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PHI-1 interacts with the catalytic subunit of myosin light chain phosphatase to produce a Ca^{2+} independent increase in MLC_{20} phosphorylation and force in avian smooth muscle

Amr El-Toukhy[a,b], Allison M Given[a], Ozgur Ogut[a], Frank V. Brozovich[a,*]

[a] *Division of Cardiovascular Diseases, Mayo Clinic, Rochester, MN 55905, USA*
[b] *Department of Physiology and Biophysics, Case Western Reserve University, Cleveland, OH 44106, USA*

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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 (MLC_{20}) phosphorylation at a constant [Ca^{2+}]. P-PHI-1, but not PHI-1, increased 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 myosin light chain phosphatase by interacting with the active site of PP1c to produce a Ca^{2+} independent increase in MLC_{20} phosphorylation and force.

Keywords: Ca^{2+} sensitization; Myosin light chain phosphatase; PP1c; PHI-1; MYPT1; Chicken gizzard

1. Introduction

Phosphorylation of the 20 kDa myosin light chain (MLC_{20}) is the hallmark of smooth muscle contraction, and is dependent on the balance between the activities of Ca^{2+}-dependent myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP) [1]. Agonist stimulation leads to an inhibition of the MLCP, or 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 (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 Ca^{2+} sensitization of avian smooth muscle tissue [8]. These data suggest that a phosphatase inhibitor protein is a likely candidate to mediate 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 PHI-1 phosphorylation mediates Ca^{2+} sensitization in chicken smooth muscle is unknown. In this study, we tested the hypothesis that phosphorylation of PHI-1 leads to a Ca^{2+} sensitization of chicken smooth muscle.

2. Materials and methods

2.1. 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: 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 Ca^{2+} free saline solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM NaH_2PO_4, 2.0 mM MOPS, 0.02 mM EDTA, 1.2 mM $MgCl_2$, 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 ($-\log_{10}[Ca^{2+}]$) 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

*Corresponding author. Fax: +1 507 284 8566.
E-mail address: brozovich.frank@mayo.edu (F.V. Brozovich).

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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, pH 8.6. The proteins were transferred to nitrocellulose membrane and probed for MLC_{20} using a monoclonal anti-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_{20} was determined as $(MLC_{20} - Pi/(MLC_{20} - Pi + MLC_{20})) \times 100\%$ 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).

2.4. Co-immunoprecipitation

Adult chicken gizzard was dissected and homogenized in lysis 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 PMSF). Samples were incubated on ice for 20 min under the following conditions: lysates only, lysate + GTPγS (100 μM), and lysates + GTPγS (100 μM) + microcystin LR (2 μM or 20 μM). The supernatant was rotated for 30 min 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 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 chemi-luminescence (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 GTPγS 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 $MgCl_2$, 5.5 ATP, 25 creatine phosphate, 56.5 KMS, pH to 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

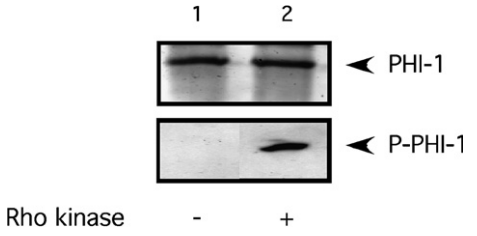

Fig. 1. Rho kinase phosphorylates PHI-1. Purified PHI-1 was phosphorylated by Rho kinase (see text for details). Lane 1 shows the western blot of the purified protein, and lane 2 after Rho kinase treatment. Western blots were probed with anti-PHI-1 and antiphospho-PHI-1 antibodies. The Thr57 phosphospecific antibody only recognized the protein after phosphorylation.

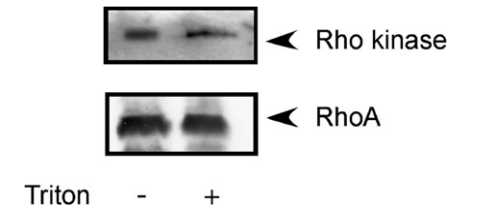

Fig. 2. Rho signaling is intact after Triton skinning. The expression of RhoA and Rho kinase was determined in intact smooth muscle and Triton skinned smooth muscle. As demonstrated, both RhoA and Rho kinase are retained following skinning of the smooth muscle.