Differential Association and Localization of Myosin Phosphatase Subunits During Agonist-Induced Signal Transduction in Smooth Muscle

Heung-Mook Shin, Hyun-Dong Je, Cynthia Gallant, Terence C. Tao, David J. Hartshorne, Masaaki Ito, Kathleen G. Morgan

Abstract—It has been known for some time that agonist-induced contractions of vascular smooth muscle are often associated with a sensitization of the contractile apparatus to intracellular \( \text{Ca}^{2+} \). One mechanism that has been suggested to explain \( \text{Ca}^{2+} \) sensitization is inhibition of myosin phosphatase activity. In the present study, we tested the hypothesis that differential localization of the phosphatase might be associated with its inhibition. Quantitative confocal microscopy of freshly dissociated, fully contractile smooth muscle cells was used in parallel with measurements of myosin light chain and myosin phosphatase phosphorylation. The results indicate that, in the smooth muscle cells, the catalytic and targeting subunits of the phosphatase are dissociated from each other in an agonist-specific manner and that the dissociation is accompanied by a slower rate of myosin phosphorylation. Targeting of myosin phosphatase to the cell membrane precedes the dissociation of subunits and is associated with phosphorylation of the targeting subunit at a Rho-associated kinase (ROK) phosphorylation site. The phosphorylation and membrane translocation of the targeting subunit are inhibited by a ROK inhibitor. This dissociation of subunits may provide a mechanism for the decreased phosphatase activity of phosphorylated myosin phosphatase. (Circ Res. 2002;90;667-673.)

Key Words: myosin phosphatase ■ smooth muscle ■ Rho-associated kinase ■ contractility ■ myosin phosphorylation

Phosphorylation of the 20-kDa myosin light chains by myosin light chain kinase (MLCK) is generally accepted to be an important mechanism by which contractility of smooth muscle is regulated. Myosin phosphatase (MP) was originally assumed to be constitutively active and not regulated. However, it has been known for some time that agonist-induced contractions can involve “Ca\(^{2+}\)” sensitization; i.e., increased force production at any given [Ca\(^{2+}\)]. One mechanism that has been suggested to explain Ca\(^{2+}\) sensitization in cases where LC20 phosphorylation is also increased is inhibition of MP by signaling pathways. MP is composed of 3 subunits: a catalytic subunit (PP1c), a large noncatalytic subunit (targeting subunit, MYPT1, M130), and a small noncatalytic subunit of unknown function (M20). The sequence of the cloned MP catalytic subunit in chicken gizzard is identical to PP1cδ, and it is generally assumed that the δ isoform is the main isoform in differentiated smooth muscle.

Binding of MP to myosin has been demonstrated in cell fractionation studies and in vitro by affinity chromatography, suggesting that the phosphatase is targeted to its substrate in cells. However, limited cell imaging studies on cultured smooth muscle cells have indicated a predominant localization of the targeting subunit in the cytoplasm or at the cell membrane. The membrane association of MP raises questions as to its function at a location distant from the myosin filaments. However, a putative upstream kinase of MP, Rho-associated kinase (ROK), has also been found to localize at the cell membrane in freshly enzymatically dissociated smooth muscle cells, and it has been suggested that the membrane may form a scaffolding site where signal transduction regulating MP activity can be organized.

Here, we provide evidence from freshly isolated, fully differentiated smooth muscle cells that the catalytic and targeting subunits can be dissociated from each other in an agonist-specific manner, and that the dissociation is accompanied by a slower rate of LC20 dephosphorylation. Furthermore, the apparent dissociation of the catalytic and targeting subunits occurs after a targeting of MP to the cell membrane that is associated with increased phosphorylation of MYPT1 at a ROK phosphorylation site and is inhibited by a ROK inhibitor.
inhibitor. This dissociation of subunits in smooth muscle cells may provide an additional mechanism for the decreased phosphatase activity of phosphorylated MP.

Materials and Methods

Tissue Preparation
All procedures were performed according to protocols approved by the Institutional Animal Care and Use Committee. Ferrets were killed by an overdose of chloroform, and the portal vein was quickly removed to a dissection dish filled with oxygenated physiological saline solution (PSS). The tissue was then cut into strips and attached to a force transducer for contractility experiments as previously described or used for single cell isolation as described in the following section.

Preparation of Single Cells
Single cells from ferret portal vein were enzymatically isolated using a modification of a previously published method. For each 50 mg of portal vein (wet weight), the digestion medium A consisted of 4.2 mg CLS 2 collagenase (type II, 228 U/mg; Worthington Biochemical), 5.6 mg elastase (Grade II, 3.65 U/mg; Boehringer Mannheim), and 5000 U soybean trypsin inhibitor (type II-S, Sigma) in 7.5 mL of Ca\(^{2+}\)-Mg\(^{2+}\)-free Hanks’ balanced salt solution (HBSS). For all experiments, isolated cells were first tested to confirm that they shortened in response to phenylephrine.

Digital Imaging
Cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with 10% goat serum, and reacted with the appropriate primary antibody at the same concentration as used for Western blots (Figure 1). This was followed either by a goat anti-rabbit Rhodamine Red-X secondary antibody (1:500 Molecular Probes) or a goat anti-mouse Rhodamine Red-X secondary antibody (1:500 Molecular Probes) and mounted with Fluorosave (Calbiochem). Images were obtained using a Kr/Ar laser (Radiance 2000) scanning confocal microscope equipped with Nikon X-60 (NA 1.4) oil immersion objectives. Images were recorded with Laser Sharp 2000 for Windows NT. A previously described ratio analysis was performed to determine the relative distribution of MYPT1 and PP1c subunits within each cell and to normalize for possible differences in staining efficiency between cells.

Measurements of LC20 Phosphorylation
Muscle strips were quick-frozen by immersion in a dry ice-acetone slurry containing 10% trichloroacetic acid (TCA) and 10 mmol/L dithiothreitol (DTT). Tissues were brought to room temperature in acetone/TCA/DTT, then ground with glass pestles, and washed with ether to remove TCA. Tissues were extracted in a urea sample buffer as previously described and run on 10% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride (PVDF) membranes and subjected to immunoblot with a specific LC20 antibody (1:1500, Sigma). Anti-mouse IgG (Goat) conjugated with horseradish peroxidase was used as a secondary antibody (1:2000, Calbiochem). Bands were detected with enhanced chemiluminescence (ECL) (Supersignal, Pierce) visualized on films and then analyzed by NIH Image or were visualized and analyzed on a Bio-Rad PhosphorImager.

Immunoblotting
Tissue samples were quick-frozen and homogenized as previously described. Protein-matched samples were electrophoresed by SDS-PAGE (ProtoGel, National Diagnostics), transferred to PVDF (Millipore) membranes, and subjected to immunostaining and densitometry as previously described using the appropriate primary antibodies. Equal lane loading was confirmed by inspection of the membrane after Napthol Blue Black staining. Any differences were corrected by normalization to the densitometry of the actin bands. An HRP-labeled secondary antibody (goat anti-mouse 1:2000, Calbiochem; or goat anti-rabbit 1:2000) was used with ECL (Supersignal CL-HRP Substrate System, Pierce) to visualize the signal. Bands were detected on film and analyzed by NIH Image or detected and analyzed with a Bio-Rad PhosphorImager.

Figure 1. A, Western blots of ferret portal vein whole-cell homogenates with antibodies used for imaging studies. M130a and M130b recognize only the MP-targeting subunit. N-PP1\(\delta\) and C-2PP1\(\delta\) recognize only the catalytic subunit. See text for details. B, Low-power confocal images of MYPT1 and PP1c distribution in freshly isolated resting ferret portal vein smooth muscle cells. Horizontal bar=10 \(\mu\)m.
Solutions and Materials
PSS contained (in mmol/L) 120 NaCl, 5.9 KCl, 2.5 CaCl_2, 1.2 MgCl_2, 25 NaHCO_3, 1.2 NaH_2PO_4, and 11.5 dextrose at pH 7.4 when bubbled with 95% O_2 15% CO_2. HBSS contained (in mmol/L) 137 NaCl, 5.4 KCl, 0.44 KH_2PO_4, 0.42 NaH_2PO_4, 4.17 NaHCO_3, 5.55 glucose, and 10 HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], pH 7.4. PBS-Tween solution contained (in mmol/L) 80 Na_2HPO_4, 20 NaH_2PO_4, 100 NaCl, and 0.05% Tween.

The following drugs were used: PGF_2\alpha (Sigma) and phenylephrine (PE) (Sigma). General laboratory reagents were of analytical grade or better and were purchased from Sigma and Fisher Scientific.

Statistics
All values given in the text are mean ± SE. Differences between means were evaluated using a Student’s t test. Significant differences were taken at the P<0.05 level. The n values given represent numbers of cells used in each experiment.

Results
Immunoblots of all antibodies used for confocal microscopy in this study (Figure 1A) showed a single band of staining at the expected molecular weight against whole-cell homogenates of ferret portal vein, indicating that the antibodies were suitable for imaging studies. Two antibodies were used to detect MYPT1, M130a (F38.130 1:10 000 polyclonal obtained from Covance, Richmond, Calif) and M130b (monoclonal 1:5000). The majority of the data, and all of the results illustrated here, were obtained with C-2PP1δ, but similar results were also obtained with N-PP1δ.

MP Localization in Ferret Portal Vein Cells in the Absence of Stimuli
Figure 1B shows a low-power view of center optical sections of whole cells in the absence of agonists, stained for MYPT1 (left panel) or PP1c (right panel). In unstimulated cells, the staining for both MYPT1 and PP1c was seen throughout the cell, with the exception of a lack of staining typically in the intranuclear space. With identical methods, we have previously been able to detect intranuclear proteins; thus, we conclude that MYPT1 and PP1c are excluded from the nuclear space in these cells.

PGF_2\alpha Causes an Apparent Dissociation of MYPT1 and Catalytic Subunits
PGF_2\alpha has previously been shown to increase LC20 phosphorylation levels at a given [Ca^{2+}]. PGF_2\alpha has been reported to cause Ca^{2+} sensitization by inhibition of the phosphatase. After the addition of a maximally effective concentration of PGF_2\alpha (10^{-7} mol/L), MYPT1 appears to translocate from the cytosol to the vicinity of the cell membrane. The translocation is detectable by 5 minutes after the addition of PGF_2\alpha and persists through 15 minutes (Figure 2A).

As is shown in Figure 2B, PP1c also appears to dock at the cell membrane 5 minutes after the addition of PGF_2\alpha, but then PP1c appears to dissociate from MYPT1 and redistribute to the center of the cell at 8 and 15 minutes. We quantitated the distribution of the staining for the subunits by measuring the ratio of the confocal fluorescence.
at the surface of the cell to that at the core of the cell. Line scans across the diameter of the cell were obtained from center optical sections of each cell. The surface value was taken as the peak pixel intensity over the outer 15% of the cell width, and the core value was taken as the peak value in the remaining central 70% of the cell (inset, Figure 3). The nuclear area was excluded from analysis, and the numbers reported for each cell are an average from 3 line scans. Cells (10 to 15) were analyzed for each experimental group. In the resting cells, the ratio for MYPT1 was $0.85 \pm 0.04$, and in the presence of PGF2α, the ratio increased significantly ($P < 0.01$ compared with resting cells) to a maximum of $2.85 \pm 0.35$ at 5 minutes and continued to be significantly increased above basal levels through 15 minutes ($P < 0.01$). The ratio for PP1c in the resting cells was $0.87 \pm 0.03$ and this increased significantly ($P < 0.01$) to $2.15 \pm 0.12$ at 5 minutes. By 8 minutes, however, the ratio was decreased to a value of $0.93 \pm 0.04$, not significantly different from the resting value ($P > 0.05$). Thus, from 8 to 15 minutes the average distribution of 2 subunits differ in a statistically significant manner in that MYPT1 is primarily at the surface of the cell and PP1c is distributed throughout the width of the cell.

**Temporal Profile of MP Subunit Targeting Is Agonist-Specific**

In contrast, when a different agonist was used to increase contractility, a different pattern of distribution of MP subunits was obtained. Phenylephrine is an α agonist that has been reported to contract ferret aorta smooth muscle by a mechanism involving both an increase in Ca$^{2+}$-dependent MLCK activity and thin filament regulation. When ferret portal vein cells were stimulated with a maximally effective concentration of phenylephrine ($10^{-5}$ mol/L), staining for MYPT1 was distributed throughout the cytoplasm at all time points investigated (Figure 4).

The localization of PP1c in the presence of phenylephrine was complex. At 2.5 and 5 minutes of exposure to phenylephrine, a pattern of staining that was clearly not homogeneous was detected. However, by 8 minutes, PP1c had a homogeneous distribution across the cell diameter, similar to...
that of resting cells (Figure 5). A montage of images of several cells is shown in Figure 5 to illustrate the general nature of the pattern.

Thus, because MYPT1 appears to be localized throughout the cell in the presence of phenylephrine (as opposed to being targeted to the cell membrane in the case of PGF2α), it seems more likely that in the presence of phenylephrine MYPT1, together with PP1c, interacts with myosin in the core of the cell.

**Temporal Profile of LC20 Phosphorylation Is Agonist-Specific**

Our laboratory has previously reported that in ferret aorta at 37°C, PGF2α induces a relatively sustained elevation of LC20 phosphorylation but the α agonist phenylephrine induces a primarily transient elevation of LC20 phosphorylation.19,20 In order to determine if the same agonist-specific pattern of LC20 phosphorylation occurs in smooth muscle from the ferret portal vein at 22°C, ie, the temperature at which the single cell studies were performed, we measured LC20 phosphorylation levels.

Resting levels of LC20 phosphorylation were somewhat higher than those previously reported by this laboratory for ferret aorta at 37°C.3,18,20 The difference could be due either to the difference in temperature or the difference in tissue or both. However, as previously seen for ferret aorta, phenylephrine increased LC20 phosphorylation levels (Figure 6A) to a high level initially (43%) followed by a relatively rapid decline in phosphorylation levels, whereas the increase in contractile force was maintained (Figure 6B). PGF2α, in contrast, produced an increase to a more sustained plateau level of 36% followed by a markedly slower decline in phosphorylation levels. At 8 and 15 minutes, the LC20 phosphorylation level in the presence of PGF2α was significantly higher than that in the presence of phenylephrine (Figure 6A).

**MYPT1 Is Phosphorylated in an Agonist- and Time-Dependent Manner at a ROK Site**

It has previously been suggested that MP activity is regulated by a signaling cascade that leads to the phosphorylation of MYPT1 on T695 by ROK and results in an inhibition of phosphatase activity.4,21 Thus, we monitored phosphorylation of MYPT1 at this ROK site using a site-specific polyclonal phosphospecific antibody raised against the gizzard MYPT1 phosphorylation site, T695 (1:500)21 (Figure 7). In tissues quick-frozen after PGF2α treatment at 37°C, there was a 2.5- to 3-fold increase in the signal with the phosphospecific antibody at 5 minutes that declined at 8 and 15 minutes (Figure 7A). In contrast, the signal did not increase in the presence of phenylephrine over the same time points (Figure 7B). To directly compare the time course of T695 phosphorylation with the imaging results, a more detailed time course was determined at 22°C. As can be seen in Figure 7C, the maximal phosphorylation occurs around 2 minutes.

**Inhibition of ROK Prevents Membrane Targeting of MYPT1 and PP1c**

Y27632 has been shown to effectively inhibit the kinase activity of ROK at 10−5 mol/L.22 In order to test the hypothesis that ROK phosphorylation of MYPT1 is involved in the membrane targeting of MP, we pretreated cells with 10−5 mol/L Y27632 before exposing them to PGF2α. As is shown in Figure 8, Y27632 caused a significant inhibition of the membrane targeting of both MYPT1 and PP1c. Thus, in the presence of Y27632 both subunits continued to be apparently homogeneously distributed throughout the cytoplasm of the cell in the presence of PGF2α. The same concentration of Y27632 also effectively prevented phosphorylation of MYPT1 (Figure 7D).

When we measured the effects of Y27632 on contractile force, we found that it had a significantly greater action to inhibit PGF2α contractions than phenylephrine contractions. Y27632 (10−5 mol/L) inhibited 10−5-mol/L PGF2α contractions by 99.8±3.6% (n=5) but inhibited 10−5-mol/L phenyl-
ephrine contractions by 88.6 ± 2.9% (n = 4) (P < 0.05). As discussed below, Y27632 is an effective inhibitor of ROK but not an entirely selective one. The inhibition of the PE contraction could be related to the known nonspecific effects of Y27632, including effects on PKC,23 or to an action of ROK on sites other than MYPT.

Discussion

The main findings of this study are that, in fully differentiated smooth muscle cells, (1) the subcellular distribution of MP is agonist-specific, (2) the MYPT1 and PP1c subunits appear to dissociate subsequent to targeting to the cell membrane and phosphorylation of MYPT1 at the ROK sites, and (3) the differential targeting of MP subunits is associated with different rates of dephosphorylation of LC20.

When the heterotrimeric composition of MP was discovered it was suggested that the 2 noncatalytic subunits might be targeting subunits.5 Although binding of MP to myosin has been demonstrated in fractionated cells8 and in vitro,7 imaging studies of intact cells have raised some questions on this issue. The subcellular distribution of the MYPT1 and PP1c subunits has been studied only in cultured rat aortic cells (passages 5 to 20)9 where staining for MYPT1 was located diffusely throughout the cytoplasm, with staining intensity increasing from the perimeter of the cell toward the perinuclear area. Myosin, in contrast, was brightest at the perimeter of the cell. Thus, most of the myosin and M130 in these cells was not distributed together. Permeabilization of the cells before fixation resulted in significant loss of M130, consistent with it being a cytoplasmic protein, but also uncovered a fraction that was associated with stress fibers as well as a fraction associated with the nucleus. In permeabilized cells, PP1cδ (the predominant isoform in differentiated smooth muscle) was found only within the nucleus. Another isoform of PP1c, the α isoform, colocalized with M130 and myosin only after permeabilization to remove the cytosolic M130. Because these studies were performed with cultured rather than differentiated smooth muscle cells, the results may not be applicable to contractile smooth muscle. However, they do raise the question of the degree to which MYPT1 is targeted to myosin in cells and also raise the possibility that the MP subunits might not always be associated with each other in vivo.

The present results, to the best of our knowledge, are the first for fully differentiated, contractile smooth muscle cells. A major finding is that the staining for the targeting subunit is not always associated with the cell core, but rather under certain conditions is associated with the cell membrane. We have previously shown that the myosin in this cell type exists in central bundles that run down the length of the cell.24 In
resting cells and in cells stimulated with phenylephrine, MYPT1 appeared to be distributed throughout the cell. However, we cannot rule out the possibility that some fraction of MYPT1 was associated with filaments but that the resolution was insufficient to resolve a filamentous pattern. In contrast, in the presence of PGF2α, essentially no MYPT1 was found in the core of the cell and thus, clearly, little of the targeting subunit was associated with the myosin filaments.

It has been reported that MYPT1 binds preferentially to phosphorylated myosin compared with nonphosphorylated myosin. In the present study, we were unable to detect any filamentous pattern to the staining for either subunit during the action of PGF2α or to the pattern of staining for MYPT1 during the action of PE. However, a nonhomogeneous pattern to the staining of PP1c was consistently seen at 5 minutes after the addition of PE, a time point where LC20 phosphorylation peaks. The predominant filamentous staining at this time point is consistent with a role of the phosphatase in the subsequent rapid decline in LC20 phosphorylation levels. It is possible that a subset of MYPT1 colocalized with PP1c on the filaments at this time point, but an excess of soluble MYPT1 obscured the bound MYPT1.

The activity of the catalytic subunit toward phosphorylated myosin is increased on binding to MYPT1 but decreased with phosphorylase a. In the present study, it is of interest that, particularly at the 8- and 15-minute time points, the subunits appear to be dissociated in the presence of PGF2α but not in the presence of PE. LC20 phosphorylation at these time points is still near its peak value in the presence of PGF2α, but LC20 phosphorylation decreased dramatically in the presence of PE. Thus, the slower dephosphorylation of myosin in the presence of PGF2α may be related to the observation that the subunits were dissociated.

It is of interest that in the presence of phenylephrine there is a sustained increase in contractile force even after the phosphorylation of LC20 declines. We assume that force is maintained in this case by thin filament regulatory mechanisms that have previously been described to include a PKC-dependent ERK1/2 activation leading to phosphoryla-

Figure 7. Effect of PGF2α (A) or PE (B) on phosphorylation of MYPT1 as determined with a phosphospecific antibody for T695. Tissues were quick-frozen at 37°C. C, Time course of phosphorylation of MYPT1 after addition of PGF2α in tissues quick-frozen at 22°C. Each data point is a mean of results from 3 to 9 separate experiments. D, Effect of pretreatment with Y27632 on 2 minutes PGF2α time point at 22°C. *P<0.05.
tion of the thin filament binding protein caldesmon in this tissue. The present study suggests that the MP holoenzyme dissociates in intact cells during a period of reduced phosphatase activity. This result was unexpected because Alessi et al.\(^5\) showed that relatively high concentrations of LiBr were required to dissociate MP in vitro and \(K_d\)s in the \(1 \times 10^{-8}\) mol/L range were reported for complexes of PP1c and the N-terminal fragments of MYPT1.\(^{26}\) Thus, in vitro, the binding of MYPT1 and PP1c is relatively strong. It is unlikely that phosphorylation of MYPT1 induces dissociation of MP. Individual subunits of MP were not detected by gel filtration following MYPT1 phosphorylation,\(^{27}\) and phosphorylation at the inhibitory site of MYPT1 by ROK did not increase activity with phosphorylase \(\alpha.\)^{21} One possibility, suggested by Gong et al.\(^{28}\) is that the gizzard MP was dissociated by arachidonic acid release. As pointed out previously,\(^{29}\) an attractive feature of this idea is the precedent involving dissociation and regulation of glycogen-associated phosphatase after phosphorylation by PKA.\(^{30}\) It is not known if PGF2\(\alpha\) induces an increase in arachidonic acid, but the results of Gong et al.\(^{28}\) offer an interesting option for dissociation of MP in ferret portal vein cells.

Several mechanisms have been suggested whereby MP activity might be inhibited in smooth muscle cells. One such mechanism is via phosphorylation of MYPT1 at an inhibitory site, ie, T695 in the larger chicken gizzard isoform.\(^{29}\) This originated with the observation of Trinkle-Mulcahy et al.\(^{31}\) that incubation of permeabilized rabbit portal vein strips with ATP\(\gamma\)S caused thiophosphorylation of MYPT1 and inhibition of MP activity. Subsequently, it was found that a kinase(s) that co-purified with the MP preparations also phosphorylated MYPT1 at T695 and inhibited phosphatase activity.\(^{27}\) It has been shown that ROK functions similarly\(^{21,32}\) and several studies have documented that ROK inhibitors prevent Ca\(^{2+}\) sensitization.\(^{33-36}\) Recently, it was suggested that the endogenous kinase of Ichikawa et al.\(^{27}\) was a ZIP-like kinase and that it functioned downstream of ROK.\(^{37}\) Another theory to account for inhibition of MP is the interaction of a phosphorylation-dependent inhibitory protein with PP1c.\(^{23}\)

The above results show that after stimulation with PGF2\(\alpha\), an early event is the phosphorylation of MYPT1 at the
inhibitory site. MP is translocated to the membrane after this inhibition and it probably provides an initial phase of MP inhibition. It seems likely that the kinase involved is dependent on RhoA activation and the previously described translocation of RhoA to the membrane in smooth muscle. The logical choice for the kinase is ROK. The results obtained with the ROK inhibitor, Y27632, are consistent with this assumption as it blocked phosphorylation of MYPT1 and translocation to the membrane. Other kinases that are inhibited by Y27632 include PKN and certain PKC isoforms. PKCδ has been shown to be inhibited by Y27632 in the presence of 0.1 mmol/L ATP with a potency similar to that for the inhibition of ROK. However, ferret portal vein contains negligible amounts of PKCδ. It contains primarily PKC α and ζ with trace amounts of the β, e, and η isoforms (Lee et al and Sougnez and K.G. Morgan, unpublished results). The ZIP-like kinase would be inhibited indirectly if it is in the Rho signaling pathway and downstream of ROK. Recent evidence suggests that PKN does not phosphorylate MYPT1 (M. Ito, unpublished data), and thus, the 2 kinases that might be involved in phosphorylation of MYPT1 are ROK and the ZIP-like kinase.

Phosphorylation of MYPT1 is maximal at 2 minutes after stimulation, and at 5 minutes, the MYPT1-PP1c complex is localized to the membrane. Our observations raise a number of points. Presumably, phosphorylation of MYPT1 is a prerequisite for the translocation to the membrane but the mechanism of translocation is not known. Also, the binding/docking site(s) at the membrane is not identified. Previously, Ito et al showed that acidic phospholipids bound to MYPT1, and thus, the interaction with the membrane may not require specific protein targets. It appears that membrane binding does not require stable MYPT1 phosphorylation as the membrane-attached state can be dephosphorylated. As mentioned above, the mechanism by which the MP complex is dissociated is a critical feature and a focus for future research. Two possibilities are that dissociation involves an interaction of MYPT1 with lipids, or that it involves competition of the N-terminal part of MYPT1 with membrane-associated proteins.

In summary, the present studies indicate that stimulation of isolated smooth muscle cells by PGF2α induces a marked change in the localization of MP. This involves an initial phosphorylation of MYPT1, probably by Rho-kinase, and this occurs in the core of the cell. Next, MP is translocated to the membrane where the subunits are dissociated. PP1c returns to the core of the cell and MYPT1 remains at the membrane location. The isolated PP1c has reduced phosphatase activity toward phosphorylated myosin, and thus, these data provide evidence for an alternative mechanism of MP inhibition.

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