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

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**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
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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 Gq and G11 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 G12 and G13 (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 Thr853 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% CO₂, 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.
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

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

Others have demonstrated Rho kinase is expressed in HEK293T cells (45, 46) and additionally, in HEK293T cells, GTP\( \gamma \)S stimulates Rho (47, 48). Further, Rho has been demonstrated to activate Rho kinase, PKN and PIP5 kinase (49). Therefore, the GTP\( \gamma \)S 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 GTP\( \gamma \)S treatment of HEK293T cells, MYPT1 phosphorylation at Thr696 and/or Thr853 is mediated by a GTP\( \gamma \)S 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 GTP\( \gamma \)S 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, GTP\( \gamma \)S 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 GTP\( \gamma \)S 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
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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.

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Conflict of Interest

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

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