Transduction of the N-Terminal Fragments of MYPT1 Enhances Myofilament Ca\(^{2+}\) Sensitivity in an Intact Coronary Artery

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Objective—The region of the 110 kDa regulatory subunit (MYPT1) of smooth muscle myosin phosphatase involved in the regulation of contraction was determined under physiological conditions.

Methods and Results—Using HIV Tat protein-mediated protein transduction, the N-terminal fragments of MYPT1 were introduced to the intact porcine coronary arterial strips. Pre-incubation with 3 \(\mu\)mol/L TAT-MYPT1\(^{1-374}\), a construct containing the Tat peptide and the residues 1 to 374 of MYPT1, for 15 minutes augmented (2.4-fold) the subsequent contraction induced by adding 1.25 mmol/L of extracellular Ca\(^{2+}\) under 118 mmol/L K\(^+\) depolarization, with no augmentation of the [Ca\(^{2+}\)], elevation. The deletion of the Tat peptide, MYPT1\(^{1-374}\), abolished the augmenting effect. TAT-MYPT1\(^{1-296}\) demonstrated a weaker but significant augmentation (1.7-fold). However, TAT-MYPT1\(^{1-171}\), TAT-MYPT1\(^{39-374}\), TAT-MYPT1\(^{19-296}\) and TAT-MYPT1\(^{297-374}\) had no augmenting activity. The myosin light chain phosphorylation level as a function of extracellular Ca\(^{2+}\) concentrations was shifted to the left in the strips pretreated with TAT-MYPT1\(^{1-374}\) compared with the control.

Conclusions—Region 1 to 296 was the minimal region involved in the enhancement of contraction, and region 297 to 374 played a supplemental role. These results suggested that the interaction mainly between catalytic subunit and MYPT1 play a critical role in the regulation of the endogenous myosin phosphatase in intact smooth muscle. (Arterioscler Thromb Vasc Biol. 2004;24:464-469.)

Key Words: myosin ■ phosphatase ■ smooth muscle ■ contraction ■ protein transduction

The increase in the vascular tone plays an important role in the pathophysiology of the vascular diseases such as hypertension and vasospasm. The tone of the vascular smooth muscle is primarily regulated by Ca\(^{2+}\)-dependent reversible phosphorylation of the 20 kDa myosin light chain (MLC). \(^{1-3}\) The enhanced MLC phosphorylation for a given level of Ca\(^{2+}\) enhances the myofilament Ca\(^{2+}\) sensitivity, and thus increases vascular tone. The level of MLC phosphorylation is determined by the balance between the activities of MLC kinase (MLCK) and MLC phosphatase (MLCP). \(^{1,4}\) Because the activity of MLCK is primarily determined by the Ca\(^{2+}\) level, the decrease in the MLCP activity is considered to play a key role in the enhancement of the Ca\(^{2+}\) sensitivity. \(^{3,4}\) MLCP is composed of three subunits: the 38 kDa catalytic subunit (PP1c), the 110 kDa regulatory myosin phosphatase target subunit (MYPT1), and the 20 kDa regulatory subunit (M20). \(^{4,9}\) MYPT1 plays an important role in targeting MLCP to myosin filaments, enhancing substrate specificity toward myosin, and regulating enzymatic activity. \(^{4}\) The phosphorylation of T696 (in human MYPT1) was shown to be associated with an inhibition of the MLCP activity, and is thus considered to be one of the important mechanisms for the regulation of the MLCP activity. \(^{4}\) This phosphorylation is catalyzed by several kinases including Rho kinase, Zip-like kinase, and myotonic dystrophy protein kinase and integrin-linked kinase. \(^{10-13}\)

Primarily, the interaction between PP1c and MYPT1 increases the activity of PP1c toward MLC while inhibiting the activity toward phosphorylase. \(^{14,16}\) The enhancement of the activity toward MLC requires the N-terminal part of MYPT1. \(^{15}\) It is conceivable that the increased activity toward MLC can be attributed to the interactions among the N-terminal part of MYPT1, PP1c, substrate MLC, and myosin filament. The N-terminal 38 residues, especially the PP1c-binding motif (K/R-V/I-x-F), \(^{17}\) and the C-terminal half of the ankyrin repeats domain play a critical role in binding to PP1c. \(^{14,15}\) The phosphorylated MLC was shown to bind to the C-terminal half of the ankyrin repeats. \(^{15}\) Both N-terminal and...
and C-terminal18 region of MYPT1 have been shown to bind to myosin. The essential region of MYPT1 for myosin binding is thus still controversial.15,16 Nevertheless, precisely which interaction plays an important role in the regulation of MLCP, and therefore in MLC phosphorylation and smooth muscle contraction, under physiological conditions remains to be elucidated.

It was reported that the covalent conjugation of protein transduction domain (PTD) of HIV transcription factor Tat19 allows the conjugated protein to enter the cells with intact plasma membrane,20–22 in a quantitative manner within 10 minutes.23 This technique is thus anticipated to be a powerful tool to investigate the physiological role of the functional proteins under physiological conditions. In the present study, using the Tat PTD-mediated protein transduction technique, we introduced the N-terminal fragments of MYPT1 into the intact smooth muscle of the isolated arterial strips and thus determined which part of the MYPT1 is important in the regulation of MLCP under physiological conditions.

**Methods**

The N-terminal fragments of MYPT1 (Figure I, available online at http://atvb.ahajournals.org) were introduced into porcine coronary artery medial strips as fusion proteins tagged with Tat PTD at their N-terminus. The recombinant proteins were expressed in bacteria and purified with a Ni2+-loaded Hi-Trap chelating column (Pharmacia Biotech, Tokyo, Japan). The arterial strips without endothelium were prepared as previously described.24,25 The effects of N-terminal fragments of MYPT1 on [Ca2+]i, isometric force, and MLC phosphorylation were examined as previously described. All data were expressed as the mean±SD. The unequal Student t test and an analysis of variance (ANOVA) evaluated statistical significance; P<0.05 were considered significant.

See Methods (available online at http://atvb.ahajournals.org) for expanded information.

**Results**

**Transduction of Tat PTD-Tagged Recombinant Proteins Into Intact Strips of Porcine Coronary Artery**

The Tat PTD-mediated transduction of protein into the intact arterial strips was verified as shown in online supplementary Figures II and III (available online at http://atvb.ahajournals.org). First, the transduction of green fluorescence protein (GFP) was observed under a confocal microscopy, which demonstrated a specific fluorescence with a pattern of smooth muscle fibers similar to that observed with the fura-2 fluorescence in the fura-2-loaded strips, as we previously reported. Furthermore, the transduction of all Tat PTD-tagged MYPT1 fragments was detected by immunoblot analysis using anti-(His)6 antibody. In both experiments, transduction of the proteins without PTD was not detected.

Enhancement of the Ca2+-Induced Contraction by Transduction of the N-Terminal Fragments of MYPT1 in the Intact Strips

The strips were stimulated with 118 mmol/L K+ in the presence of 1.25 mmol/L extracellular Ca2+, and the contractile response was recorded as a reference response (Figure 1a). The strips were then exposed to the Ca2+-free PSS with and then without 2 mmol/L EGTA for 5 minutes each and stimulated with 118 mmol/L K+ in the Ca2+-free PSS for 15 minutes. The stimulation with 118 mmol/L K+ in the absence of extracellular Ca2+ did not induce any contraction, whereas the subsequent addition of extracellular Ca2+ induced a contraction (Figure 1a). In the vehicle-treated strips, the tension (111.7%±9.2%, n=9) obtained by the addition of 1.25 mmol/L of Ca2+ was similar (P>0.05) to the reference response. Pretreatment with 3 μmol/L TAT-MYPT1–374 during the 15-minute stimulation with 118 mmol/L K+ in the Ca2+-free PSS augmented the subsequent contraction (Figure 1b). This effect of TAT-MYPT1–374 was reversible and reproducible (Figure 1e). Tension induced by replenishing 1.25 mmol/L Ca2+ was 267.4%±22.1% (n=10) in the strips treated with 3 μmol/L TAT-MYPT1.1–374. The protein was removed from the bathing solution when the extracellular Ca2+ was added, because the continuous presence of elution buffer at 10-fold dilution, regardless of the presence or absence of the proteins, inhibited the tension development (data not shown). Imidazole in the elution buffer was considered to be responsible for inhibition of contractility. However, removal of imidazole by dialysis caused precipitation of the protein. We thus used this experimental protocol to
examine the effect of TAT-proteins on the contraction. Pretreatment with MYPT1\(^{1–374}\) without Tat PTD demonstrated no significant augmenting effect (Figure 1c). Pretreatment with 3 \(\mu\)mol/L TAT-GFP did not have any augmenting effect on the contraction (Figure 1d). The simultaneous measurement of \([Ca^{2+}]_i\), and tension revealed that the augmentation of tension by TAT-MYPT1\(^{1–374}\) was not associated with the augmentation of \([Ca^{2+}]_i\) elevation (Figure 2). The replenishment of 1.25 mmol/L Ca\(^{2+}\) produced a similar \([Ca^{2+}]_i\) elevation to the reference response in TAT-MYPT1\(^{1–374}\)-treated and vehicle-treated strips (Figure 2c).

The augmentation of the contraction by TAT-MYPT1\(^{1–374}\) was dependent on the incubation time and the concentration, as shown in online supplementary Figure IV (available online at http://atvb.ahajournals.org). The level of tension at rest (in the normal PSS containing 5.9 mmol/L K\(^+\), 1.25 mmol/L Ca\(^{2+}\)) and that obtained with the first contraction induced by 118 mmol/L K\(^+\) were assigned to be 0% and 100%, respectively. The resting level of \([Ca^{2+}]_i\) is indicated by the broken lines. Data are the mean±SD (n=3). *Significantly different \((P<0.05)\); NS indicates not significantly different.

Determination of the MYPT1\(^{1–374}\) Region Required for the Enhancement of Ca\(^{2+}\)-Induced Contraction

The effect of various fragments of MYPT1 on the Ca\(^{2+}\)-induced contraction was examined to determine the essential region for the enhancement of contraction (Figure 3). We compared the effects of truncation mutants at a fixed concentration (3 \(\mu\)mol/L) and at incubation time (15 minutes). TAT-MYPT1\(^{1–296}\), which lacks a region containing an acidic cluster, still enhanced the Ca\(^{2+}\)-induced contraction (Figure 3a). The extent of tension obtained with 1.25 mmol/L Ca\(^{2+}\) (183.4±11.0%, n=9) was significantly smaller than that obtained with TAT-MYPT1\(^{1–374}\). TAT-MYPT1\(^{1–171}\), which lacks a C-terminal half of the ankyrin repeats, had no significant effect (Figure 3b), and the extent of tension obtained with 1.25 mmol/L Ca\(^{2+}\) was 99.4±10.4% (n=9).

Enhancement of the Ca\(^{2+}\)-induced contraction required TAT-MYPT1\(^{1–374}\) concentrations of 3 \(\mu\)mol/L and higher (Figure IVb).
The deletion of N-terminal 38 residues containing the PP1c-binding motif, TAT-MYPT1-374 and TAT-MYPT1-296 abolished the augmenting effects of TAT-MYPT1-374 and TAT-MYPT1-296, respectively (Figure 3c and 3d). However, TAT-MYPT1-297-374, containing the acidic cluster but not the N-terminal ankyrin repeats, did not enhance the Ca\(^{2+}\)-induced contraction (Figure 3e). As a result, region 1 to 296 was the minimal sequence required for augmentation of the Ca\(^{2+}\)-induced contraction in the intact tissue.

The effects of the MYPT1 fragments on the Ca\(^{2+}\)-induced contraction were quantitatively evaluated by inducing contraction with various concentration of Ca\(^{2+}\) (Figure 3f). In the vehicle-treated strips, a significant contraction developed by adding 0.125 mmol/L and higher concentrations of Ca\(^{2+}\), and the extent of tension obtained with 1.25 mmol/L of Ca\(^{2+}\) was similar to the reference response. TAT-MYPT1-1-374 augmented the contraction seen with 0.125 mmol/L of Ca\(^{2+}\) and higher concentrations. TAT-MYPT1-1-296 demonstrated a weaker enhancement, while the other constructs, MYPT1-1-374, TAT-MYPT1-1-171, TAT-MYPT1-297-374, TAT-MYPT1-199-374, and TAT-MYPT1-19-296 did not demonstrate significant enhancement (Figure 3f).

The correlation between in vivo effect of MYPT1 fragments on the contraction and the in vitro effect on the activity of the isolated MLCP holoenzyme was studied. TAT-MYPT1-1-374 significantly inhibited the activity of the isolated MLCP toward MLC, whereas TAT-MYPT1-1-171 and TAT-GFP also demonstrated an inhibitory effect (data not shown). However, the inhibitory effect seen with TAT-MYPT1-1-374 was greater than that seen with TAT-MYPT1-1-171 and TAT-GFP.

Enhancement of MLC Phosphorylation by TAT-MYPT1-1-374

The effect of TAT-MYPT1-1-374 transduction on the MLC phosphorylation was examined under the same experimental conditions as those used in the tension measurements. The resting level of MLC phosphorylation (21.3±1.8%, \(n=7\)) obtained in the normal PSS (5.9 mmol/L K\(^{+}\), 1.25 mmol/L Ca\(^{2+}\)) decreased significantly \((P<0.05)\) to 7.3±3.9% \((n=7)\) after exposing the strips to the Ca\(^{2+}\)-free PSS and just before replenishing the extracellular Ca\(^{2+}\) in the vehicle-treated strips (Figure 4a and 4b). The subsequent Ca\(^{2+}\) replenishment induced a concentration-dependent increase in MLC phosphorylation, as determined at the peak of contraction (3 to 4 minutes after initiation of contraction) (Figure 4a and 4b). The level of MLC phosphorylation obtained with replenishment of 1.25 mmol/L Ca\(^{2+}\) was 43.8±5.5% \((n=7)\), and this value was similar to that obtained with the reference contraction \((41±2.7\%, n=7)\). However, when the strips were pretreated with 3 μmol/L TAT-MYPT1-1-374, the level of MLC phosphorylation obtained just before the Ca\(^{2+}\) replenishment did not significantly differ from that obtained in the control strips. The MLC phosphorylation levels obtained with 0.125 mmol/L and 0.25 mmol/L Ca\(^{2+}\) were significantly higher than the control levels, whereas the MLC phosphorylation obtained with 0.5 mmol/L and 1.25 mmol/L Ca\(^{2+}\) did not significantly differ from the control. The analysis at the earlier time point also revealed that the phosphorylation level obtained with 0.5 mmol/L and 1.25 mmol/L Ca\(^{2+}\) in the TAT-MYPT1-1-374-treated strips was similar to the control (data not shown). The concentration-dependent increases in MLC phosphorylation as a function of the extracellular Ca\(^{2+}\) obtained in the TAT-MYPT1-1-374-treated strips shifted to the left of that obtained in the vehicle-treated strips (Figure 4b). The tension obtained with 0.5 mmol/L and 1.25 mmol/L Ca\(^{2+}\) in the TAT-MYPT1-1-374-treated strips was thus not associated with an increase in MLC phosphorylation.

Discussion

We herein demonstrated that Tat PTD-mediated protein transduction was successfully applied to intact arterial strips. It was reported earlier that Clostridium botulinum exoenzyme C3 tagged with Tat PTD inhibited the urotensin-induced contraction in the rat aorta.\(^{29}\) In the present study, the introduction of the protein into the cells by Tat PTD was clearly proved by the observation of GFP fluorescence in the Tat-GFP-treated strips, and by immunoblot detection of Tat PTD-tagged MYPT1 fragments in the extract of the strips. The introduction of protein is also supported by the observation that the MYPT1 fragments enhanced the Ca\(^{2+}\)-induced contraction only when tagged with Tat PTD. The time needed to obtain a significant enhancement of contraction suggested that the transduction of functional protein into the intact

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![Figure 4.](http://atvb.ahajournals.org/)

Enhancement of MLC phosphorylation by TAT-MYPT1-1-374 in intact medial strips of the porcine coronary artery. Representative photos (a) showing MLC immunoblot analysis in the strips treated with 3 μmol/L TAT-MYPT1-1-374 and vehicle (control). The samples were obtained at the peak of contraction during the tension measurement with the experimental protocol the same as in Figure 1, except for the fact that the contraction was initiated by replenishing 0 mmol/L (just before replenishing Ca\(^{2+}\)), 0.125 mmol/L, 0.25 mmol/L, 0.5 mmol/L, and 1.25 mmol/L Ca\(^{2+}\). The resting level (resting) was determined in the normal PSS (5.9 mmol/L K\(^{+}\), 1.25 mmol/L Ca\(^{2+}\)) before incubation with proteins. MLC indicates unphosphorylated MLC; MLC-P, monophosphorylated MLC. The extent of MLC phosphorylation obtained at the peak of contraction (b) induced by replenishment of different concentrations of Ca\(^{2+}\) in the strips treated with 3 μmol/L TAT-MYPT1-1-374 and the vehicle (control). *P<0.05; NS indicates not significantly different. Data are the mean±SD \((n=7)\).
arterial strips takes place within 10 minutes, which is consistent with previous reports.22,23

Treatment with TAT-MYPT1-1–374 enhanced the Ca$^{2+}$-induced contraction with no effect on the [Ca$^{2+}$], elevation, suggesting that the Ca$^{2+}$ sensitivity of the contractile apparatus was potentiated. The following two control experiments excluded the possibilities that TAT-MYPT1-1–374 acted from outside of the cells and that Tat PTD per se enhanced the contraction. First, the deletion of Tat PTD abolished the augmenting effect of TAT-MYPT1-1–374 (Figure 1b). Second, TAT-GFP and some of the truncation mutants of MYPT1 had no effect on the contraction (Figures 1d and 3). We thus conclude that MYPT1-1–374 was introduced into the cells and enhanced the myofilament Ca$^{2+}$ sensitivity in the intact muscle. This observation is consistent with previous observations of Triton X-100 permeabilized muscle.26 However, the enhanced contraction seen in the strips pretreated with TAT-MYPT1 fragments was not sustained. According to the experimental protocol, the strips were no longer exposed to the TAT-MYPT1 fragments during the contraction initiated by replenishing extracellular Ca$^{2+}$. The decline of the contraction may reflect the leak of the transduced protein from the strips. Indeed, the augmenting effect seen with TAT-MYPT1 fragments was reversible and reproducible (Figure 1e).

The enhancement of Ca$^{2+}$ sensitivity by TAT-MYPT1-1–374 was associated with an increase in MLC phosphorylation, especially in the contractions induced by replenishing Ca$^{2+}$ at levels less than 0.5 mmol/L. The Ca$^{2+}$–MLC phosphorylation curve was thus shifted to the left (Figure 4). The level of MLC phosphorylation is determined mainly by the balance between MLCK activity and MLCP activity.4 It is conceivable that this balance shifted to the kinase-dominated state by the introduction of TAT-MYPT1-1–374, namely either the MLCP activity decreased or the MLCK activity increased. The concentration of intracellular TAT-MYPT1-1–374 was estimated to be in μmol/L order by quantitative immunoblot analysis with a purified recombinant protein as reference (data not shown), and this estimation is consistent with the concentration of endogenous MYPT1 (3 μmol/L) as reported in the literature.4 We thus suggest that the introduced TAT-MYPT1-1–374 served as a dominant negative mutant against endogenous MYPT1 and inhibited the MLCP activity. The dominant negative effect of TAT-MYPT1-1–374 may be supported by its inhibitory effect on the isolated MLCP. However, in vitro phosphatase assay also demonstrated a lesser inhibitory effect of TAT-MYPT1-1–371 and TAT-GFP on MLCP. We thus suggest that the in vitro effect of TAT-MYPT1-1–371 and TAT-GFP was insufficient to exert an inhibitory effect in vivo, while the additional inhibition seen with TAT-MYPT1-1–374 correlated to the dominant negative effect seen in vivo.

However, the contraction induced by replenishing 0.5 mmol/L and higher concentrations of Ca$^{2+}$ was not associated with an increase in MLC phosphorylation (Figure 4). This enhancement of contraction was thus suggested to be independent of MLC phosphorylation. The Ca$^{2+}$ sensitivity of the contractile apparatus can be regulated in a manner either dependent on or independent of MLC phosphorylation.20–23 It is thus possible that the transduced MYPT1 might modulate such MLC phosphorylation-independent mechanism and thereby enhance the Ca$^{2+}$ sensitivity. The precise mechanism for enhancement of contraction seen with high Ca$^{2+}$ concentrations remains to be determined.

The present study suggests that region 1 to 296 is the minimal sequence required for a significant enhancement of Ca$^{2+}$ sensitivity in intact artery. Region 1 to 296 contains PP1c-binding motif and ankyrin repeats,4,14,15,17 and the C-terminal half of the ankyrin repeats are involved in PP1c binding and substrate binding.15 It is thus suggested that the introduced MYPT1 fragments primarily impaired the endogenous interaction between PP1c and MYPT1 mediated by region 1 to 296 and inhibited the endogenous MLCP. A dissociation of PP1c from the holoenzyme was reported to play an important role in inhibiting the myosin phosphatase activity,35,36 However, the augmenting effect of TAT-MYPT1 fragments was demonstrated to be reversible and reproducible (Figure 1e). It is thus unlikely that exogenous MYPT1 fragments caused substantial dissociation of PP1c from the holoenzyme and thereby inhibited the myosin phosphatase activity. MYPT1 fragments are rather suggested to cause subtle changes in protein interactions involved in the regulation of MLCP activity.

Both the present study and our previous study26 demonstrated MYPT1-1–374 to enhance contraction. However, there is a discrepancy in the region of MYPT1-1–374 that played an essential role in enhancing contraction between two studies. In the previous study, the Triton X-100–permeabilized strips of the porcine interlobular renal artery were used, and the recombinant proteins tagged with (His)$_n$ were introduced. The analysis of the various truncated fragments determined region 304 to 374 to be necessary and sufficient for enhancement of contraction, thus suggesting this region to play an essential role in regulation of endogenous MLCP in the Triton X-100–permeabilized strips. The region 1 to 296 played no significant role in the permeabilized strips. However, the present study found that region 1 to 296 was necessary for enhancing the contraction and thus played an essential role in regulating endogenous MLCP, whereas region 297 to 374 played supplemental role. As a result, the relative importance of two regions 1 to 296 and 297 to 374 within MYPT1-1–374 seems to differ with experimental conditions. Both regions play an important role in intact strips, although region 1 to 296 is essential. However, in the permeabilized preparation, region 297 to 374 plays an essential role. One possible explanation for this discrepancy is the effect of Triton X-100 on the protein interaction. The region 1 to 296 contains the ankyrin repeats domain,7 which is predicted to form a protein-interacting platform consisting of repeated α-helices and β-turns.37 The region 297 to 374 contains an acidic cluster and is thus considered to mediate ionic interaction. Triton X-100, a nonionic detergent, may disturb some of the nonionic interactions. We thus speculate that the interaction mediated by region 1 to 296 is more susceptible to Triton X-100 than is the interaction mediated by the acidic cluster; therefore, the functional importance of the acidic cluster may be magnified in the permeabilized muscle. However, this possibility remains to be examined.
In conclusion, we herein demonstrate that the introduction of the N-terminal fragment of MYPT1 using Tat PTD enhanced the myofilament Ca$^{2+}$ sensitivity by primarily inhibiting the endogenous MLCP activity toward MLC and possibly other substrates. The protein interactions mediated by region 1 to 296 of MTPT1, such as the interaction between PP1c and MYPT1, are thus suggested to play a critical role in the regulation of MLCP in intact muscle. The interactions mediated by the acidic cluster are thus suggested to play a supplemental role under physiological conditions. We therefore propose that Tat-mediated protein transduction is a powerful tool for investigating the physiological role of functional protein in the cells with an intact plasma membrane.

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