Abstract  Herein, we provide evidence that in chicken smooth muscle, G-protein stimulation by a Rho-kinase pathway leads to an increase in myosin light chain phosphorylation. Additionally, G-protein stimulation did not increase MYPT1 phosphorylation at Thr695 or Thr850, and CPI-17, was not expressed in smooth muscle. However, PHI-1 was present in smooth muscle tissues. Both agonist and GTP-γS stimulation result in an increase in PHI-1 phosphorylation, which is inhibited by inhibitors to both Rho-kinase (Y-27632) and (PKC) GF109203x. These data suggest that PHI-1 may act as a CPI-17 analog in chicken smooth muscle and inhibit myosin phosphatase activity during G-protein stimulation to produce Ca2+ sensitization.

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Keywords: Myosin light chain phosphatase; CPI-17; PHI-1; Zip kinase; Integrin-linked kinase; MYPT1

1. Introduction

The regulation of force in smooth muscle involves the phosphorylation of the 20 kDa myosin light chain (MLC20). MLC20 phosphorylation is determined by the activities of Ca2+-calmodulin dependent MLCK and MLC phosphatase [1]. Changes in [Ca2+]i and the resulting change in MLCK activity had been thought to regulate the level of MLC20 phosphorylation and force [2]. However, recent evidence has demonstrated that a Ca2+-independent pathway can modulate MLC20 phosphorylation, as it has been well documented that both agonist [3] and G-protein [4,5] stimulation lead to a Ca2+ sensitization of the contractile filaments. Further, Ca2+ sensitization has been demonstrated at normal resting levels of Ca2+ (100 nM), which suggests that there is Ca2+-independent MLCK activity that increases MLC20 phosphorylation with inhibition of MLC phosphatase (reviewed in [31]).

MLC phosphatase is a trimeric protein consisting of a catalytic subunit, a myosin binding or myosin phosphatase targeting subunit (MYPT1) and a 20 kDa subunit of unknown function (reviewed in [6]). Activation of G-proteins has been shown to result in MYPT1 phosphorylation [7], and Rho-kinase has been demonstrated to phosphorylate MYPT1 at Thr695 in the avian [8,9], or Thr697 in the mammalian sequence [10], which results in an inhibition of MLC phosphatase activity [8] and a Ca2+ independent increase in MLC20 phosphorylation and force (reviewed in [6,11–14]). Evidence also suggests another pathway, involving PKC, exists for the inhibition of MLC phosphatase [15,16]. PKC phosphorylates CPI-17, which in turn binds to the active site of the catalytic subunit of MLC phosphatase to inhibit phosphatase activity [15]. In addition, there is evidence that several proteins including Rho-kinase [17], Zip kinase [18] and integrin-linked kinase [19] can directly phosphorylate MLC20 to produce a Ca2+ independent increase in force.

In avian smooth muscle, agonist stimulation has been shown to inhibit MLC phosphatase activity [7], and there are studies that implicate both MYPT1 phosphorylation [7] and other regulatory pathways [18–20] in the mechanism for agonist induced force enhancement. In this study, we investigated the mechanisms for G-protein mediated regulation of MLC phosphatase activity in chicken smooth muscle to determine the signaling molecules and/or pathways involved in the mechanism for G-protein mediated force enhancement.

2. Material and methods

2.1. Tissue preparation

Thin strips (~1500 x 500 x 400 μm) of gizzard and aortic smooth muscle were dissected from either embryonic day (ED) 16/17 or adult (hatched) chicken. These small strips of tissue were directly placed into skinning solution or sample buffer and used in subsequent experiments.

2.2. Western blotting

The level of MLC20 phosphorylation was determined as previously described [20,21]. Briefly, the excised tissue was skinned in relaxing solution (pCa 9) containing 400 μM β-escin and then transferred to a tube containing either a pCa 9 solution or a pCa 9 solution with 100 μM GTP;S 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 DTT. After the final wash, the tissue was dried and cut into fine pieces. MLC20 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 phenylmethanesulfonyl fluoride.

The samples were run in the absence of SDS through the use of 19:1ipase substrate buffer with NitroBlue Tetrazo-

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To determine the level of PHI-1 phosphorylation, using polyclonal PHI-1 antisera. Also, in order to determine the effect of inhibition of the PKC and Rho-kinase pathways, 5 μM GF109203x or 10 μM Y27632 were added to the GTP-S solutions.

To demonstrate that the intensities of non- and phosphorylated protein from densitometric analysis of the blots of both MLC20 and PHI-1 were in the linear range of the detection system, we determined phosphorylation from the same samples of intact aortic smooth muscle loaded at our standard as well as both one-half and double our standard protein loading. As demonstrated for PHI-1 (Fig. 1), there was no significant difference (P > 0.05) in the phosphorylation of either PHI-1 or MLC20 (data not shown) determined when protein loading was varied, demonstrating that the immunoblots are in the linear range of the detection system. Thus, protein phosphorylation determined from the ratios of the intensities of the phosphorylated and unphosphorylated proteins on the immunoblots are not influenced by differences in protein loading.

To determine the level of MYPT1 phosphorylation, embryonic and adult gizzard and aortic tissues were removed and stimulated as in the MLC20 phosphorylation assay, using pCa 9, with or without 100 μM GTP·S. MYPT1 was solubilized by vortexing the samples in SDS sample buffer containing 150 mM Tris, pH 6.8, 6% SDS, 0.3% Bromphenol Blue, and 30% glycerol. The samples were resolved on 18% acrylamide: bisacrylamide 14% SDS-PAGE. The proteins were transferred to nitrocellulose membrane and probed for either MYPT1 or phosphorylated MYPT1 using a polyclonal anti-MYPT1 antibody (Upstate Biotechnologies) or two different phosphorylation-specific anti-MYPT1 antibodies specific for phosphorylation at Thr 695 or at Thr 850 (Upstate Biotechnologies). Similar methods were used to determine the level of PHI-1 phosphorylation using polyclonal anti-PHI-1 and a phosphorylation specific (Thr57) anti-PHI-1 antibodies.

The samples were used to determine the expression of integrin-linked kinase (ILK), Zip kinase, CPI-17, Rho A and Rho-kinase. These proteins were resolved on 18% acrylamide:bisacrylamide 14% SDS-PAGE for ILK and Zip kinase and 29.1 acrylamide:bisacrylamide 12% SDS-PAGE for CPI-17, PKC, Rho A and Rho-kinase, and were then transferred to nitrocellulose membrane and probed with anti-ILK (Sigma), anti-Zip kinase, anti-CPI-17 antibodies, anti-PKC (Sigma), anti-Rho A (Upstate Biotechnologies), and anti-Rho-kinase (Upstate Biotechnologies). Blots were developed with alkaline phosphatase or chemio-luminescence (Amersham).

2.3. Protein expression and purification

E. coli BL21 transformed with an expression vector encoding untagged, full length PHI-1 DNA (a kind gift from Matsumi Eto) were used to inoculate 4 L of LB TY media and grown to OD 600 = 0.4. Protein expression was induced by the addition of 0.4 mM IPTG and the bacteria were allowed to grow for an additional 3 h. The bacteria were then collected by centrifugation and lyzed. The resulting supernatant underwent ammonium sulphate precipitation first at 30% then 60% saturation, each stirred at 4 °C for 25 min to collect the precipitate. As determined by 15% SDS-PAGE, the 30% and 60% ammonium sulphate precipitates contained the PHI-1 protein and were dialyzed against three changes of 4 L of 250 mM NaCl, 10 mM MES, 0.1 mM EDTA, 14 mM 2-mercaptoethanol, pH 6. Following dialysis, the samples were centrifuged and the precipitates containing PHI-1 were collected. These were dialyzed two times against 4 L of 10 mM MES, 0.1 mM EDTA, 14 mM 2-mercaptoethanol to remove any excess salts. The dialyzed protein was dissolved in 6 M urea, 0.1 mM EDTA, 0.1 mM PMSF, 2 mM 2-mercaptoethanol and resolved by an SP Sepharose ion exchange column developed with 0–500 mM NaCl. The column fractions were resolved by SDS-PAGE and Western blots were used to determine those containing PHI-1. These fractions were dialyzed in 0.01% formic acid, 2 mM 2-mercaptoethanol (4 L total) three times and then freeze-dried to minimize the sample volume for gel filtration. The lyophilized protein was dissolved in 6 M urea, 0.1 mM EDTA, 150 mM NaCl, 30 mM phosphate buffer, pH 7, and resolved by Mr using Superdex 75 media. The fractions containing PHI-1 were then collected, dialyzed as before and lyophilized.

3. 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 [22]. The ionic strength for all solutions was 200 mM and the experiments were carried out at a temperature of 22 °C in pCa 9.0 solution containing (in mM): 25 BES, 10 EGTA, 0.02 CaCl2, 7.2 MgCl2, 5.5 ATP, 25 creatine phosphate, 56.5 KMS, pH to 7.0 with 1 M KOH, and creatine kinase was added prior to the experiments.

Intact tissue was bathed in a standard physiologic saline solution containing (in mM): NaCl, 140; KCl, 4.7; MgSO4, 1.2; CaCl2, 1.6; NaH2PO4, 1.2; MOPS, 2.0; pH, 7.4; d-glucose, 5.0; and EDTA, 0.2. Tissue was stimulated with the agonist angiotensin II (All, 0.1 μM), and PHI-1 phosphorylation was determined as described above.

3.1. Statistics

All values are given as the means ± S.E.M. of between three and eight experiments, and significance was taken at P < 0.05.

4. Results

In the present study, we demonstrate that GTP·S stimulation of skinned chicken smooth muscle increases MLC20 phosphorylation (Fig. 2 and Table 1), and this increase in MLC20 phosphorylation was inhibited by Y27632, but not GF109203x (Fig. 3 and Table 1). These data are in agreement with our previous report demonstrating that in chicken smooth muscle GTP·S dependent force enhancement was mediated by a Rho-kinase, not a PKC dependent mechanism [20].

To determine if the G-protein induced increase in MLC20 phosphorylation is mediated by a Rho-kinase dependent phosphorylation of MYPT1, we determined MYPT1 phosphorylation using phospho-specific antibodies. These studies demonstrate that there is a baseline level of MYPT1 phosphorylation at Thr695 and Thr850 that does not change after GTP·S stimulation (Fig. 4). To confirm that these antibodies were able to detect phosphorylation at their specific sites, we phosphorylated MYPT1 in vitro using Rho-kinase [23], and
Fig. 2. GTP\(_s\) treatment leads to an increase in MLC\(_{20}\) phosphorylation. GTP\(_s\) was added to chicken smooth muscle tissues skinned with \(\beta\)-escin to determine the effect of G-protein stimulation on MLC\(_{20}\) phosphorylation. ED 16 and adult aorta (A) and gizzard (G) tissues were at pCa 9 in the presence (+) or absence (−) of 100 \(\mu\)M GTP\(_s\). The lysates were prepared and Western blotting was performed, and the visualized proteins were analyzed by densitometry (\(n = 5–8\)).

Table 1
MLC\(_{20}\) phosphorylation in response to GTP\(_s\), Y27632 and GF109203x

<table>
<thead>
<tr>
<th>Tissue</th>
<th>pCa 9</th>
<th>pCa 9 + GTP(_s)</th>
<th>pCa 9 + Y27632</th>
<th>pCa 9 + GF109203x</th>
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<tr>
<td>ED16 Gizzard</td>
<td>20 ± 3.7</td>
<td>42 ± 2.9</td>
<td>36 ± 0.4</td>
<td>38 ± 2.0</td>
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<tr>
<td>ED16 Aorta</td>
<td>18 ± 3.6</td>
<td>38 ± 2.5</td>
<td>26 ± 2.7</td>
<td>36 ± 1.8</td>
</tr>
<tr>
<td>Adult Gizzard</td>
<td>16 ± 4.1</td>
<td>35 ± 3.6</td>
<td>24 ± 3.6</td>
<td>39 ± 3.6</td>
</tr>
<tr>
<td>Adult Aorta</td>
<td>18 ± 2.3</td>
<td>47 ± 1.4</td>
<td>36 ± 2.5</td>
<td>44 ± 1.4</td>
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Embryonic and adult aorta and gizzard tissues were skinned with 5 mM \(\beta\)-escin, and stimulated with pCa 9 alone or pCa 9 with either GTP\(_s\) alone, with GTP\(_s\) and Y27632, or with GTP\(_s\) and GF109203x. The samples were then resolved and analyzed as in Fig. 2, with the means \(±\) S.E.M. (MLC\(_{20}\)-Pi (%), \(n = 5–8\)). In all tissues, GTP\(_s\) stimulation significantly (\(P < 0.05\)) increased the level of MLC\(_{20}\) phosphorylation, and the increase was (\(P < 0.05\)) partially inhibited by Y27632, but not GF109203x.

Fig. 3. A Rho-kinase dependent pathway mediates the GTP\(_s\) stimulated increase in MLC\(_{20}\) phosphorylation. Gizzard tissue was treated with GTP\(_s\) in pCa 9 and stimulated with Y-27632 or GF109203x. The lysates were prepared and analyzed as in Fig. 1 (\(n = 3–6\)).

Fig. 4. Stimulation of G-protein pathways does not affect the level of MYPT1 phosphorylation. Embryonic and adult gizzard (G) and aortic (A) tissues were at pCa 9 in the presence (+) or absence (−) of GTP\(_s\). The samples were then solubilized with SDS sample buffer, resolved by SDS-PAGE and analyzed using Western blotting with either anti-MYPT1 antibody or with an anti-phospho-MYPT1 antibody (\(n = 4–5\)). Control lanes (right) show Western blots of purified MYPT1 and MYPT1 after Rho-kinase phosphorylation probed with anti-MYPT1 and either the antiphospho-MYPT1 (Thr580) or MYPT1 (Thr695) antibody, respectively.

Fig. 5. MLC\(_{20}\) phosphorylation in response to GTP\(_s\), Y27632 and GF109203x

only the phosphorylated protein was detected by the phospho-specific antibodies. The results also show that MYPT1 phosphorylation is isoform specific. The expression of MYPT1 isoforms is developmentally regulated (Fig. 4): the aorta expresses the splice-in (M133) MYPT1 isoform throughout development. However, the gizzard expresses M133 during its embryonic period and both isoforms (M133 and M130) shortly after hatching, with the final ratio of M133:M130 reaching 30:70 in >D7 tissue. The phospho-specific antibodies detected a single phosphorylated band in aortic tissue, which corresponds to the splice-in (M133) isoform, and similarly in gizzard smooth muscle a single band is seen at the molecular weight of the splice-in (M133) MYPT1 isoform in aorta, which corresponds to the higher Mr band in adult gizzard tissue (Fig. 4). In our previous reports [20,21,24], we found that the gizzard expressed exclusively the M130 MYPT1 isoform shortly after hatching. This difference in isoform expression could be due to the increased sensitivity of the polyclonal MYPT1 antibody used in the present study. Furthermore, a different strain of chicken was used in the present study. Others [25] have demonstrated variability in the expression of CPI-17 among different avian species (chicken vs. pigeon), and thus the change in the strain of chicken could also account for the difference in M133/M130 MYPT1 expression between our previous reports [20,21,24] and the present study.

An alternative mechanism for G-protein induced force enhancement would be a Rho-kinase or PKC mediated phosphorylation of CPI-17 [26]. We could not detect the expression of CPI-17 at any developmental stage (Fig. 5). However, small signaling molecules are known to be lost after Triton X-100 permeabilization [16]. But, CPI-17 was detected in \(\beta\)-escin permeabilized rat aorta (Fig. 5, lane 1), demonstrating that it is not lost during \(\beta\)-escin permeabilization of rat tissue making it unlikely that it was lost during permeabilization of the chicken smooth muscle. If neither MYPT1 phosphorylation nor CPI-17 phosphorylation participates in a Rho/Rho-kinase mediated smooth muscle signaling pathway for Ca\(^{2+}\) independent force enhancement, there must be an alternative signaling molecule and/or pathway.

It has been widely appreciated that another phosphoprotein phosphatase inhibitor is present in tissues where CPI-17 is low or absent [25,27]. This protein, PHI-1 is an inhibitor of type-1 protein phosphatases with a high degree of homology to protein phosphatases with a high degree of homology to type-2 protein phosphatases.
crease in its inhibition of MLC phosphatase [27]. To confirm the increase in PHI-1 phosphorylation (Fig. 6) and a resulting in-
crease of PHI-1 phosphorylation. Using this technique, GTP
/C24 stimulation of skinned chicken smooth muscle produced an increase in di-phosphorylated form (Thr57 + Ser142) of PHI-1 (Fig. 7 and Table 2) consistent with a GTP
/C0 mediated increase in PHI-1 phosphorylation (Fig. 6); at pCa 9, phosphorylation was not detected while following GTP
S stimulation significantly (P < 0.05) increased the level of PHI-1 phosphorylation, and the increase was inhibited (P < 0.05) by both Y27632 and GF109203x.

Table 2
| Tissue            | pCa 9 | pCa 9 + GTP
/S | pCa9 + Y27632 | pCa9 + GF109203x |
<table>
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<tr>
<td>ED16 Gizzard</td>
<td>16 ± 1.2</td>
<td>25 ± 2.1</td>
<td>19 ± 2.5</td>
<td>13 ± 1.5</td>
</tr>
<tr>
<td>ED16 Aorta</td>
<td>14 ± 1.0</td>
<td>23 ± 2.0</td>
<td>17 ± 2.2</td>
<td>15 ± 1.6</td>
</tr>
<tr>
<td>Adult Gizzard</td>
<td>19 ± 0.7</td>
<td>24 ± 0.8</td>
<td>19 ± 0.2</td>
<td>19 ± 1.3</td>
</tr>
<tr>
<td>Adult Aorta</td>
<td>13 ± 1.3</td>
<td>22 ± 1.7</td>
<td>18 ± 1.8</td>
<td>19 ± 1.1</td>
</tr>
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</table>

Skinned embryonic and adult aorta and gizzard samples were treated with pCa9 and stimulated with either GTP/S alone, with GTP/S and Y27632, or with GTP/S and GF109203x. The samples were then solubilized and analyzed (see text for details), means ± S.E.M. (PHI-1-Pi (%), n = 4–6). In all tissues, GTP/S stimulation significantly (P < 0.05) increased the level of PHI-1 phosphorylation, and the increase was inhibited (P < 0.05) by both Y27632 and GF109203x.

CPI-17 and is ubiquitously expressed in many cell types [27]. In chicken aortic and gizzard smooth muscle GTP/S stimulation increased the level of PHI-1 phosphorylation (Fig. 6); at pCa 9, phosphorylation of PHI-1 at Thr57 was not detected while following GTP/S stimulation, phosphorylation was apparent. However, this technique requires the use of two different antibodies with differing substrate affinities and only a relative level of PHI-1 phosphorylation can be determined. Therefore, the method described by Eto et al. [27] was used to determine the level of PHI-1 phosphorylation. Using this technique, GTP/S stimulation of skinned chicken smooth muscle produced an ~55–60% increase in di-phosphorylated form (Thr57 + Ser142) of PHI-1 (Fig. 7 and Table 2) consistent with a GTP/S mediated increase in PHI-1 phosphorylation (Fig. 6) and a resulting increase in its inhibition of MLC phosphatase [27]. To confirm that the lowest protein band (labeled PHI1-2P in Fig. 7) detected on the urea/glycerol gels is the diphosphorylated form of PHI-1, we purified the PHI-1 and phosphorylated the protein in vitro using Rho-kinase [23]. Both the native protein and phosphorylated PHI-1 were run on an urea/glycerol gel and subsequently probed using an anti-PHI-1 or anti-phosphospecific PHI-1 (Thr57) antibody, respectively. PHI-1 was detected as a single protein band corresponding to the upper band (labeled PHI1 in Fig. 7) while the anti-phosphospecific PHI-1 (Thr57) antibody detected a single band corresponding with the lowest band (labeled PHI1-2 in Fig. 7) on the urea/glycerol gels probed with the polyclonal PHI-1 antibody, suggesting that the three bands detected using urea/glycerol gels (Fig. 7) represent un-, mono- and di-phosphorylated PHI-1, as previously demonstrated by others [27]. To further delineate the signaling pathway for the GTP/S induced increase in PHI-1 phosphorylation, we used Y-27632 and GF109203x. Both Y-27632 and GF109203x inhibited the G-protein induced phosphorylation of PHI-1 (Fig. 7 and Table 2).

To demonstrate that the increase in PHI-1 phosphorylation is part of a physiologically significant pathway for agonist induced force enhancement, we determined PHI-1 phosphorylation at rest and following AII stimulation of intact smooth muscle using phospho-specific PHI-1 antibodies (Fig. 8A), as well as PHI-1 antibodies and urea/glycerol gels (Fig. 8B). At rest PHI-1 phosphorylation for aorta and gizzard, was 17 ± 3.8% and 17 ± 3.5%, respectively, which increased (P < 0.05) to 24 ± 3.8% and 24 ± 4.1% following agonist stimulation. Both the resting and agonist stimulated level of PHI-1
MLC20 by Zip kinase [18], Rho-kinase [17] or ILK [19] has been force enhancement. In addition, a direct phosphorylation of CPI-17 participates in the mechanism for agonist induced MLC20 phosphorylation by these kinases could participate in enhancement, it would be difficult to envision how a direct activity is a common theme for G-protein mediated force shown to occur. However, if inhibition of MLC phosphatase phosphorylates MYPT1 at Thr850, which results in a dissoci-

activity of the MLC phosphatase complex from myosin, thus inhibiting MLC phosphatase activity. However, in the present study, MYPT1 phosphorylation at Thr850 did not change after GTP;S stimulation (Fig. 4). Similar to MYPT1 phosphorylation at Thr695, only phosphorylation of the M133 MYPT1 isoform at Thr850 could be detected, which suggests that MYPT1 phosphorylation by Rho-kinase may be MYPT1 isoform specific. Nonetheless, these results strongly suggest that in chicken smooth muscle MYPT1 phosphorylation at either Thr695 or Thr850 does not participate in the mechanism for agonist induced force enhancement.

Our results are similar to those reported for rabbit femoral arterial smooth muscle strips, in which MYPT1 phosphorylation at Thr641 did not change during histamine or ET-1 stimulation [26]. These investigators showed that although there is no change in MYPT1 phosphorylation at its inhibitory site, there is an increase in phosphorylation at Thr799. However, the results of the present study (Fig. 4) show that phosphorylation of the chicken MYPT1 is isoform specific. Thus, phosphorylation may not occur at Thr 641, but rather at Thr 697 of the mammalian MYPT1 sequence. These results could explain the increase in MYPT1 phosphorylation observed during G-protein stimulation in experiments using radio-labeled ATP [7,20] and phosphorylation specific antibodies in some [28,29] but not all [26] studies, as increases of MYPT1 phosphorylation during G-protein stimulation may depend on the relative expression of the M133/M130 MYPT1 isoforms. Consistent with this is the demonstration that carbachol stimulation of rabbit bladder smooth muscle leads to a Rho-kinase dependent increase in MYPT1 phosphorylation at its inhibitory site (Thr697) and force enhancement [29]. Similarly, Shin et al. [35] demonstrated that PDGF2z stimulation of ferret portal vein led to MYPT1 phosphorylation at Thr695.

An alternative mechanism for agonist induced force enhancement would be a Rho-kinase or PKC mediated phosphorylation of CPI-17. Niiro et al. [26] demonstrated that CPI-17 phosphorylation increased after agonist stimulation and the change in Thr38 phosphorylation was inhibited with Y27632. Similar results have been reported for rabbit vas deferens and portal vein stimulated with GTP;S [36]. Others [32] have demonstrated that histamine stimulation of rabbit femoral artery increased CPI-17 phosphorylation (Thr38) and produced force enhancement, which was inhibited by 70% with a PKC inhibitor and partially reversed by Y27632. Phenylephrine stimulation of this tissue produced a similar degree of force enhancement, but less of an increase in CPI-17 phosphorylation, in which inhibitors of PKC and Rho-kinase were equally effective for inhibition. However, we could not detect the expression of CPI-17 at any developmental stage (Fig. 5); making a strong case that CPI-17 does not participate in the regulation of MLC phosphatase activity in chicken smooth muscle. Our results are similar to those reported by Kitazawa's group [25] who were also unable to detect CPI-17 in chicken smooth muscles using either immunoblotting or RT-PCR, but the polyclonal antisera detected CPI-17 in pigeon tissues, suggesting that the antisera cross-reacts with avian CPI-17. Thus these data suggest that neither MYPT1 phosphorylation nor CPI-17 phosphorylation participates in a Rho/Rho-kinase mediated pathway for Ca2+ independent force enhancement.

It has been widely appreciated that another phosphoprotein phosphatase inhibitor is present in tissues where CPI-17 is low or absent [25,27]. This protein, PHI-1 is an inhibitor of type-1 phosphorylation are similar to that observed in skinned tissue at pCa 9 ± GTP;S (Table 2), demonstrating that the magnitude of the increase in PHI-1 phosphorylation in response to GTP;S stimulation of permeabilized smooth muscle and agonist stimulation of intact tissue is similar.

5. Discussion

In general, G-proteins stimulation has been shown to inhibit MLC phosphatase activity (reviewed in [6,11,13,14]). Several investigators have demonstrated that agonist stimulation leads to a Rho/Rho-kinase mediated phosphorylation of MYPT1 [28,29]. However, others have demonstrated that either a PKC [16,30,31] or Rho-kinase [32,33] induced phosphorylation of CPI-17 participates in the mechanism for agonist induced force enhancement. In addition, a direct phosphorylation of MLC20 by Zip kinase [18], Rho-kinase [17] or ILK [19] has been shown to occur. However, if inhibition of MLC phosphatase activity is a common theme for G-protein mediated force enhancement, it would be difficult to envision how a direct MLC20 phosphorylation by these kinases could participate in a physiologically relevant mechanism for force enhancement.

In contrast to several reports [7,29], but similar to others [26], we found there is a baseline level of MYPT1 phosphorylation at Thr695 that does not change after GTP;S stimulation (Fig. 4). Recently, Cohen [34] demonstrated that Rho-kinase phosphorylates MYPT1 at Thr850, which results in a dissoci-

Fig. 8. PHI-1 phosphorylation after agonist stimulation. (A) PHI-1 phosphorylation was determined in intact gizzard and aortic (data not shown) tissues at rest or after AII stimulation. The samples were then solubilized with SDS sample buffer, resolved by SDS-PAGE and analyzed using Western blotting with either an anti-PHI1 antibody or with an anti-phospho-PHI-1 antibody (n = 3). (B) PHI-1 phosphorylation was determined in intact gizzard (top panel) and aortic (lower panel) tissues at rest and following AII stimulation. The samples were solubilized, resolved on urea/glycerol gels and the phospho-PHI-1 protein was identified on immunoblots (n = 3).
protein phosphatases with a high degree of homology to CPI-17 and is ubiquitously expressed in many cell types [27]. Additionally, the inhibitory potency of PHI-1 for MLC phosphatase is increased several fold upon phosphorylation [27]. Furthermore, in vitro phosphorylation of recombinant PHI-1 by PKC revealed three forms of the protein, unphosphorylated, mono-phosphorylated (predominately at Ser142 [27]), and di-phosphorylated at Ser142 and Thr57 [27]. The inhibition of MLC phosphatase activity by PHI-1 is dependent on its phosphorylation at Thr57 [27]. Both agonist stimulation of intact smooth muscle strips (Figs. 8 and 9) and GTP/S stimulation of skinned chicken smooth muscle (Figs. 6 and 7 and Table 2) produced a significant increase in PHI-1 phosphorylation, consistent with G-protein stimulation mediating an increase in PHI-1 inhibitory potency toward MLC phosphatase. Both Y-27632 and GF109203x inhibited the G-protein induced phosphorylation of PHI-1 (Fig. 7 and Table 2). It is unclear why GF109203x decreases GTP/S induced PHI-1 phosphorylation (Fig. 7 and Table 2), but not MLC20 phosphorylation (Fig. 3 and Table 1) in these tissues. Others have demonstrated that G-protein stimulation activates both PKC and Rho kinase dependent pathways, and activation of both pathways lead to force enhancement [32]. However, it is not surprising that GF109203x inhibits the G-protein mediated increase in PHI-1 phosphorylation as the sequence of PHI-1 surrounding the inhibitory site, Thr57 (aa 54–59, GKVTVK), is a consensus sequence for PKC [37] and PHI-1 is known to be a substrate for PKC [27,37]. However, GF109203x did not attenuate the G-protein mediated increase in phosphorylation of MLC20 (Fig. 3 and Table 1), implying that the activation of PKC does not participate in a physiologically relevant pathway for force enhancement in smooth muscle. On the other hand, the GTP/S induced increase in both PHI-1 and MLC20 is inhibited by Y-27632. These results suggest that agonist induced force enhancement is due to a G-protein mediated activation of Rho/Rho kinase, which induces PHI-1 phosphorylation leading to inhibition of MLC phosphatase and an increase in MLC20 phosphorylation. Our results with PHI-1 phosphorylation are consistent with recent reports of others [25]. These investigators demonstrated that in avian smooth muscle GTP/S stimulation leads to a Rho kinase dependent Ca2+ sensitization, and that phorbol ester stimulation of rabbit and pigeon smooth muscle resulted in a Ca2+ sensitization, while in chicken smooth muscle it produced a Ca2+ desensitization. Taken together with our results, the data could suggest that in chicken smooth muscle G-protein stimulation leads to a Rho kinase dependent increase in PHI-1 phosphorylation, which in turn binds to the catalytic subunit of MLC phosphatase to inhibit its activity to produce an increase in MLC20 phosphorylation and force. Further, our results are the first to demonstrate that PHI-1 phosphorylation increases with agonist stimulation of intact smooth muscle (Fig. 8), consistent for a role of PHI-1 phosphorylation in a physiologically relevant pathway for inhibition of MLC phosphatase during agonist induced force enhancement. Additionally, both integrin-linked kinase (ILK) [19,38] and Zip-kinase [29] may participate in smooth muscle force enhancement. Zip kinase is expressed in chicken smooth muscle during both embryonic and adult stages (data not shown), and has been demonstrated to phosphorylate MYPT1 [29]. However, we did not see an increase in MYPT1 phosphorylation, which would suggest that if Zip kinase participated in the pathway for force enhancement, it must be directly phosphorylating MLC20 [18]. Similarly, ILK is expressed in both adult and embryonic chicken smooth muscle (data not shown), consistent with its ubiquitous expression in all cell types [38]. Muranyi et al. [38] have demonstrated ILK is associated with the contractile apparatus of smooth muscle and can phosphorylate both MYPT1 [38] and MLC20 [39]. Additionally, ILK has been shown to phosphorylate both CPI-17 and PHI-1 [37,39], which could implicate ILK in a signaling pathway for force enhancement. Thus, our results are consistent with a physiologically relevant pathway for agonist induced force enhancement in smooth muscle resulting from G-protein induced activation of Rho/Rho-kinase leading to a phosphorylation of PHI-1, possibly by Rho-kinase, ILK or even another unidentified kinase. The phosphorylated PHI-1 would then bind to the catalytic subunit of MLC phosphatase to inhibit the MLC phosphatase activity and produce a Ca2+ independent increase in MLC20 phosphorylation and force. A Rho/Rho kinase pathway leading to PHI-1 phosphorylation and an inhibition of MLC phosphatase could represent a mechanism for increasing force in smooth muscle as well as non-muscle cell motility.

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References


