PHI-1 interacts with the catalytic subunit of myosin light chain phosphatase to produce a Ca²⁺ independent increase in MLC₂₀ phosphorylation and force in avian smooth muscle

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Abstract In avian smooth muscles, GTPγS 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 myosin light chain (MLC₂₀) phosphorylation at a constant [Ca²⁺]. P-PHI-1, but not PHI-1, increased MLC₂₀ 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 myosin light chain phosphatase by interacting with the active site of PP1c to produce a Ca²⁺ independent increase in MLC₂₀ phosphorylation and force.

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1. Introduction

Phosphorylation of the 20 kDa myosin light chain (MLC₂₀) is the hallmark of smooth muscle contraction, and is dependent on the balance between the activities of Ca²⁺-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) [1]. Agonist stimulation leads to an inhibition of the MLCP, or Ca²⁺ 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 (PP1cδ), and a 20 kDa subunit of an 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 Ca²⁺ sensitization of avian smooth muscle tissue [8]. These data suggest that a phosphatase inhibitor protein is a likely candidate to mediate Ca²⁺ 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 PHI-1 phosphorylation mediates Ca²⁺ sensitization in chicken smooth muscle is unknown. In this study, we tested the hypothesis that phosphorylation of PHI-1 leads to a Ca²⁺ sensitization of chicken smooth muscle.

2. Materials and methods

2.1. Preparation of phospho-PHI-1

Purified PHI-1 (Upstate Biotecnologies) was phosphorylated using a previously described protocol [7]. Briefly, PHI-1 was phosphorylated in an assay buffer containing: 25 mM MOPS-NaOH pH 7.0, 10 mM magnesium acetate, 0.3 mg/ml PHI-1 (Upstate Biotechnologies), 2 µg/ml Rho kinase (Upstate Biotechnologies), and 0.1 mM ATP for 120 min 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].

2.2. Force

Following an institutionally approved IACUC protocol, the chicken gizzard was removed and placed into cold Ca²⁺ free saline solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM Na₂HPO₄, 2.0 mM MOPS, 0.02 mM EDTA, 1.2 mM MgCl₂, 5.6 mM glucose, and 0.5 mM EGTA, pH 7.0). Small pieces of gizzard smooth muscles were cut into strips approximately 200–700 µm long, 100–150 µm wide, and 50–150 µm 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 min at 4 °C in pCa9 (−log[Ca²⁺]) solution containing 1% Triton X-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 L₀, 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 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.

2.3. Western blotting

The level of 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 μg/ml PHI-1 or P-PHI-1 for 15 min. The tissues were then denatured in 10% TCA in acetone with 10 mM dithiothreitol and stored at −80 °C overnight. Samples were removed and brought to room temperature for 1 h. After centrifuging for 1 min, the TCA was removed and the tissues were washed three times in acetone with 10 mM dithiothreitol. After the final wash, the tissue was dried and cut into fine pieces. 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. After gel separation, the proteins were transferred to nitrocellulose membrane and probed for MLC\(_{20}\) with a monoclonal anti-MLC\(_{20}\) antibody (Sigma). The blot was developed using alkaline phosphatase or chemiluminescence (Amersham).

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 chemiluminescence (Amersham).

2.4. Co-immunoprecipitation

Adult chicken gizzard was dissected and homogenized in lysin buffer (8 M Urea, 10 mM Tris–HCl, 0.1 mM EDTA, 1X EDTA-free Complete Protease Inhibitor (Roche), pH 8). The lysate was rotated for 15 min at 4 °C, spun (14,000 rpm) and the supernatant was collected and stored at −80 °C. Aliquots (250–300 μl) of the homogenates were added to 1.5 ml of immunoprecipitation buffer (50 mM Tris–HCl (pH 8), 7 mM MgCl\(_2\), 2 mM EDTA, and 1 mM PMSE). Samples were incubated on ice for 20 min under the following conditions: lysates only, lysate + GTP\(_{S}\) (100 μM), and lysates + GTP\(_{S}\) (100 μM) + microcystin (12 μM or 20 μM). The supernatant was rotated for 30 min at 4 °C, centrifuged and separated from the precipitate. An antibody to PPlc (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 min 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 200 μl 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: [PHI-1 band intensity/(IgG band intensity)]. Then, the intensity of the band for lysates treated with GTP\(_{S}\) was set to 100%, and for the other conditions, intensities were normalized accordingly.

2.5. 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 200 mM 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\(_2\), 7.2 MgCl\(_2\), 5.3 ATP, 25 creatine phosphate, 56.5 KMS, pH 7.0 with 1 M KOH and pCa4.0 solution (in mM): 25 BES, 10 EGTA, 10.2 CaCl\(_2\), 6.9 MgCl\(_2\), 5.6 ATP, 25 creatine phosphate, 35.8 KMS, pH to 7.0 with 1 M KOH. The solution of pCa6.2 was prepared by proportionate mixing of pCa9.0 and pCa4.0 solutions.

2.6. Statistics

All values are given as the means ± S.E.M. 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.

3. Results

These experiments were designed to investigate whether phosphorylation of PHI-1 leads to force enhancement at a constant Ca\(^{2+}\), or Ca\(^{2+}\) 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 (Fig. 1). As is demonstrated, the anti-Thr57 phosphospecific PHI-1 antibody \([7]\) does not recognize the non-phosphorylated, 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 (Fig. 2).

The effect of PHI-1 and P-PHI-1 on force in skinned gizzard strips is demonstrated in Fig. 3 and summarized in Table 1. 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 μg/ml PHI-1 did not lead to force enhancement (0.0 ± 3.4 mN/mm\(^2\), n = 5, P > 0.05). However, at pCa6.2, 3 μg/ml of P-PHI-1 increased force by 7.4 ± 1.7 mN/mm\(^2\) (n = 5, P < 0.05), which is ∼50% of the level for maximal Ca\(^{2+}\) activation (18.4 ± 4.4 mN/mm\(^2\), 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\(_{20}\) phosphorylation is the hallmark of smooth muscle contraction, and to determine if phosphorylation of PHI-1 enhances force by increasing MLC\(_{20}\) phosphorylation, we measured MLC\(_{20}\) 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\(_{20}\) phosphorylation in skinned gizzard strips was 45 ± 3.9% \((n = 4)\), and the addition of PHI-1 did not result in an increase in MLC\(_{20}\) phosphorylation (45 ± 1.9%, \(n = 4\), \(P > 0.05\)). In contrast, at pCa6.2, the addition of 3 \( \mu \)g/ml P-PHI-1 increased MLC\(_{20}\) phosphorylation to 56 ± 3% \((n = 4\), \(P < 0.05\)) compared to 63 ± 4.3% \((n = 3\), \(P < 0.05\)) at pCa4.0 (Fig. 4 and Table 2). This result is consistent with P-PHI-1 enhancing force by producing an increase in MLC\(_{20}\) 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\(_{S}\) stimulation in avian smooth muscles [8], this suggests that P-PHI-1 could inhibit...
MLC20 phosphorylation (%)

<table>
<thead>
<tr>
<th>Condition</th>
<th>Value (± S.E.M.)</th>
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<tbody>
<tr>
<td>pCa 6.2</td>
<td>45 ± 4</td>
</tr>
<tr>
<td>pCa 6.2 + PHI-1</td>
<td>45 ± 2</td>
</tr>
<tr>
<td>pCa 6.2 + P-PHI-1</td>
<td>56 ± 3</td>
</tr>
<tr>
<td>pCa 4</td>
<td>63 ± 4</td>
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Table 2

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 (mean ± S.E.M., 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 MLC20 phosphorylation compared to pCa 6.2, and MLC20 phosphorylation was further increased at pCa 4 (P < 0.05).

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 Ca2+-independent increase in MLC20 phosphorylation and force [20,21]. Lysates were treated with microcystin after adding GTPγS. 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 (Fig. 5), which suggests that microcystin and PHI-1 could compete for the same binding site on PP1c.

4. Discussion

G-protein stimulation has been shown to inhibit MLCP with a resultant increase in MLC20 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 GTPγS stimulation [8]. These results suggest that a mechanism, other than MYPT1 phosphorylation, mediates Ca2+ sensitization in avian smooth muscle. One possibility is that a phosphatase inhibitor protein could inhibit MLCP to produce Ca2+ 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 GTPγS 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 Ca2+ 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 Ca2+ 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 MLC20 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 (Fig. 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 (Fig. 5). This is consistent with the results 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 μg/ml of P-PHI-1, but not PHI-1, increases force, while higher concentrations (>6 μg/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$_{20}$ and PHI-1 phosphorylation during Ca$^{2+}$ 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$_{20}$ 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 [33]. With further progression of the disease, this is thought to be an initial compensatory mechanism for the failing heart [33].

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Appendix A. Supplementary data


References


