Biphasic Regulation of Myosin Light Chain Phosphorylation by p21-activated Kinase Modulates Intestinal Smooth Muscle Contractility

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Running Title: P21-activated Kinase in Mechanotransduction Pathway and Cell Contractility

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Keywords: myosin light chain (MLC); p21-activated kinase (PAK); MLC phosphatase targeting subunit (MYPT1); mechanotransduction
**Background:** Myosin Light Chain (MLC) phosphorylation is the driving force of cell contractility.

**Results:** P21-activated kinase (PAK) can either activate or inhibit myosin light chain phosphorylation depending on the level of PAK activation.

**Conclusion:** Inhibition of intestinal motility by supra-physiological mechanical stretch is attributable to increased PAK activity.

**Significance:** The bi-phasic regulation of PAK may provide an explanation for the conflicting observations on PAK-mediated MLC phosphorylation.

**SUMMARY**

Supra-physiological mechanical stretching in smooth muscle results in decreased contractile activity. However, the mechanism is unclear. Previous studies indicated that intestinal motility dysfunction after edema development is associated with increased smooth muscle stress and decreased myosin light chain (MLC) phosphorylation *in vivo*, providing an ideal model for studying mechanical stress-mediated decrease in smooth muscle contraction. Primary human intestinal smooth muscle cells (hISMCs) were subjected to either control cyclical stretch (CCS) or edema (increasing) cyclical stretch (ECS), mimicking the biophysical forces in non-edematous and edematous intestinal smooth muscle *in vivo*. ECS induced significant decreases in phosphorylation of MLC and MLC phosphatase targeting subunit (MYPT1) and a significant increase in p21-activated kinase (PAK) activity compared to CCS. PAK regulated MLC phosphorylation in an activity-dependent bi-phasic manner. PAK activation increased MLC and MYPT1 phosphorylation in CCS but decreased MLC and MYPT1 phosphorylation in hISMC subjected to ECS. PAK inhibition had the opposite results. SiRNA studies showed that PAK1 plays a critical role in regulating MLC phosphorylation in hISMCs. PAK1 enhanced MLC phosphorylation via phosphorylating MYPT1 on Thr696; whereas PAK1 inhibited MLC phosphorylation via decreasing MYPT1 on both Thr696 and Thr853. Importantly, *in vivo* data indicated increased PAK activity in edematous tissue and inhibition of PAK in edematous intestine improved intestinal motility. We conclude that PAK1 positively regulates MLC phosphorylation in intestinal smooth muscle through increasing inhibitory phosphorylation of MYPT1 under physiologic conditions; whereas PAK1 negatively regulates MLC phosphorylation via inhibiting MYPT1 phosphorylation when PAK activity is increased under pathologic conditions.

**INTRODUCTION**

As a primary site for food digestion and transduction, intestinal smooth muscle is constantly receiving external pressure and mechanical stress. Therefore, the response of the intestinal smooth muscle to mechanical stimulation is among the most important adaption responses to the outside environment. Increased contraction in response to mechanical stretch of smooth muscle is a common response observed in multiple systems, including the intestine (1-5), which is important in maintaining normal organ function. However, persistent or supra-physiological stretch has been shown to decrease smooth muscle cell contraction and may trigger dysfunction leading to severe clinical consequences (5-7). The mechanism of stretch-mediated decrease of smooth muscle contraction is unclear.

Intestinal edema development is one instance when intestinal smooth muscle cells are subjected to persistently increased stretch. Intestinal edema develops under various pathologic conditions, such as trauma with fluid resuscitation, abdominal surgery, heart failure or traumatic brain injury (8-10). Intestinal edema causes a 6-fold increase in strain in the smooth muscle layer of the intestine compared to non-edematous tissue (11). This increased strain,
resulting in increased stretching force to the intestinal smooth muscle cells, is associated with decreased intestinal contractile activity via decreased myosin light chain (MLC) phosphorylation (12-15). This is accomplished by edema-induced attenuation of the constitutive inhibition of MLC phosphatase in intestinal smooth muscle (7). The mechanism by which intestinal mechanical stress alters the regulation of MLC phosphorylation is poorly understood. Understanding the response of intestinal smooth muscle cells to stretch will help in understanding the mechanotransduction pathway in regulating MLC phosphorylation; it will also aid in identifying therapeutic targets for treating edema-induced motility disorders.

Thr18 and Ser19 are two common phosphorylation sites of MLC. The interaction between myosin and actin, regulated by MLC phosphorylation especially at Ser19, is the driving force for smooth muscle cell contraction (16,17). MLC phosphorylation is precisely regulated by Ca\(^{2+}\)/calmodulin-dependent MLC kinase (MLCK) and MLC phosphatase (MLCP) (18-20). MYPT1, the myosin binding subunit of MLCP, is regulated via phosphorylation of two sites, threonine-696 (Thr696) and threonine-853 (Thr853) (21). Phosphorylation of these two sites inhibits MLCP activity. Numerous kinases have been reported to phosphorylate MYPT1 including Rho kinase (ROCK), zipper-interacting protein kinase (ZIPK), integrin-linked kinase (ILK), and p21-activated kinase (PAK) (22-24).

PAKs, a group of Rac- and cdc42-regulated serine/threonine protein kinases participating in many mechanotransduction pathways and involved in multiple biologic processes including cytoskeletal dynamics, cell motility, angiogenesis, and cancer metastasis (25-27). There are two subgroups of PAK, group I PAK (PAK1-3) and group II (PAK 4-6). Group I PAK activity is critical to cytoskeletal organization, cell motility, movement, and migration which all involve MLC phosphorylation. The regulatory role of PAK in MLC phosphorylation has been reported in several studies (28-31). However, the results are controversial. PAK has been shown to increase MLC phosphorylation and cell motility through enhanced inhibitory MYPT1 phosphorylation or by directly phosphorylating MLC (22,28,30). In contrast, PAK has been shown to inhibit MLC phosphorylation by phosphorylating and inhibiting MLCK (32).

In the current studies, the role of PAK in the mechanotransductive regulation of MLC phosphorylation and intestinal smooth muscle contraction has been investigated. These findings will help us to further understand PAK-regulated MLC phosphorylation, identify new drug targets and design better therapeutic methods to treat motility disorders. It may also help us to understand the regulation of smooth muscle contraction in response to mechanical stimuli.

**EXPERIMENTAL PROCEDURES**

**Human primary cell isolation and culture**

Disease-free small intestinal tissue was collected from organ donor patients with the generous consent of a family member and approval by the Committee for the Protection of Human Subjects at University of Texas Health Science Center at Houston. The human ileal muscularis externa was separated from mucosal and submucosal tissue, cut into small pieces, and incubated in Ca\(^{2+}\) free Hank’s balanced salt solution (pH 7.4) (Cellgro\textsuperscript{®}, Manassas, VA) (37°C, 15 minutes) followed by incubation in Ca\(^{2+}\) free Hank’s balanced salt solution containing 0.38 mg/ml papain and 15 ng/ml DTT until tissue became loose and sticky. After washing with HEPES buffer (NaCl 120mM, KCl 4mM, KH\(_2\)PO\(_4\) 2.6mM, CaCl\(_2\) 2mM, MgCl\(_2\) 0.6mM, HEPES 25mM, Glucose 14mM, essential amino-acid mixture 2.1%), muscle tissue was digested with 0.21 mg/ml collagenase type II (Worthington, Lakewood, NJ) and 0.1 mg/ml soybean trypsin inhibitor (30 minutes, 31°C) (Worthington, Lakewood, NJ). After washing with HEPES buffer again, human intestinal smooth muscle cells (hISMCs) were harvested by filtration through 500μm Nitex mesh and cultured in Smooth Muscle Cell Growth Medium 2 with 5% Fetal Calf Serum.
(PromoCell®, Heidelberg, Germany), 100 units/ml penicillin, 100µg/ml streptomycin and 0.25 µg/ml Amphotericin B in a 5% CO₂ incubator. To avoid de-differentiating cells, only passages 1-3 hISMCs were used in the current studies.

Cell stretching
HISMCs were seeded onto 6-well BioFlex® culture plates with flexible silicone elastomer bottoms (Flexcell, Hillsborough, NC) coated with 1 mg/ml poly-D-lysine (Sigma, St. Louis, MO). During stretching, computer programmed vacuum created negative pressure under the BioFlex® culture plates, deforming the flexible membrane in an equibiaxial manner to stretch the cells (Fig. 1 A) (FX-4000™ Tension System, Flexcell, Hillsborough, NC). The cyclical stretch protocols were designed using the spontaneous contraction amplitude and frequency measured in vivo in previous experiments (11). Control cells were subjected to basal cyclical stretch consisting of 0.4 Hz sinusoidal strain with a minimum 1% and a maximum 3% elongation (Control cyclical stretch, CCS), mimicking basal spontaneous contractions. Edema Cyclical Stretch (ECS) protocol consisted of gradually escalating 0.4 Hz cyclical stretch starting at a minimum 1% and a maximum 3% elongation and reaching a minimum 18% and maximum 20% elongation at 30 minutes that was maintained for 3.5 hours, mimicking the increased stress experienced by smooth muscle cells during edema development. Graphic representation of the two stretching protocols is shown in Figure 1B. Stretched cells were collected at 4 hours for further analysis.

Western blot
After cell stretching or agonist treatment, cells were lysed with whole cell lysis buffer (Cell Signaling, Danvers, MA) with 1X phosphatase inhibitor (Active Motif®, Carlsbad, CA) and 1X protease inhibitor cocktail (Sigma, St. Louis, MO). After centrifugation, proteins were separated by SDS-PAGE. For the Phos-Tag, 12.5% Phos-tag SDS-PAGE with 25mM Mn²⁺ was prepared as described in (33). For all other Western blotting, a 4-15% gradient precast SDS-PAGE (Bio-Rad, Hercules, CA) or a 4-12% gradient precast SDS-PAGE (Invitrogen, Grand Island, NY) and 15% regular SDS-PAGE were used. Antibodies used were MLC Thr18/Ser19 and MLC Ser19 (Cell Signaling, Danvers, MA), MLC Thr18 and total MLC (Santa Cruz, Santa Cruz, CA), MYPT1 Thr696 and MYPT1 Thr850 (Millipore, Billerica, MA), total MYPT1 (BD, Franklin Lakes, NJ), PAK1 and PAK2 (Cell Signaling, Danvers, MA), GAPDH (Santa Cruz, Santa Cruz, CA). ImageJ from NIH (34) was used to quantify luminescence intensities.

For Western blotting of tissues, mucosa was removed immediately after collection of intestine and smooth muscle tissue was washed with 10% trichlororoacetic acid in acetone with 10mM DTT once and followed with 100% acetone with 10mM DTT for three times. The tissues were then frozen in liquid nitrogen. After lyophilization, the tissue was ground over liquid nitrogen, then suspended in 200µl 1X sample buffer (100mM DTT, 10% glycerol, 0.01% bromphenol blue, 60 mM Tris-HCL, 2%SDS, pH6.8), boiled at 95°C for 5 minutes. Then the samples were rotated in 4°C for overnight before loaded in SDS-PAGE (35).

PAK activity assay
PAK activity in hISMCs was determined by HTScan® PAK1 Kinase Assay Kit (Cell Signaling, Danvers, MA) following the protocol provided by manufacturer. Briefly, whole cell lysates were co-incubated with vehicle or 10µM IPA-3 for 30 minutes. Lysates were then incubated with Biotin-conjugated Phosphor-Tyrosine Hydroxylase (Ser40) in reaction buffer provided by manufacturer for 30 minutes at room temperature before incubating on streptavidin-coated plate. After incubating with Phosphor-Tyrosine Hydroxylase (Ser40) antibody and HRP-conjugated secondary antibody, absorbance was measured (VersaMax™ plate reader, Molecular Devices, Sunnyvale, CA) at 450nm wavelength.

To determine PAK activity level in intestinal smooth muscle tissues, distal small intestine (ileum) was collected in ice-cold Kreb’s solution (NaCl 119mM, KCl 9.6mM,
NaHCO₃ 15mM, CaCl₂ 1.5mM, MgCl₂ 1.2mM, NaH₂PO₄ 1.2mM, D-glucose 11mM, albumin 0.5%, pH 7.4). After removing the mucosa, samples were quickly frozen and pulverized over liquid nitrogen. Muscle tissue lysates were prepared using Protein Extract Kit following manufacturer’s protocol (Active Motif®, Carlsbad, CA). Kinase activity towards MYPT1 was measured by co-incubating tissue lysates with MYPT1 peptide (MYPT1654-880; Cell Signaling, Beverly, MA) in reaction buffer (Tris-HCl 25mM, β-glycerophosphate 5mM, DTT 2mM, sodium orthovanadate 0.1mM, MgCl₂ 10mM, ATP 250μM) with 2.5μCi [γ-³²P]-ATP. Specific PAK activity towards MYPT1 was determined by pre-treating tissue lysates with the PAK specific inhibitor, IPA-3 (Tocris®, Ellisville, MO).

To determine PAK activation with BPIPP (5-(3-Bromophenyl)-5,11-dihydro-1,3-dimethyl-1H-indeno[2',1':5,6]pyrido[2,3-d]pyrimidine-2,4,6(3H)-trione) treatment, PAK1 (1.25μg/ml), PAK2 (1.25μg/ml; ProQuinase, Freiburg, Germany) or PAK5 (2.5μg/ml; Abcam, Cambridge, MA) were pretreated for 10 min with 50μM BPIPP or 0.1% DMSO and GST-tagged MYPT1654-880 (25μg/ml; Millipore, Billerica, MA) was phosphorylated in reaction buffer containing 40mM triethanolamine, pH 8.0, 2mM DTT, 1mM EDTA, 10mM MgCl₂, and 50 μM ATP with 800,000 cpm/nmol of [γ-³²P]-ATP (Perkin Elmer) at 30°C for 10 minutes. Reactions were stopped by spotting onto p81 filters. MYPT1 phosphorylation was measured in triplicate and normalized to total protein kinase activity. BPIPP was a generous gift from Scott Gilbertson (Dept. Chemistry, University of Houston).

DNA and siRNA transfection of hISMCs

hISMCs were seeded in 6-well BioFlex® culture plates one day before transfection to reach desired confluence (70%) on day of transfection. Fugene6 (Roche, Indianapolis, IN) was used to transfet 2 μg/well DNA plasmids into hISMCs according to manufacturer’s protocol. Briefly, 3μl Fugene6 reagent was pipetted into 97 μl serum-free medium and incubated for 5 minutes before 2 μg DNA was added. After incubating for additional 15 minutes in room temperature, the medium containing Fugene6 reagent and DNA was added into one well of a six-well plate. The constitutively active (pCMV6M-hPAK1L107F) and kinase negative (pCMV6M-hPAK1K299R) PAK1 plasmids were generous gifts from Dr. Jonathan Chernoff (36). Lipofectamine™ RNAiMAX (Invitrogen, Grand Island, NY) was used to transfet 3 pmol/well siRNA into hISMCs following manufacturer’s protocol. Briefly, 3 pmol/well siRNA was added into another 250μl serum free Opti-MEM medium (Invitrogen, Grand Island, NY). At the same time, 3μl lipofectamine™ RNAiMAX was mixed into another 250μl serum free Opti-MEM medium. After incubating for 5 minutes at room temperature, medium containing siRNA or lipofectamine™ RNAiMAX was mixed together and incubated at room temperature for 15 minutes. The mixture was added into one well in 6-well plate. Fresh medium was added after 4 hours; and cells were collected after 48 hours. 3 pre-designed PAK1 siRNAs and 3 pre-designed PAK2 siRNAs were used (Sigma, St. Louis, MO). Two days after transfection, cells were collected for analysis.

Rat intestinal edema model

All procedures were approved by University of Texas Medical School Institutional Animal Care and Use Committee and are consistent with the NIH "Guide for the Care and Use of Laboratory Animals". Intestinal edema was induced in male Sprague Dawley rats weighing between 250-350g as described previously (14). Briefly, intestinal edema was induced by a combination of mesenteric venous hypertension and fluid administration (80 ml/kg, 0.9% NaCl). Control-operated animals underwent laparotomy and gut manipulation but no mesenteric venous hypertension and saline infusion. 6 hours after surgery, the animals were sacrificed and small intestines were collected for contractile function and kinase assays.

Intestinal Tissue Contractile Activity.

Intestinal contractile activity, in the longitudinal axis, was measured 6 hours after
surgery as described previously (14). Isometric force was monitored by an external force displacement transducer (Experimetria Ltd., Budapest, Hungary) connected to a PowerLab (AD Instruments, Colorado Springs, CO). The force displacement transducer was calibrated in grams using a known weight. Each strip was stretched to 0.5 g tension and allowed to equilibrate for 30 min. After equilibration, 10 min of basal contractile activity data were recorded. IPA-3 or Vehicle (DMSO) was added to the chamber and 5 minutes of additional data was collected. A dose response curve for IPA-3 was performed to select the optimal dose. After recording contractile activity, length of each strip was measured and tissue was removed, dried and weighed. Contractile activity parameters were all calculated over 5 minutes of recorded data. Total contractile activity was calculated as area under the curve. Basal tone was defined as average minimum of the contraction cycle. Amplitude was calculated as average cycle height. All contractile activity parameters were normalized to tissue cross-sectional area of the intestinal strip calculated using measured weight and length and an assumed density of 1.05 g/cm³.

Statistical analyses
At least three independent repetitions were conducted for each cell experiment and presented as means ± standard deviations. Data were analyzed by unpaired Student’s t-tests or ANOVA to determine significance.

RESULTS

Effect of mechanical stretching on MLC phosphorylation

To study the detailed mechanism of cellular response to mechanical stimulation, a primary human intestinal smooth muscle cell model (hISMC) was developed. HISMCs were isolated and characterized by flow cytometry using the smooth muscle cell markers, smooth muscle actin and desmin, and the endothelium cell marker, CD34. Our hISMC were compared to commercially available hISMC (ScienCell, Carlsbad, California) as a positive control.

According to flow cytometry data, 93% of cells from the first passage of hISMC were positive for both smooth muscle actin (Sup. Fig. 1) and desmin (data not shown). Less than 0.1% of the cells were positive for CD34.

As described in the methods, hISMCs were subjected to either control cyclical stretch (CCS) or edema cyclical stretch (ECS) protocols (Fig. 1B), mimicking non-edematous and edematous conditions in vivo (11). We first determined the time course of MLC phosphorylation changes after cells were subjected to CCS or ECS. MLC phosphorylation decreased within 2 hours of ECS compared to CCS, and further decreased after 4 hours ECS (Sup. Fig. 2A). MLC was separated into un-phosphorylated MLC, mono-phosphorylated, and di-phosphorylated MLC using phos-tag SDS-PAGE (33). Both single- and di-phosphorylation bands were detected (Sup. Fig. 2B). Decreases in Di-phosphorylation bands were observed after 4 hours ECS compared to CCS treatment (Sup. Fig. 2B).

Thr18 and Ser19 are the two major MLC phosphorylation sites (37,38). In order to determine the effect of the mechanical stimulation, MLC phosphorylation in the CCS group were compared to the static cells using antibodies which specifically recognize di-phosphorylated MLC (Thr18 and Ser19), Ser19 phosphorylated MLC and Thr18 phosphorylated MLC were used. No significant differences were observed in di-phosphorylation and phosphorylation at the Thr18 site in the CCS group compared to static cells (Sup. Fig. 3A and C). However, Ser19 phosphorylation increased significantly in the CCS group compared to static cells (Sup. Fig. 3B).

Similar to the observation in phos-tag SDS-PAGEs, diphosphorylation of MLC after 4 hours ECS treatment was decreased to 65±11% compared to CCS treatment (Fig. 2A). Ser19 phosphorylation of MLC was decreased to 42±12% after 4 hours ECS compared to CCS (Fig. 2B). In contrast, Thr18 phosphorylation was not significantly changed in the ECS group.
compared to CCS (Fig. 2C). These data suggested that Ser19 phosphorylation which exists in both di-phosphorylated MLC and single Ser19 phosphorylated MLC is more sensitive to mechanical stimulation in hISMCs. Thus, it is the major phosphorylation site we monitored in all subsequent studies. Since hISMCs experience continued mechanical stress under physiological conditions similar to CCS treatment, in all subsequent experiments CCS was the control for ECS treatment.

Effect of mechanical stretching on MYPT1 phosphorylation and PAK activity

MLC phosphatase is inhibited by phosphorylation of its MYPT1 subunit (18); thus, decreased MYPT1 phosphorylation will increase phosphatase activity. Inhibitory phosphorylation of MYPT1 was monitored after 4 hours ECS or CCS. ECS significantly decreased MYPT1 phosphorylation at both Thr696 and Thr853 (Fig. 3A and B). These data suggest that MLC phosphatase activity is increased. These data agree with our findings in the in vivo intestinal edema model in which intestinal smooth muscle MLC and MYPT1 phosphorylation is decreased (7,14). Previous studies have indicated that PAK is responsive to mechanical signals and can regulate MLCP activity (22,27,39,40). PAK activity was measured in hISMCs after stretching (CCS or ECS). A significant increase in kinase activity towards the PAK substrate was observed in the ECS group compared to CCS (Fig. 3C). Pre-incubating the whole cell lysates with 10µM IPA-3, a group 1 PAK (PAK1-3) inhibitor (34), for 30 minutes, significantly decreased kinase activity towards the PAK substrate in both groups. After IPA-3 treatment, the basal kinase activity was not significantly different between CCS and ECS suggesting that the increased kinase activity in ECS group resulted from the PAK activity (Fig. 3C).

Effects of IPA-3 and BPIIPP treatment

To determine PAK involvement in regulation of MLC and MYPT1 phosphorylation, IPA-3 (41) and a PAK activator, BPIIPP, were utilized. Inhibition of PAK with IPA-3 decreased MLC Ser19 phosphorylation in hISMCs in a dose-dependent manner in the CCS group (Fig. 4A). In contrast, low dose IPA-3 treatment (100 nM) rescued the decreased MLC Ser19 phosphorylation in ECS group (Fig. 4A). Moreover, IPA-3 regulated MLC Ser19 phosphorylation in a bi-phasic manner in the ECS group, first increasing MLC Ser19 phosphorylation at the 100 nM concentration and then returning to vehicle control levels with application of higher inhibitor concentrations.

Similar to IPA-3, activation of PAK with BPIIPP also regulated MLC Ser19 phosphorylation in a biphasic manner in the CCS group (Fig. 4B). Low dose BPIIPP treatment increased MLC Ser19 phosphorylation in CCS; however, high BPIIPP concentration decreased MLC Ser19 phosphorylation. The same phenomenon was observed in static cells where low concentrations of BPIIPP increased but higher concentrations decreased MLC Ser19 phosphorylation in hISMCs (Sup Fig. 4A). Changes in MLC phosphorylation with BPIIPP treatment were confirmed by immunofluorescence (Sup. Fig. 4B-D). In contrast to the CCS group, BPIIPP treatment in ECS, in which PAK activity is higher (Fig. 3C), decreased MLC phosphorylation at Ser19 (Fig. 4B).

Determining PAK subtypes involved in regulation of MLC phosphorylation

As shown in Figure 5A, group 1 PAKs (PAK1-3), but not group 2 PAKs (PAK4-6), are activated by BPIIPP and can directly phosphorylate MYPT1 in vitro. To determine which group 1 PAK is involved in regulating MLC phosphorylation, PAK1 and PAK2 siRNA were utilized to knockdown PAK activity. Both PAK1 and PAK2 siRNA specifically knocked-down their corresponding proteins in hISMCs (Fig. 5B). Transfection with PAK1 siRNA, but not PAK2 siRNA, significantly down-regulated MLC Ser19 phosphorylation in static hISMC (Fig. 5C). Furthermore, PAK1 knockdown had opposite effects in the ECS group compared to the CCS group. While PAK1 knockdown decreased MLC Ser19 phosphorylation in hISMC subjected to CCS, PAK1 knockdown...
attenuated ECS-induced decreases in MLC Ser19 phosphorylation (Fig. 5D).

**Effects of ca-PAK1 or kn-PAK1 on MLC phosphorylation**

Constitutively active PAK1 (ca-PAK) and kinase negative PAK1 (kn-PAK) were utilized to further investigate the role of PAK in regulating MLC phosphorylation. PAK1 levels after transfection with ca-PAK or kn-PAK are shown in Figure 5E. In CCS, transfection with ca-PAK1 increased MLC Ser19 phosphorylation compared to vector only transfection. Decreasing PAK1 activity with kn-PAK1 over-expression in CCS inhibited MLC Ser19 phosphorylation (Fig. 5E). In contrast, ca-PAK1 expression did not alter MLC Ser19 phosphorylation compared to vector only transfection in ECS, in which PAK activity is already high (Figure 3C). However, kn-PAK1 over-expression attenuated the ECS-induced decrease in MLC Ser19 phosphorylation. Thus, MLC phosphorylation in the ECS transfected with control plasmid is significantly decreased compared to the CCS group, whereas the ECS transfected with kn-PAK1 is not significantly different from CCS control (Fig. 5E). These data indicate that PAK1 differentially regulates MLC phosphorylation in cells subjected to ECS compared to CCS.

**Effects of PAK on MYPT1 phosphorylation**

In our *in vivo* model, MLC phosphorylation was decreased via decreased inhibitory phosphorylation of MYPT1 (14). Thus, effects of PAK activation on MYPT1 phosphorylation in hISMCs were investigated. Inhibiting PAK1 activity using PAK1 siRNA significantly decreased MYPT1phosphorylation at Thr696 in static hISMCs (Fig. 6A); however, MYPT1 phosphorylation at Thr853 was not affected (Fig. 6B). PK2 siRNA had no effect on MYPT1 phosphorylation in static hISMCs (Fig. 6A and B).

Although ca-PAK expression tended to increase MYPT1 phosphorylation at Thr696 in hISMC subjected to CCS, the differences were not significant. However, decreasing PAK activity with kn-PAK significantly decreased MYPT1 phosphorylation at Thr696 after CCS (Fig. 6C). MYPT1 phosphorylation at Thr696 was significantly decreased in hISMC subjected to ECS after transfection with vector only compared to CCS group (Fig. 6C). Transfection with kn-PAK in the ECS group attenuated the decreased MYPT1 phosphorylation at Thr696 (Fig. 6C).

Interestingly, MYPT1 phosphorylation at Thr853 was significantly decreased in the CCS group after transfection with ca-PAK; however, transfection with kn-PAK had no effect on MYPT1 phosphorylation at Thr853. MYPT1 phosphorylation at Thr853 was significantly decreased in hISMC subjected to ECS after transfection with vector only (Fig. 6D). While transfection with ca-PAK had little effect on MYPT1 phosphorylation at Thr853 compared to vector only in the ECS group, transfection with the kn-PAK prevented ECS-induced decrease in MYPT1Thr853 (Fig. 6D).

**PAK involvement in edema-induced intestinal smooth muscle contractile dysfunction**

To determine if our findings in the hISMC stretch model extend to an animal model of mechanotransduction, we tested the effects of PAK inhibition in our intestinal edema model. We have shown previously that MLC phosphorylation was significantly decreased in edematous intestinal smooth muscle compared to non-edematous tissue (12-15). As shown in Figure 7A, kinase activity towards MYPT1 also significantly decreased after induction of edema compared to non-edematous control tissue as previously described, suggesting that MLC phosphatase activity was increased. As result, the MYPT1 phosphorylation at both Thr696 and Thr853 significantly decreased *in vivo* (Fig. 7B). To determine the involvement of PAKs, tissue lysates were treated with IPA-3 to inhibit PAK activity. Treatment with IPA-3 significantly increased kinase activity towards the MYPT1 substrate in edematous tissue lysates but not non-edematous tissue (Fig. 7C). These data indicate that the decreased kinase activity towards MYPT1 phosphorylation in edematous tissue resulted from increased PAK activity in edematous tissue.
To confirm the involvement of PAK in intestinal smooth muscle contractile dysfunction, the effects of PAK inhibition on intestinal contractile activity were measured (Fig. 8A). We have previously published that contractile activity, including basal tone and amplitude, is decreased in edematous intestinal smooth muscle compared to non-edematous tissue, as can be observed in Figure 8A (7,14). Inhibition of PAK with IPA-3 significantly increased the amplitude of intestinal contractions in edematous tissue compared to control (Figure 8B). Basal tone was not significantly different in the edematous tissue after IPA-3 treatment compared to the control tissue (p=0.11) (Figure 8D). These data confirm the critical role of increased PAK activity in mediating contractile dysfunction in edematous tissue.

Figure 9 shows our hypothetical model for the biphasic effects of PAK on regulation of intestinal smooth muscle MLC phosphorylation. Our current studies demonstrate a novel PAK function in regulating MLC phosphorylation in human smooth muscle. Under physiologic conditions, PAK positively regulates MLC phosphorylation through phosphorylation of MYPT1 at Thr696. However, under pathologic conditions, over-stimulated PAK switches to a different signaling pathway that negatively regulates MLC phosphorylation via decreased MYPT1 phosphorylation at Thr696 and Thr853.

**DISCUSSION**

PAK is an important regulator of mechanotransduction, cell motility and cytoskeleton formation. Although MLC is one of the most important downstream effectors of PAK, PAK’s role in regulating MLC phosphorylation is still controversial. Our data show that increased PAK activity in stretched intestinal smooth muscle cells negatively regulates MYPT1 phosphorylation and inhibits MLC phosphorylation. MLC phosphorylation strongly correlates with contractility; thus, decreased MLC phosphorylation is likely to result in decreased contractile activity. Along with the in vivo studies, these data support the conclusion that edema-induced alterations in PAK activity inhibit intestinal smooth muscle contractile activity. Moreover, using a hISMC model, we further demonstrate that PAKs, especially PAK1, differentially regulate MLC phosphorylation in edematous versus non-edematous conditions. This is the first study that shows dual roles for PAK in regulating the downstream effector, MLC, giving us a better understanding of PAK’s role in regulating smooth muscle contraction.

To better understand PAK’s functions in mechanotransduction and the regulation of MLC phosphorylation, a primary hISMC model was utilized. Intestinal smooth muscle cells are not static in vivo and MLC phosphorylation is different for static cells versus contracting cells. Therefore, we designed two stretching protocols to mimic conditions in control (non-edematous) and edematous intestinal smooth muscle, CCS and ECS, respectively. Although diphosphorylation of MLC was not changed after CCS compared to static hISMC, the Ser19 phosphorylation of MLC was significantly increased in response to the CCS (Sup. Fig.2). This observation correlates to previous reports that MLC Ser19 is the primary phosphorylation site for the smooth muscle tissue force generation (16,17). The increase in Ser19 phosphorylation of MLC may be due to increased Rac/PAK activity with mechanical stimulation (27,42) or an activation of MLCK (43). Because hISMC are usually under mechanical stress in vivo and we showed that CCS affected MLC phosphorylation, we used CCS as the control for ECS.

The stretching protocols utilized in these studies incorporate data from the in vivo intestinal edema model including increased mechanical stress measured during edema development and spontaneous contraction frequency and amplitude measured in the organ bath system (7,11,14). While we have attempted to reproduce the increased stretch that occurs during edema development in intestinal smooth muscle, intestinal edema is a complex phenomenon and we cannot completely reproduce the effects of edema in a cell model. Furthermore, although cyclical stretching more closely mimics in vivo smooth muscle than
static cultures, passive stretching does not recapitulate the effects of membrane depolarizations and calcium influx in vivo. Thus, this is a mechanotransduction cell model only. Nevertheless, the regulation of MLC phosphorylation in the ECS model parallels observations in edematous intestinal smooth muscle tissue in vivo (12-15). Importantly, PAK negatively regulates MLC phosphorylation in hISMC subjected to ECS just as in the in vivo edematous intestinal smooth muscle (Fig. 2). Subjecting cells to ECS significantly increases PAK activity while significantly decreasing both MLC and MYPT1 phosphorylation compared to CCS (7,14). Thus, subjecting primary hISMC to ECS is a good model for studying molecular mechanisms by which edema induces decreased MLC phosphorylation. Furthermore, these data suggest that the mechanical changes in intestinal smooth muscle induced by edema may trigger the signaling cascade leading to decreased MLC phosphorylation and eventually decreased intestinal contractile activity. This is supported by Shah et al. in which stretching intestinal tissue alone resulted in decreased MLC phosphorylation (15).

MLC phosphorylation is regulated via both Ca\(^{2+}\)-dependent and independent mechanisms. Ca\(^{2+}\)-dependent regulation, involving Ca\(^{2+}\)/calmodulin-dependent MLCK, is transient and primarily regulates initiation of smooth muscle contraction (44). In contrast, Ca\(^{2+}\)-independent regulation of MLC phosphorylation involves regulation of MLC phosphatase by Ca\(^{2+}\)/calmodulin-independent kinases, including Rho Kinase (ROCK), zipper-interacting protein kinase (ZIPK) and integrin-linked kinase (ILK) (45,46). Phosphorylation of the MLC binding subunit of MLC phosphatase, MYPT1, negatively regulates phosphatase activity. Our laboratory has previously demonstrated that edema development in small intestine induces a significant and sustained decrease in intestinal smooth muscle contractile activity via decreased MYPT1 phosphorylation (7,14). Our current study supports the idea that increased intestinal smooth muscle stretch (edema development) decreases MLC phosphorylation via decreased MYPT1 phosphorylation.

PAK1 has been shown to regulate MLC phosphorylation; however, whether PAK positively or negatively regulates MLC phosphorylation was unclear. An in vitro study suggested that PAK can directly phosphorylate MYPT1 at Thr696, but not Thr853, to increase MLC phosphorylation (22). PAK activation was also associated with increased MLC phosphorylation in several other studies; however, the involvement of MYPT1 phosphorylation was unclear (30,31). Negative regulation of MLC phosphorylation by PAKs has also been reported. Several studies have shown that PAK can phosphorylate and inhibit MLCK (28,29). PAK may also antagonize ROCK which can phosphorylate MYPT1 to decrease MLC phosphatase activity and, therefore, increase MLC phosphorylation (21,47). PAK was shown to inhibit ROCK-mediated cytoskeletal rearrangement in several studies (48-50). We present evidence that PAK both positively and negatively regulates MLC phosphorylation depending on PAK activity level. Moderately increasing PAK activity with the PAK activator, BPIPP, or ca-PAK in static hISMC or hISMC subjected to CCS resulted in increased MLC phosphorylation. Inhibition of PAK either with IPA-3 or kn-PAK inhibited MLC phosphorylation under control conditions. In contrast, in ECS and edematous intestinal smooth muscle, PAK activity is increased and negatively regulates MLC phosphorylation (Fig. 3 and 7). Furthermore, inhibition of PAK in cells subjected to ECS attenuated ECS-induced decreases in MLC phosphorylation indicating that PAK is negatively regulating MLC phosphorylation under these conditions (Fig. 4A, 5D). Thus, our study may partially reconcile conflicting reports concerning PAK effects on MLC phosphorylation. PAK inhibition in edematous tissue improved increased contracton ammpulse suggesting that inhibition of PAK can attenuate edema-induced decreases in intestinal contractility. These data support a critical role of PAK in the regulation of smooth muscle contraction under pathological conditions. IPA-3 treatment did not significantly change the
smooth muscle contraction in control tissue. This data combined with the data showing a greater change in kinase activity towards the MYPT1 substrate after IPA-3 treatment in tissue lysates from edematous tissue compared to control (Fig. 7 and 8) suggest that PAK activity is higher in edematous tissue.

PAK-mediated phosphorylation of MYPT1 has been demonstrated in vitro (22), but our study is the first to show PAK-mediated phosphorylation of MYPT1 in vivo in smooth muscle cells. Moreover, PAK activation increases MYPT1 phosphorylation at Thr696, but not Thr853, in hISMC subjected to CCS. Knockdown of PAK1 or kn-PAK expression decreases phosphorylation at Thr696 in static hISMC or CCS (Fig.6). These data indicate that under physiologic conditions, PAK activation only affects Thr696. In contrast, subjecting hISMCs to ECS decreases phosphorylation at both Thr696 and Thr853. In a previous study, ROCK activity decreased in edematous intestinal smooth muscle tissues (7). ROCK has been shown to phosphorylate MYPT1 at both Thr696 and Thr853 (21,47). Therefore, we speculate that PAK1 positively regulates MLC phosphorylation through phosphorylation of MYPT1 at Thr696, possibly through direct phosphorylation; however, under pathologic conditions, PAK negatively regulates MLC phosphorylation via inhibition of ROCK-mediated phosphorylation of MYPT1 at both Thr696 and Thr853.

We show that increased PAK activity decreases MYPT1 phosphorylation. We speculate that the decreased MYPT1 phosphorylation is due to decreased kinase activity towards MYPT1; however, another possibility is that PAK increases phosphatase activity towards MYPT1. There is some evidence that the catalytic subunit of MLC phosphatase dephosphorylates MYPT1 (51); however, other phosphatases could be involved. While we give evidence for changes in MLC phosphatase activity, altered MLCK activity is not excluded. There is evidence that PAK can phosphorylate MLCK to inhibit its activity (28,52). However, in the intestinal edema animal model, no changes in MLCK activity were detected in tissue lysates (7).

The PAK inhibitor, IPA-3, specifically inhibits group I PAKs (PAK1-3) (41). Both PAK1 and PAK2 are expressed in intestinal smooth muscle cells, whereas PAK3 is predominantly expressed in neuronal tissue (53) and was not detected in intestinal smooth muscle (data not shown). Previous studies have shown that both PAK1 and PAK2 can regulate MLC phosphorylation (29,54,55). We found that siRNA knockdown of PAK1, but not PAK2, inhibits MLC phosphorylation in static cells. Furthermore, PAK1 knockdown in hISMC prevents ECS induced decreases in MLC phosphorylation (Fig. 5D). PAK1 knockdown also decreased MYPT1 phosphorylation at the Thr696 site but not the Thr853 site in static cells. The current study is the first to show a positive regulation of MLC phosphorylation by PAK1 via phosphorylation of MYPT1 at Thr696 in smooth muscle cells. Although these data suggest a critical role for PAK1 in regulating MLC phosphorylation, the participation of PAK2 cannot be excluded. PAK1 and 2 are highly homologous kinases (53) and regulation of MLC phosphorylation by PAK2 was also reported (54). Interestingly, a recent study has shown that PAK1 and PAK2 have opposing roles in regulating MLC phosphorylation in tumor cells (56).

In conclusion, current studies clearly demonstrate a dual function of PAK in regulating MLC phosphorylation under physiological versus pathological conditions. Under physiologic conditions, PAK positively regulates MLC phosphorylation through phosphorylation of MYPT1 at Thr696. However, increased activation of PAK under pathologic conditions, like edema development, switches PAK signaling to a different pathway involving inhibition of MYPT1 phosphorylation at Thr696 and Thr853. The mechanism by which PAK switches from a positive to a negative regulatory role in MLC phosphorylation is unclear. A better understanding of the PAK-mediated MLC phosphorylation regulatory mechanism will help
in identifying drug targets for the treatment of motility disorders.

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Figure Legends

Figure 1. Schematic diagram of hISMCs cyclical stretching model. (A) Flexible bottom plates were placed on the Flexcell® FX-4000™ tension station. Computer-regulated vacuum applied negative pressure under the flexible bottom to produce equibiaxial cyclic strain to hISMCs cultured on the plate. (B) Upper panel: Schematic diagram shows ECS and CCS stretching protocols. Each cyclical stretching protocol is a sinusoidal wave with a frequency of 0.4 Hz and an amplitude of 2% elongation over 4 hours with no prestretch. The hISMCs in the CCS group were subjected to continual cyclical stretch with a min 1%, max 3% elongation for the entire 4 hour time period. The hISMCs in the ECS group were subjected to a gradually increasing elongation but the same frequency and waveform beginning at min 1%, max 3% elongation, increasing until reaching min 18%, max 20% elongation at 30 min, and remaining at this level of cyclical strain for the rest of the time course (3.5 hours). Lower panel: Schematic diagram shows cyclical stretching in 2.5 second time frame. HISMCs in both CCS and ECS groups were subjected to a sinusoidal (0.4 Hz, amplitude 2%) radial strain during the whole stretching time course.

Figure 2. Phosphorylation of MLC is decreased significantly after 4 hours ECS compared to CCS. MLC di-phosphorylation (Thr18/Ser19) (A), MLC(Ser19) phosphorylation (B) and MLC(Thr18) phosphorylation (C) were monitored by Western blotting. Representative Western blots are shown for each protein as indicated, and the ratio of the phosphorylated protein to the total protein is shown in the lower panels. (*, p<0.05 vs. CCS; n=3 per group)

Figure 3. 4 hours ECS significantly decreased phosphorylation of MYPT1 and increased PAK activity. MYPT1(Thr696) phosphorylation (A) and MYPT1(Thr853) phosphorylation (B) were monitored by Western blotting. Representative Western blots are shown for each protein as indicated, and the ratio of the phosphorylated protein to the total protein is shown in the lower panels. (*, p<0.05 vs. CCS; n=4 per group) (C) PAK activity increased in ECS group compared to CCS group. hISMCs lysates from CCS and ECS groups were incubated with vehicle (Veh) or IPA-3 (10µM) for 30 min before kinase activity towards a PAK substrate was measured. (*, p<0.05 vs. Veh treated CCS group. n=4 per group).
Figure 4. Change of MLC phosphorylation with PAK inhibition or activation. (A) Change of MLC phosphorylation with IPA-3 treatment in stretched hISMCs. HISMCs were pretreated with varying concentrations of IPA-3 for 30 minutes before subjecting cells to CCS or ECS. (B) Change of MLC phosphorylation with BPIPP treatment in stretched hISMCs. HISMCs were pretreated with varying concentrations of BPIPP, as indicated, for 30 minutes before subjecting cells to CCS or ECS. The ratio of phosphorylated to total MLC phosphorylation is shown. (*, p<0.05 vs. CCS group at the same concentrations; +, p<0.05 vs. Veh treatment in CCS; ¶, p<0.05 vs. Veh treatment in ECS; n=3 per group)

Figure 5. Determination of PAK subtypes that regulate MLC phosphorylation. (A) BPIPP treatment increased MYPT1 phosphorylation through activation of group 1 PAKs. The kinase activity of PAK1, PAK2 and PAK5 towards an MYPT1 peptide was determined with or without 50 μM BPIPP treatment. (*, p<0.05 vs. Veh; n=3 per group). (B) Change of PAK1 or PAK2 protein expression level in static hISMCs after PAK1 or PAK2 siRNA transfection. HISMCs were transfected with 1nM PAK1, PAK2 or scrambled siRNA for 48 hours before collection. PAK1 or PAK2 protein levels were monitored by Western blotting and normalized to GAPDH. The ratio of PAK siRNA to scramble siRNA is shown for each PAK isoform. (*, p<0.05 vs. Scramble siRNA transfected group; n=3 per group). (C) Change of MLC phosphorylation in static hISMCs after PAK1 or PAK2 siRNA transfection. HISMCs were transfected with 1nM PAK1, PAK2 or scrambled siRNA for 48 hours before collection for analysis. MLC(Ser19) phosphorylation was measured by Western blotting and normalized to total MLC amount. The ratio of MLC(Ser19) phosphorylation after PAK1 or PAK2 siRNA transfection compared to scramble siRNA transfection is shown. (*, p<0.05 vs. Scramble siRNA transfected group; n=3 per group). (D) Change of MLC phosphorylation with PAK1 siRNA transfection in CCS or ECS. HISMCs were transfected with 1nM PAK1 or scramble siRNA for 48 hours before cell stretching. MLC(Ser19) phosphorylation was measured by Western blotting and normalized to GAPDH. The ratio of MLC(Ser19) phosphorylation after PAK1 siRNA transfection compared to scramble siRNA transfection is shown. (*, p<0.05 vs. Scramble siRNA transfected CCS group; n=3 per group). (E) Change of MLC phosphorylation with ca-PAK1(L107F), kn-PAK1(K299E) or CMV6 vector transfection. HISMCs were transfected with 2μM plasmids 48 hours before cell stretching. MLC(Ser19) phosphorylation was measured by Western blotting and normalized to total MLC. The ratio of MLC(Ser19) level with ca-PAK or kn-PAK to vector only CCS group is shown. (*, p<0.05 vs. control vector transfected CCS group; n=3 per group)

Figure 6. Determination of MYPT1 phosphorylation changes. Change of MYPT1(Thr696) phosphorylation (A) or MYPT1(Thr853) phosphorylation (B) with PAK1 siRNA, PAK2 or scrambled siRNA transfection. HISMCs were transfected with 1nM PAK1 siRNA, PAK2 siRNA or 1nM scramble siRNA for 48 hours before collection for analysis. MYPT1(Thr696) and MYPT1(Thr853) phosphorylation were measured by Western blotting and normalized to total MYPT1 amount. Values are shown as a ratio of the scrambled siRNA transfection. (*, p<0.05 vs. scramble siRNA transfected group; n=3 per group). Change of MYPT1(Thr696) phosphorylation (C) or MYPT1(Thr853) phosphorylation (D) after transfection with ca-PAK1(L107F), kn-PAK1(K299E) or CMV6 vector. HISMCs were transfected with 2μM plasmids 48 hours before cell stretching. MYPT1(Thr696) and MYPT1(Thr853) phosphorylation were measured by Western blotting and normalized to total MYPT1. Values are shown as a ratio of the vector only CCS group. (*, p<0.05 vs. CCS in control vector transfected group; +, p<0.05 vs. indicated CCS group; n=3 per group)
Figure 7. PAK inhibition attenuated the decreased kinase activity towards MYPT1 in edematous tissue. (A) Kinase activity towards MYPT1 decreases 6 hours after edema development. Kinase activity towards MYPT1 peptide in control or edematous intestinal smooth muscle tissues was determined. (*, p<0.05 vs. Control; n=6 per group). (B) Change of both MYPT1(Thr696) phosphorylation and MYPT1(Thr853) phosphorylation 6 hours after edema development. Rat intestinal smooth muscle tissues from both control and edema groups were isolated and processed as described in Experimental Procedures. Representative blots are shown along with quantification of the MYPT1 phosphorylation. (*, p<0.05 vs. Control; n=12 per group). (C) Inhibition of PAK with IPA-3 increased kinase activity towards MYPT1 in edematous tissue. Control or edematous intestinal smooth muscle tissue lysates were pretreated with IPA-3 (10 μM, 30 minutes) before measuring kinase assay towards an MYPT1 peptide. (*, p<0.05 vs. veh; n=6 per group).

Figure 8. PAK inhibition increased the intestinal smooth muscle contraction in edematous tissues. Original tracings of intestinal tissue spontaneous contractions before and after addition of IPA-3 (10 μM) in Control (A) and Edema (B) groups. Intestinal contractile activity after surgery was monitored in an organ bath. Representative force tracings shows the basal intestinal contractile activity before IPA-3 treatment and intestinal contractile activity 5 minutes after IPA-3 (10μM) treatment. Effects of PAK inhibition on contraction amplitude (C) and basal tone (D). Contractile activity was measured 5 minutes after treatment with vehicle (0.005% DMSO) or IPA-3 (10μM) in non-edematous (Control) and edematous (Edema) distal small intestine. Data is shown as percent change from vehicle treatment (*, p<0.05 vs. Control; n=8 per group).

Figure 9. Theoretical model of the dual function of PAK in regulating intestinal smooth muscle myosin light chain phosphorylation. (A) Under physiologic conditions, PAK positively regulates MLC phosphorylation through phosphorylation of MYPT1 at the Thr696 site. (B) Under pathologic condition, over-stimulated PAK switches its signaling to a different pathway, which negatively regulates MLC phosphorylation via decreased MYPT1 phosphorylation at both the Thr696 and Thr853 sites.
Figure 1.
Figure 2.
Figure 3.

A

\[
\begin{array}{c|c|c}
\text{CCS} & \text{ECS} \\
\hline
\text{MYPT1 (thr696)} & \text{MYPT1 (total)} & \text{GAPDH} \\
\end{array}
\]

\[
\frac{\text{MYPT1 (thr696)}}{\text{MYPT1 (total)}} \times 100\%
\]

B

\[
\begin{array}{c|c|c}
\text{CCS} & \text{ECS} \\
\hline
\text{MYPT1 (thr853)} & \text{MYPT1 (total)} & \text{GAPDH} \\
\end{array}
\]

\[
\frac{\text{MYPT1 (thr853)}}{\text{MYPT1 (total)}} \times 100\%
\]

C

\[
\text{Kinase Activity towards PAK substrate (Absorbance)}
\]

\[
\begin{array}{c|c|c|c}
\text{Veh} & \text{IPA-3} & \text{Veh} & \text{IPA-3} \\
\hline
\end{array}
\]

\*

\text{CCS}

\text{ECS}
Figure 4.
Figure 6.
Figure 8.
Figure 9.
Biphasic regulation of myosin light chain phosphorylation by p21-activated kinase modulates intestinal smooth muscle contractility

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