JOURNAL OF SMOOTH MUSCLE RESEARCH

J. Smooth Muscle Res. 2016; 52: 66–77
Published online: October 7, 2016; doi: 10.1540/jsmr.52.66

Original

MYPT1 isoforms expressed in HEK293T cells are differentially phosphorylated after GTPγS treatment

Simon Lin and Frank V Brozovich

Mayo Medical School, Department of Cardiovascular Disease, Rochester, MN 55905, USA

Submitted July 12, 2016; accepted in final form August 24, 2016

Abstract

Agonist stimulation of smooth muscle is known to activate RhoA/Rho kinase signaling, and Rho kinase phosphorylates the myosin targeting subunit (MYPT1) of myosin light chain (MLC) phosphatase at Thr696 and Thr853, which inhibits the activity of MLC phosphatase to produce a Ca^{2+} independent increase in MLC phosphorylation and force (Ca^{2+} sensitization). Alternative mRNA splicing produces four MYPT1 isoforms, which differ by the presence or absence of a central insert (CI) and leucine zipper (LZ). This study was designed to determine if Rho kinase differentially phosphorylates MYPT1 isoforms. In HEK293T cells expressing each of the four MYPT1 isoforms, we could not detect a change in Thr853 MYPT1 phosphorylation following GTPγS treatment. However, there is differential phosphorylation of MYPT1 isoforms at Thr696; GTPγS treatment increases MYPT1 phosphorylation for the CI+LZ- and CI-LZ- MYPT1 isoforms, but not the CI+LZ+ or CI-LZ+ MYPT1 isoforms. These data could suggest that in smooth muscle Rho kinase differentially phosphorylates MYPT1 isoforms.

Key words: MLC phosphatase, Rho kinase, Ca^{2+} sensitization

Introduction

Phosphorylation of the smooth muscle 20 kDa myosin light chain (MLC) regulates smooth muscle contraction or vascular tone (1, 2), and MLC phosphorylation is determined by the balance of the activities of MLC kinase and MLC phosphatase (3). There are a number of important signaling pathways in smooth muscle that regulate vascular tone, and the vast majority influence the activity of MLC phosphatase (4, 5).

The signaling pathways converging on MLC phosphatase either increase phosphatase activity to decrease MLC phosphorylation and force, which is referred to as Ca^{2+} desensitization, or decrease phosphatase activity to increase MLC phosphorylation and force, which is referred to as Ca^{2+} sensitization (4). MLC phosphatase is a holoenzyme consisting of enzymatic, 20 kDa and myosin targeting (MYPT1) subunits (5). Alternative

Corresponding author: Frank V Brozovich, Mayo Clinic, CV Research, 200 1st ST SW, Rochester, MN 55905, USA
Phone: +1 507 266 0324 Fax: +1 507 538 6418 e-mail: brozovich.frank@mayo.edu

mRNA splicing of a central and a 3' exon produce a MYPT1 central insert (CI) and a COOH-terminal leucine zipper (LZ), respectively (5); there are four MYPT1 isoforms (CI+LZ+, CI+LZ-, CI-LZ+ and CI-LZ-), and MYPT1 isoform expression is developmentally regulated, tissue specific (6–8), and is modulated in disease (9–13).

The MYPT1 LZ domain is important for mediating Ca^{2+} desensitization during NO mediated vasodilatation: during NO/cGMP signaling, LZ+, but not LZ-, MYPT1 isoforms are phosphorylated by PKG (14, 15). Therefore, LZ+/LZ- MYPT1 isoform expression, in part, underlies the heterogenous response of the vasculature to NO and NO based vasodilators (14–17). For G-protein coupled agonists, the stimulation of G_q and G_{11} activates phospholipase C, and the subsequent increase in intracellular Ca^{2+} activates MLC kinase (4). However, a number of agonists also activate RhoA/Rho kinase through G_{12} and G_{13} (4). Rho kinase phosphorylates MYPT1 at both Thr696 and Thr853 (18), which decreases the activity of the MLC phosphatase (18–22) to produce Ca^{2+} sensitization (21, 23, 24). However, similar to Ca^{2+} desensitization, there is variability in the response of the vasculature to agonists; in smooth muscles, there is heterogeneity in the magnitude and sensitivity of Ca^{2+} sensitization (25, 26), and the mechanism to explain this variability is unknown.

Therefore, this study was designed to determine if MYPT1 isoforms are differentially phosphorylated by Rho kinase. To investigate this question, we determined the time course of MYPT1 phosphorylation (Thr696 and Thr853) following activation of Rho kinase with GTPγS in HEK293T cells lines that express each of the MYPT1 isoforms.

Methods

For the present study, we used our human embryonic cell (HEK293T) lines that express the four avian MYPT1 isoforms (CI+LZ+, CI+LZ-, CI-LZ+, CI-LZ-). These HEK293T cell lines have the SV40 Large T-antigen, which allows an episomal plasmid to be replicated, and the cloning and transfection techniques to generate these HEK293T cell lines have been previously described (15). Untransfected HEK293T cells expresses the four isoforms of human MYPT1 (15), and in the present experiments, the density of the ratio of CI+/CI- bands was 1.2–2x higher for the band containing the exogenous avian MYPT1 isoform, which is consistent with an overexpression of the exogenous avian MYPT1 isoform and our previous data (15).

The sequences of the avian and mammalian MYPT1 are highly homologous and there are multiple regions of sequence identity (27). Rho kinase mediated MYPT1 phosphorylation occurs at Thr696 and Thr853 of the mammalian sequence, which corresponds to Thr695 and Thr850 of the avian sequence. For consistency, we will refer to the numbering of the mammalian sequence throughout the manuscript.

Cell culture

As previously described (15), HEK293T cells lines were grown in P100 plates using 1x Dulbecco's Modified Eagle's Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 0.004% zeocin at 37°C with 5% CO_2, and cells were split at 80–100% confluence. To determine the time course of MYPT1 phosphorylation, cells were grown to 70–90% confluence, and then the HEK293T cells were starved for 24h in low serum media (1% FBS, 1% penicillin/streptomycin and 0.004% zeocin) at 37°C. As described in previous publications (28), cells were placed in skinning solution (0.1% Triton X-100) for 10 min and then the solution was changed to a low Ca^{2+} solution and the cells were treated at 37°C with 0.1 mM guanosine 5′-O-(3-thiotriphosphate) tetralithium (GTPγS). At 0 min, 1 min, 5 min and 30 min of GTPγS treatment, cells scraped off the plate, sonicated, spun and placed in fresh tubes, and stored at –80°C prior to immunoblotting.

Table 1. Relative Thr853 MYPT1 phosphorylation

MYPT1 Isoform	0 min	30 min
CI+LZ-MYPT1	0.7 ± 0.3	1.0 ± 0.4
CI+LZ+MYPT1	0.7 ± 0.1	1.0 ± 0.3
CI-LZ-MYPT1	0.9 ± 0.1	1.0 ± 0.4
CI-LZ+MYPT1	0.7 ± 0.1	1.0 ± 0.3

Following treatment with GTPγS, relative Thr853 phosphorylation, computed as the density of both bands on the Western blot in Fig. 1 (total phospho-Thr853/total MYPT1) did not change (*, $P<0.05$, n=4–6).

Table 2. Relative Thr696 MYPT1 phosphorylation

MYPT1 Isoform	0 min	30 min
CI+LZ-MYPT1	0.2 ± 0.1	1.0 ± 0.3*
CI+LZ+MYPT1	0.6 ± 0.2	1.0 ± 0.3*
CI-LZ-MYPT1	0.5 ± 0.1	1.0 ± 0.2*
CI-LZ+MYPT1	0.8 ± 0.2	1.0 ± 0.2

Following treatment with GTPγS, relative Thr696 phosphorylation, computed as the density of both bands on the Western blot in Fig. 2 (total phospho-Thr696/total MYPT1) increased for every MYPT1 isoform, except CI-LZ+MYPT1 (*, $P<0.05$, n=4–6). Note, the data in this table represent the results for both bands in Fig. 2, while the time course of Thr696 MYPT1 phosphorylation in Fig. 3 is only for the single MYPT1 band expressing the exogenous MYPT1 isoform (CI+ or CI-), which is indicated by the arrowhead.

Immunoblotting

As we have described (15), MYPT1 phosphorylation was determined using phospho-specific antibodies and normalized to MYPT1 expression. Gels were run in pairs, one for MYPT1 and the other for phospho-MYPT1. MYPT1 was detected using a polyclonal anti-MYPT1 antibody (Epitomics), while MYPT1 phosphorylation at Thr696 or Thr853 was detected using phospho-specific antibodies to either phospho-Thr696 or phospho-Thr853 (Cell Signaling). To determine relative Thr696 and Thr853 MYPT1 phosphorylation, images were scanned (Epson Perfection V750 PRO) and band density was determined using ImageQuant TL software (GE Healthcare). Relative phosphorylation was computed as the density of the phosphorylated signal (Thr696 or Thr853) divided by the density of the MYPT1 protein band(s). The data for relative phosphorylation for both Thr696 and Thr853 were normalized. To normalize the data for each analysis, the density of the ratio of phospho-MYPT1/MYPT1 for the 30 min time point was averaged and all time points were subsequently normalized to this mean.

Data are presented as mean ± SEM with n representing the number of experiments. The Student's *t*-test was used to determine the significance of the difference between the relative phosphorylation between the 0 and 30 min time points, and $P<0.05$ was considered significant.

Results

MYPT1 phosphorylation at both Thr853 and Thr696 was detected prior to treatment with GTPγS. However, the signal representing phospho-Thr853 was faint. For the HEK293T cells over expressing CI+ MYPT1 isoforms (CI+LZ+MYPT1 & CI+LZ-MYPT1), GTPγS did not increase relative MYPT1 phosphorylation at Thr853 (phospho-Thr853/MYPT1; Table 1, Fig. 1A). Similarly, for the HEK293T cells expressing CI- MYPT1 isoforms, there was no significant increase in relative Thr853 MYPT1 phosphorylation with GTPγS treatment (Table 1, Fig. 1B).

In contrast to the results for Thr853 MYPT1 phosphorylation, the phospho-Thr696 bands were readily apparent. We first analyzed total relative MYPT1 phosphorylation (Fig. 2, both bands). In the HEK293T cells expressing CI+ MYPT1 isoforms (CI+LZ+MYPT1 & CI+LZ-MYPT1), there was a significant increase in total relative Thr696 MYPT1 phosphorylation following treatment with GTPγS (Table 2). However in the HEK293T

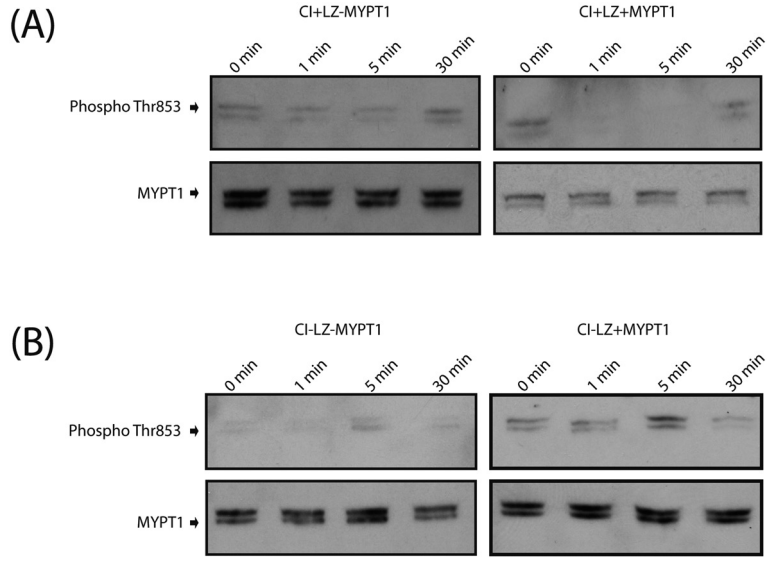

Fig. 1. GTPγS treatment does not increase MYPT1 phosphorylation at Thr853. (A) Western blots demonstrating time course of Thr853 phosphorylation for CI+LZ-MYPT1 and CI+LZ+MYPT1. (B) Western blots demonstrating time course of Thr853 phosphorylation for the CI-LZ-MYPT1 and CI-LZ+MYPT1. The arrowhead denotes the MYPT1 band containing the overexpressed MYPT1 isoform. Relative total MYPT1 Thr853 phosphorylation (total phospho-Thr853/total MYPT1) did not significantly change after GTPγS for any MYPT1 isoform (Table 1).

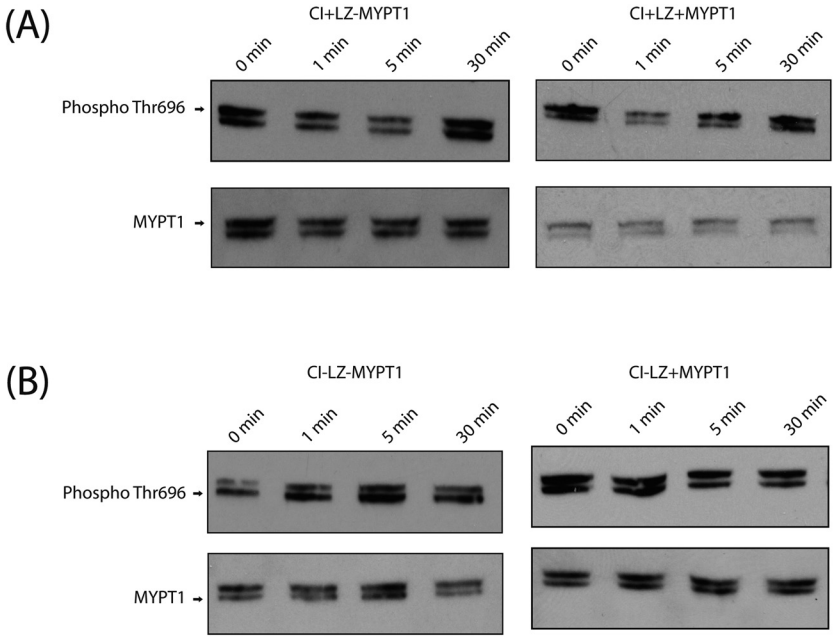

Fig. 2. GTPγS increases MYPT1 isoforms at Thr696. (A) Western blots demonstrating time course of Thr696 phosphorylation for CI+LZ-MYPT1 and CI+LZ+MYPT1. (B) Western blots demonstrating time course of Thr696 phosphorylation for the CI-LZ-MYPT1 and CI-LZ+MYPT1. The arrowhead denotes the MYPT1 band containing the overexpressed MYPT1 isoform. Relative total MYPT1 Thr696 phosphorylation (total phospho-Thr696/total MYPT1) increased after GTPγS for all MYPT1 isoforms, except CI-LZ+ MYPT1 (Table 2).

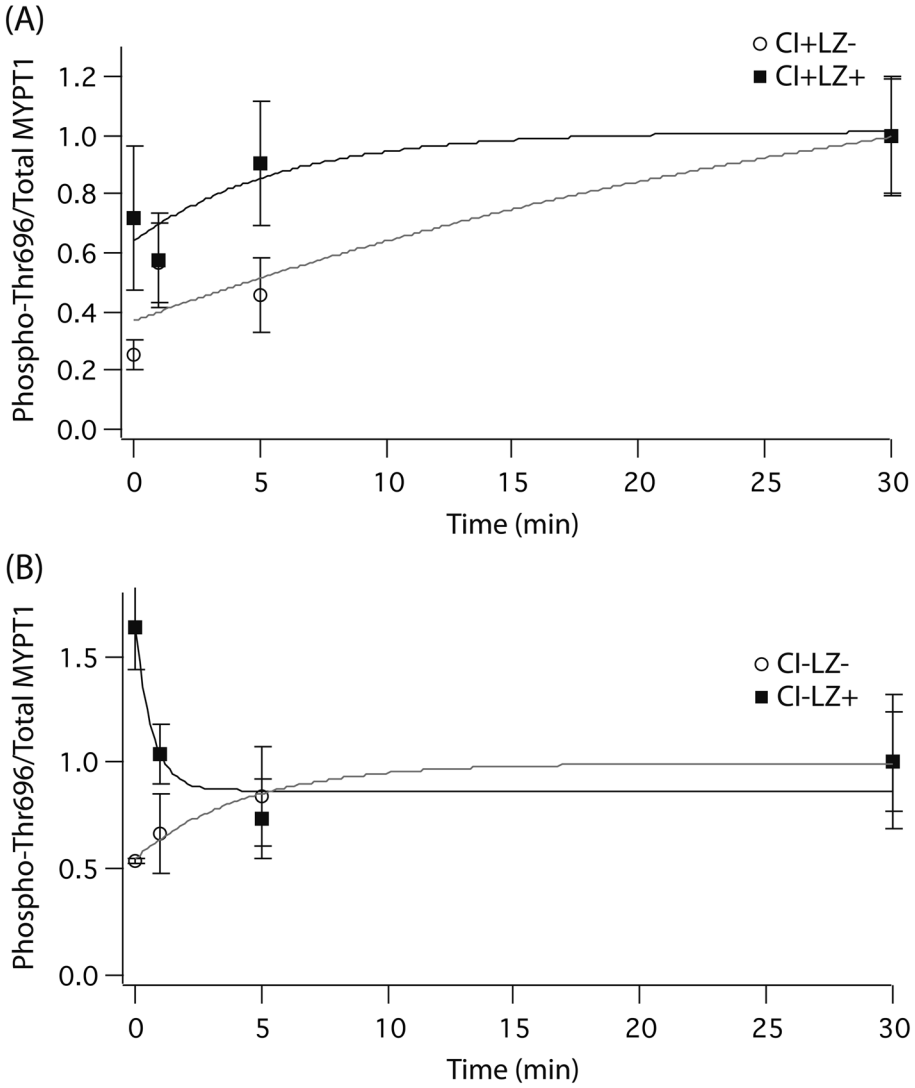

Fig. 3. GTPγS treatment results in differential phosphorylation of MYPT1 isoforms at Thr696. The time course of relative Thr696 MYPT1 phosphorylation was computed for only the single band containing the exogenous MYPT1 isoform (CI+ or CI-), which is indicated by the arrowhead in Fig 2. (A) Time course of relative Thr696 phosphorylation for CI+LZ-MYPT1 (○, n=4) and CI+LZ+MYPT1 (■, n=4). The increase in Thr696 phosphorylation was significant for CI+LZ- MYPT1 ($P<0.05$), but not the CI+LZ+MYPT1 isoform. (B) Time course of relative Thr696 phosphorylation of CI-LZ-MYPT1 (○, n=6) and CI-LZ+MYPT1 (■, n=4). There is a significant increase in Thr696 phosphorylation for CI-LZ- MYPT1 ($P<0.05$) and a significant decrease ($P<0.05$) in Thr696 phosphorylation for CI-LZ+ MYPT1. The solid lines represent a single exponential fit of the time course of phosphorylation.

cells expressing CI- MYPT1 isoforms, the results were variable; in HEK293T cells expressing CI-LZ-MYPT1, GTPγS stimulation increased total relative MYPT1 phosphorylation at Thr696, while in the HEK293T cells expressing CI-LZ+MYPT1, total relative Thr696 MYPT1 phosphorylation did not change (Table 2).

To further define isoform specific MYPT1 phosphorylation in response to GTPγS, we examined the time course of Thr696 phosphorylation for only the single MYPT1 band containing the exogenous MYPT1 isoform (indicated by the arrowhead in Fig. 2). For both LZ- MYPT1 isoforms (CI+LZ- & CI-LZ-), GTPγS produced a significant increase in relative Thr696 MYPT1 phosphorylation (Fig. 3). For the CI+LZ+ MYPT1, the increase

in relative Thr696 phosphorylation was not significant (Fig. 3A, $P>0.05$), while for the CI-LZ+ MYPT1 isoform, relative Thr696 phosphorylation decreased ($P<0.05$) following GTPγS (Fig. 3B).

Discussion

In smooth muscle, in addition to MLC kinase (1) and RhoA/Rho kinase (21, 24), there are multiple other signaling pathways that modulate force including PKC (29–31), Zip kinase (32), integrin-linked kinase (33) and Rac1 (34), and further, the physiologically important signaling pathways that mediate Ca^{2+} sensitization are both agonist as well as tissue specific (4, 35). The Rho kinase inhibitor fasudil reduces blood pressure in animal models of hypertension (36–38) and in humans, is effective in treating both cerebral vasospasm (39, 40) and pulmonary hypertension (41, 42), which suggests that Rho kinase mediated signaling is important in both health and disease. Rho kinase has been demonstrated to phosphorylate both MYPT1 as well as CPI-17 (43, 44), and similar to the other signaling pathways which influence MLC phosphatase activity, the importance of each of these proteins for Ca^{2+} sensitization is tissue specific (44). In the present study, we examined whether GTPγS treatment results in isoform specific MYPT1 phosphorylation.

Others have demonstrated Rho kinase is expressed in HEK293T cells (45, 46) and additionally, in HEK293T cells, GTPγS stimulates Rho (47, 48). Further, Rho has been demonstrated to activate Rho kinase, PKN and PIP5 kinase (49). Therefore, the GTPγS stimulated increase in MYPT1 phosphorylation could be mediated by Rho kinase, PKN, PIP5-kinase or another unknown kinase. However, only Rho kinase and ILK have been demonstrated to phosphorylate MYPT1 at Thr696 and Thr853 (50), and ILK is not activated by Rho kinase, PKN or PIP5 kinase (51). These data strongly suggest that following GTPγS treatment of HEK293T cells, MYPT1 phosphorylation at Thr696 and/or Thr853 is mediated by a GTPγS induced activation of Rho kinase.

We have previously demonstrated that an overexpressed exogenous MYPT1 isoform replaces the endogenous isoform in the MLC phosphatase holoenzyme (17) and alters the activity of MLC phosphatase (15, 17). Similar to previous reports (15, 17), in the present experiments, the ratio of CI+/CI- changed consistent with overexpression of the exogenous MYPT1. These data suggest that MYPT1 phosphorylation determined in these HEK293T cell lines reflects the phosphorylation of the exogenous MYPT1 isoform (15). However compared to our prior results, the expression of the exogenous avian MYPT1 isoform is lower in the present study, which could result in an underestimation of the magnitude of the changes in MYPT1 phosphorylation.

In the present study, MYPT1 phosphorylation at Thr696 increased after treatment with GTPγS for the CI+LZ-MYPT1 and CI-LZ-MYPT1 isoforms (Fig. 3). Others have demonstrated that activation of PKG increases MYPT1 phosphorylation at both Ser695 and Ser852, which then inhibits Rho kinase mediated MYPT1 phosphorylation at Thr696 and Thr853 (52, 53). The LZ domain is hypothesized to mediate the interaction of MYPT1 and PKG (16) and is required for PKG mediated MYPT1 phosphorylation (14, 15, 17). These data could suggest that LZ- MYPT1 isoforms, but not LZ+ MYPT1 isoforms, are phosphorylated by Rho kinase, which is consistent with our results. For the CI+LZ+ MYPT1 isoform, GTPγS treatment increased Thr696 phosphorylation, but the increase did not reach statistical significance (Fig. 3), which could suggest the CI domain modulates Rho kinase phosphorylation. For the CI-LZ+MYPT1 isoform, it is unclear why relative Thr696 phosphorylation decreased with GTPγS stimulation (Fig. 3). One possibility is that Rho kinase cannot access Thr696 due to differences in the three-dimensional structure of CI-LZ+MYPT1 and the other MYPT1 isoforms, and thus for CI-LZ+ MYPT1, Thr696 is only subject to autodephosphorylation (54). Alternatively, this may be due to an interaction of CI-LZ+MYPT1 and another protein, such as M-RIP (55) or prostate apoptosis response 4 (56), which could alter the kinetics of Rho kinase mediated MYPT1 phosphorylation.

In contrast to the signal for Thr696 phosphorylation, the signal for phospho-Thr853 MYPT1 was low, and we could not detect a significant increase in relative Thr853 MYPT1 phosphorylation after GTPγS treatment (Table 1, Fig. 1). These results contrast to the significant increase in MYPT1 Thr853 phosphorylation observed in smooth muscle (44, 57). The mechanism to explain the lack of Thr853 phosphorylation in the HEK293T cells expressing avian MYPT1 isoforms is beyond the scope of the present study. In our HEK293T cells, GTPγS could have activated a G-protein pathway that inactivated Rho or even activated a kinase other than Rho kinase. Since GTPγS treatment increased phosphorylation at Thr696, a well documented MYPT1 residue for Rho kinase (4), this possibility is unlikely. In isolated 500 aa MYPT1 protein fragments, we have demonstrated that Rho kinase does not phosphorylate Thr853 (14). However, in untransfected control HEK293T cells, we have previously demonstrated that the phosphorylation of the endogenous human CI+ MYPT1 at both Thr696 and Thr853 (15), which differs from the present results. These data could also suggest that for avian MYPT1, phospho-Thr853 is poorly recognized by the anti-phosphoThr853 antibody. However, there is a high degree of identity between the aa sequences of avian and mammalian MYPT1 (27); 85% identity for the 60 aa flanking the Thr850/Thr853 phosphorylation site and we also detected a phospho-Thr853 signal, albeit faint (Fig. 1), which suggests that this possibility is unlikely.

Similar to our results, there is also considerable variability in Thr696 vs. Thr853 MYPT1 phosphorylation in mammalian smooth muscle; MYPT1 phosphorylation is both tissue and agonist dependent. Studies have demonstrated that 1) a Rho kinase mediated MYPT1 phosphorylation at Thr853, but not Thr696, is responsible for the sustained phase of the force response in rat uterine smooth muscle (58), 2) a Rho kinase mediated increase in both Thr696 and Thr853 MYPT1 phosphorylation occurs during serotonin induced vasoconstriction of cerebral arteries (59), 3) a Rho kinase mediated increase in MYPT1 phosphorylation at Thr853, but not Thr696, contributes to the myogenic response of cerebral vessels (60), 4) a Rho kinase mediated increase in Thr696 MYPT1 phosphorylation occurs during Ca^{2+} sensitization of rat ileal smooth muscle (61) and 5) a Rho kinase mediated increase in Thr696 MYPT1 phosphorylation is responsible for PGF2α induced Ca^{2+} sensitization in rabbit aorta (62). Additionally, during activation of mouse bladder smooth muscle, MYPT1 phosphorylation at Thr853 is mediated predominantly by Rho kinase, while phosphorylation at Thr696 did not change (63). Using MYPT1 Ala mutants, these investigators demonstrated that the increase in Thr853 phosphorylation did not contribute to the force response, while the force maintenance was reduced in the Ala696Thr MYPT1 mutant, suggesting that MYPT1 phosphorylation at Thr696 is important for the regulation of force maintenance (63).

Our current results demonstrate that GTPγS treatment of HEK293T cells increased Thr696 phosphorylation of CI+LZ- and CI-LZ-, but not CI+LZ+ and CI-LZ+, MYPT1 isoforms. These data suggest that following agonist activation, Rho kinase will differentially phosphorylate MYPT1 isoforms. We have previously demonstrated that MYPT1 isoform expression determines the sensitivity of NO mediated vasodilatation (14, 15, 17); our previous results show that smooth muscle tissues expressing LZ- MYPT1 isoforms (CI+LZ-, CI-LZ-) are not phosphorylated by PKG (14, 15). Taken together, these results suggest that the LZ MYPT1 domain is an important determinant for the regulation of MLC phosphatase; LZ+ MYPT1 isoforms are phosphorylated by PKG (14, 15), but poor substrates for Rho kinase (Fig. 3), while LZ- MYPT1 isoforms are phosphorylated by Rho kinase (Fig. 3), but not PKG (14, 15). The CI domain appears to modulate Rho kinase phosphorylation of LZ+ MYPT1 isoforms; following GTPγS treatment MYPT1 phosphorylation decreased for CI-LZ+MYPT1, but did not change for CI+LZ+MYPT1 (Fig. 3), which provides another mechanism to tune the vasculature's response to vasoactive agents. These data demonstrate that alternative splicing to produce CI+/CI- and LZ+/LZ- MYPT1 isoforms could contribute to the molecular mechanism producing the variable sensitivity of smooth

muscle to signaling pathways for both Ca^{2+} sensitization (RhoA/Rho kinase) and desensitization (NO/cGMP/PKG). MYPT1 isoform expression is both developmentally regulated and tissue specific (6–8). Changes in relative CI+/CI- MYPT1 isoform expression have only been examined in an animal model of portal hypertension (10), but relative LZ+/LZ- MYPT1 isoform expression is well documented to decrease in a number of diseases including heart failure (9, 11, 64, 65) and pulmonary hypertension (13, 66). Therefore, modulation of MYPT1 isoform expression may represent a mechanism to tune the vasculature's response to both NO/PKG and G-protein coupled agonists/Rho kinase signaling, which will influence vascular tone and/or resistance in both health and disease.

Acknowledgments

We would like to thank Gresin Hawes, Samantha Yuen and Dr Young Soo Han for their help with the protocols for cell culture and immunoblotting. This study was supported by grants from the Mayo Foundation for Medical Research and a Mayo/Karolinska Institute Collaborative Award.

Conflict of Interest

Neither Simon Lin nor Frank V Brozovich have a conflict of interest.

References

1. Ikebe M, Hartshorne DJ. Effects of Ca2+ on the conformation and enzymatic activity of smooth muscle myosin. J Biol Chem. 1985 Oct 25; 260(24): 13146–53.
2. Barsotti RJ, Ikebe M, Hartshorne DJ. Effects of Ca2+, Mg2+, and myosin phosphorylation on skinned smooth muscle fibers. Am J Physiol. 1987 May; 252(5 Pt 1): C543–54.
3. Gong MC, Cohen P, Kitazawa T, Ikebe M, Masuo M, Somlyo AP, Somlyo AV. Myosin light chain phosphatase activities and the effects of phosphatase inhibitors in tonic and phasic smooth muscle. J Biol Chem. 1992 Jul 25; 267(21): 14662–8.
4. Somlyo AP, Somlyo AV. Ca2+ sensitivity of smooth muscle and nonmuscle myosin II: modulated by G proteins, kinases, and myosin phosphatase. Physiol Rev. 2003 Sep 25; 83(4): 1325–58.
5. Hartshorne DJ, Ito M, Erdodi F. Myosin light chain phosphatase: subunit composition, interactions and regulation. J. Muscle Res Cell Motil. 1998 May; 19(4): 325–41.
6. Dirksen WP, Vladic F, Fisher SA. A myosin phosphatase targeting subunit isoform transition defines a smooth muscle developmental phenotypic switch. Am J Physiol Cell Physiol. 2000 Mar; 278(3): C589–600.
7. Khatri JJ, Joyce KM, Brozovich FV, Fisher SA. Role of myosin phosphatase isoforms in cGMP-mediated smooth muscle relaxation. J Biol Chem. 2001 Oct 5; 276(40): 37250–7.
8. Payne MC, Zhang H-Y, Prosdocimo T, Joyce KM, Koga Y, Ikebe M, Fisher SA. Myosin phosphatase isoform switching in vascular smooth muscle development. J Mol Cell Cardiol. 2006 Feb; 40(2): 274–82.
9. Karim SM, Rhee AY, Given AM, Faulx MD, Hoit BD, Brozovich FV. Vascular reactivity in heart failure: role of myosin light chain phosphatase. Circ Res. 2004 Sep 17; 95(6): 612–8.
10. Payne MC, Zhang H-Y, Shirasawa Y, Koga Y, Ikebe M, Benoit JN, Fisher SA. Dynamic changes in expression of myosin phosphatase in a model of portal hypertension. Am J Physiol Heart Circ Physiol. 2004 May; 286(5): H1801–10.

11. Chen FC, Ogut O, Rhee AY, Hoit BD, Brozovich FV. Captopril prevents myosin light chain phosphatase isoform switching to preserve normal cGMP-mediated vasodilatation. J Mol Cell Cardiol. 2006 Sep; 41(3): 488–95.
12. Lu Y, Zhang H, Gokina N, Mandala M, Sato O, Ikebe M, Osol G, Fisher SA. Uterine artery myosin phosphatase isoform switching and increased sensitivity to SNP in a rat L-NAME model of hypertension of pregnancy. Am J Physiol Cell Physiol. 2008 Feb; 294(2): C564–71.
13. Konik EA, Han YS, Brozovich FV. The role of pulmonary vascular contractile protein expression in pulmonary arterial hypertension. J Mol Cell Cardiol. 2013 Oct 23; 65C: 147–55.
14. Yuen S, Ogut O, Brozovich FV. MYPT1 protein isoforms are differentially phosphorylated by protein kinase G. J Biol Chem. 2011 Oct 28; 286(43): 37274–9.
15. Yuen SL, Ogut O, Brozovich FV. Differential phosphorylation of LZ+/LZ- MYPT1 isoforms regulates MLC phosphatase activity. Arch Biochem Biophys. 2014 Aug 26; 562C: 37–42.
16. Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM, Mendelsohn ME. Regulation of myosin phosphatase by a specific interaction with cGMP- dependent protein kinase Ialpha. Science. 1999 Nov 24; 286(5444): 1583–7.
17. Huang QQ, Fisher SA, Brozovich FV. Unzipping the role of myosin light chain phosphatase in smooth muscle cell relaxation. J Biol Chem. 2004 Jan 2; 279(1): 597–603.
18. Murányi A, Derkach D, Erdödi F, Kiss A, Ito M, Hartshorne DJ. Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: inhibitory effects and occurrence in A7r5 cells. FEBS Lett. 2005 Dec 5; 579(29): 6611–5.
19. Trinkle-Mulcahy L, Ichikawa K, Hartshorne DJ, Siegman MJ, Butler TM. Thiophosphorylation of the 130-kDa subunit is associated with a decreased activity of myosin light chain phosphatase in alpha-toxin-permeabilized smooth muscle. J Biol Chem. 1995 Aug 4; 270(31):18191–4.
20. Ichikawa K, Ito M, Hartshorne DJ. Phosphorylation of the large subunit of myosin phosphatase and inhibition of phosphatase activity. J Biol Chem. 1996 Mar 1; 271(9): 4733–40.
21. Kitazawa T, Masuo M, Somlyo AP. G protein-mediated inhibition of myosin light-chain phosphatase in vascular smooth muscle. Proc Natl Acad Sci USA. 1991 Oct 15; 88(20): 9307–10.
22. Velasco G, Armstrong C, Morrice N, Frame S, Cohen P. Phosphorylation of the regulatory subunit of smooth muscle protein phosphatase 1M at Thr850 induces its dissociation from myosin. FEBS Lett. 2002 Sep 11; 527(1-3): 101–4.
23. DeFeo TT, Morgan KG. Calcium-force relationships as detected with aequorin in two different vascular smooth muscles of the ferret. J Physiol (Lond). 1985 Dec; 369: 269–82.
24. Kitazawa T, Gaylinn BD, Denney GH, Somlyo AP. G-protein-mediated Ca2+ sensitization of smooth muscle contraction through myosin light chain phosphorylation. J Biol Chem. 1991 Jan 25; 266(3): 1708–15.
25. Anabuki J, Hori M, Hayakawa K, Akahane S, Ozaki H, Karaki H. Muscarinic stimulation does not induce rhoA/ROCK-mediated Ca2+ sensitization of the contractile element in chicken gizzard smooth muscle. Pflugers Arch. 2000 Dec; 441(2-3): 189–99.
26. Solaro RJ. Myosin light chain phosphatase: a Cinderella of cellular signaling. Circ Res. 2000 Aug 4; 87(3): 173–5.
27. Chen YH, Chen MX, Alessi DR, Campbell DG, Shanahan C, Cohen P, Cohen PT. Molecular cloning of cDNA encoding the 110 kDa and 21 kDa regulatory subunits of smooth muscle protein phosphatase 1M. FEBS Lett. 1994 Dec 12; 356(1): 51–5.
28. Richards CT, Ogut O, Brozovich FV. Agonist-induced force enhancement: the role of isoforms and phosphorylation of the myosin-targeting subunit of myosin light chain phosphatase. J Biol Chem. 2002 Feb 8; 277(6): 4422–7.
29. Ikebe M, Brozovich FV. Protein kinase C increases force and slows relaxation in smooth muscle: evi-

dence for regulation of the myosin light chain phosphatase. Biochem Biophys Res Commun. 1996 Aug 14; 225(2): 370–6.

30. Kitazawa T, Takizawa N, Ikebe M, Eto M. Reconstitution of protein kinase C-induced contractile Ca2+ sensitization in triton X-100-demembranated rabbit arterial smooth muscle. J Physiol (Lond). 1999 Oct 1; 520 Pt 1: 139–52.
31. Eto M, Kitazawa T, Yazawa M, Mukai H, Ono Y, Brautigan DL. Histamine-induced vasoconstriction involves phosphorylation of a specific inhibitor protein for myosin phosphatase by protein kinase C alpha and delta isoforms. J Biol Chem. 2001 Aug 3; 276(31): 29072–8.
32. MacDonald JA, Borman MA, Muranyi A, Somlyo AV, Hartshorne DJ, Haystead TA. Identification of the endogenous smooth muscle myosin phosphatase-associated kinase. Proc Natl Acad Sci USA. 2001 Feb 27; 98(5): 2419–24.
33. Deng JT, Sutherland C, Brautigan DL, Eto M, Walsh MP. Phosphorylation of the myosin phosphatase inhibitors, CPI-17 and PHI-1, by integrin-linked kinase. Biochem J. 2002 Oct 15; 367(Pt 2): 517–24.
34. Rahman A, Davis B, Lövdahl C, Hanumaiah VT, Feil R, Brakebusch C, Arner A. The small GTPase Rac1 is required for smooth muscle contraction. J Physiol (Lond). 2014 Mar 1; 592(Pt 5): 915–26.
35. Puetz S, Lubomirov LT, Pfitzer G. Regulation of smooth muscle contraction by small GTPases. Physiology (Bethesda). 2009 Dec; 24: 342–56.
36. Uehata M, Ishizaki T, Satoh H, Ono T, Kawahara T, Morishita T, Tamakawa H, Yamagami K, Inui J, Maekawa M, Narumiya S. Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension. Nature. 1997 Oct 30; 389(6654): 990–4.
37. Mukai Y, Shimokawa H, Matoba T, Kandabashi T, Satoh S, Hiroki J, Kaibuchi K, Takeshita A. Involvement of Rho-kinase in hypertensive vascular disease: a novel therapeutic target in hypertension. FASEB J. 2001 Apr; 15(6): 1062–4.
38. Seko T, Ito M, Kureishi Y, Okamoto R, Moriki N, Onishi K, Isaka N, Hartshorne DJ, Nakano T. Activation of RhoA and inhibition of myosin phosphatase as important components in hypertension in vascular smooth muscle. Circ Res. 2003 Mar 7; 92(4): 411–8.
39. Tanaka K, Minami H, Kota M, Kuwamura K, Kohmura E. Treatment of cerebral vasospasm with intra-arterial fasudil hydrochloride. Neurosurgery. 2005 Feb; 56(2): 214–23.
40. Zhao J, Zhou D, Guo J, Ren Z, Zhou L, Wang S, Xu B, Wang R. Effect of fasudil hydrochloride, a protein kinase inhibitor, on cerebral vasospasm and delayed cerebral ischemic symptoms after aneurysmal subarachnoid hemorrhage. Neurol Med Chir (Tokyo). 2006 Sep; 46(9): 421–8.
41. Ishikura K, Yamada N, Ito M, Ota S, Nakamura M, Isaka N, Nakano T. Beneficial acute effects of rho-kinase inhibitor in patients with pulmonary arterial hypertension. Circ J. 2006 Feb; 70(2): 174–8.
42. Archer SL, Weir EK, Wilkins MR. Basic science of pulmonary arterial hypertension for clinicians: new concepts and experimental therapies. Circulation. 2010 May 11; 121(18): 2045–66.
43. Kitazawa T, Eto M, Woodsome TP, Brautigan DL. Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. J Biol Chem. 2000 Apr 7; 275(14): 9897–900.
44. Kitazawa T, Eto M, Woodsome TP, Khalequzzaman M. Phosphorylation of the myosin phosphatase targeting subunit and CPI-17 during Ca2+ sensitization in rabbit smooth muscle. J Physiol (Lond). 2003 Feb 1; 546(Pt 3): 879–89.
45. Shao J, Welch WJ, Diamond MI. ROCK and PRK-2 mediate the inhibitory effect of Y-27632 on polyglutamine aggregation. FEBS Lett. 2008 May 28; 582(12): 1637–42.
46. Gallo RM, Khan MA, Shi J, Kapur R, Wei L, Bailey JC, Liu J, Brutkiewicz RR. Regulation of the actin cytoskeleton by Rho kinase controls antigen presentation by CD1d. J Immunol. 2012 Aug 15; 189(4): 1689–98.
47. Anastasiadis PZ, Moon SY, Thoreson MA, Mariner DJ, Crawford HC, Zheng Y, Reynolds AB. Inhibi-

tion of RhoA by p120 catenin. Nat Cell Biol. 2000 Sep; 2(9): 637–44.

48. Kukar T, Murphy MP, Eriksen JL, Sagi SA, Weggen S, Smith TE, Ladd T, Khan MA, Kache R, Beard J, Dodson M, Merit S, Ozols VV, Anastasiadis PZ, Das P, Fauq A, Koo EH, Golde TE. Diverse compounds mimic Alzheimer disease-causing mutations by augmenting Abeta42 production. Nat Med. 2005 May; 11(5): 545–50.
49. Van Aelst L, D'Souza-Schorey C. Rho GTPases and signaling networks. Genes Dev. 1997 Sep 15; 11(18): 2295–322.
50. Murányi A, MacDonald JA, Deng JT, Wilson DP, Haystead TAJ, Walsh MP, Erdödi F, Kiss E, Wu Y, Hartshorne DJ. Phosphorylation of the myosin phosphatase target subunit by integrin-linked kinase. Biochem J. 2002 Aug 15; 366(Pt 1): 211–6.
51. Wu C, Dedhar S. Integrin-linked kinase (ILK) and its interactors: a new paradigm for the coupling of extracellular matrix to actin cytoskeleton and signaling complexes. J Cell Biol. 2001 Nov 12; 155(4): 505–10.
52. Nakamura K, Koga Y, Sakai H, Homma K, Ikebe M. cGMP-dependent relaxation of smooth muscle is coupled with the change in the phosphorylation of myosin phosphatase. Circ Res. 2007 Sep 28; 101(7): 712–22.
53. Wooldridge AA, MacDonald JA, Erdodi F, Ma C, Borman MA, Hartshorne DJ, Haystead TAJ. 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. J Biol Chem. 2004 Jun 15; 279(33): 34496–504.
54. Khasnis M, Nakatomi A, Gumpper K, Eto M. Reconstituted human myosin light chain phosphatase reveals distinct roles of two inhibitory phosphorylation sites of the regulatory subunit, MYPT1. Biochemistry. 2014 Apr 29; 53(16): 2701–9.
55. Surks HK, Richards CT, Mendelsohn ME. Myosin phosphatase-Rho interacting protein. A new member of the myosin phosphatase complex that directly binds RhoA. J Biol Chem. 2003 Dec 19; 278(51): 51484–93.
56. Vetterkind S, Lee E, Sundberg E, Poythress RH, Tao TC, Preuss U, Morgan KG. Par-4: a new activator of myosin phosphatase. Mol Biol Cell. 2010 Apr 1; 21(7):1214–24.
57. Grassie ME, Sutherland C, Ulke-Lemée A, Chappellaz M, Kiss E, Walsh MP, MacDonald JA. Cross-talk between Rho-associated kinase and cyclic nucleotide-dependent kinase signaling pathways in the regulation of smooth muscle myosin light chain phosphatase. J Biol Chem. 2012 Oct 19; 287(43): 36356–69.
58. Wang T, Kendig DM, Smolock EM, Moreland RS. Carbachol-induced rabbit bladder smooth muscle contraction: roles of protein kinase C and Rho kinase. Am J Physiol Renal Physiol. 2009 Dec; 297(6): F1534–42.
59. El-Yazbi AF, Johnson RP, Walsh EJ, Takeya K, Walsh MP, Cole WC. Pressure-dependent contribution of Rho kinase-mediated calcium sensitization in serotonin-evoked vasoconstriction of rat cerebral arteries. J Physiol (Lond). 2010 May 15; 588(Pt 10): 1747–62.
60. Johnson RP, El-Yazbi AF, Takeya K, Walsh EJ, Walsh MP, Cole WC. Ca2+ sensitization via phosphorylation of myosin phosphatase targeting subunit at threonine-855 by Rho kinase contributes to the arterial myogenic response. J Physiol (Lond). 2009 Jun 1; 587(Pt 11): 2537–53.
61. Ihara E, Moffat L, Ostrander J, Walsh MP, MacDonald JA. Characterization of protein kinase pathways responsible for Ca2+ sensitization in rat ileal longitudinal smooth muscle. Am J Physiol Gastrointest Liver Physiol. 2007 Oct; 293(4): G699–710.
62. Ito K, Shimomura E, Iwanaga T, Shiraishi M, Shindo K, Nakamura J, Nagumo H, Seto M, Sasaki Y, Takuwa Y. Essential role of rho kinase in the Ca2+ sensitization of prostaglandin F(2alpha)-induced contraction of rabbit aortae. J Physiol (Lond). 2003 Feb 1; 546(Pt 3): 823–36.
63. Chen C-P, Chen X, Qiao Y-N, Wang P, He W-Q, Zhang C-H, Zhao W, Gao Y-Q, Chen C, Tao T, Sun J,

Wang Y, Gao N, Kamm KE, Stull JT, Zhu M-S. In vivo roles for myosin phosphatase targeting subunit-1 phosphorylation sites T694 and T852 in bladder smooth muscle contraction. J Physiol (Lond). 2015 Feb 1; 593(3): 681–700.

64. Ararat E, Brozovich FV. Losartan decreases p42/44 MAPK signaling and preserves LZ+ MYPT1 expression. PLoS ONE. 2009; 4(4): e5144.
65. Han YS, Brozovich FV. Altered reactivity of tertiary mesenteric arteries following acute myocardial ischemia. J Vasc Res. 2013; 50(2): 100–8.
66. Singh DK, Sarkar J, Raghavan A, Reddy SP, Raj JU. Hypoxia modulates the expression of leucine zipper-positive MYPT1 and its interaction with protein kinase G and Rho kinases in pulmonary arterial smooth muscle cells. Pulm Circ. 2011 Oct; 1(4): 487–98.