Role of Myosin Phosphatase Isoforms in cGMP-mediated Smooth Muscle Relaxation*

Received for publication, June 7, 2001
Published, JBC Papers in Press, August 2, 2001, DOI 10.1074/jbc.M105275200

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In vitro experiments showing the activation of the myosin phosphatase via heterophilic leucine zipper interactions between its targeting subunit (MYPT1) and cGMP-dependent protein kinase I suggested a pathway for smooth muscle relaxation (Surks, H. K., Mochizuki, N., Kasai, Y., Georgescu, S. P., Tang, K. M., Ito, M., Lincoln, T. M., and Mendelsohn, M. E. (1999) Science 286, 1583–1587). The relationship between MYPT1 isoform expression and smooth muscle responses to cGMP signaling in vivo has not been explored. MYPT1 isoforms that contain or lack a C-terminal leucine zipper are generated in birds and mammals by cassette-type alternative splicing of a 31-nucleotide exon. The avian and mammalian C-terminal isoforms are highly conserved and expressed in a tissue-specific fashion. In the mature chicken the tonic contracting aorta and phasic contracting gizzard exclusively express the leucine zipper positive and negative MYPT1 isoforms, respectively. Expression of the MYPT1 isoforms is also developmentally regulated in the gizzard, which switches from leucine zipper positive to negative isoforms around the time of hatching. This switch coincides with the development in the gizzard of a cGMP-resistant phenotype, i.e. inability to dephosphorylate myosin and relax in response to 8-bromo-cGMP after calcium activation. Furthermore, association of cGMP-dependent protein kinase I with MYPT1 is detected by immunoprecipitation only in the tissue that expresses the leucine zipper positive isoform of MYPT1. These results suggest that the regulated splicing of MYPT1 is an important determinant of smooth muscle phenotypic diversity and the variability in the response of smooth muscles to the calcium desensitizing effect of cGMP signaling.

Smooth muscle contraction is initiated by the phosphorylation of the regulatory myosin light chain (MLC\textsubscript{20}) by the calcium/calmodulin-dependent activation of the myosin light chain kinase (MLCK) (1). Relaxation is effected by the dephosphorylation of MLC\textsubscript{20} by the smooth muscle myosin phosphatase (SMMP). Complexity is brought to this system by accessory proteins and signaling pathways that regulate the smooth muscle contractile state (reviewed in Refs. 2 and 3).

The SMMP is a target of signals that are positive and negative modulators of smooth muscle tone. SMMP is a heterotrimERIC protein composed of the 37-kDa catalytic subunit (PP1c\textsubscript{6}), the 130/133-kDa myosin targeting subunit (MYPT1, also referred to as MBS), and the 21-kDa M21 subunit (4–6). MYPT1 targets the catalytic subunit to MLC\textsubscript{20} (7, 8) and in this way confers substrate specificity to the phosphatase, whereas the function of the M21 subunit is unknown. Activation of the Rho kinase signaling pathway leads to phosphorylation of the MYPT1 subunit, resulting in inhibition of myosin phosphatase activity and an increase in smooth muscle tone (9–13). This signaling pathway is thought to determine the calcium-sensitizing effect of \textalpha-ADRENERGIC stimulation, for example, in which greater force is produced at a given calcium concentration than when force is activated by calcium alone (10, 12, 14, 15).

Activation of guanylate cyclase by nitric oxide (NO) or atrial natriuretic peptide and the increase in intracellular cGMP has the opposite effect (16–18), desensitizing the contractile apparatus to activating concentrations of calcium (19, 20). Calcium desensitization is one of several proposed mechanisms by which the cGMP-dependent protein kinase (cGKI) effects smooth muscle relaxation (reviewed in Refs. 21 and 22). SMMP was recently suggested to be one of the final mediators of this calcium desensitization signaling pathway. SMMP activity is enhanced in vitro by dimerization of MYPT1 with the cGMP-dependent protein kinase \textalpha (cGK\textalpha) via leucine zipper motifs present in the C terminus of MYPT1 and the N terminus of cGK\textalpha (23).

It has long been appreciated that smooth muscle tissues display considerable diversity with respect to both their basic contractile properties as well as their responses to modulating signals (reviewed in Ref. 3). As the NO/cGMP-mediated relaxation pathway was elucidated, it was also appreciated that smooth muscle tissues differ in their sensitivity to this pathway (24–27). The molecular basis of this smooth muscle functional diversity is not well understood. Since SMMP is a target of signaling pathways that modulate smooth muscle tone, we hypothesized that SMMP isoform differences may play an important role in the variable response of smooth muscle tissues to this signal. In mammals isoforms of the MYPT1 subunit of SMMP have been identified that either contain or lack the hexahydro-1,4-diazepine; IP, immunoprecipitation; RT-PCR, reverse transcriptase-polymerase chain reaction; BES, N,N-bis(2-hydroxy-ethyl)-2-aminoethanesulfonic acid; DTT, dithiothreitol; SMCs, smooth muscle cells; PSS, physiological saline solution.

* This work was presented in part at the Biophysical Society Meeting, 2000 (72) and 2001 (73). 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.

§ Supported by American Heart Association Ohio Valley Affiliate Postdoctoral Research Fellowship Award 0020332B and funds from University Hospitals of Cleveland.

¶ Supported by National Institutes of Health Grant RO1 HL64137.

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1 The abbreviations used are: MLC\textsubscript{20}, regulatory myosin light chain; MLCK, myosin light chain kinase; SMMP, smooth muscle myosin phosphatase; MYPT1, myosin targeting subunit; NO, nitric oxide; cGKI, cGMP-dependent protein kinase; nt, nucleotide; bp, base pair; 8-Br-cGMP, 8-bromo-cGMP; ML-9, 1-(5-chloronaphthalenesulfonyl)-1-fluor...
The terminal leucine zipper (8, 28). Surprisingly, the terminal leucine zipper motif has not been identified previously in chicken MYPT1, yet it is highly conserved in mammals and the worm (reviewed in Ref. 29). In this study we demonstrate that chicken MYPT1 isoforms that contain or lack the terminal leucine zipper are generated by cassette-type alternative splicing of a 31-nucleotide (nt) alternative exon. The MYPT1 isoforms are expressed in a tissue-specific and developmentally regulated fashion. Tissues that express MYPT1 isoforms that lack the terminal leucine zipper fail to calcium desensitize in response to cGMP, and the phenotypic switch of the gizzard at hatching from MYPT1 leucine zipper positive to negative coincides with a switch from sensitivity to resistance to cGMP-mediated calcium desensitization. These results provide a mechanistic explanation for the differential sensitivity of smooth muscle tissues to the calcium-desensitizing (relaxing) effect of the NO/cGMP signaling pathway.

EXPERIMENTAL PROCEDURES

Isolation and Analysis of RNA—Fertile White Leghorn chicken (Gallus gallus) eggs were obtained from Squire Valleevue Farm (Cleveland, OH). Eggs were incubated at 38°C in humidified incubators (after decapitation) from embryonic chicks from 8 to 21 days of incubation (hatching is 21 days) and from chicks 1–12 days after hatching. Organs from Harlan Sprague-Dawley adult rats (Rattus norvegicus) were harvested after lethal CO2 inhalation. After organs were stripped of adventitia, the smooth muscle tissues were dissected, frozen in liquid nitrogen, and total RNA isolated as described previously (30). Quantification of MYPT1 and M21 isoform splice variants was performed by RT-PCR using total RNA as described previously (30) with minor modifications. Oligonucleotide primer sets were designed with the PRIMER EXPRESS (Version 1.0) program, using cDNA sequences. Primer sets were tested by ethidium bromide staining, and images captured with a GelDoc 1000 (Bio-Rad). Bands were quantified using MultiAnalyt/Macintosh software (Bio-Rad). Omission of RT in PCRs yielded no product (data not shown). The accuracy of the measured isoform ratios was determined by varying the amount of input RNA and observing a constant ratio, as described previously (30, 31). Selected products of the RT-PCR reactions were subcloned into a plasmid and sequenced by standard techniques (30) to determine their identity.

Cloning of the Chicken MYPT1 Gene—Total DNA was isolated from chicken liver tissue using the Puregene DNA isolation kit (Gentra Systems, Inc.). A PCR-amplified genomic fragment of the MYPT1 gene was generated using the Expand Long Template PCR System (Roche Molecular Biochemicals) with the oligonucleotide primers listed above. The ~4.6-kilobase fragment was subcloned and sequenced by standard methods. Greater than 95% of the genomic clone was sequenced in both directions, with the remainder sequenced in one direction. The sequence of the exon splice sites was confirmed by sequencing these portions from a second, independent PCR clone.

Force Measurement in Skinned Smooth Muscle Strips—Chicken gizzard and aorta strips (290–1000 × 80–150 × 20–150 μm) were prepared as described previously (30). The strips were mounted between two aluminum foil T clips and permeabilized (skinned) at room temperature for 15 (gizzard) or 30 min (aorta) in relaxing solution (pCa 9 in mM) containing activation solution (pCa 4 in mM), 58.33 KMS, 5 EGTA, 5.19 CaCl2, 4.43 MgCl2, 5.27 ATP, 25 creatine phosphate, 25 BES, final pH to 7.1 with 1 x KOH or pCa 6 (in mM), 61 KMS, 5 EGTA, 3.92 CaCl2, 8.94 MgCl2, 5.20 ATP, 25 creatine phosphate, 25 BES, final pH to 7.1 with 1 x KOH. After force reached steady state, the strips were transferred to solution containing activating solution (pCa 4) and progressively increasing concentrations of 8-Br-cGMP (Sigma) (10–7−10−4 m). Finally, strips were transferred to relaxing solution (pCa 9) to demonstrate complete relaxation. A force versus time tracing was recorded on a digital oscilloscope (Nicolet). This tracing was used to calculate the mean percent force reduction in response to 8-Br-cGMP from each experiment (pCa 4) and submaximal (pCa 6) activation. Results are reported as the mean force reduction ± S.D. Additional activated strips were transferred to a well containing activating solution (pCa 4) and 1-(5-chloronaphthalenesulfonfonyl)-1H-hexahydro-1,4-diazepine (ML-9, 200 μM), a selective MLCK inhibitor (32) in the presence or absence of 8-Br-cGMP (10−4 m). A force versus time tracing was used to determine the rate of relaxation. Results are reported as the mean ± S.D.

Measurement of Myosin Light Chain Phosphorylation—To determine the extent of phosphorylation of MLC20, a glycerol/urea-polyacrylamide gel electrophoresis procedure (33) was adapted to resolve the phosphorylated and unphosphorylated forms of MLC20 using a mini-gel apparatus ( Hoeffer). Chicken aortic and gizzard strips were prepared and subjected to skinned muscle permeabilization as described above. Strips were washed in acetone containing 10 mM DTT three times to remove excess trichloroacetic acid and air-dried for 30 min after the final wash. Samples were frozen in liquid nitrogen, pulverized, placed in 50 μl of sample buffer (8 M urea, 10 mM DTT, glycerol (5% v/v), bromphenol blue (0.1% w/v)), and allowed to extract for 1 h, with frequent vortexing, at room temperature. The samples were resolved by electrophoresis through a 10% polyacrylamide (18:1 acrylamide/bisacrylamide), 40% glycerol (v/v) gel (20 μm Tris, pH 8.6; 22 μm glycine) for 3 h at 400 V. Resolved proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech) in transfer buffer (25 mM Tris, pH 8.2; 192 mM glycine, 10% methanol (v/v)) at 220 mA for 20 min. Membranes were blocked with 1% bovine serum albumin (w/v) in wash buffer (10 mM Tris, pH 7.4; 150 mM NaCl, 0.1% Tween 20 (v/v)) for 1 h, incubated with a monoclonal antibody against MLC20 (mouse IgM clone MY-21, Sigma) for 1 h, washed for 1 h, and incubated with the secondary antibody (goat anti-mouse IgM conjugated with alkaline phosphatase, Sigma) for 1 h. Both primary and secondary antibodies were diluted at 1:3000 with 0.1% bovine serum albumin (w/v) in wash buffer. After final washing, phosphorylated and unphosphorylated forms of MLC20 were detected by developing the membrane in alkaline phosphatase substrate buffer (100 mM NaCl, 50 mM Na2HPO4, pH 9.5, 50 mM MgCl2, 0.01% bromophenol blue, 3-indolyl phosphate, 400 μM nitro blue tetrazolium). Membranes were digitally scanned (UMAX), and bands were subsequently quantified using Scion Image Beta 4.0.2 software (Scion Corp.). The percentage of MLC20 phosphorylated was calculated by determining the ratio of the phosphorylated MLC20 band versus the total MLC20 (phosphorylated + unphosphorylated species) identified by the monoclonal antibody. Results are reported as mean phosphorylated MLC20 ± S.D.

Immunoprecipitation of MYPT1 and cGKI—Chicken aortic and gizzard strips were harvested and permeabilized as described above and subsequently incubated in pCa 4 activating solution treated with 8-Br-cGMP (10−4 m) or an equal volume of vehicle alone (PSS) for 15 min. In addition, aortas were harvested from Harlan Sprague-Dawley adult rats (R. norvegicus) after lethal CO2 inhalation. Vessels were quickly removed, rinsed in PSS containing 1.6 mM CaCl2 (PSS-Ca), stripped of fat and adventitia, and dissected into small smooth muscle strips (∼5 × 2 × 2 mm). Rat strips were activated by incubation in PSS-Ca containing KCl (80 mM) substituted isotonically for NaCl in the presence or absence of 8-Br-cGMP (10−4 m) for 15 min. Both rat and chicken strips were homogenized in 150 μl of lysis buffer (20 mM Tris, pH 7.5; 150 mM NaCl, Triton X (1% v/v), protease inhibitor mixture (10% v/v, Sigma)) on ice. Lysates were sonicated for 10 s on ice and clarified by centrifugation at 10,000 × g for 10 min. Total protein of the clarified lysates was determined by the Bradford method (Bio-Rad). A 100-μg aliquot of lysate was preincubated with 10% v/v protein G-agarose bead solution (Sigma, 50% v/v in phosphate-buffered saline) for 2 h at 4°C. Samples were centrifuged at 10,000 × g for 1 min, and supernatant was col-

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isolated. Precleared lysates were incubated overnight at 4 °C with either a monoclonal antibody against chicken MYPT1 (mouse IgG1 clone ASC.M130, Babco, 1:1000) or a polyclonal antibody against the common C terminus of cGKI (rabbit IgG clone anti-PKG CT, Stressgen, 1:100). A 10% v/v protein G-agarose bead solution was added to the lysates and incubated for 1 h at 4 °C. The samples were centrifuged at 10,000 × g for 1 min, and the pellet washed twice with lysis buffer. Pellets were resuspended in 20 μl of SDS sample buffer (62.5 mM Tris-HCl, pH 6.8; 2% w/v SDS, 10% v/v glycerol, 50 mM DTT, 0.01% w/v bromphenol blue), heated to 95 °C for 5 min, and separated by electrophoresis through a 7.5% polyacrylamide gel (29:1 acrylamide/bisacrylamide) for 1 h at 25 mA. Resolved proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech) in transfer buffer (25 mM Tris, pH 8.2; 192 mM glycine, 10% methanol (v/v)) at 230 mA for 45 min. Membranes were blocked with 5% nonfat dry milk (w/v) in wash buffer for 1 h, incubated with primary antibody for 1 h, washed for 30 min, and incubated with the secondary antibody for 1 h. Blots of lysates immunoprecipitated with anti-PKG CT were probed with primary antibody anti-PKG CT (1:800) followed by secondary antibody goat anti-rabbit (1:1600). After the final washing, the signals were visualized with a chemiluminescent reaction (Kirkegaard & Perry Laboratories).

FIG. 1. The chicken MYPT1 C-terminal leucine zipper variable isoforms are generated by cassette-type alternative splicing of a 31-nucleotide exon. a, the 3′ region of chicken MYPT1 genomic DNA was compared with RT-PCR products obtained using oligonucleotide primers flanking this alternative exon. This is represented in schematic form with amino acid sequences indicated above and below the nucleotide sequence. Skipping of the alternative exon (shaded box) codes for the leucine zipper motif, whereas exon inclusion shifts the reading frame and codes for a C terminus that lacks the leucine zipper motif. A cryptic 5′ donor splice site located within the alternative exon can generate an additional MYPT1 splice variant indicated by gray lines. Exons and introns are not to scale; the numbers of nucleotides are shown below each. * = termination codon. b, the MYPT1 C-terminal leucine zipper amino acid sequence is identical in chicken, rat, and human and is highly conserved in the worm homologue and chicken and human M21. Identical residues are indicated in bold and leucine residues are underlined. C, chicken; R, rat; H, human; the position of the first amino acid shown is indicated.

### Isoforms of Chicken Smooth Muscle MYPT1 Are Generated by Cassette-type Alternative Splicing of a 3′ Exon

To characterize the MYPT1 isoforms, we cloned a chicken MYPT1 3′ genomic fragment. Comparison with cDNA obtained by RT-PCR of transcripts from chicken smooth muscle tissues indicated that cassette-type alternative splicing of a 31-nt exon gave rise to MYPT1 leucine zipper positive and negative isoforms (Fig. 1). Skipping of the 3′ alternative exon coded for the leucine zipper positive isoform of MYPT1. The amino acid sequence of the C-terminal leucine zipper was identical in birds and mammals and shared 75% identity (18/24 residues) with the worm homologue mel-11 (34) (Fig. 1). Inclusion of the 31-nt alternative exon shifted the reading frame and introduced a premature stop codon, resulting in an MYPT1 C terminus lacking a leucine zipper. This amino acid sequence showed 85% identity (17-20 residues) between birds and mammals and was not found in the worm. The M21 subunit of SMMP appears to have a similar scheme for the generation of C-terminal leucine zipper positive and negative isoforms (35). The M21 leucine zipper amino acid sequences were identical in birds and mammals (Fig. 1) and were highly similar to the MYPT1 leucine zipper sequence (96% identity, 23:24 residues). A gene homologous to M21 has not been identified in the worm.

### Expression of Leucine Zipper Positive and Negative SMMP Subunit Isoforms Is Tissue-specific and Developmentally Regulated

We examined the expression of C-terminal variant MYPT1 and M21 isoforms in the mature and developing chicken as a first step in determining the relationship between SMMP isoform expression and sensitivity to cGMP-mediated signaling. We demonstrated previously that 1) the mature tonic aorta and phasic gizzard tissues are the most pure and distinct with respect to the expression of contractile protein isoforms and 2) developmental changes in gene expression in these tissues correlate with their contractile properties (30, 31, 36), thus providing a dynamic model for examining the functional significance of contractile protein isoform expression.

In the adult chicken the aorta, a tonic smooth muscle tissue, expressed exon-excluded transcripts (coding for the leucine zipper) of MYPT1 and M21 nearly exclusively (Fig. 2). The phasic gizzard smooth muscle expressed exon-included transcripts (coding for absence of the leucine zipper) almost exclusively. Other smooth muscle-containing tissues, such as intestine and lung, contained combinations of exon-included and exon-excluded transcripts. This pattern of expression was conserved in mammalian smooth muscle tissues. The rat aorta (tonic) expressed predominantly exon-excluded MYPT1 transcripts, and other tissues such as the portal vein, uterus, and intestine contained combinations of exon-included and exon-excluded transcripts. This pattern of expression was conserved in mammalian smooth muscle tissues. The rat aorta (tonic) expressed predominantly exon-excluded MYPT1 transcripts, and other tissues such as the portal vein, uterus, and intestine contained combinations of exon-included and exon-excluded transcripts. This pattern of expression was conserved in mammalian smooth muscle tissues.
and out/in (central/C terminus alternative exons) transcripts (data not shown).

The MYPT1 isoform switching was recapitulated by a growth stimulus in vitro (data not shown). Gizzard smooth muscle cells (SMCs) in culture skipped the 3' alternative exon when processing the MYPT1 transcript, resulting in the MYPT1 leucine zipper positive isoform. Aortic SMCs in culture also skipped the alternative exon, as they do in vivo. Thus, as in the case of myosin heavy chain, myosin light chain, and the MYPT1 central alternative exon (30), the splicing of the C-terminal alternative exon of MYPT1 reverted to the embryonic phenotype when SMCs were placed in culture.

Expression of MYPT1 Isoforms and Sensitivity to cGMP-mediated Smooth Muscle Relaxation—The sensitivity of smooth muscle tissues to cGMP was examined in mature tissues that were pure in their expression of the SMMP subunit isoforms, as well as developing smooth muscle tissues where the phenotype was modulating. Smooth muscle strips were permeabilized and pre-contracted at fixed calcium concentrations in order to obviate cGMP effects on calcium fluxes, thereby isolating the effect of cGMP to calcium desensitization of the contractile apparatus (19, 20). Aortic smooth muscle strips activated at pCa 4 relaxed completely in response to 8-Br-cGMP (10^-7 - 10^-4 M), force reduction from peak activation = 100%, n = 6) independent of developmental stage (Fig. 5, a and d). In contrast, the adult gizzard showed no response to 8-Br-cGMP (10^-7 M to 10^-4 M) at pCa 4 (n = 6, Fig. 5, b and d) or pCa 6 (data not shown). Gizzard tissue from intermediate ages when MYPT1/M21 isoforms were switching from leucine zipper positive to negative showed an intermediate response to 8-Br-cGMP (10^-4 M, mean force reduction from peak activation 20 ± 5%, n = 4) (Fig. 3d and data not shown). The concentrations of 8-Br-cGMP required to achieve smooth muscle relaxation in these experiments were consistent with the measured $K_a$ of 8-Br-cGMP for cGKI in vitro (2.6 x 10^-8 and 2.1 x 10^-7 M for L and Ia, respectively (38)).

Since smooth muscle tone is primarily determined by the opposing activities of MLCK and SMMP, two sets of experiments were performed to localize the differential sensitivity to 8-Br-cGMP to an effect on SMMP. First, the effect of 8-Br-cGMP (10^-4 M) on the rate of relaxation of calcium-activated strips (pCa 4) in the presence of a specific inhibitor of MLCK, ML-9 (32), was tested. Adult gizzard strips treated with ML-9 (200 μM) completely relaxed ($t_{50} = 1645 ± 120$ s, n = 3). In the presence of 8-Br-cGMP (10^-4 M), there was no change in the rate of relaxation of ML-9-treated adult gizzard strips ($t_{50} = 1603 ± 85$ s, n = 3, p > 0.05). In contrast, an ~40% increase in the rate of relaxation of ML-9-treated embryonic gizzard strips was observed in the presence of 8-Br-cGMP (control, $t_{50} = 936 ± 58$ s, n = 3; 8-Br-cGMP treated, $t_{50} = 547 ± 138$ s, n = 3, p < 0.01). These experiments suggested that the smooth muscle relaxant effect of 8-Br-cGMP is not mediated by an inhibition of MLCK but rather by activation of SMMP.

The steady state level of myosin phosphorylation in these tissues was measured to localize further the effect of cGMP to activation of SMMP. Phosphorylation of the regulatory myosin light chain (MLC$_{my}$) was undetectable in aortic and gizzard smooth muscle strips in pCa 9 relaxing solution and increased to ~50% upon activation at pCa 4 (Fig. 6). 8-Br-cGMP (10^-4 M) caused a decrease in MLC$_{my}$ phosphorylation from 51 ± 1 to 11 ± 1% (n = 3, p < 0.001) in aortic strips independent of developmental stage and from 47 ± 5 to 9 ± 3% (n = 3, p <
relax to the cGMP analogue, whereas d, graph of 8-Br-cGMP concentration peak activation in calcium-activated (Ca^{2+}) AORTA (ED16 GIZ) show significant reductions in the level of MLC20 AORTA, ED16 GIZ (detected by Western blot as shown. Tissues that relax to 8-Br-cGMP (10^{-4} M) were separated by 7.5% polyacrylamide gel electrophoresis. A band of ~130-kDa corresponding to MYPT1 was detected by Western blot only in aorta strips treated with 8-Br-cGMP. b, either cGKI or MYPT1 were immunoprecipitated from intact rat aorta strips activated by potassium depolarization and treated with 8-Br-cGMP (10^{-4} M) or vehicle. MYPT1 association with cGKI was then detected by Western blotting with the appropriate antibody. Bands of ~130- and 75-kDa corresponding to MYPT1 and cGKI, respectively, were detected. * = faster migrating cGKI band observed with 8-Br-cGMP treatment only. In both a and b IP performed without the primary antibody served as the negative control. Mouse stomach lysates served as the positive control. C, control.

Fig. 7. MYPT1 is detected in association with cGKI in vivo. a, cGKI was immunoprecipitated from pCa 4 activated adult chicken gizzard and aorta strips treated with 8-Br-cGMP (10^{-4} M) or vehicle. Proteins were separated by 7.5% polyacrylamide gel electrophoresis. A band of ~130-kDa corresponding to MYPT1 was detected by Western blot only in aorta strips treated with 8-Br-cGMP. b, either cGKI or MYPT1 were immunoprecipitated from intact rat aorta strips activated by potassium depolarization and treated with 8-Br-cGMP (10^{-4} M) or vehicle. MYPT1 association with cGKI was then detected by Western blotting with the appropriate antibody. Bands of ~130- and 75-kDa corresponding to MYPT1 and cGKI, respectively, were detected. * = faster migrating cGKI band observed with 8-Br-cGMP treatment only.

In this case, immunoprecipitation with MYPT1 followed by cGKI detection by Western blotting. We were not able to confirm the observed association of cGKI and MYPT1 in the chicken aorta using MYPT1 in the immunoprecipitate followed by cGKI detection by Western blotting. We therefore repeated the experiments with intact rat aorta strips, in which force was activated by KCl depolarization followed by complete relaxation with 8-Br-cGMP (10^{-4} M) (data not shown). In this case, immunoprecipitation with MYPT1 followed by immunodetection of cGKI or vice versa yielded positive results (Fig. 7b). In contrast to the chicken aorta, the MYPT1 was found in association with cGKI in the rat aorta in the presence or absence of exogenous 8-Br-cGMP. Of note, a second band of greater mobility was detected with the cGKI antibody only in the samples treated with 8-Br-cGMP, consistent with a post-translational modification of the protein (likely phosphorylation).

FIG. 6. Effect of 8-Br-cGMP on MLC20 phosphorylation levels. Proteins from permeabilized smooth muscle strips in relaxing (pCa 4) or activating solution (pCa 4) in the presence or absence of 8-Br-cGMP (10^{-4} M) were separated by 10% glycerol-urea-polyacrylamide gel electrophoresis. Phosphorylated and unphosphorylated MLC20 species were detected by Western blot when cGKI was immunoprecipitated from lysates of calcium-activated adult chicken aortic strips that had been treated with 8-Br-cGMP (10^{-4} M) (Fig. 7a). MYPT1 was not detected in the cGKI immunoprecipitates of calcium-activated aortic strips alone or in the control assay in which the cGKI antibody was omitted. In contrast, MYPT1 was not detected in cGKI immunoprecipitates of calcium-activated adult chicken gizzard smooth muscle strips in the presence or absence of 8-Br-cGMP (10^{-4} M), although this tissue expressed abundant amounts of MYPT1 (30). Because the polyclonal anti-cGKI antibody would not react with the chicken cGKI in Western blotting, we were not able to confirm the observed association of cGKI and MYPT1 in the chicken aorta using MYPT1 in the immunoprecipitate followed by cGKI detection by Western blotting.

The MYPT1 Subunit of SMMP Associates with cGKI in Vivo—A previous study (23) showed that the leucine zipper motif of MYPT1 is critical for its association with cGKIα and activation of the SMMP in vitro. We performed immunoprecipitation (IP) assays to determine if the leucine zipper positive and negative isoforms of MYPT1 associated with cGKI in vivo. Force was activated in smooth muscle strips followed by treatment with 8-Br-cGMP or vehicle only. MYPT1 was detected by Western blot when cGKI was immunoprecipitated from lysates of calcium-activated chicken aortic strips that had been treated with 8-Br-cGMP (10^{-4} M) (Fig. 7a). MYPT1 was not detected in the cGKI immunoprecipitates of calcium-activated aortic strips alone or in the control assay in which the cGKI antibody was omitted.

In contrast, MYPT1 was not detected in cGKI immunoprecipitates of calcium-activated adult chicken gizzard smooth muscle strips in the presence or absence of 8-Br-cGMP (10^{-4} M), although this tissue expressed abundant amounts of MYPT1 (30). Because the polyclonal anti-cGKI antibody would not react with the chicken cGKI in Western blotting, we were not able to confirm the observed association of cGKI and MYPT1 in the chicken aorta using MYPT1 in the immunoprecipitate followed by cGKI detection by Western blotting. We therefore repeated the experiments with intact rat aorta strips, in which force was activated by KCl depolarization followed by complete relaxation with 8-Br-cGMP (10^{-4} M) (data not shown). In this case, immunoprecipitation with MYPT1 followed by immunodetection of cGKI or vice versa yielded positive results (Fig. 7b). In contrast to the chicken aorta, the MYPT1 was found in association with cGKI in the rat aorta in the presence or absence of exogenous 8-Br-cGMP. Of note, a second band of greater mobility was detected with the cGKI antibody only in the samples treated with 8-Br-cGMP, consistent with a post-translational modification of the protein (likely phosphorylation).
tion). These results support a model in which expression of a MYPT1 isoform containing a leucine zipper motif is required for the association of cGKIα with MYPT1 (1), SMMP activation, and desensitization of the contractile apparatus to calcium leading to smooth muscle relaxation.

**DISCUSSION**

Reversible phosphorylation of serine/threonine (Ser/Thr) residues of proteins by specific kinases and phosphatases is commonly used to propagate signals and alter protein function within cells. In many instances substrate specificity is conferred to common catalytic subunits via unique targeting subunits (reviewed in Ref. 39). The targeting subunits are also targets of signaling pathways that may modulate the activity of the enzyme toward its substrate. In the case of the myosin phosphatase, two genes encoding for the targeting subunit have been identified, one of which is expressed ubiquitously in smooth muscle and non-muscle cells (MYPT1) and a second that is predominantly expressed in striated muscle and brain (MYPT2). MYPT1 and MYPT2 are highly related, sharing 61% amino acid identity among a species (40). The MYPT sequence has also been conserved through evolution with nearly 90% amino acid identity among mammalian MYPT1s and ~80% identity between mammalian and avian MYPT1. A more distantly related MYPT1 homologue termed mel-11 is present in the worm and shares 35% amino acid identity with the higher vertebrate form (5, 28, 34, 41).

Further diversity is generated in the MYPT isoforms by cassette-type alternative splicing of exons. This strategy is commonly used to add and remove specific functional modules from proteins (reviewed in Ref. 42). In the current study we have demonstrated the tissue-specific and developmentally regulated cassette-type alternative splicing of a 31 nt 3’ MYPT1 exon in the chicken. This results in MYPT1 isoforms that either contain or lack a C-terminal leucine zipper. These isoforms have also been identified in mammalian MYPT1, and examination of the Celera human genome data base reveals similarity in exon-intron sizes and conservation of the alternative splicing mechanism for generation of the isoforms. The pattern of expression of the MYPT1 C-terminal isoforms is also similar in birds and mammals. The MYPT2 pre-mRNA (43) and presumptively the M21 subunit pre-mRNA are also alternatively spliced to produce isoforms with variable presence of the C-terminal leucine zipper. The highly conserved nature of the leucine zipper sequences (100% identity among chicken, rat, and human sequences and 75% identity with worm) and conserved patterns of isoform expression are consistent with the proposed functional significance of this motif.

We have used the developing and mature chicken as a model to understand the relationship between contractile protein isoform expression and smooth muscle diversity. The chicken aorta and gizzard are particularly attractive smooth muscle tissues for this analysis because of their distinct contractile properties and patterns of gene expression, as well as their accessibility during development. The aorta throughout development is always phenotypically and functionally slow (tonic) (30, 31, 36), expresses leucine zipper positive isoforms of MYPT1 and M21, and relaxes to cGMP in the presence of activating concentrations of calcium. The evidence that the calcium desensitization is due to cGMP-dependent activation of the SMMP includes 1) the parallel decrease in force and MLC20 phosphorylation levels in the presence of calcium concentrations that maximally activate MLCK, 2) an acceleration of relaxation by cGMP when MLCK was specifically inhibited with ML-9 (20), and 3) the demonstration by immunoprecipitation that the cGKI associates with the MYPT1 subunit in aortic tissues in vivo. This in vivo observation supports the initial observation in vitro that SMMP/MYPT1 is a target of cGKIα via heterophilic leucine zipper interactions (23). In our experiments MYPT1 and cGKI were associated in calcium-activated chicken aorta strips only in the presence of 8-Br-cGMP. However, in rat smooth muscle strips that were activated by KCl depolarization, MYPT1 and cGKI were associated prior to the addition of 8-Br-cGMP. Whether the difference between chicken and rat aorta in the requirement of cGMP for association of MYPT1 with cGKI is due to differences in the activation of the two tissues (Ca2+ versus KCl depolarization, respectively) or reflects species differences will require further study. The mechanism by which cGMP/cGKI enhances the activity of the SMMP has not been determined.

In contrast to the tonic aorta the gizzard undergoes a series of phenotypic transitions during development before acquiring its mature phasic contractile properties. From ED10 to just before hatching there is a marked increase in the rate of force development that coincides with a switch in the myosin heavy and light chain isoforms (31, 36). From ED16 to shortly after hatching there is a marked change in the relaxation response of the gizzard to cGMP, from complete calcium desensitization to complete resistance to calcium desensitization. This coincides with a switch in the SMMP subunit isoforms from leucine zipper positive to negative. The transition from cGMP-sensitive to cGMP-resistant is attributed to a failure to activate the SMMP for the following reasons: 1) a failure to dephosphorylate MLC20 in the presence of activating calcium concentrations, 2) a failure to speed relaxation in the presence of the specific MLCK inhibitor ML-9, and 3) an inability of cGKI to associate with MYPT1 as determined by immunoprecipitation.

In this study we focused on phenotypically distinct smooth muscle tissues, the aorta and gizzard. This enabled us to show a relationship between the expression of the myosin phosphatase isoforms and a single component of the NO signaling pathway, cGMP mediated calcium desensitization. However, the physiological role of NO/cGMP signaling in all vascular (tonic) or visceral (phasic) tissues cannot be inferred from this study for a number of reasons. First, NO may signal through cGMP-independent mechanisms, including S-nitrosylation of proteins (44–47). However, the signaling of NO via cGMP-dependent activation of cGKI appears to be the predominant pathway for physiological smooth muscle relaxation given the minimal response in cGKI-null mice (48).

Second, this study as well as others (49–52) have shown considerable diversity in the expression of the contractile protein isoforms throughout both the vascular and intestinal systems. The variation in isoform expression is observed at both the tissue and single cell level and is also evident during smooth muscle maturation by the variation in the timing of isoform switching (Ref. 30 and this study). Thus smooth muscle phenotypic diversity approaches that of striated muscle, while how it is generated is much less well understood (reviewed in Refs. 53–55). How tissues that express various mixtures of MYPT1 isoforms respond to cGMP was not a goal of this study. However, the partial response of the gizzard around the time of hatching to higher concentrations of cGMP suggests that tissues with mixed isoform expression may show intermediate responses.

Third, several targets of cGMP/cGKI signaling other than MYPT1 have also recently been identified (reviewed in Ref. 22), but an association between their expression and sensitivity to NO/cGMP signaling has not been demonstrated. Telokin, an MLCK-related protein, is detected at much higher levels in embryonic and adult visceral smooth muscle, including gizzard, as compared with vascular smooth muscle (56–58), i.e. in a pattern opposite to that of the cGMP sensitivity. The vasodila-
tor-stimulated phosphoprotein (reviewed in Ref. 59) and heat shock protein-20 (60) appear to be ubiquitously expressed, although their expression in this developing chick model has not been examined. Interestingly, a recent study of NO donor activation of cGMP sensitive (aorta) and resistant (myometrium and vas deferens) rat tissues reports seven phosphorylated substrates of cGK in the aorta, none of which were found in the cGMP-resistant tissues (61). The identity of these proteins was not determined.

Fourth, cGMP has as targets not only proteins that determine calcium sensitivity but also proteins that regulate calcium flux (62–64). This aspect of cGMP signaling was obviated in our experiments by clamping calcium concentrations. Interestingly the two cGKI-dependent components of the NO/cGMP response appear to be mediated by different cGKI isoforms that vary only in their N-terminal leucine zipper sequences (reviewed in Ref. 65). The cGKβ association with the inositol 1,4,5-triphosphate receptor-associated cGKI substrate (66) is proposed as a mediator of the reduction in calcium, whereas the cGKls association with MYPT1 is proposed as a mediator of calcium desensitization (23). Thus it is plausible that cGMP-dependent signaling may independently regulate the phasic nature of smooth muscle contractions via an effect on ion flux and regulate smooth muscle tone via an effect on calcium sensitivity of the myofilaments.

Finally, isoforms variations in the proteins that determine cGMP steady state levels (guanylate cyclase and phosphodies-
terase) and in the cGMP-binding proteins (e.g. cGKI) may also determine the smooth muscle response to a given signal. This upstream component of the cGMP signaling pathway is targeted by sildenafil, a selective phosphodiesterase type 5 inhibitor that somewhat selectively regulates blood flow in the corpus cavernosum. Although some studies (67, 68) have reported differences in the relative expression of the two cGKI isoforms that could influence the response to cGMP, no smooth muscle tissue has been identified that expresses one isoform exclusively. The lack of an antibody against chicken cGKI isoforms precluded a determination of cGKI isoform expression, but semi-quantitative RT-PCR indicated no significant change in the embryonic gizzard requires higher concentrations of cGMP for harvesting rat tissues.

Acknowledgments—We thank Albert Rhee for technical assistance with myosin light chain phosphorylation assays and Sarah MacFarland for harvesting rat tissues.

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Role of Myosin Phosphatase Isoforms in cGMP-mediated Smooth Muscle Relaxation
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J. Biol. Chem. 2001, 276:37250-37257.
doi: 10.1074/jbc.M105275200 originally published online August 2, 2001

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