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## PHI-1 Interacts with the Catalytic Subunit of Myosin Light Chain Phosphatase to Produce a $\text{Ca}^{2+}$ Independent Increase in $\text{MLC}_{20}$ Phosphorylation and Force in Avian Smooth Muscle

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### Abstract

In avian smooth muscles,  $\text{GTP}\gamma\text{S}$  produces a Rho kinase mediated increase in PHI-1 phosphorylation and force, but whether this correlation is causal is unknown. We examined the effect of phosphorylated PHI-1 (P-PHI-1) on force and  $\text{MLC}_{20}$  phosphorylation at a constant  $[\text{Ca}^{2+}]$ . P-PHI-1, but not PHI-1, increased  $\text{MLC}_{20}$  phosphorylation and force, and phosphorylation of PHI-1 increased the interaction of PHI-1 with PP1c. Microcystin induced a dose-dependent reduction in the binding of PHI-1 to PP1c. These results suggest PHI-1 inhibits MLCP by interacting with the active site of PP1c to produce a  $\text{Ca}^{2+}$  independent increase in  $\text{MLC}_{20}$  phosphorylation and force.

### Keywords

$\text{Ca}^{2+}$  sensitization; Myosin light chain phosphatase; PP1c; PHI-1; MYPT1; Chicken Gizzard

### Introduction

Phosphorylation of the 20 kDa myosin light chain ( $\text{MLC}_{20}$ ) is the hallmark of smooth muscle contraction, and is dependant on the balance between the activities of  $\text{Ca}^{2+}$ -dependant myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) [1]. Agonist stimulation leads to an inhibition of the MLCP, or  $\text{Ca}^{2+}$  sensitization of smooth muscles, a G-protein mediated process, which involves many different signaling pathways and molecules (reviewed in [2]). MLCP is a trimeric protein consisting of three subunits; a myosin binding subunit (MYPT1), a catalytic subunit (PP1c $\delta$ ), and a 20 kDa subunit of a unknown function [3]. Although little is known about the physiologically relevant mechanism for MLCP inhibition, two are widely accepted; 1) the phosphorylation of MYPT1 [4,5] and 2) the binding of phosphatase inhibitor proteins to the catalytic subunit of the enzyme [6,7].

We have previously demonstrated that phosphorylation of MYPT1 does not participate in  $\text{Ca}^{2+}$  sensitization of avian smooth muscle tissue [8]. These data suggest that a phosphatase inhibitor protein is a likely candidate to mediate  $\text{Ca}^{2+}$  sensitization. Phosphatase inhibitor proteins are a family of proteins that specifically inhibit MLCP, and their inhibitory potency for the phosphatase is increased upon phosphorylation [9]. They are termed inhibitor-1, in contrast to inhibitor-2 proteins that are effective without phosphorylation [7]. Phosphatase inhibitor-1 proteins include a 17 kDa PKC and/or Rho-kinase potentiated protein (CPI-17),

phosphoprotein holoenzyme inhibitor-1 (PHI-1), and dopamine and cAMP regulated phosphoprotein of 32 kDa (DARPP-32) that is expressed in brain [7]. It has previously been demonstrated that CPI-17 is not expressed in chicken smooth muscles [8,10], suggesting that another inhibitor-1 type protein may serve an analogous role. We have previously demonstrated in avian smooth muscle that a Rho-kinase mediated pathway phosphorylates PHI-1 during both G-protein stimulation of skinned smooth muscles and agonist stimulation of intact preparations [8]. However, whether in chicken smooth muscle, PHI-1 phosphorylation mediates  $\text{Ca}^{2+}$  sensitization is unknown. In this study, we tested the hypothesis that phosphorylation of PHI-1 leads to a  $\text{Ca}^{2+}$  sensitization of chicken smooth muscle.

## Materials and Methods

### Preparation of phospho-PHI-1

Purified PHI-1 (Upstate Biotechnologies) was phosphorylated using a previously described protocol [7]. Briefly, PHI-1 was phosphorylated in an assay buffer containing: 25mM MOPS-NaOH pH 7.0, 10mM magnesium acetate, 0.3mg/ml PHI-1 (Upstate Biotechnologies), 2 $\mu$ g/ml Rho kinase (Upstate Biotechnologies), and 0.1 mM ATP for 120 minutes at 30°C. Phosphorylation of PHI-1 was confirmed by SDS-PAGE and western blotting using a phosphospecific antibody which recognizes PHI-1 phosphorylated at Thr 57 [7].

### Force

Following an institutionally approved IACUC protocol, the chicken gizzard was removed and placed into cold  $\text{Ca}^{2+}$  free saline solution (140mM NaCl, 4.7mM KCl, 1.2mM  $\text{NaH}_2\text{PO}_4$ , 2.0mM MOPS, 0.02mM EDTA, 1.2mM  $\text{MgCl}_2$ , 5.6mM glucose, and 0.5mM EGTA, pH 7.0). Small pieces of gizzard smooth muscles were cut into strips approximately 200–700 $\mu$ m long, 100–150 $\mu$ m wide, and 50–150 $\mu$ m thick. As previously described [11,12], aluminum foil T-clips were attached to each end of a gizzard strip, and then the preparation was skinned for 30 minutes at 4°C in pCa9 ( $-\log_{10}[\text{Ca}^{2+}]$ ) solution containing 1% TritonX-100. Skinned gizzard strips were then transferred to a mechanics workstation (Aurora Scientific, Aurora, Canada). In pCa9 solution, one end was hooked to a force transducer (Akers AE 801 MEMSCAP, San Jose, USA) and the other to a servomotor (Aurora Scientific). The tissue was stretched to  $L_0$ , the length where force is maximum as previously described [12]. Strips were then moved to pCa6.2 solution, and varying concentrations of PHI-1 or P-PHI were added and the resulting change in force was recorded. Finally, the tissue was moved to pCa4 solution to determine the maximum  $\text{Ca}^{2+}$  activated force for each strip. The force at pCa9 was set to zero, and all forces are given relative to the baseline at pCa9.

### Western blotting

The level of  $\text{MLC}_{20}$  phosphorylation was determined as previously described [8,11–14]. After skinning, tissue was placed in a pCa6.2 solution with and without 3 $\mu$ g/ml PHI-1 or P-PHI-1 for 15 minutes. The tissues were then denatured in 10% TCA in acetone with 10 mM dithiothreitol and stored at  $-80^\circ\text{C}$  overnight. Samples were removed and brought to room temperature for 1 hour. After centrifuging for 1 minute, the TCA was removed and the tissues were washed three times in acetone with 10mM dithiothreitol. After the final wash, the tissue was dried and cut into fine pieces.  $\text{MLC}_{20}$  was solubilized by vortexing the tissue in 8 M urea, 20 mM Tris, 22 mM glycine, pH 8.6, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. The samples were run in the absence of SDS using 19:1 acrylamide:bisacrylamide 10% gels containing 40% v/v glycerol. The running buffer contained 20 mM Tris, 22 mM glycine, 1 mM dithiothreitol, and 1 mM thioglycolic acid, pH 8.6. The proteins were transferred to nitrocellulose membrane and probed for  $\text{MLC}_{20}$  using a monoclonal anti- $\text{MLC}_{20}$  antibody (Sigma). The blot was developed using alkaline phosphatase substrate buffer with NitroBlue Tetrazolium and 5-Bromo-4-choloro-3-indolyl phosphate disodium salt. The level of

phosphorylation of MLC<sub>20</sub> was determined as  $(\text{MLC}_{20}\text{-Pi}/(\text{MLC}_{20}\text{-Pi}+\text{MLC}_{20}))\times 100\%$  using densitometric analysis (Scion Image). All blots were in the linear range of the detection system [8].

The same samples were used to determine the expression of RhoA and Rho-kinase as well as PHI-1 phosphorylation. These proteins were resolved on 29:1 acrylamide:bisacrylamide 12% SDS-PAGE, and were then transferred to nitrocellulose membrane and probed with anti-Rho A (Upstate Biotechnologies), anti-Rho kinase (Upstate Biotechnologies), anti-PHI-1 [7] or anti-Thr57 phosphospecific PHI-1 antibody [7]. Blots were developed with alkaline phosphatase or chemo-luminescence (Amersham).

### Co-immunoprecipitation

Adult chicken gizzard was dissected and homogenized in lysis buffer (8M Urea, 10mM Tris-HCl, 0.1 mM EDTA, 1X EDTA-free Complete Protease Inhibitor (Roche), pH 8). The lysate was rotated for 15 minutes at 4°C, spun (14,000 rpm) and the supernatant was collected and stored at -80°C. Aliquots (250–300µl) of the homogenates were added to 1.5 ml of immunoprecipitation buffer (50mM Tris-HCl (pH 8), 7mM MgCl<sub>2</sub>, 2mM EDTA, and 1mM PMSF). Samples were incubated on ice for 20 minutes under the following conditions: lysates only, lysate + GTPγS (100 µM), and lysates + GTPγS (100 µM) + microcystin LR (2µM or 20µM). The supernatant was rotated for 30 minutes at 4°C, centrifuged and separated from the precipitate. An antibody to PP1c (Transduction Laboratories), the catalytic subunit of MLCP, was added to the lysates and the samples were rotated overnight. Lysates were also rotated overnight in absence of the antibody as a negative control. Samples were centrifuged for 5 minutes at 4°C, and the supernatant was removed. The antibody-protein complex was recovered using Protein G sepharose beads (Amersham Biosciences). Samples were washed twice with 200µl of immunoprecipitation buffer, 40 µl of SDS sample buffer was added, the samples were heated, and the protein was resolved by 29:1, 12% SDS-PAGE, and Western blotted with the antibody to PHI-1 [7]. Blots were developed with alkaline phosphatase or chemiluminescence (Amersham). Protein loading was normalized by the IgG band, and the PHI-1 band intensity was expressed using the following formula:  $[\text{PHI-1 band intensity}/(\text{IgG band intensity})]$ . Then, the intensity of the band for lysates treated with GTPγS was set to 100%, and for the other conditions, intensities were normalized accordingly.

### Solutions

Calcium solutions were prepared using a computer program designed to give a set of free ion concentrations that are adjusted for both temperature and ionic strength [15]. The ionic strength for all solutions was 200mM and the experiments were carried out at a temperature of 22°C. The relaxing solution (pCa9.0) contained (in mM): 25 BES, 10 EGTA, 0.02 CaCl<sub>2</sub>, 7.2 MgCl<sub>2</sub>, 5.5 ATP, 25 creatine phosphate, 56.5 KMS, pH to 7.0 with 1M KOH and pCa4.0 solution (in mM): 25 BES, 10 EGTA, 10.2 CaCl<sub>2</sub>, 6.9 MgCl<sub>2</sub>, 5.6 ATP, 25 creatine phosphate, 35.8 KMS, pH to 7.0 with 1M KOH. The solution of pCa6.2 was prepared by proportionate mixing of pCa9.0 and pCa4.0 solutions.

### Statistics

All values are given as the mean ± SEM of between three and six experiments. Means were compared with an ANOVA and the Tukey HSD test, and statistical significance was taken at  $p < 0.05$ .

### Results

These experiments were designed to investigate whether phosphorylation of PHI-1 leads to force enhancement at a constant Ca<sup>2+</sup>, or Ca<sup>2+</sup> sensitization in avian smooth muscle strips. We

phosphorylated PHI-1, in vitro, with Rho kinase using a previously published protocol [7], and confirmed PHI-1 phosphorylation with Western blotting (Figure 1). As is demonstrated, the anti-Thr57 phosphospecific PHI-1 antibody [7] does not recognize the nonphosphorylated, purified protein, while after Rho kinase treatment, Thr57 phosphorylated PHI-1 is easily detected. Further, both RhoA and Rho kinase are retained after skinning of the avian smooth muscle strips with TritonX-100 (Figure 2).

The effect of PHI-1 and P-PHI-1 on force in skinned gizzard strips is demonstrated in Figure 3 and summarized in Table I. When skinned gizzard strips were placed in pCa6.2 solution, there was no increase in force compared to pCa9.0. Further at pCa6.2, the addition of 3 $\mu$ g/ml PHI-1 did not lead to force enhancement ( $0.0 \pm 3.4$  mN/mm<sup>2</sup>, n=5, p>0.05). However at pCa6.2, 3 $\mu$ g/ml of P-PHI-1 increased force by  $7.4 \pm 1.7$  mN/mm<sup>2</sup> (n=5, p<0.05), which is ~ 50% of the level for maximal Ca<sup>2+</sup> activation ( $18.4 \pm 4.4$  mN/mm<sup>2</sup>, p<0.05). In some instances, adding higher concentrations of PHI-1 also produced an increase in force. However, unlike P-PHI-1, which always increased force at 3 $\mu$ g/ml, higher concentrations of PHI-1 (> 6 $\mu$ g/ml) produced an increase in force only in 2 of 8 cases. Because the force increase at high concentrations of unphosphorylated PHI-1 occurred infrequently, it did not reach statistical significance.

MLC<sub>20</sub> phosphorylation is the hallmark of smooth muscle contraction, and to determine if phosphorylation of PHI-1 enhances force by increasing MLC<sub>20</sub> phosphorylation, we measured MLC<sub>20</sub> phosphorylation in skinned gizzard smooth muscle strips at pCa6.2 alone, pCa6.2 + PHI-1, or pCa6.2 + P-PHI-1. At pCa6.2, the level of MLC<sub>20</sub> phosphorylation in skinned gizzard strips was  $45 \pm 3.9\%$  (n=4), and the addition of PHI-1 did not result in an increase in MLC<sub>20</sub> phosphorylation ( $45 \pm 1.9\%$ , n=4, p> 0.05). In contrast, at pCa6.2, the addition of 3 $\mu$ g/ml P-PHI-1 increased MLC<sub>20</sub> phosphorylation to  $56 \pm 3\%$  (n=4, p<0.05), compared to  $63 \pm 4.3\%$  (n=3, p< 0.05) at pCa4.0 (Figure 4 and Table II). This result is consistent with P-PHI-1 enhancing force by producing an increase in MLC<sub>20</sub> phosphorylation.

Two mechanisms have been reported for MLCP inhibition. The first mechanism is phosphorylation of MYPT1 [4,5,16,17], and the second mechanism is direct binding of phosphatase inhibitors to the catalytic subunit of the enzyme [6,7,18]. Since we have previously demonstrated that MYPT1 phosphorylation does not change during GTP $\gamma$ S stimulation in avian smooth muscles [8], this suggests that P-PHI-1 could inhibit MLCP activation by binding to PP1c, similar to CPI-17 [18]. To investigate this possibility, we utilized a co-immunoprecipitation protocol. The data show that PHI-1 and PP1c interact in avian smooth muscles (Figure 5). G-protein stimulation is known to enhance the inhibitory potency of PHI-1 by Thr57 phosphorylation [7], which could occur if G-protein stimulation enhanced the binding of PHI-1 to the catalytic subunit. To study this, we added GTP $\gamma$ S, which increases PHI-1 phosphorylation [8], to the tissue lysates prior to co-immunoprecipitation. The addition of GTP $\gamma$ S resulted in an increase in the interaction of PHI-1 and PP1c (p<0.05), as evidenced by increase in the signal detected after probing with PHI-1 antibody (Figure 5). The increase in the interaction of PHI-1 and PP1c was not due to an increase in PP1c that is immunoprecipitated, as PP1c was similar before and after GTP $\gamma$ S treatment (Supplemental Figure).

To further investigate the mechanism of PHI-1 inhibition of MLCP, we used microcystin LR. Microcystin has been demonstrated to inhibit MLCP by binding to the active site of PP1c [18,19], which results in a Ca<sup>2+</sup>-independent increase in MLC<sub>20</sub> phosphorylation and force [20,21]. Lysates were treated with microcystin after adding GTP $\gamma$ S. The addition of microcystin to the co-immunoprecipitation experiments resulted in a dose dependent reduction (p<0.05) in the signal detected with the PHI-1 antibody (Figure 5), which suggests that microcystin and PHI-1 could compete for the same binding site on PP1c.

## Discussion

G-protein stimulation has been shown to inhibit MLCP with a resultant increase in MLC<sub>20</sub> phosphorylation and force [22]. Phosphorylation of MYPT1 has been identified both in vivo and in vitro as a mechanism for the inhibition of MLCP [4,5,16,23,24]. However, a G-protein stimulation induced increase in MYPT1 phosphorylation appears to be species, tissue and agonist dependent [4,8,16,23–29]. In chicken smooth muscle, we have demonstrated that MYPT1 phosphorylation does not change during GTP $\gamma$ S stimulation [8]. These results suggest that a mechanism, other than MYPT1 phosphorylation, mediates Ca<sup>2+</sup> sensitization in avian smooth muscle. One possibility is that a phosphatase inhibitor protein could inhibit MLCP to produce Ca<sup>2+</sup> sensitization. Phosphorylation of protein-1 phosphatase inhibitors increases their inhibitory potency towards the phosphatase [6,7], and it has been previously demonstrated that the small phosphatase inhibitor protein, CPI-17, is phosphorylated by both PKC and Rho kinase mediated pathways [29,30]. Phosphorylation of CPI-17 results in CPI-17 binding to the active site of PP1c [18], and this leads to an inhibition of MLCP. However, CPI-17 expression is tissue and species specific [10], and is not expressed in avian smooth muscle tissue [8,10]. These findings raise the possibility that another phosphatase inhibitor protein exists in tissues with low levels of CPI-17 expression. PHI-1 belongs to the family of protein 1 phosphatase inhibitors and is homologous to CPI-17, with 29% sequence identity [7]. We have previously demonstrated that in chicken smooth muscles, a Rho-kinase mediated pathway leads to an increase in PHI-1 phosphorylation at Thr 57 during both agonist stimulation of intact smooth muscles, and GTP $\gamma$ S stimulation of skinned smooth muscle strips [8]. Phosphorylation of PHI-1 at Thr 57 is known to increase the inhibition of MLCP [7]. Our data demonstrate that the addition of P-PHI-1 to skinned avian smooth muscle results in a Ca<sup>2+</sup> independent increase in force, and are similar to results reported for rat tail arterial strips [31].

Our results are the first demonstration that PHI-1 increases force and mediates Ca<sup>2+</sup> sensitization in avian smooth muscle, and further extend the results of Deng *et al.* [31] by demonstrating that the P-PHI-1 induced increase in force is concomitant with an increase in MLC<sub>20</sub> phosphorylation. Further, we have demonstrated that P-PHI-1 inhibits MLCP by interacting with the active site of PP1c. Our data demonstrate that PHI-1 can also interact with PP1c (Figure 5), consistent with data suggesting that both PHI-1 and P-PHI-1 inhibit MLCP. However, phosphorylation of PHI-1 increases the interaction of PHI-1 with PP1c (Figure 5). This is consistent with the results of the demonstrating that phosphorylation of PHI-1 at Thr57 increases the inhibitory potency of PHI-1 towards MLCP [7], as well as our data showing that 3  $\mu$ g/ml of P-PHI-1, but not PHI-1, increases force, while higher concentrations (>6  $\mu$ g/ml) of PHI-1 can occasionally lead to force enhancement.

We have previously demonstrated that a RhoA/Rho kinase pathway mediates the increase in MLC<sub>20</sub> and PHI-1 phosphorylation during Ca<sup>2+</sup> sensitization of skinned and intact avian smooth muscles [8]. Similarly, agonist stimulation of intact smooth muscle has been demonstrated to result in PHI-1 phosphorylation [8,32], suggesting that PHI-1 is part of a physiologically relevant pathway for force regulation. In the present study, the data demonstrate that the increase in PHI-1 phosphorylation increases its interaction with the active site of PP1c to inhibit MLCP and increase MLC<sub>20</sub> phosphorylation and force.

In heart failure, there is an increase in renin angiotensin system activity together with the increase in vascular tone, which is thought to be an initial compensatory mechanism for the failing heart [33]. With further progression of the disease, this mechanism and others will contribute to remodeling of the failing heart. However, the molecular mechanism for the increase in vascular tone in heart failure is unknown. An increase in Angiotensin II activity could lead to a generalized increase in PHI-1 phosphorylation, Ca<sup>2+</sup> sensitization, and a resting vasoconstriction, which could contribute to the increase in vascular tone observed in heart failure.

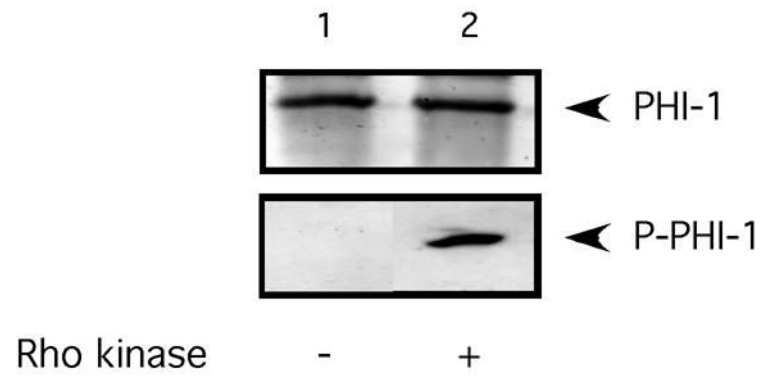
### Acknowledgements

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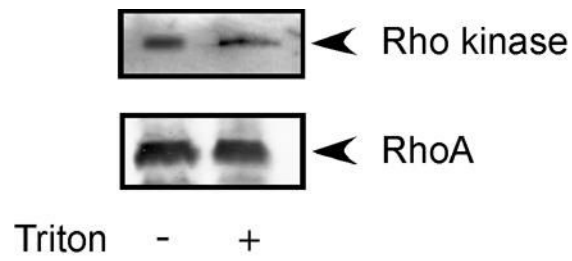
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**Figure 1. Rho kinase phosphorylates PHI-1**

Purified PHI-1 was phosphorylated by Rho kinase (see text for details). Lane 1 shows the western blot of the purified protein, and lane two after Rho kinase treatment. Western blots were probed with anti-PHI-1 and antiphospho-PHI-1 antibodies. The Thr57 phospho-specific antibody only recognized the protein after phosphorylation.

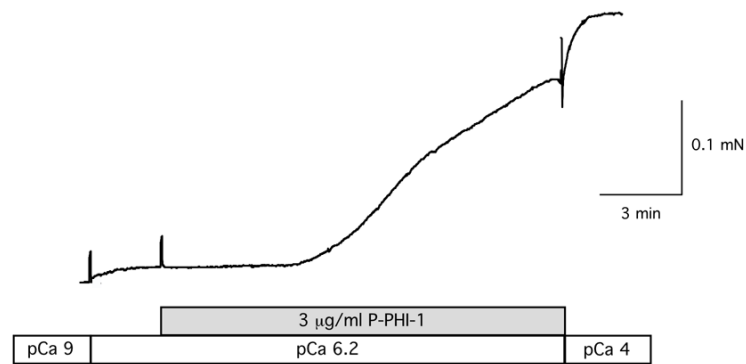




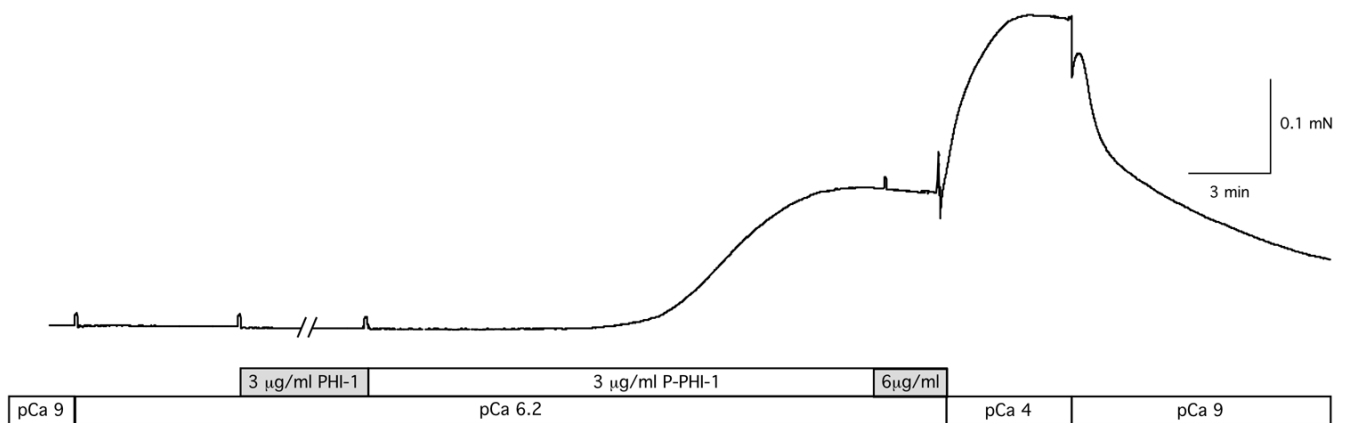
**Figure 2. Rho signaling is intact after Triton skinning**

The expression of RhoA and Rho kinase was determined in intact smooth muscle and Triton skinned smooth muscle. As demonstrated, both RhoA and Rho kinase are retained following skinning of the smooth muscle.

(A)

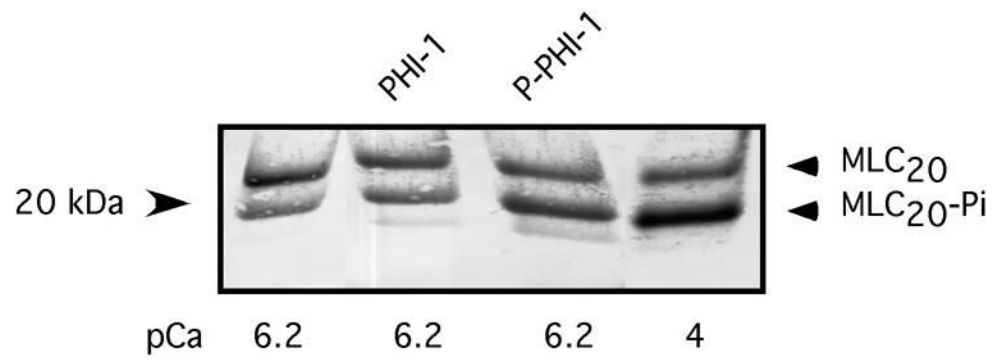


(B)



### Figure 3. P-PHI-1 leads to $\text{Ca}^{2+}$ sensitization

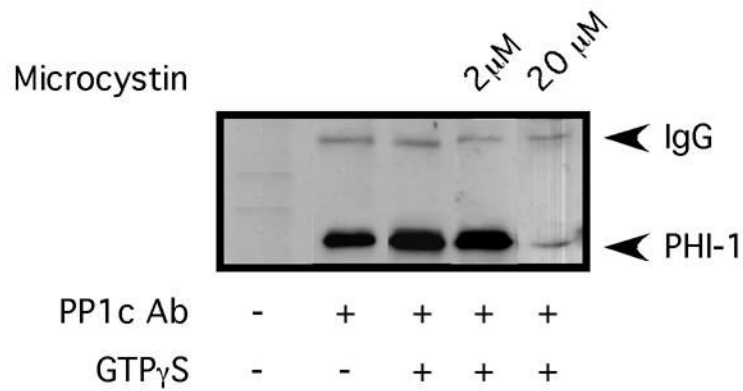
(A) A skinned gizzard strip was placed in relaxing solution (pCa9) and then at the first transferred to pCa6.2. P-PHI-1 (3 µg/ml) was added to the pCa6.2 and the preparation was then transferred to pCa4 solution (without PHI-1). The pCa solutions changes and the addition of P-PHI-1 are indicated below the data trace. (B) A skinned gizzard strip was placed in relaxing solution (pCa9) and then transferred to pCa6.2. PHI-1 (3 µg/ml) and then P-PHI-1 (1<sup>st</sup>, 3 µg/ml and then 6 µg/ml) was added to the pCa6.2 solution. The fiber was then transferred to pCa4 solution (without PHI-1 or P-PHI-1) and then to pCa9 solution (without PHI-1 or P-PHI-1). The pCa solutions changes and the addition of PHI-1 and P-PHI-1 are indicated below the data trace. As is demonstrated, the addition of P-PHI-1, but not PHI-1, resulted in a significant increase in force.



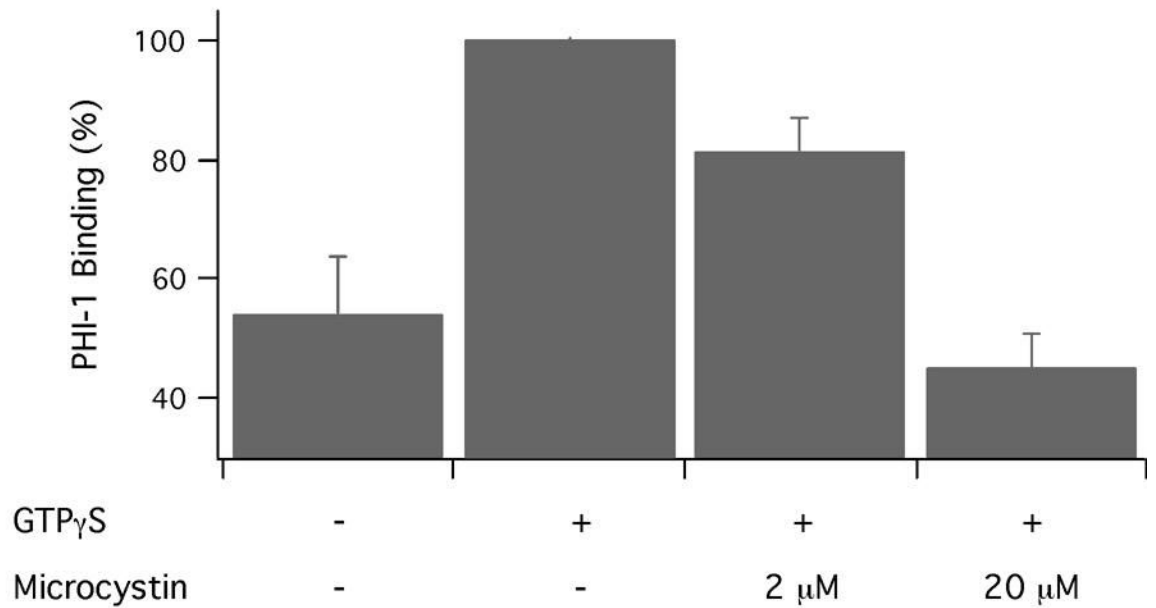
**Figure 4. P-PHI-1 increases MLC<sub>20</sub> phosphorylation**

MLC<sub>20</sub> phosphorylation was determined in skinned gizzard strips (n=4 for each condition) at pCa6.2 alone, pCa6.2 with PHI-1, pCa6.2 with P-PHI-1, as well as pCa4 (see Table II).

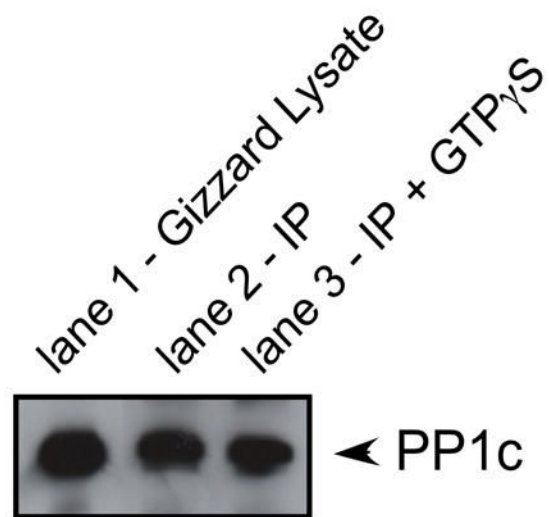
(A)



(B)

**Figure 5. P-PHI-1 binds to the active site of PP1c**

(A) Representative immunoprecipitation with anti-PP1c antibody and Western blot for PHI-1, which demonstrate the interaction of PP1c and PHI-1. Immunoprecipitation was also performed following the addition of GTP $\gamma$ S and microcystin to the tissue lysates. (B) Western blots were analyzed using densitometry, and the bar graph summarizing the results (n=3–6). The immunoprecipitations demonstrate an interaction between PP1c and PHI-1, which increases with phosphorylation of PHI-1. The addition of Microcystin LR results in a dose dependent decrease in the interaction of PP1c and PHI-1.



**Supplemental Figure. GTP $\gamma$ S does not affect immunoprecipitation of PP1c**

Western blot for PP1c in gizzard lysate (lane 1). Endogenous PP1c was immunoprecipitated from gizzard lysates in the absence (lane 2) and presence (lane 3) of GTP $\gamma$ S. Similar amounts of PP1c were detected in the immunoprecipitation with and without GTP $\gamma$ S.

**Table I**

Force	(mN/mm <sup>2</sup> )
pCa 6.2	0 ± 0
pCa 6.2 + PHI-1	0.0 ± 3.4
pCa 6.2 + P-PHI-1	7.4 ± 1.7
pCa4	18.4 ± 4.4

Adult gizzard tissues were skinned with 1% Triton-X100, and stimulated with pCa 6.2 alone or pCa 6.2+PHI-1, or pCa 6.2+P-PHI-1. Force is given as the mean ± SEM ( $n = 4-5$ ). P-PHI-1 significantly ( $p < 0.05$ ) increased force, but not to the level achieved with maximal  $\text{Ca}^{2+}$  activation (pCa 4).

**Table II**MLC<sub>20</sub> Phosphorylation (%)

	(%)
pCa 6.2	45 ± 4
pCa 6.2 + PHI-1	45 ± 2
pCa 6.2 + P-PHI-1	56 ± 3
pCa4	63 ± 4

Adult gizzard tissues were skinned with 1% Triton-X100, and stimulated with pCa 6.2 alone, pCa 6.2+PHI-1, or pCa 6.2+P-PHI-1 (n=3-4). The samples were then resolved and analyzed (see the text for details). P-PHI-1 stimulation significantly ( $p < 0.05$ ) increased the level of MLC<sub>20</sub> phosphorylation compared to pCa 6.2, and MLC<sub>20</sub> phosphorylation was further increased at pCa 4 ( $p < 0.05$ ).