechanism for explaining Ca^{2+} sensitization/desensitization in smooth muscle. Ca^{2+} sensitization induced by activation of G protein-coupled receptors acting through RhoA involves phosphorylation of Thr-696 (of the human isoform) of the myosin targeting subunit (MYPT1) of SMPP-1M inhibiting activity. In contrast, agonists that elevate intracellular cGMP and cAMP promote Ca^{2+} desensitization in smooth muscle through apparent activation of SMPP-1M. We show that cGMP-dependent protein kinase (PKG)/cAMP-dependent protein kinase (PKA) efficiently phosphorylates MYPT1 *in vitro* at Ser-692, Ser-695, and Ser-852 (numbering for human isoform). Although phosphorylation of MYPT1 by PKA/PKG has no direct effect on SMPP-1M activity, a primary site of phosphorylation is Ser-695, which is immediately adjacent to the inactivating Thr-696. *In vitro*, phosphorylation of Ser-695 by PKA/PKG appeared to prevent phosphorylation of Thr-696 by MYPT1K. In ileum smooth muscle, Ser-695 showed a 3-fold increase in phosphorylation in response to 8-bromo-cGMP. Addition of constitutively active recombinant MYPT1K to permeabilized smooth muscles caused phosphorylation of Thr-696 and Ca^{2+} sensitization; however, this phosphorylation was blocked by preincubation with 8-bromo-cGMP. These findings suggest a mechanism of Ca^{2+} desensitization in smooth muscle that involves mutual exclusion of phosphorylation, whereby phosphorylation of Ser-695 prevents phosphorylation of Thr-696 and therefore inhibition of SMPP-1M.

Contraction and relaxation of smooth muscle are primarily determined by the level of phosphorylation of the myosin light chain (MLC-20).[1] To initiate contraction, an action potential or binding of a contractile agonist causes an increase in intracellular Ca^{2+}, which activates myosin light chain kinase (MLCK), a Ca^{2+}/calmodulin-dependent enzyme. MLCK phosphorylates MLC-20 on serine 19, resulting in contraction of smooth muscle through increases in myosin ATPase activity and cross-bridge cycling (1, 2). Smooth muscle myosin phosphatase (SMPP-1M) dephosphorylates MLC-20 resulting in relaxation of smooth muscle. Contraction can also occur in response to certain signals in the absence of changes in intracellular Ca^{2+}, a phenomenon called Ca^{2+} sensitization. Inhibition of SMPP-1M activity is a primary mechanism of Ca^{2+} sensitization (3–7). One hypothesis is that SMPP-1M activity is inhibited by phosphorylation of the targeting subunit, MYPT1, at Thr-696 (of the human isoform). Several kinases have been shown to phosphorylate this site including Rho kinase (ROCK) (6), MYPT1-associated kinase (MYPT1K or ZIPK) (5, 8), myotonic dystrophy kinase (9), and integrin-linked kinase (9, 10).

Relaxation of smooth muscle is achieved by either removal of the contractile agonist (passive relaxation) or through agonists that activate guanylyl or adenylyl cyclase and stimulate the production of cyclic nucleotides, cGMP or cAMP, thus activating their targets, cGMP-dependent protein kinase (PKG) and cAMP-dependent protein kinase (PKA) (2, 3). These kinases lower intracellular Ca^{2+} through multiple mechanisms, but they also raise the Ca^{2+} threshold for contraction, thus causing Ca^{2+} desensitization (3, 11). This is illustrated by the observation that GTPγS and carbachol-contracted smooth muscle relax in response to 8-bromo-cGMP at constant Ca^{2+} (12, 13). These findings suggest the mechanism of Ca^{2+} desensitization involves direct activation of SMPP-1M by the cyclic nucleotide-dependent kinases PKA and PKG. Indeed, several studies in permeabilized muscle have suggested that cGMP/PKG stimulates endogenous SMPP-1M activity; however, none have shown a direct activation (12–14). *In vitro* the MYPT1 phosphorylation data suggest that multiple serine residues are phosphorylated by PKA/PKG; specifically Ser-849 (M133 isoform) was suggested because it is in a PKG phosphorylation consensus sequence, and the C-terminal portion of the protein

* This work was supported in part by National Institutes of Health Grants HL19242-24, DK52378-06 (to T. A. J. H.), and HL23615 (to D. J. H.), a National Science Foundation graduate research fellowship (to A. A. W.), and by Hungarian Science Research Fund Grant OTKA T043296 (to F. E.). 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.

This paper is dedicated to the memory of Andrew Somlyo. Andrew Somlyo more than anyone else built our current concepts of smooth muscle function, and in particular, he discovered the molecular basis underlying Ca^{2+} sensitivity of smooth muscle. Andrew will be sadly missed by his many friends and colleagues.

¶ Holder of a Protein Engineering Network of Centres of Excellence (PENCE) Chair in Protein Sciences.

‡‡ To whom correspondence should be addressed: Duke University Medical Center, Box 3813, Durham, NC 27710. Tel.: 919-613-8609/8606; Fax: 919-668-0977; E-mail: hayst001@mc.duke.edu.

[1] The abbreviations used are: MLC, myosin light chain; MLCK, MLC kinase; PKG, cGMP-dependent protein kinase; PKA, cAMP-dependent protein kinase; ROCK, Rho kinase; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate; GST, glutathione *S*-transferase; CRP, cleaved radioactive peptide; HPLC, high pressure liquid chromatography; ATPγS, adenosine 5′-*O*-(thiotriphosphate).

was found to be phosphorylated (15). In smooth muscle, the presence of the leucine zipper domain of both PKG and MYPT1 has been shown to be essential for the protein kinase to mediate relaxation as determined by MLC-20 phosphorylation, although the binding of PKG-1 to MYPT1 does not absolutely require the leucine zipper domain (16–18). Pretreatment of vascular smooth muscle cells with 8-bromo-cGMP before treatment with angiotensin II reduces MYPT1 Thr-696 phosphorylation (4). Activation of SMPP-1M was suspected, but activity was measured indirectly by assessing MLC dephosphorylation. The study suggested that phosphorylation by PKA/PKG may inhibit Thr-696 phosphorylation and thus a decrease in the level of inhibition of SMPP-1M rather than an activation.

To investigate the mechanisms by which PKA/PKG might bring about Ca^{2+} desensitization through direct interactions with SMPP-1M, we first identified all the major sites phosphorylated by these kinases on MYPT1 *in vitro*. We identified three serine residues on MYPT1 that are phosphorylated by PKA/PKG (Ser-692, Ser-695, and Ser-852, human isoform numbering), and we showed specifically that phosphorylation of Ser-695 precludes phosphorylation at the inactivating site Thr-696 by MYPT1K and ROCK *in vitro*. The reverse situation also occurred. Treatment of permeabilized ileal smooth muscles with 8-bromo-cGMP induced phosphorylation of Ser-695 and greatly attenuated the Ca^{2+}-sensitizing effects of exogenous MYPT1K. These findings suggest that one way cyclic nucleotides mediate their Ca^{2+}-desensitizing effects in smooth muscle is by blocking the inhibitory phosphorylation mechanism for SMPP-1M.

EXPERIMENTAL PROCEDURES

Materials—Affinity-purified MYPT1 antibody was made by Quality Control Biochemicals (Hopkington, MA) in rabbits by using a recombinant N-terminal fragment of rat MYPT1 as the antigen. Affinity-purified MYPT1^{pS695} antibody (Invitrogen) was produced in rabbits by using the peptide KLHRQSRRpSTQGVT (where pS is phosphoserine). This peptide was synthesized by Biomolecules Midwest (St. Louis, MO). Affinity-purified pMYPT1^{T696} antibody and ROCK (an N-terminal fragment) were from Upstate Group, Inc. (Charlottesville, VA). The MYPT1K substrate peptide from MYPT1 (^{689}RQSRRSTQGVTL^{700}) and the serine to alanine mutant peptide (^{689}RQARRSTQGVTL^{700}) (chicken M133 numbering) were synthesized by Biomolecules Midwest. cGMP-dependent protein kinase and the catalytic subunit of PKA were from Calbiochem, and 8-bromo-cGMP was from Sigma. Native PP-1c from rabbit skeletal muscle was a gift from Shirish Shenolikar (Durham, NC), and ^{32}P-labeled myosin from pig bladder (19) or ^{32}P-labeled gizzard MLC-20 (9) was prepared as described.

Expression and Purification of Recombinant Proteins—Recombinant MYPT1 kinase (rMYPT1K) encoding the N-terminal portion of the protein was produced as described from I.M.A.G.E. clone A1660136 (8). The cDNA clone was inserted in-frame into pGEX-6-P-1 in the NotI/BamHI sites (Amersham Biosciences). *Escherichia coli* cells were grown in LB broth in 50 μg/ml ampicillin overnight at 37 °C. Cells were incubated in 300 μM isopropyl-1-thio-β-D-galactopyranoside for 2 h to induce protein expression, and GST-MYPT1K was isolated by using glutathione-Sepharose. The GST moiety was cleaved by an overnight digestion with Precission Protease® according to the manufacturer's protocol (Amersham Biosciences). A hexahistidine-tagged C-terminal fragment of MYPT1 (sequence 514–963 of the M130 chicken isoform; termed C130), full-length chicken GST-MYPT1 (M133), and rat GST-MYPT1 (M110) were expressed and purified as described (6, 20, 21).

Muscle Tension Measurement—For *in situ* contractile experiments, adult male New Zealand White rabbits were anesthetized with halothane and exsanguinated according to approved animal protocols. Ileum smooth muscle was removed, and strips of muscle (5 mm × 200 μm) were cut and the ends tied with silk suture. The muscle strips were then mounted in 140-μl "bubble chambers." One end of each strip was attached to a force transducer (SensorOne AE801, Sausalito, CA), and tension was set to 1.3 times resting length. The strips were permeabilized with β-escin (50 μM) for 30 min at room temperature in Ca^{2+}-free solution containing 1 mM EGTA (G1). Muscles were treated with the Ca^{2+} ionophore A23187 (10 μM) for 10 min to deplete intracellular Ca^{2+} stores. After extensive washing in G1, muscles were stimulated with 10 mM Ca^{2+} solution (CaG) for 5 min and then washed extensively and placed in G1. Either vehicle (water) or 8-bromo-cGMP (100 μM) was administered for 5 min, followed by rMYPT1K (10 μM) for 10 min. The strips were washed extensively again and then contracted in CaG. Force and rate of contraction were measured as a percent of the maximum contraction in CaG.

Western Blot Analysis of Smooth Muscle Tissue—Rabbit ileum was collected as described above, and squares (5 × 5 mm) were mounted in a silicone bottom dish. The muscles were permeabilized and washed as described above. To test the phosphorylation of Ser-695, the ileum was treated with vehicle (water) or 8-bromo-cGMP (100 μM) for 10 min and then flash-frozen in liquid N_2-cooled Freon. To test the level of Thr-696 phosphorylation after pretreatment with 8-bromo-cGMP, muscles were treated with vehicle or 8-bromo-cGMP (100 μM) for 5 min, followed by 10 μM rMYPT1K for 10 min, and then flash-frozen in liquid N_2-cooled Freon. The frozen squares were stored in 10% trichloroacetic acid in acetone, thawed, washed in acetone, and then homogenized in buffer containing 600 mM NaCl, 25 mM Tris-HCl, pH 7.0, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM dithiothreitol, 2 mM EGTA, and 1 μM microcystin-LR. Homogenates were resolved on a 10% SDS-polyacrylamide gel and transferred to polyvinylidene difluoride membranes. Nonspecific binding sites were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.5% Tween 20. Blots were incubated overnight with primary antibody directed against phospho-Ser-695 of MYPT1 (1:1,000) or with primary polyclonal antibody directed against nonphosphorylated MYPT1 (1:2000). The blots were washed and incubated with horseradish peroxidase-conjugated rabbit secondary antibody (1:2000) for 1 h and developed with enhanced chemiluminescence (Amersham Biosciences). Bands were quantitated by densitometry, and the relative phosphorylation was determined as a function of the density of total MYPT1. Equal protein loading was determined by Amido Black staining of the polyvinylidene difluoride membranes after blotting.

In Vitro Phosphorylation, Kinase, and Phosphatase Assays—All phosphorylation reactions for site analysis were carried out by using 100 μg of MYPT1 (c130), 5 μg of PKAc or PKG, 50 mM HEPES, pH 7.2, 1 mM $MgCl_2$, and 0.2 mM [γ-^{32}P]ATP (2,500 cpm/nmol). For stoichiometry reactions, 2 μM GST-MYPT1 (or C130) was phosphorylated by PKA (1 μg/ml), ROCK (0.4 units/ml), or rMYPT1K (44 μg/ml) in the presence of 50 mM HEPES, pH 7.2, 1 mM $MgCl_2$, and 0.2 mM [γ-^{32}P]ATP (2500 cpm/nmol). For phosphatase assays, GST-MYPT1 (M110) was mixed with an equimolar amount of native rabbit PP1C. Smooth muscle myosin phosphatase assays were performed by using ^{32}P-labeled myosin as described previously (19). Kinase assays with the substrate peptide were carried out at 25 °C in 50-μl reactions with 50 mM HEPES, pH 7.2, 1 mM $MgCl_2$, 0.2 mM [γ-^{32}P]ATP (2500 cpm/nmol), 100 μM MYPT1 Ser-691 to Ala substrate peptide, and 0.3 μM rMYPT1K. Reactions were terminated by spotting on P81 paper after a time course. P81 papers were washed three times in 20 mM H_3PO_4, placed in 1.5-ml Eppendorf tubes, and Cerenkov counted. Kinase assays for sequential phosphorylation of MYPT1 by PKA, ROCK, or rMYPT1K were carried out as described previously (9) by using GST-MYPT1 (2 μM), PKA (1 μg/ml), or ROCK (0.4 units/ml) or rMYPT1K (44 μg/ml). Phosphorylation was initiated with one kinase, and then part of the reaction mixture was removed and continued with a second kinase. Incorporation of ^{32}P was estimated by Cerenkov counting as above. GST-MYPT1 (2 μM) was thiophosphorylated by PKA (1 μg/ml) for 150 min with 0.5 mM ATPγS (other conditions as in Ref. 9) and dialyzed against 20 mM Tris-HCl, pH 7.5, 85 mM KCl, 5 mM $MgCl_2$, 10 mM EGTA, and 4 mM dithiothreitol. The thiophosphorylated GST-MYPT1 was then subjected to phosphorylation with ROCK (0.4 units/ml) in 0.2 mM [γ-^{32}P]ATP. A non-thiophosphorylated GST-MYPT1 was phosphorylated by ROCK, as a control. Aliquots of each reaction were removed at intervals to measure phosphate incorporation and also for use in Western blots with the MYPT1^{pT696} antibody. At the end of the reaction period, the two samples were assayed for inhibition of native PP1c (0.5 nM) by using ^{32}P-MLC-20 (5 μM) as substrate (9).

Phosphorylation Site Analysis—Phosphorylated MYPT1 was digested either with endoprotease Lys-C (6 μg/mg) or V8 endoprotease E (V8 protease) (6 μg/mg) overnight, and phosphopeptides were separated by reverse phase HPLC. Radioactive peptides were collected and cross-linked to Immobilon P filters (Applied Biosystems) as described (22). The filters were placed in an ABI 474cLC Edman sequenator configured to collect the phenylthiohydantoin-derivatives immediately following cleavage of the N-terminal amino acid with trifluoroacetic acid. The amount of radioactivity released after each Edman cycle was determined by Cerenkov counting. The cleaved radioactive peptide (CRP) algorithm (fasta.bioch.virginia.edu/crp/) using lysine as the cleavage

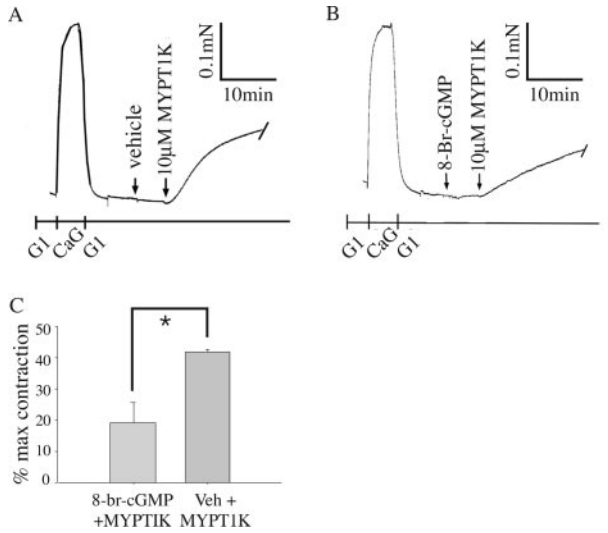

FIG. 1. ***In situ* treatment of smooth muscle strips with 8-bromo-cGMP reduces the calcium sensitization effect of exogenous MYPT-1 kinase (MYPT1K).** *A* and *B*, longitudinal β-escin-permeabilized ileum smooth muscle strips (200 μm [times] 4 mm) were isolated and placed in an intracellular solution containing 1 mM EGTA (G1), 10 mM EGTA (G10), or 30 μM Ca^{2+} (CaG). Strips were treated with vehicle (*A*) or 100 μM 8-bromo-cGMP (*B*) for 5 min and then contracted with 10 μM MYPT1K. *C*, pretreatment with 8-bromo-cGMP reduced the maximum contraction elicited by addition of MYPT1K. * indicates significance at $p < 0.04$.

point was used to identify all possible serine/threonine residues in the MYPT1K sequence that corresponded to release of radioactivity during Edman sequencing. To precisely identify each site, the experiment was repeated following digestion with V8 protease. CRP analysis was repeated using glutamic acid as the cleavage site. Cross-referencing release of radioactivity after cleavage at lysine with glutamic acid unambiguously identified three PKA/PKG phosphorylation sites on MYPT1.

Statistical Analysis—All results are reported as mean ± S.E. A Student's unpaired *t* test was used to determine the statistical significance of Ser-695 and Thr-696 phosphorylation in smooth muscles and of changes in smooth muscle contraction. $p < 0.05$ was considered significant.

RESULTS

Treatment of Smooth Muscle Strips with 8-Bromo-cGMP Attenuates the Ca^{2+}-sensitizing Effect of MYPT1K—Treatment of isolated smooth muscles with 8-bromo-cGMP is known to bring about relaxation in a Ca^{2+}-independent manner (13). The primary target for cGMP is PKG, yet the mechanisms by which the protein kinase brings about Ca^{2+} desensitization are unclear. We sought to determine whether phosphorylation of MYPT1 by PKG reduced the level of Ca^{2+} sensitization induced by MYPT1K. Addition of constitutively active rMYPT1K to permeabilized smooth muscle is known to phosphorylate MYPT1 (at Thr-696), inhibit endogenous SMPP-1M activity, and produce a marked Ca^{2+} sensitization (5, 8). The effect of pretreatment with 8-bromo-cGMP on contraction elicited by rMYPT1K was analyzed. Treatment of permeabilized muscle with rMYPT1K elicited a contraction that was 41.8 ± 0.9% of the maximum Ca^{2+} (CaG) contraction (Fig. 1, *A* and *C*). Pretreatment with 100 μM 8-bromo-cGMP before rMYPT1K administration significantly reduced the contraction to 19 ± 6.54% ($n = 5$, $p < 0.04$) of the maximum Ca^{2+} contraction (Fig. 1, *B* and *C*). The findings suggest a possible mechanism of Ca^{2+} desensitization, whereby phosphorylation of SMPP-1M or other proteins attenuates the Ca^{2+}-sensitizing actions of MYPT1K in smooth muscle.

Characterization of the Site Phosphorylated on rMYPT1 by PKA/PKG in Vitro—To investigate the hypothesis that PKG/PKA may bring about their Ca^{2+}-desensitizing effects through phosphorylation of MYPT1, we identified the phosphorylation sites by using a recombinant fragment of MYPT1 (C130). Fig. 2*A* (*inset*) shows that PKG efficiently phosphorylated C130 to ~3.5 mol of phosphate/mol of C130. Phosphoamino acid analysis revealed that the fully phosphorylated protein contained phosphoserine. Some phosphothreonine was seen if the *in vitro* phosphorylation reaction was carried out to 2.5 h. To identify the phosphorylation sites, C130 was phosphorylated with PKA or PKG in the presence of [γ-^{32}P]ATP, and the phosphorylated protein was digested with endolysine protease C. The digest was separated by reverse phase HPLC, and phosphopeptides were identified in column fractions by Cerenkov counting (Fig. 2*A*). Three peaks of radioactivity were identified and cross-linked to Immobilon P filters (Applied Biosystems, Inc.) as described (22). The Immobilon filters were placed in an automated Edman sequenator, and after each Edman cycle, the amount of radioactivity released from each cross-linked peptide was determined by Cerenkov counting (Fig. 2, *B–D*). Peak 1 yielded radioactivity at cycle 3; peak 2 at cycles 4, 9, and 12; and peak 3 at cycles 4 and 12. To confirm the identity of the sites, this cycle of analysis was repeated on phosphopeptides recovered from reverse phase HPLC separation after digestion of PKA/PKG-phosphorylated C130 with V8 endoprotease E (Fig. 2*E*). These three peptides were subjected to Edman cycling (Fig. 2 *F–H*). CRP analysis was then carried out using cleavage at glutamic acid as the reference point. Cross-referencing CRP analysis at lysine with glutamic acid unambiguously identified Ser-692, Ser-695, and Ser-852 (of the human isoform, corresponding to Ser-650, Ser-653, and Ser-808 on the chicken M130 protein) as sites phosphorylated by PKA/PKG on rMYPT1. Peak I (endoprotease Lys-C) was the peptide containing the Ser-852 site, and peak II (endoprotease Lys-C) contained Ser-692 and Ser-695. Analysis of a time course of phosphorylation of peptide II showed that the phosphorylation of Ser-692 and Ser-695 was not ordered, *i.e.* one site does not need to be phosphorylated before the other one can be. Some phosphorylation of threonine in peak 2 (endolysine protease C) and peak 3 (V8 E) was noted at later time points.

PKA/PKG Phosphorylation of MYPT1 Does Not Affect SMPP-1M Activity in Vitro—The effect of phosphorylation of MYPT1 by PKA/PKG on SMPP-1M activity was determined. Purified full-length rMYPT1 (M110) that had been incubated with the phosphatase catalytic subunit PP-1C was phosphorylated by PKG. As a control, parallel reactions using rMYPT1K were performed. Phosphatase assays were carried out with ^{32}P-labeled smooth muscle myosin as the substrate. Phosphorylation of recombinant SMPP-1M by PKG does not affect phosphatase activity (Fig. 3*A*). In contrast, phosphorylation of the enzyme by rMYPT1K dramatically inhibits activity, which corroborates previous work (8) showing that MYPT1K preferentially phosphorylates Thr-696, inhibiting SMPP-1M activity.

The Phosphorylation Sites for PKA, ROCK, and rMYPT1K Are Mutually Dependent—Because the *in situ* data suggested that pre-phosphorylation of MYPT1 by PKA was affecting calcium sensitization, we investigated the effect of pre-phosphorylation of GST-MYPT1 (M133 isoform) by PKA on the ability of known SMPP-1M inhibitory kinases, ROCK and MYPT1K, to phosphorylate rMYPT1. Pre-phosphorylation of GST-MYPT1 (M133) by PKA to 2 mol of phosphate/mol of GST-MYPT1 caused a dramatic decrease in the subsequent phosphorylation of MYPT1 by ROCK and 0.26 mol/mol in contrast to 1.60 mol/mol phosphorylation of MYPT1 by ROCK without PKA pre-phosphorylation (Fig. 3, *B* and *C*). The reverse reaction also occurs in which pre-phosphorylation of MYPT1 by ROCK decreased further phosphorylation by PKA (Fig. 3*C*). Note that the extent of phosphorylation by PKA in the sample

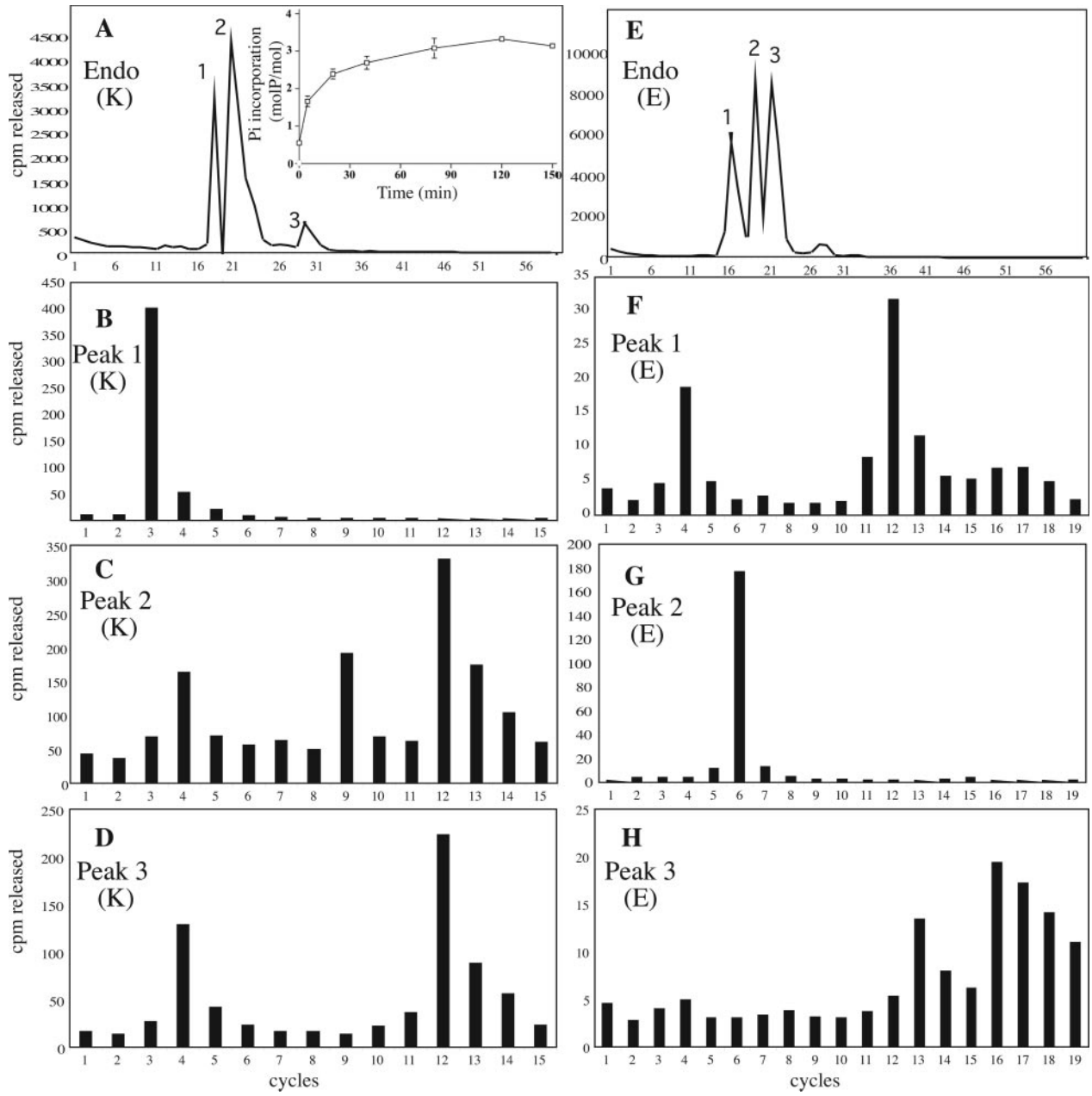

FIG. 2. **Identification of *in vitro* PKA/PKG phosphorylation sites on recombinant MYPT1 (C130).** *A*, *inset*, C130 was phosphorylated by PKG (shown) and PKA. The stoichiometry of phosphorylation was 3.5 mol of phosphate/mol of C130. *A*, three phosphopeptides were isolated from PKG-phosphorylated C130 on reverse phase HPLC following digestion with endoproteinase Lys-C. *B–D*, the PKG phosphorylation sites on ^{32}P-labeled C130 were identified by differential protease treatment followed by Edman sequencing with ^{32}P-release. Precise sites were identified using the CRP method. *E*, PKG-phosphorylated MYPT1 was cleaved with V8 endoproteinase E, and three peaks of radioactivity were identified. *F–H*, Edman cycle analysis was repeated by using cleavage at glutamic acid as the reference point to identify precisely the sites identified with endoproteinase Lys-C cleavage. Cross-referencing CRP analysis at lysine with glutamic acid identified Ser-692, Ser-695, and Ser-852 (human numbering) as the sites phosphorylated by PKA/PKG on MYPT1.

pre-phosphorylated by ROCK is about 0.95 mol of phosphate/mol (over a 150-min incubation) compared with about 2.2 mol of phosphate/mol (over a similar time period) with PKA alone (Fig. 3*B*). Similar experiments were carried out with PKA and rMYPT1K. Pre-phosphorylation of GST-MYPT1K by PKA reduced subsequent phosphorylation by rMYPT1K to about 0.4 mol phosphate/mol (Fig. 3*D*). By using the opposite sequence, pre-phosphorylation by rMYPT1K (to about 1.7 mol of phosphate/mol) attenuated phosphorylation by PKA (increase of only 0.25 mol of phosphate/mol) and essentially blocked phosphorylation by ROCK (Fig. 3*E*). These data suggest that the adjacent phosphorylation sites on MYPT1 for PKA, ROCK, and rMYPT1K are dependent on phosphorylation of the partner site. Although the emphasis in this article is on the pair of sites containing the inhibitory site (*i.e.* Ser-695 and Thr-696), it is evident that the adjacent sites containing the second major ROCK site (*i.e.* Ser-852 and Thr-853) may be subject to the same mutual dependence.

Another approach to assess the influence of the adjacent Ser-695 and Thr-696 sites was to use the synthetic substrate peptide (residues 689–700). Because Ser-691 (avian numbering) was identified as a PKA/PKG site, the Ser-691 to Ala mutant was used (see "Experimental Procedures") to simplify analysis. Both PKA and MYPT1K readily phosphorylated the peptide to 1 mol/mol. Phosphorylation site analysis using radioactivity release during Edman sequence analysis confirmed

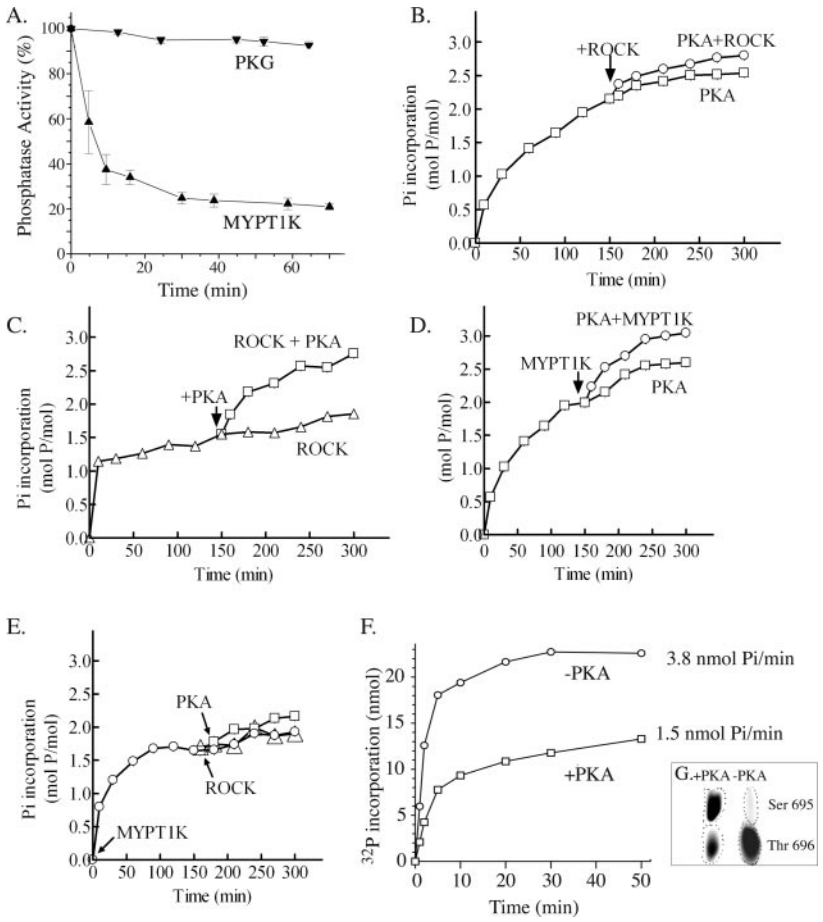

FIG. 3. **PKG/PKA phosphorylation does not affect SMPP-1M activity *in vitro*, pre-phosphorylation of MYPT1 by PKA decreases phosphorylation of MYPT1 by ROCK and MYPT1K *in vitro*, and the phosphorylation of Ser-695 and Thr-696 is mutually exclusive *in vitro*.** *A*, full-length recombinant MYPT1 was thiophosphorylated by PKG (and PKA, not shown) or MYPT1K prior to performing SMPP-1M phosphatase activity assays. *B*, phosphorylation of MYPT1 first by PKA and then by ROCK. 2 μM GST-MYPT1 was phosphorylated by PKA (1 μg/ml), and aliquots taken at indicated time intervals were assayed for ^{32}P incorporation (□). After 150 min, part of the reaction mixture was removed, and the phosphorylation reaction continued in the presence of ROCK (0.4 units/ml) up to 300 min (○). *C*, phosphorylation of 2 μM GST-MYPT1 first by ROCK (△) and then by PKA (□). *D*, phosphorylation of GST-MYPT1 first by PKA (□) and then by MYPT1K (44 μg/ml) (○). *E*, phosphorylation of GST-MYPT1 first by MYPT1K (○) and then by PKA (□) or ROCK (△). *F*, phosphorylation at Ser-695 by PKG/PKA precludes Thr-696 phosphorylation by MYPT-1 kinase. A peptide, ^{689}RQARRSTQGVTL^{700} (avian numbering), encoding the inhibitory phosphorylation region of MYPT1 with a Ser to Ala exchange at 691, was phosphorylated to 0.8 mol/mol with PKA and nonradioactive ATP. This phosphorylation substantially reduced the subsequent rate of phosphorylation by MYPT1K. The rates of phosphorylation with and without PKA treatment are indicated. *G*, phosphoamino acid analysis confirmed that increased Ser-695 phosphorylation by PKA reduced the Thr-696 phosphorylation by MYPT1K.

that PKA phosphorylated Ser-695, whereas rMYPT1K phosphorylated Thr-696. To test the ability of Ser-695 phosphorylation to interfere with phosphorylation of Thr-696, the peptide was pre-phosphorylated in the presence and absence of PKA by using nonradioactive ATP. After 30 min, rMYPT1K was added with [γ-^{32}P]ATP, and the reaction was followed over the indicated times (Fig. 3*F*). Phosphorylation of Thr-696 by rMYPT1K was considerably reduced after pre-phosphorylation of Ser-695. The rate of phosphorylation of the peptide without PKA pretreatment was 3.8 nmol of P_i/min *versus* 1.5 nmol of P_i/min with PKA pre-phosphorylation (Fig. 3*F*). Phosphoamino acid analysis confirmed a significant reduction in phosphothreonine content in the PKA-treated peptide compared with non-PKA-treated peptide (Fig. 3*G*). The reverse sequence, *i.e.* rMYPT1K followed by PKA, also blocked the ability of PKA to phosphorylate the substrate peptide at Ser-695 (data not shown).

In the final *in vitro* experiment, GST-MYPT1 (M133 isoform) was thiophosphorylated with PKA, dialyzed, and then subjected to phosphorylation by ROCK. (Further prolonged phosphorylation by PKA and [γ-^{32}P]ATP resulted only in the incorporation of 0.11 mol of phosphate/mol, suggesting saturation by thiophosphorylation.) Phosphorylation of non-thiophosphorylated GST-MYPT1 by ROCK resulted in the incorporation of 1.9 mol of phosphate/mol, but in the thiophosphorylated sample only 0.4 mol of phosphate/mol was incorporated (Fig. 4*A*). These data again suggested that both pairs of sites (Ser-695/Thr-696 and Ser-852/Thr853) were influenced. Western blots carried out over the reaction time course (with the pMYPT1^{T696} antibody) indicated a reduced level of phosphorylated Thr-696 in the thiophosphorylated sample (Fig. 4*B*). Phosphatase assays were carried out (using ^{32}P-labeled MLC-20) to determine the extent of inhibition of PP1c by the two GST-MYPT1 samples (*i.e.* aliquots were removed at the end of the reaction shown in Fig. 4*A*). The IC_{50} for GST-MYPT1 phosphorylated only by ROCK was about 0.25 nM compared with about 2.4 nM for the PKA/ROCK sample (Fig. 4*C*). These data indicate the reduced level of phosphorylated Thr-696 in the sample thiophosphorylated with PKA.

Overall, the *in vitro* data are consistent with a potential regulatory mechanism, in which phosphorylation of Ser-695 by PKA/PKG could influence SMPP-1M activity by reducing phosphorylation of the inhibitory site by rMYPT1K or other kinases. In effect, the phosphorylation by PKA would extend a more active state for SMPP-1M.

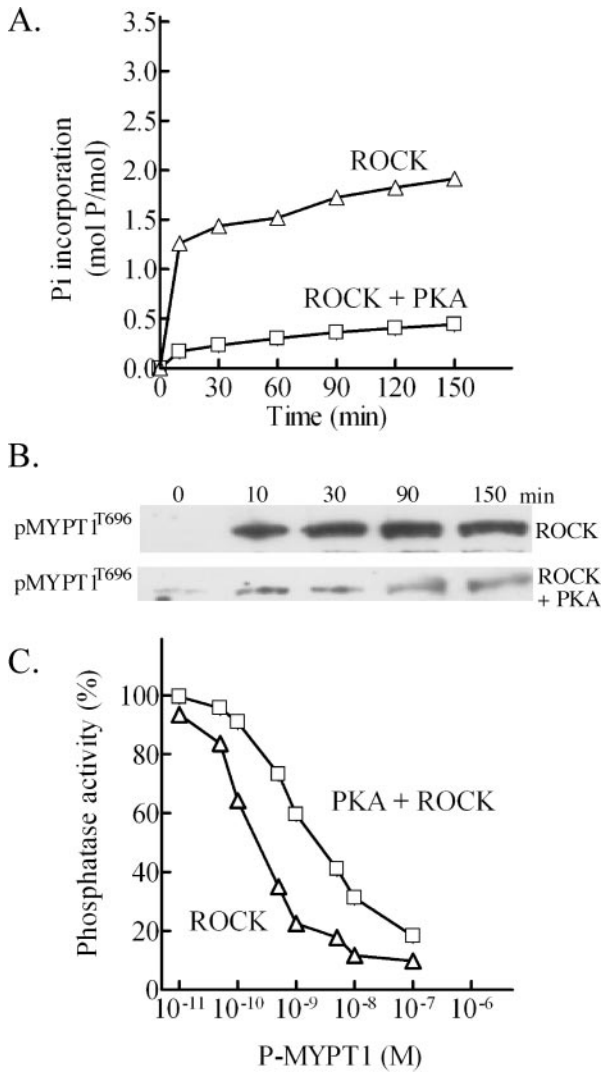

FIG. 4. **Pre-phosphorylation with PKA reduces Thr-696 phosphorylation by ROCK *in vitro* and increases the IC_{50} for ROCK inhibition of SMPP-1M activity.** *A*, GST-MYPT (2 μM) was thiophosphorylated by PKA for 150 min in the presence of 0.5 mM ATPγS and 5 mM Mg^{2+}. Thiophosphorylated GST-MYPT1 was then phosphorylated by ROCK (0.4 units/ml) in the presence of [^{32}P]ATP for 150 min (□) and compared with phosphate incorporation of GST-MYTP1 phosphorylated by ROCK alone (△). *B*, aliquots of GST-MYPT1 thiophosphorylated by PKA or GST-MYPT1 phosphorylated by ROCK alone were taken at the indicated time points and immunoblotted with pMYPT1^{T696} antibody. *C*, inhibition of PP-1c by GST-MYPT1 phosphorylated by ROCK alone (△) or with thiophosphorylation by PKA (□). The IC_{50} for ROCK alone was 0.25 nM compared with 2.4 nM with PKA thiophosphorylation. The phosphatase activity in the absence of phospho-MYPT1 was taken as 100%.

Ser-695 Is Phosphorylated in Smooth Muscle in Response to 8-Bromo-cGMP, and Phosphorylation of This Site Blocks Phosphorylation of Thr-696—To determine whether SMPP-1M is regulated by a mutual exclusion mechanism involving phosphorylation of Ser-695 under *in vivo* conditions in smooth muscle, it was necessary to generate a phosphospecific antibody to this site. This was raised in rabbits using the synthetic phosphopeptide ^{689}RQSRRpSTQGVTL^{700} as the antigen. The antibody was affinity-purified over a ^{689}RQSRRpSTQGVTL^{700} peptide affinity column. To determine the selectivity and sensitivity of the antibody, recombinant MYPT1 was phosphorylated to completion with MYPT1K (~2 mol/mol) and PKA (2.5–3 mol/mol). Radioactive release by Edman sequencing was used to confirm that Thr-696 and Ser-695 were phosphorylated, respectively. The phosphoproteins were diluted and

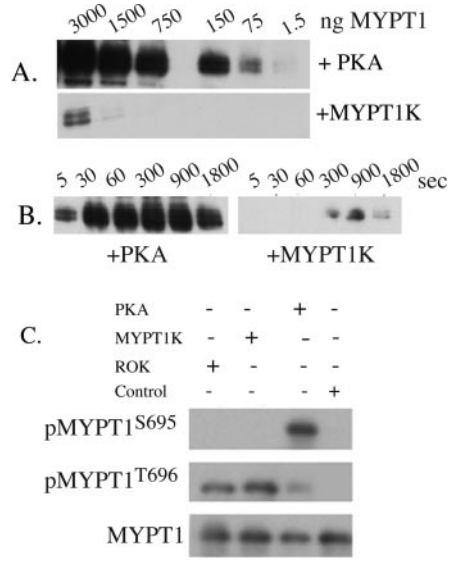

FIG. 5. **Characterization of a polyclonal phosphospecific antibody directed against Ser-695 of MYPT-1.** *A*, decreasing amounts of MYPT1 (3000 to 1.5 ng) phosphorylated with MYPT1K or PKA were blotted for pMYPT1^{S695}. *B*, 3 μg of MYPT1 was phosphorylated with MYPT1K or PKA, and the reaction was stopped at the indicated time points and immunoblotted using the pMYPT1^{S695} antibody. *C*, MYPT1 was phosphorylated with MYPT1K, Rho kinase, PKA, or vehicle control and blotted with pMYPT1^{S695} antibody, pMYPT1^{T696} antibody, or antibody against total MYPT1.

tested against the pMYPT1^{S695} antibody. Fig. 5*A* shows that the pMYPT1^{S695} antibody has a high degree of selectivity toward phosphorylated Ser-695 over phosphorylated Thr-696 at a 1/10,000-fold dilution (or ~0.1 μg of antibody/ml). The figure shows that at the highest concentration of MYPT1 tested (3 μg), the pMYPT1^{S695} antibody shows an ~100-fold selectivity toward pMYPT1^{S695} over pMYPT1^{T696}. At <0.75 μg of MYPT1, no cross-reactivity with pMYPT1^{T696} was detected. A time course study shows the sensitivity and selectivity of the pMYPT1^{S695} antibody toward Ser-695 over Thr-696 at 3 μg of MYPT1 (Fig. 5*B*). A commercial pMYPT1^{T696} antibody recognizes Thr-696 phosphorylation by MYPT1K and ROCK, and there is slight recognition of PKA phosphorylated MYPT1 by this antibody (Fig. 5*C*). The pMYPT1^{S695} antibody only recognized PKA-phosphorylated MYPT1.

By having demonstrated the selectivity of the pMYPT1^{S695} antibody *in vitro*, we used the antibody to examine the phosphorylation state of Ser-695 in smooth muscle. For the ileal smooth muscle experiments, squares of muscle (5 mg w/w) were permeabilized in silicone dishes with β-escin (5). The intracellular concentration of MYPT1 in smooth muscle has been determined to be between 0.8 and 2 μM (19). By assuming an intracellular space of 30% (w/w), the estimated amount of MYPT1 in smooth muscle is 62–154 ng/mg (w/w.). Based on these calculations, 5 mg of smooth muscle provided sufficient levels of MYPT1 to measure the phosphorylation state of Ser-695 at stoichiometries of phosphorylation <0.1 mol/mol. After maximum Ca^{2+} contraction, the muscles were washed extensively in G10 and treated for 10 min with 100 μM 8-bromo-cGMP or vehicle. The muscles were flash-frozen in liquid N_2-cooled Freon, and extracts were prepared for Western blot analysis with the pMYPT1^{S695} antibody. Ser-695 is basally phosphorylated in smooth muscle at varying levels, and treatment with 8-bromo-cGMP significantly ($n = 38$, $p < 0.01$) increases the Ser-695 phosphorylation state (Fig. 6, *A* and *C*). To determine directly the effects of phosphorylation of Ser-695 and Thr-696 in smooth muscle, squares of ileum were permeabilized with β-escin and treated with either vehicle (water) or

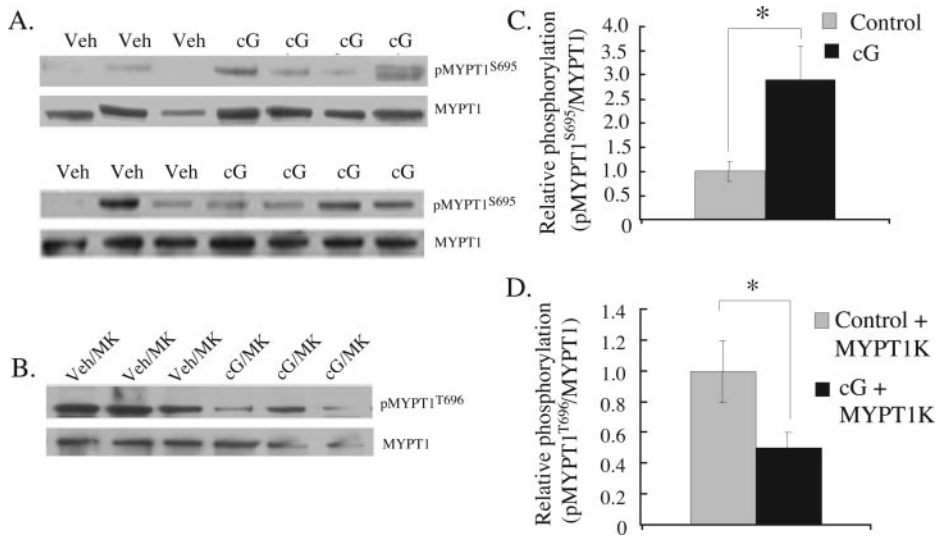

FIG. 6. **Ser-695 phosphorylation of native MYPT1 is increased by cGMP stimulation, and phosphorylation of this site blocks phosphorylation of Thr-696.** *A*, permeabilized rabbit ileum was stimulated with 100 μM 8-bromo-cGMP (*cG*) or vehicle (*veh*) for 10 min and immunoblotted with the pMYPT1^{S695} antibody or antibody against total MYPT1. Representative Western blot results of multiple experiments are shown. *B*, permeabilized rabbit ileum was stimulated with 100 μM 8-bromo-cGMP (*cG*) or vehicle (*veh*) for 5 min and then treated with 10 μM MYPT1K and immunoblotted with the pMYPT1^{T696} antibody or antibody against total MYPT1. *C*, summarized data of densitometric analyses of Ser-695 phosphorylation in permeabilized ileum. *Control*, ileum without stimulation; *cG*, ileum stimulated for 10 min with 100 μM 8-bromo-cGMP. (* indicates significance at $p < 0.01$.) *D*, summarized data of densitometric analysis of Thr-696 phosphorylation in permeabilized ileum. *Control + MYPT1K*, no stimulation before MYPT1K administration; *cG + MYPT1K*, 100 μM 8-bromo-cGMP administered before MYPT1K for 5 min. (* indicates significance at $p < 0.04$.) Mean density of phosphorylated MYPT1 *versus* the total MYPT1 density for the controls was expressed as 1 arbitrary unit.

8-bromo-cGMP for 5 min, followed by rMYPT1K for 10 min. Muscles were flash-frozen in liquid N_2-cooled Freon, and extracts were blotted for phosphorylation of Thr-696 and total MYPT1. Pretreatment of muscles with 8-bromo-cGMP significantly ($n = 17$, $p < 0.04$) reduced Thr-696 phosphorylation as compared with vehicle-treated tissues (Fig. 6, *B* and *D*). The finding that phosphorylation of Ser-695 reduces phosphorylation of Thr-696 by MYPT1K explains the attenuation of force development shown in Fig. 1 following addition of the kinase to 8-bromo-cGMP-treated muscles.

DISCUSSION

The results obtained in this work show that Ser-695 on MYPT1 is phosphorylated in smooth muscle in response to cGMP activation, and phosphorylation of this site reduces subsequent phosphorylation of Thr-696. These findings suggest a possible mechanism of Ca^{2+} desensitization involving mutual exclusion of phosphorylation. Activation of PKG/PKA leads to phosphorylation of MYPT1, which prevents phosphorylation of Thr-696 by MYPT1K and likely ROCK, thereby blocking inactivation of SMPP-1M (Fig. 7*A*). Such a mechanism of regulation is consistent with the fine level of control of contraction that is required of smooth muscle under normal physiological conditions.

The contractile state of any smooth muscle is governed by the balance of opposing activities of MLCK and SMPP-1M, which controls the steady state phosphorylation of MLC-20. Activation or inhibition of either the kinase or phosphatase results in profound effects on the level of phosphorylation of MLC-20 and, therefore, the contractile state of the muscle. Regulation of SMPP-1M through phosphorylation of MYPT1 has been established as a primary mechanism by which the contractile state can be controlled independently of [Ca^{2+}]. Several protein kinases that specifically target Thr-696 have been identified in addition to MYPT1K, including ROCK, myotonic dystrophy kinase, and integrin-linked protein kinase (5, 6, 8–10). Phosphorylation of Thr-696 by these kinases in smooth muscle has been shown to inhibit SMPP-1M and cause Ca^{2+} sensitization

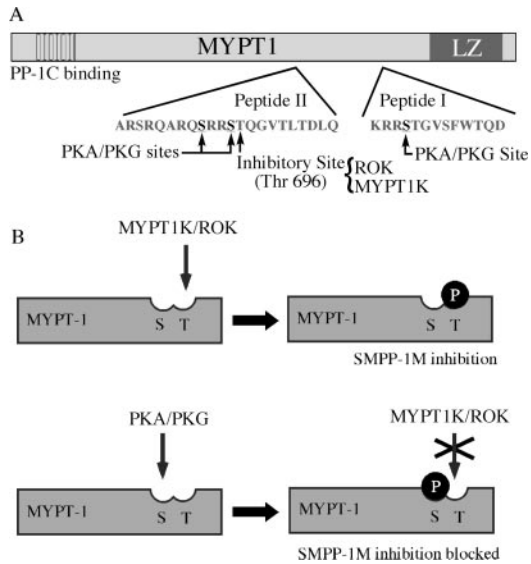

FIG. 7. *A*, PKA/PKG phosphorylation sites identified on MYPT1 *in vitro*. Ser-692 and Ser-695 were found in peptide II, and phosphorylation of Ser-695 was found to inhibit phosphorylation of the adjacent Thr-696 MYPT1K/ROK inhibitory site. Ser-852 was also identified as a PKA/PKG phosphorylation site in peptide I and may lead to inhibition of phosphorylation of the adjacent Thr-853 site. *LZ*, leucine zipper. *B*, proposed mechanism for calcium desensitization induced by cyclic nucleotide kinases in smooth muscle. Calcium-independent contraction elicited by kinases such as ROCK or MYPT1K is caused by inhibition of myosin phosphatase activity by phosphorylation of Thr-696 on MYPT1. Phosphorylation of Thr-696 is inhibited by Ser-695 phosphorylation by PKA/PKG, thus effectively leading to indirect activation of SMPP-1M activity by cyclic nucleotide kinases.

(4–6, 9, 10, 23). Ca^{2+} sensitization/desensitization pathways are essential for maintenance of normal smooth muscle tone, and abnormalities in these pathways have been shown to be key components in the pathogenesis of diseases such as hypertension and asthma (3, 4, 24). Signaling through G protein-coupled Ca^{2+} sensitization pathways has been shown to be

up-regulated in multiple models of hypertension, cerebral vasospasm, bronchial asthma, and erectile dysfunction, and RhoA or ROCK inhibitors can be effective for treatment of these conditions (3, 24–28).

We have shown herein that 8-bromo-cGMP greatly attenuates the Ca^{2+}-sensitizing actions of MYPT1K, demonstrating the capacity for PKG to regulate SMPP-1M in smooth muscle. Although MYPT1 is clearly phosphorylated by PKA/PKG in smooth muscle, this does not affect SMPP-1M activity directly. The question then arises, how can activation of PKG/PKA affect contractile activity through altering SMPP-1M activity? *In vitro* data showed that phosphorylation of recombinant full-length MYPT1 by PKA reduced subsequent phosphorylation by ROCK and MYPT1K. The reverse situation also occurred. *In vitro* data also showed that thiophosphorylation of MYPT1 by PKA reduced the ability of ROCK to inhibit phosphatase activity, supporting an indirect mechanism of phosphatase activation. We found three PKA/PKG phosphorylation sites on MYPT1 (Ser-692, Ser-695, and Ser-852) (Fig. 7*A*). We chose to pursue Ser-695 because this site was adjacent to the inhibitory Thr-696 site. Our hypothesis was that phosphorylation at this site could affect SMPP-1M activity indirectly through mutual exclusion of phosphorylation. A similar mechanism could apply to Ser-852 and Ser-853, but that was not pursued in this study. The level of reduction in phosphorylation observed *in vitro* on full-length recombinant MYPT1 suggested that both the inhibitory Thr-696 and the Th-r853 sites on MYPT1 might be affected by mutual exclusion of phosphorylation. *In vitro* data using synthetic peptides and phosphoamino acid analysis also showed that PKA phosphorylation reduces the rate of MYPT1 phosphorylation by MYPT1K, and Thr-696 phosphorylation is reduced dramatically as Ser-695 phosphorylation increases.

Regulation of enzyme activity by mutual exclusion of phosphorylation is well described. Examples of this mechanism of regulation occur with both hormone-sensitive lipase and 3-hydroxy-3-methylglutaryl-CoA reductase (29–31). Based on our data, we suggest that phosphorylation of Ser-695 in response to cGMP/cAMP alters the balance of SMPP-1M activity through competition with opposing RhoA-mediated Ca^{2+}-sensitizing pathways acting through MYPT1K or ROCK (or other kinases). In the mechanism outlined in Fig. 7*B*, the balance of Ser-695 and Thr-696 phosphorylation acts as a homeostatic system to check the impact of opposing Ca^{2+}-desensitizing/sensitizing pathways. Such a mechanism provides exquisite control of the smooth muscle contractile state, allowing muscles to respond in a highly coordinate fashion to a given physiological situation. If this mechanism is accepted then a critical corollary is the role of the phosphatase(s) involved in dephosphorylation of the various sites. Obviously, the lifetime of any blocking effect would reflect the activity of the phosphatase involved. Intuitively, it might be predicted that the activity of the phosphatase for Ser(P)-695 (or Ser(P)-852) would differ from that for Thr(P)-696 (or Thr(P)-853). Equal phosphatase activity for Ser(P)-695 and Thr(P)-696 seems to be counter productive.

We noted some Ser-695 phosphorylation in control tissues. In the permeabilized ileum smooth muscle, some of this could have been due to inconsistencies in stretch of the muscle. There is also likely a basal level of phosphorylation, which further supports the idea that Ca^{2+} desensitization is not necessarily an "on or off" mechanism of smooth muscle regulation, rather a method of making fine adjustments to various physiologic conditions, which can lead to pathologic conditions if the system goes awry (3, 4, 24).

Several mechanisms probably are involved in the Ca^{2+} desensitization of smooth muscle induced by an increase in cAMP/cGMP concentrations. Our results suggest one possibility, but other mechanisms should be considered. Perhaps most interesting in light of the above data is the suggestion that phosphorylation of Thr-853 (*i.e.* the second major site for ROCK) of MYPT1 reduces binding to myosin leading to decreased SMPP-1M activity (32). The *in vitro* evidence, presented above, suggests that both ROCK sites would be blocked by phosphorylation of adjacent PKA sites. Another mechanism may involve different cellular locations of MYPT1, depending on its state of phosphorylation (15, 16). With respect to other proteins, additional targets for PKG/PKA in smooth muscle include Hsp20 and telokin (33). In the case of telokin the *in vitro* PKG/PKA sites have been identified, and phosphorylation promotes Ca^{2+} desensitization in permeabilized smooth muscle (34–37). Thus it is possible that phosphorylation of Ser-695 acts in collaboration with other mechanisms to promote the relaxation of smooth muscle. The combination of different PKG/PKA targets may provide an attractive explanation underlying the varied contractile responses in different smooth muscles.

In summary, we identified three PKA/PKG phosphorylation sites on MYPT1. Phosphorylation has no direct effect on SMPP-1M activity, so we pursued the Ser-695 phosphorylation site as a potential mechanism of SMPP-1M activation through mutual exclusion of phosphorylation. This work shows that Ser-695 on MYPT1 is phosphorylated in smooth muscle in response to 8-bromo-cGMP, and phosphorylation of this site prevents phosphorylation of the inhibitory site Thr-696. Our proposed mechanism of Ca^{2+} desensitization is activation of PKG/PKA, leading to phosphorylation of Ser-695 of MYPT1, which prevents phosphorylation of Thr-696 by MYPT1K and likely RhoA-activated kinases, thereby blocking inactivation of SMPP-1M. This mechanism of Ca^{2+} desensitization is a potential mechanism of fine control over smooth muscle tone, which is essential for maintenance of normal physiology.

Acknowledgment—We thank to Elizabeth H. Snyder for help with manuscript preparation.

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