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Decreased myosin phosphatase target subunit 1(MYPT1) phosphorylation via attenuated rho kinase and zipper-interacting kinase activities in edematous intestinal smooth muscle

Ji Chu, PhD[1], **Christopher T. Miller, MD**[1], **Karina Kistlitsyna, BS**[1], **Glen A. Laine, PhD**[2], **Randolph H. Stewart, DVM**[2], **Charles S. Cox, MD**[1,2], and **Karen S. Uray, PhD**[1,2]

[1]Department of Pediatric Surgery, University of Texas Medical School at Houston

[2]Michael E. DeBakey Institute, Texas A&M University

Abstract

Background—Intestinal edema development after trauma resuscitation inhibits intestinal motility which results in ileus, preventing enteral feeding and compromising patient outcome. We have shown previously that decreased intestinal motility is associated with decreased smooth muscle myosin light chain (MLC) phosphorylation. The purpose of the present study was to investigate the mechanism of edema-induced decreases in MLC in a rodent model of intestinal edema.

Methods—Intestinal edema was induced by a combination of resuscitation fluid administration and mesenteric venous hypertension. Sham operated animals served as controls. Contractile activity and alterations in the regulation of MLC including the regulation of MLC kinase (MLCK) and MLC phosphatase (MLCP) were measured.

Key Results—Contraction amplitude and basal tone were significantly decreased in edematous intestinal smooth muscle compared to non-edematous tissue. Calcium sensitivity was also decreased in edematous tissue compared to non-edematous intestinal smooth muscle. Although inhibition of MLCK decreased contractile activity significantly less in edematous tissue compared to non-edematous tissue, MLCK activity in tissue lysates was not significantly different. Phosphorylation of MYPT was significantly lower in edematous tissue compared to non-edematous tissue. In addition, activities of both rho kinase and zipper-interacting kinase were significantly lower in edematous tissue.

Conclusions and Inferences—We conclude from these data that interstitial intestinal edema inhibits MLC phosphorylation predominantly by decreasing inhibitory phosphorylation of the MLC targeting subunit (MYPT1) of MLC phosphatase via decreased ROCK and ZIPK activities, resulting in more MLC phosphatase activity.

Keywords

intestinal smooth muscle; myosin light chain; MYPT1; edema; intestinal motility; rho kinase

Corresponding author-K S Uray, PhD, UT Med Sch. MSB 5.254, 6431 Fannin, Houston, TX 77030, 713-500-6196, Fax: 713-500-7296, karen.l.davis@uth.tmc.edu.
Ji Chi- 713-500-7323, FAX: 713-500-7296, ji.chu@uth.tmc.edu
Christopher T. Miller- 713-500-6525, FAX: 713-500-7296, Christopher.t.miller@uth.tmc.edu
Karina Kislitsyna- 713-500-7879, FAX: 713-500-7296, karina.kislitsyna@uth.tmc.edu
Glen A. Laine- 979-845-7261, FAX: 979-845-6544, glaine@cvm.tamu.edu
Randolph H. Stewart- 979-862-7764, rstewart@cvm.tamu.edu
Charles S. Cox- 713-500-7307, FAX: 713-500-7296, Charles.s.cox@uth.tmc.edu
Karen S. Uray- 713-500-6196, FAX: 713-500-7296, Karen.l.davis@uth.tmc.edu

Intestinal edema occurs in a number of pathological conditions, including ischemia/reperfusion injury, congestive heart failure, spinal cord injury, and trauma resuscitation. Interstitial intestinal edema develops in trauma patients due to damage control surgical procedures that include abdominal packing, which increases abdominal venous pressures, and fluid resuscitation, which results in hemodilution and, thus, decreased plasma oncotic pressure (1–6). Ileus often occurs after abdominal trauma, preventing enteral feeding and leading to prolonged hospital stays and increased morbidity (4, 7, 8). Intestinal edema contributes to ileus by suppressing both spontaneous and agonist induced contractile activity (2, 3).

To date, there are few drugs that effectively treat resuscitation-induced ileus. Understanding the signal transduction pathways affected by edema development in trauma patients is important in identifying drug targets for the treatment of ileus. Edema may affect intestinal contractile function in a number of ways, including effects on the contractile apparatus, enteric nervous system or cell-cell and cell-matrix interactions. Given the known effects of edema on tissue architecture, edema is likely to affect a combination of these factors. We have shown that intestinal edema affects the contractile apparatus by suppressing phosphorylation of intestinal smooth muscle myosin light chain (MLC) (2). Thus, the kinase cascade that regulates MLC phosphorylation in intestinal smooth muscle may be a promising target for treating ileus. The purpose of the present study was to examine the regulation of MLC phosphorylation, the obligatory step in initiation of smooth muscle contraction, in edematous intestine.

The tightly regulated phosphorylation of MLC depends predominantly on the balance between phosphorylation by MLC kinase (MLCK) and dephosphorylation by MLC phosphatase (MLCP) (9). MLC phosphorylation is responsive to intracellular calcium; in particular, calcium/calmodulin dependent MLCK activity (9). However, MLC phosphorylation can also be modulated via calcium-independent pathways through direct phosphorylation of MLC or through regulation of MLCP activity. MLC can be directly phosphorylated by a number of kinases other than MLCK, including rho Kinase (ROCK) and zipper-interacting protein kinase (ZIPK) (10–12). MLCP activity is regulated via phosphorylation of the regulatory MLC targeting subunit, MYPT1. MYPT1 contains two inhibitory phosphorylation sites (threonine-696 and threonine-853) (13–15). Numerous kinases have been shown to phosphorylate MYPT1 including ROCK, ZIPK and p21 activated kinase (PAK) (14, 16–20).

In general, the signaling cascade regulating MLC phosphorylation is well understood; however, mechanisms of pathological alterations in MLC phosphorylation are less clear. The purpose of this study was to determine how interstitial edema, known to inhibit contractile activity in the intestine, alters the regulation of MLC phosphorylation. Thus, the effects of intestinal edema on spontaneous contractions, which reflect the state of the contractile apparatus, and MLC phosphorylation were examined using an established hydrostatic intestinal edema animal model (1, 2). This model of intestinal edema was chosen because no ischemia or cellular inflammation occurs in the intestine, allowing us to determine the effects of edema alone on the regulation of MLC phosphorylation (3, 21–26).

Materials and methods

Animal Model

250–350 g male Sprague Dawley rats were used for all experiments. All procedures were approved by University of Texas Medical School Institutional Animal Care and Use Committee and consistent with National Institute of Health "Guide for the Care and Use of Laboratory Animals". Intestinal edema was induced as described previously (2). The

EDEMA group had both administration of saline and induction of mesenteric venous hypertension. Control animals (CONTROL) underwent sham surgeries, including exteriorizing the intestines, but no saline infusion or venous constriction. At 0.5, 2 and 6 hours post-surgery, animals were sacrificed and small intestines were collected. The 0 hour time point consisted of untreated animals. The 6 hour time point was selected for functional studies to minimize the effects of anesthesia. The mid and proximal segments were used for wet to dry analysis. In the distal segment (i.e. ileum) mucosa was removed and the muscularis layers were immediately frozen for subsequent analysis.

Intestinal Contractile Activity

Intestinal contractile activity, in the longitudinal axis, was measured 6 hours after surgery as described previously (2). 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. Inhibitors were added to the chamber and 10 minutes of additional data were collected. The following inhibitors were used: MLCK inhibitor, W-7, 50 uM; MLCP inhibitor, okadaic acid, 1 nM; ROCK inhibitor, Y-27632, 10 uM. A dose response curve for each inhibitor was performed to select optimal doses. 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.

Calcium sensitivity curves were generated as follows. After 30 minutes of equilibration, the organ bath solution was exchanged for Kreb's plus the lowest calcium concentration. After 5 minutes of equilibration, the solution was then exchanged in the organ bath chamber to increasing calcium concentrations. Contractile activity parameters were calculated as described above.

All functional studies were performed on intact tissues. While the authors recognize the advantages of permeabilized or stripped intestinal smooth muscle studies, we wished to study the physiological state of the intestinal smooth muscle contractile apparatus. Thus, the tissue was manipulated as little as possible before mounting in the organ baths, allowing us to examine the spontaneous contractions of the edematous intestine.

Wet to Dry Analysis

Samples were weighed immediately after collection and dried in a 60°C oven. The wet to dry ratio was calculated as: (wet weight – dry weight)/ dry weight.

Tissue Extract Preparation

For MYPT1 Western blotting, tissue extracts were prepared as follows. Tissue was collected and immediately placed in ice cold Kreb's solution. Mucosa was removed and muscle layers were placed in ice-cold acetone with 10%TCA and 10 mM DTT. Samples were washed twice with acetone + 10 mM DTT and lyophilized overnight, then stored at −80°C. Before separating protein by electrophoresis, tissue was ground over liquid nitrogen, Laemmli's buffer was added, the sample was boiled for 10 minutes, and then incubated overnight at 4°C while rotating.

For all other assays and western blots, whole cell lysates were prepared by suspending ground tissue in hypotonic buffer with protease and phosphatase inhibitors and incubated on ice for 15 minutes. Incubation was followed by centrifugation (4°C, 14000 ×g, 5 minutes). All samples were aliquoted and frozen until use.

Myosin Light Chain Kinase Activity Assay

MLCK activity was measured by phosphorylation of a smooth muscle MLC substrate as described by Gilbert et al. (27). Whole cell lysates were added to reaction mixture containing the following (in mM): 40 HEPES, 3 $Mg(CH_3COO)_2$, 0.1 EGTA, 0.55 $CaCl_2$, 0.001 okadaic acid, 0.5 peptide substrate (Biomol, Plymouth Meeting, PA), with 0.1 % Tween 20 and 1 mg/ml BSA. Immediately before assay, 150 uM ATP with 0.25 uCi [γ-^{32}P]-ATP per reaction was added. After 20 minutes, an aliquot was spotted on P81 filters, washed (1% phosphoric acid) and dried for measurement of radioactivity. Samples were assayed in duplicate and normalized to total protein. MLCK was calculated as the difference between total phosphorylation activity and activity after treatment with a specific MLCK inhibitor, ML-7.

MYPT1 Phosphorylation Assay

MYPT1 phosphorylation activity in whole cell tissue lysates were measured using an *in vitro* radioactive kinase assay. Briefly, 10 ul of tissue lysate were added to the following reaction mixture to start the reaction (in mM): 25 Tris-HCl, 5 beta-glycerophosphate, 2 dithiothreitol, 0.1 sodium orthovanadate, 10 MgCl, 0.25 ATP, plus 3 ug MYPT1 peptide (MYPT1 654–880, Cell Signaling, Beverly, MA) and 2.5 uCi [γ-^{32}P]-ATP. After 15–30 minutes incubation at 30°C, reactions were stopped by spotting onto p81 filters. After washing filters with 1% phosphoric acid, radioactivity was measured. Samples were measured in duplicate and normalized to total protein. The following inhibitors/activators were used: ROCK inhibitor, 10 uM Y-27632 (Calbiochem, San Diego, CA); ZIPK inhibitor, 50 uM SM-1 peptide (28); active Rac1 (Cytoskeleton, Denver, CO). The specificities of inhibitors were confirmed using commercially available enzymes: ROCK and ZIPK/DAPK (Cell Signaling, Beverly, MA). Specific kinase activity was calculated as the difference between activity with no inhibitor and specific kinase inhibitor.

Rho GTPase activity

Tissue was cut into 1 mm pieces and frozen in liquid nitrogen for later processing. To extract proteins, cell lysis buffer (Cytoskeleton, Inc. Denver, CO) with phosphatase inhibitors (Sigma) and protease inhibitors (Cytoskeleton, Inc. Denver, CO) was added to each sample and homogenized briefly. After centrifugation at 4°C, samples were aliquoted and stored in −80°C freezer. Immediately before assay, samples were thawed and protein concentration measured quickly. The assay was performed following manufacturer's instructions.

Western Blotting

Standard Western blotting techniques were used to detect ROCK, ZIPK, MYPT1, and rhoA. The following antibodies were used: Anti-MYPT1, anti-phospho-MYPT1(thr696) and anti-phospho-MYPT1(thr853) (Millipore, Billerica, MA); ZIPK and ROCK2 antibodies (AbCam, Cambridge, MA); rhoA (Cell Signaling Technology, Inc. Danvers, MA). After blotting for specific proteins, membranes were stripped and blotted with glutaraldehyde 3-phosphate dehydrogenase (GAPDH) antibody for protein normalization.

Statistics

Data are presented as mean ± standard error of the mean. Differences between CONTROL and EDEMA groups were tested using ANOVA, followed by a Fishers LSD to compare groups at individual time points if differences were detected by ANOVA. Significance was accepted as $p \leq 0.05$.

Results

We have previously shown that intestinal edema development causes decreased intestinal contractile activity without significant changes in contraction frequency. To understand the mechanism of edema-induced contractile dysfunction, the effects of edema on the amplitude of spontaneous contractions, basal tone, and calcium sensitivity were examined as shown in Figure 1. Intestinal edema caused a significant decrease in basal tone of intestinal smooth muscle compared to non-edematous intestine measured 6 hours after induction of edema as shown in Figure 1A (0.41 ± 0.05 vs. 0.32 ± 0.03 g/cm^2). The average contraction amplitude was also significantly decreased in edematous small intestine, as shown in Figure 1C (6hr time point) (0.12 ± 0.02 vs. 0.07 ± 0.02). Although the basal tone was lower in edematous intestine compared to non-edematous tissue at all concentrations, there were no significant differences between calcium concentrations (Figure 1B). In contrast to basal tone, amplitude was calcium sensitive and calcium sensitivity was significantly decreased in edematous intestine compared to control (i.e. slopes significantly different) (Figure 1D).

The extent of MLC phosphorylation and, therefore, smooth muscle contractility, is predominantly due to a balance between MLCK and MLCP activities. Thus, the contribution of each of these enzymes to edema-induced suppression of intestinal contractile activity was examined. Figure 2 shows the effects of the MLCK inhibitor, W-7, on contractile activity in CONTROL and EDEMA groups. Figures 2A and 2B show representative tracings of contractile activity before and after inhibition of MLCK (W-7) in CONTROL and EDEMA groups. Panel C shows the average percent inhibition of contractile activity from baseline after treatment with W-7 including decreased amplitude and basal tone. MLCK inhibition decreased contraction amplitude and basal tone significantly less in the EDEMA group compared to CONTROL (Amplitude, 67.73 ± 3.92 vs. 25.19 ± 7.44%; Basal tone, 33.99 ± 5.38 vs. 16.62 ± 3.01%). However, no differences in MLCK activity were detected when measured *in vitro* in tissue lysates using a radiolabeled phosphate incorporation assay (similar results obtained from 3 separate samples sets) (Figure 2D).

As shown in Figure 3, Panel A, inhibition of MLCP with okadaic acid had very little effect on contractile activity in the CONTROL group. In contrast, MLCP inhibition increased contractile activity in the EDEMA group indicating significantly more MLCP activity in edematous tissue compared to non-edematous tissue. MLCP activity is regulated in a calcium-independent manner by inhibitory phosphorylation of its myosin binding subunit, MYPT1, at thr696 and thr853. Thus, we examined the effects of edema development on MYPT1 phosphorylation at these sites. Figure 3, Panel C shows representative Western blots of MYPT1 phosphorylation at both phosphorylation sites. The average MYPT1 phosphorylation, normalized to unphosphorylated MYPT1, is shown in Figure 3, Panel D. Phosphorylation at both the thr696 site and the thr853 site was significantly decreased in edematous intestinal smooth muscle compared to normal tissue. Total MYPT1 protein normalized to GAPDH did not change significantly in the EDEMA group compared to CONTROL, as shown in Figure 3, Panel E (CONTROL vs. EDEMA, 1.13 ± 0.06 vs 1.28 ± 0.07, $p=0.63$). Kinase activity towards an exogenous MYPT1 substrate (containing the two inhibitory phosphorylation sites) was measured in intestinal smooth muscle tissue lysates using a radioactive phosphate incorporation assay. As shown in Panel B of Figure 3, after incubation with tissue lysates from the EDEMA group, MYPT1 phosphorylation was

significantly lower compared to CONTROL at both the 2 and 6 time points. MYPT1 phosphorylation activity significantly decreased in lysates from both the CONTROL and EDEMA tissues between 30 minutes and 2 hours compared to baseline (0 hours) but recovers by 6 hours after surgery in the CONTROL group. MYPT1 phosphorylation activity in the edematous tissue is still decreased at 6 hours after surgery compared to baseline.

MYPT1 is phosphorylated by several kinases including ROCK and ZIPK. Thus, the role of these kinases in edema-induced alterations in contractile activity and MYPT1 phosphorylation were examined. While treatment with Y-27632, a ROCK inhibitor, induced a significant decrease of 10–25% in contractile activity from baseline, the difference between CONTROL and EDEMA was not significant (26.25 ± 2.37 vs. 10.62 ± 7.50 %, p=0.08) (Figure 4, Panel A). However, when intestines were pretreated with an MLCK inhibitor (W-7), ROCK inhibition caused significantly less suppression of contractile activity in the EDEMA group (36.19 ± 4.04 %) compared to the CONTROL group (59.97 ± 4.37%), indicating less ROCK activity in edematous intestine compared to CONTROL. In contrast, Y-27632-inhibited activity was not significantly different after pretreatment with okadaic acid, an inhibitor of the catalytic subunit of MLCP (32.87 ± 3.98 vs. 26.16 ± 1.72%, CONTROL vs. EDEMA, p=0.16). Corresponding to the decreased effect of ROCK inhibition on contractile activity in edematous intestine, ROCK activity (measured *in vitro* as MYPT1 phosphorylation inhibited by Y-27632) in tissue lysates from the EDEMA group was significantly less than in the CONTROL group (1113.81 ± 301.92 vs. 505.21 ± 223.81 CPM/ug protein, Figure 4, Panel B). Alterations in ROCK protein levels were not responsible for decreased ROCK activity, as ROCK protein levels were actually increased in the EDEMA group compared to CONTROL (Figure 4D and E). However, rho-GTPase activity (0.23 ± 0.03 vs. 0.15 ± 0.002 ABS) and rhoA protein levels (1.56 ± 0.32 vs. 0.92 ± 0.17) were both significantly decreased in edematous tissue (EDEMA) compared to CONTROL (Figure 4, Panels C, F, and G).

ZIPK activity towards MYPT1 was measured as the difference between total MYTP1 phosphorylation and phosphorylation after treatment with the ZIPK inhibitor, SM1. ZIPK activity towards MYPT1 was significantly decreased in edematous tissue (EDEMA) compared to CONTROL as demonstrated in Figure 5, Panel A (1377 ± 373.38 vs. 312.03 ± 170.56 CPM/ug protein). However, ZIPK protein levels were not significantly different (Figure 5B). To determine if ZIPK and ROCK activities towards MYPT1 were additive, we compared combined ROCK and ZIPK inhibition of MYPT1 phosphorylation to the effects of either inhibitor alone. As shown in Figure 5C, inhibition of MYPT1 phosphorylation in tissue lysates after combined treatment with Y-27632 and SM1 (55.40 ± 5.07%) was not significantly different from treatment with SM1 alone (54.65 ± 3.51%). However, MYPT1 phosphorylation after combined treatment was significantly less than Y-27632 treatment alone (79.39 ± 1.47%).

Discussion

Intestinal interstitial edema develops in a number of different pathological states, including heart failure, ischemic injury, and trauma resuscitation. These pathological states usually involve a combination of edema development and ischemic injury or inflammation. Thus, effects of edema alone on intestinal contractile activity are difficult to discern. We have developed a model of hydrostatic intestinal edema mimicking trauma resuscitation, where intestinal edema develops in a reproducible manner with no cellular inflammation, ie no infiltration of inflammatory cells, and no ischemia. In addition to slowing transit and decreasing intestinal contractile activity, we have shown previously that edema inhibits intestinal smooth muscle MLC phosphorylation (1, 2). We assume that decreased MLC phosphorylation results in decreased contractile activity in edematous tissue. To understand

the mechanism by which intestinal edema alters smooth muscle MLC phosphorylation, we have examined the effects of edema on MLCK and MLCP activities in the present study.

Figure 6 summarizes our theoretical model. We found that intestinal edema modulates MLC phosphorylation predominantly by decreasing the inhibitory phosphorylation of the MLC targeting subunit (MYPT1) of MLC phosphatase via decreased ROCK and ZIPK activities. MYPT1 phosphorylation is inhibitory; therefore, decreased MYPT1 phosphorylation should increase phosphatase activity, resulting in decreased MLC phosphorylation.

As shown in Figure 1, both basal tone and amplitude of contractions are significantly diminished in edematous tissue compared to non-edematous tissue. Intestinal basal tone represents a combination of MLC phosphorylation and/or changes in tissue stiffness. Radhakrishnan has demonstrated previously that intestinal edema decreases tissue stiffness (24). Alterations in stiffness with edema development may be due to changes in cytoskeleton, cell-matrix interactions, or cell-cell contacts, all of which serve to facilitate the transmission of force throughout the tissue (29–31). Edema-induced changes to the actin-cytoskeleton, as well as changes in focal adhesion proteins, have been demonstrated in edematous intestine and may contribute to decreased basal tone (23). However, MLCK inhibition changes basal tone in non-edematous intestine to a much larger extent than in edematous tissue, indicating that changes in the regulation of MLC also contribute to decreased basal tone in edematous smooth muscle (Figure 2).

In contrast to basal tone, contraction amplitude was sensitive to alterations in calcium concentrations in both non-edematous and edematous small intestine. Significantly, calcium sensitivity was lower in edematous intestine compared to non-edematous intestine indicating calcium independent alterations in the regulation of MLC phosphorylation. Both MLCK and MLCP are targets of intracellular signaling cascades that can modulate their activity, resulting in changes in calcium sensitivity (9, 32). Based on our data, it appears that both MLCK and MLCP activities are altered in edematous intestinal smooth muscle tissue. Inhibition of MLCK affects both contraction amplitude and basal tone to a lesser extent in edematous tissue than in control, non-edematous tissue. These data suggest that MLCK activity is down-regulated in edematous intestinal smooth muscle. However, we could not detect significant changes in MLCK activity in tissue lysates using a radiolabeled phosphate incorporation assay. Of note, several other kinases that are present in tissue lysates can also phosphorylate the MLC substrate. Thus, we used a specific MLCK inhibitor and phosphatase inhibitors to assure that we were measuring MLCK mediated phosphorylation. There are several explanations for this discrepancy in the *in vivo* versus *in vitro* data. Decreased MLCK activity in edematous tissue may be due to changes in MLCK localization, which would not be reflected in MLCK activity measurements in tissue lysates. Changes in actin-binding activity of MLCK, which have been reported, may also account for the disparity between the *in vivo* and *in vitro* results and are the focus of further studies (33, 34).

Inhibition of MLCP increased contractile activity in the EDEMA group more than in the CONTROL group suggesting that MLCP activity is higher in edematous intestine (Figure 3A). MYPT1 phosphorylation negatively regulates MLCP activity. In edematous tissue, we found decreased MYPT1 phosphorylation at both phosphorylation sites compared to non-edematous tissue (Figure 3C and D). Furthermore, we found decreased kinase activity towards MYPT1 in tissue lysates isolated from edematous compared to non-edematous intestinal smooth muscle (Figure 3B). Of note, MYPT1 phosphorylation decreased in both CONTROL and EDEMA groups 30 minutes to 2 hours after surgery. The CONTROL group was subjected to sham operation including gut manipulation. Gut manipulation has been shown to cause a local inflammatory response which may be responsible for decreased

MYPT1 phosphorylation at these time points. However, MYPT1 phosphorylation was significantly decreased even 6 hours after surgery in the EDEMA group compared to CONTROL. Although we did not measure MLCP activity directly, these results all support a decrease in MLCP inhibition and, therefore, more MLCP activity, culminating in less MLC phosphorylation.

Numerous kinases, including ROCK and ZIPK, have been shown to phosphorylate MYPT1 (14, 16–20). ROCK and ZIPK activities were both down-regulated in edematous tissue compared to non-edematous tissue (Figure 4 and 5). Although inhibition of ROCK alone inhibited contractile activity in the small intestine in both CONTROL and EDEMA groups, pretreatment with a MLCK inhibitor, unmasked significant differences in ROCK activity. Y-27632 inhibited contractile activity in edematous tissue to a significantly lesser extent than non-edematous tissue, indicating less ROCK activity in edematous tissue. After pretreatment with a MLCP inhibitor, ROCK inhibition still suppressed contractile activity, indicating that ROCK has a direct affect on MLC phosphorylation in both CONTROL and EDEMA groups. However, pretreatment with the MLCP inhibitor abrogated the differences in ROCK activity between CONTROL and EDEMA groups indicating that edema-induced decreases in ROCK activity inhibited motility via MLCP, not MLCK. ROCK activity towards MYPT1 was also significantly decreased in edematous tissue, as shown by the *in vitro* MYPT1 phosphorylation assay (Figure 4B). ROCK has been shown to phosphorylate both MLC and MYPT1 to affect intestinal smooth muscle contractile activity. Our data indicate that while ROCK phosphorylates both MLC and MYPT1 in the rat intestinal smooth muscle, the suppression of constitutive ROCK-mediated inhibition of MLCP is responsible for edema-induced decreases in MLC phosphorylation and motility.

ROCK is activated predominantly via activation of rhoA, a small GTPase. While there is no decrease in total ROCK protein, there are decreases in both rhoA protein and rhoA GTPase activity in edematous tissue that account for decreased ROCK activity (Figures 4C–G). Interestingly, ROCK protein increases in edematous tissue, perhaps as a compensatory mechanism. Increased ROCK expression and decreased rhoA expression were also found in a microarray analysis of edematous intestinal smooth muscle compared to non-edematous tissue (microarray data previously published and deposited at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE13193) suggesting that changes in protein levels of ROCK and rho are due at least in part to changes in gene expression (26).

ZIPK activity is also decreased in intestinal smooth muscle lysates from edematous intestine. There are no changes in ZIPK protein levels; therefore, changes in ZIPK activity are likely due to changes in either post-translational modifications or cellular localization. ZIPK and ROCK mediated phosphorylation of MYPT1 do not appear to be additive, as indicated in Figure 5C. MYPT1 phosphorylation after ZIPK inhibition alone was not significantly different from combined inhibition of ROCK and ZIPK. However, ROCK inhibitor treatment alone inhibited MYPT1 phosphorylation significantly less than either ZIPK inhibitor or combined treatment with both inhibitors. One possible explanation for these data is that ROCK does not directly phosphorylate MYPT1, but rather ROCK phosphorylates and activates ZIPK, which subsequently phosphorylates MYPT1. Published data support the interaction of ROCK with ZIPK. First, Haystead and colleagues have shown that ROCK can phosphorylate and activate ZIPK (35). Second, ROCK inhibition does not prevent ZIPK-induced contraction and MLC phosphorylation, suggesting that ROCK is upstream of ZIPK (36, 37). Thus, our data, supported by published literature, suggest that ROCK and ZIPK interact to regulate phosphorylation of MYPT1.

The mechanism by which ROCK activity, and therefore MYPT1 phosphorylation, is decreased in edematous tissue is unknown. However, we hypothesize that edema

development causes a mechanical stress that triggers a signaling cascade eventually leading to decreased ROCK activity. We have previously shown that edema induces more than a 6-fold increase in mechanical stress in intestinal smooth muscle (38). The mechanism by which edema causes decreased ROCK activity is the subject of future studies in our laboratory.

In summary, Figure 6 shows our proposed model for the mechanism of edema–induced decreases in MLC phosphorylation in intestinal smooth muscle, based on our data and previously published data from other investigators. In this model, we postulate that edema-induced decreases in rhoA activation leads to decreased ROCK activity. Decreases in ROCK activity result in decreased ZIPK activation and eventually decreased MYPT1 phosphorylation and, therefore, more MLCP activity. A combination of increased MLCP and decreased MLCK activities results in decreased MLC phosphorylation and, thus, decreased intestinal contractile activity. While many studies have focused on the regulation of MLC phosphorylation in smooth muscle, few studies have focused on pathological alterations in the regulation of MLC phosphorylation. Understanding the mechanism of edema-induced ileus in trauma patients is critical to preventing and/or treating ileus to improve outcomes in these patients.

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JC performed the research and analyzed the data; CM performed the research; KK performed the research; GL, RH and CC contributed to the design of the study; KU designed the study, performed the research, analyzed the data, and wrote the paper.

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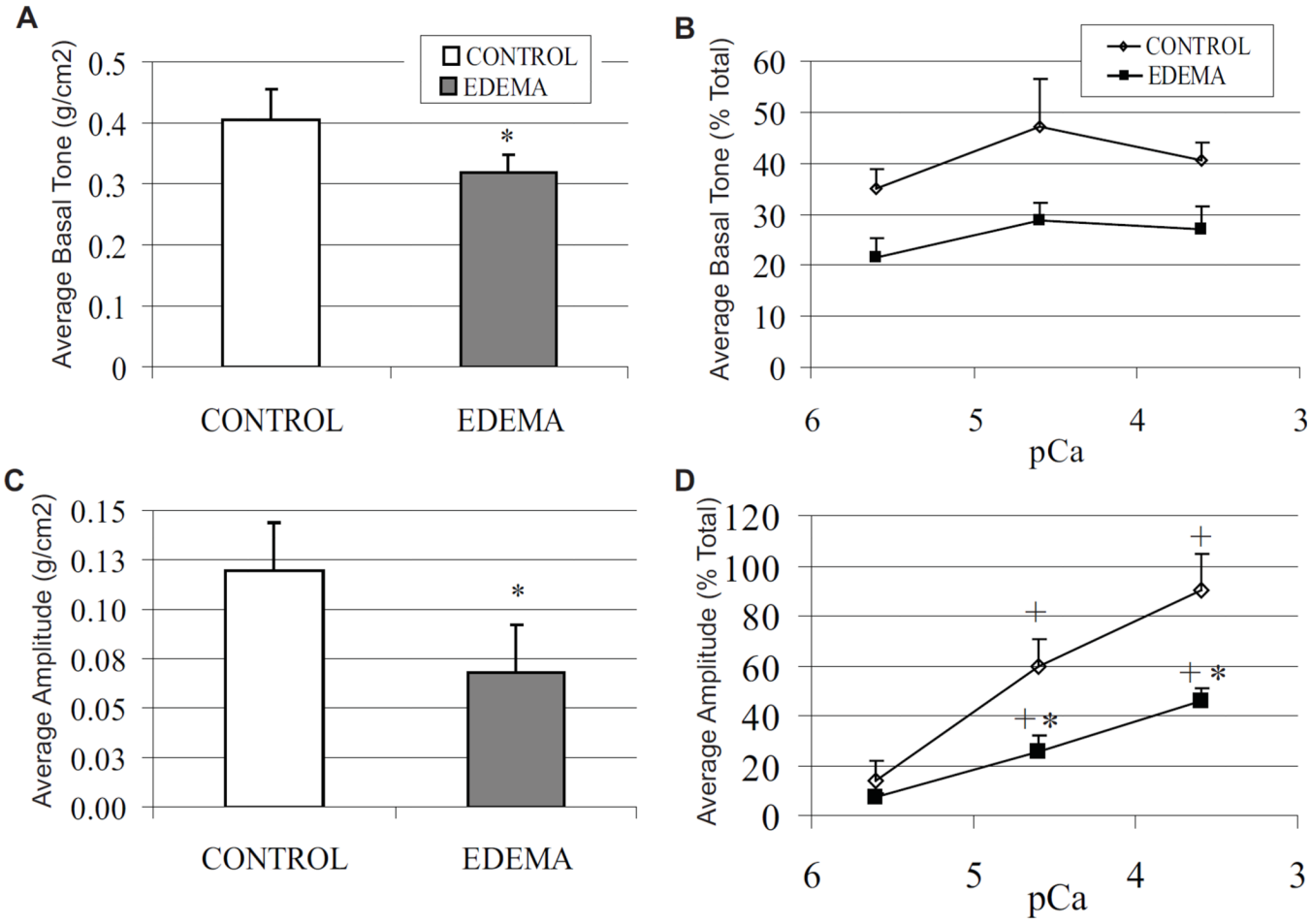

Figure 1.

Contractile activity measured 6 hours after surgery in the distal small intestine in EDEMA and CONTROL groups is shown as follows: Panel A. Average basal tone; Panel B. Average basal tone with changes in calcium concentrations; Panel C. Average cycle amplitude; Panel D. Average cycle amplitude with changes in calcium concentration. (n= 19 per group in Panels A and C; n= 4–5 per group in Panels B and D; pCa= $-\log[Ca^{2+}]$; *, $p<0.05$ vs. CONTROL; +, $p<0.05$ vs. pCa=5.5)

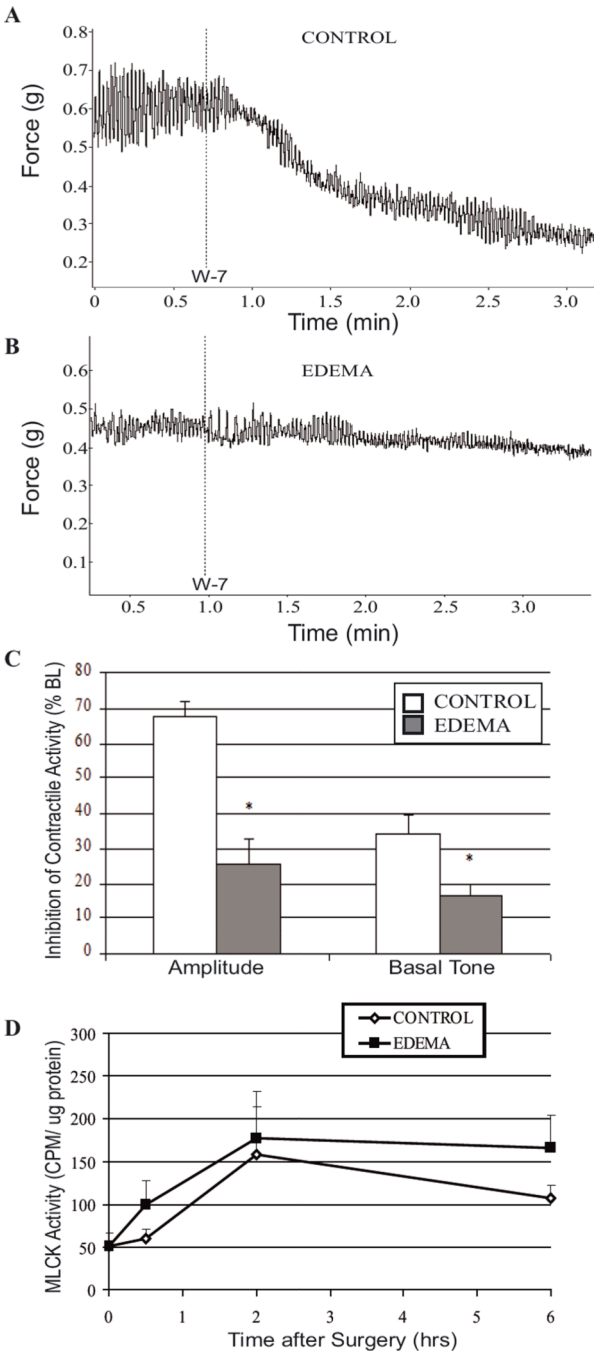

Figure 2.

The effects of myosin light chain kinase activity on contractile activity are shown. Panels A and B. Samples tracing of spontaneous contractions in the distal small intestine 6 hours after surgery in CONTROL and EDEMA groups, respectively, before and after treatment with the myosin light chain kinase inhibitor, W-7 (50 uM). Panel C. Inhibition of contractile amplitude and basal tone after treatment with W-7 in CONTROL and EDEMA groups. Panel D. Changes in myosin light chain kinase activity over time measured in tissue lysates from CONTROL and EDEMA groups. (Panel C, n= 4 per group; Panel D, n= 5 per group in 0–2 hour time points, n= 10 per group at 6 hour time point; *, $p<0.05$ vs. CONTROL)

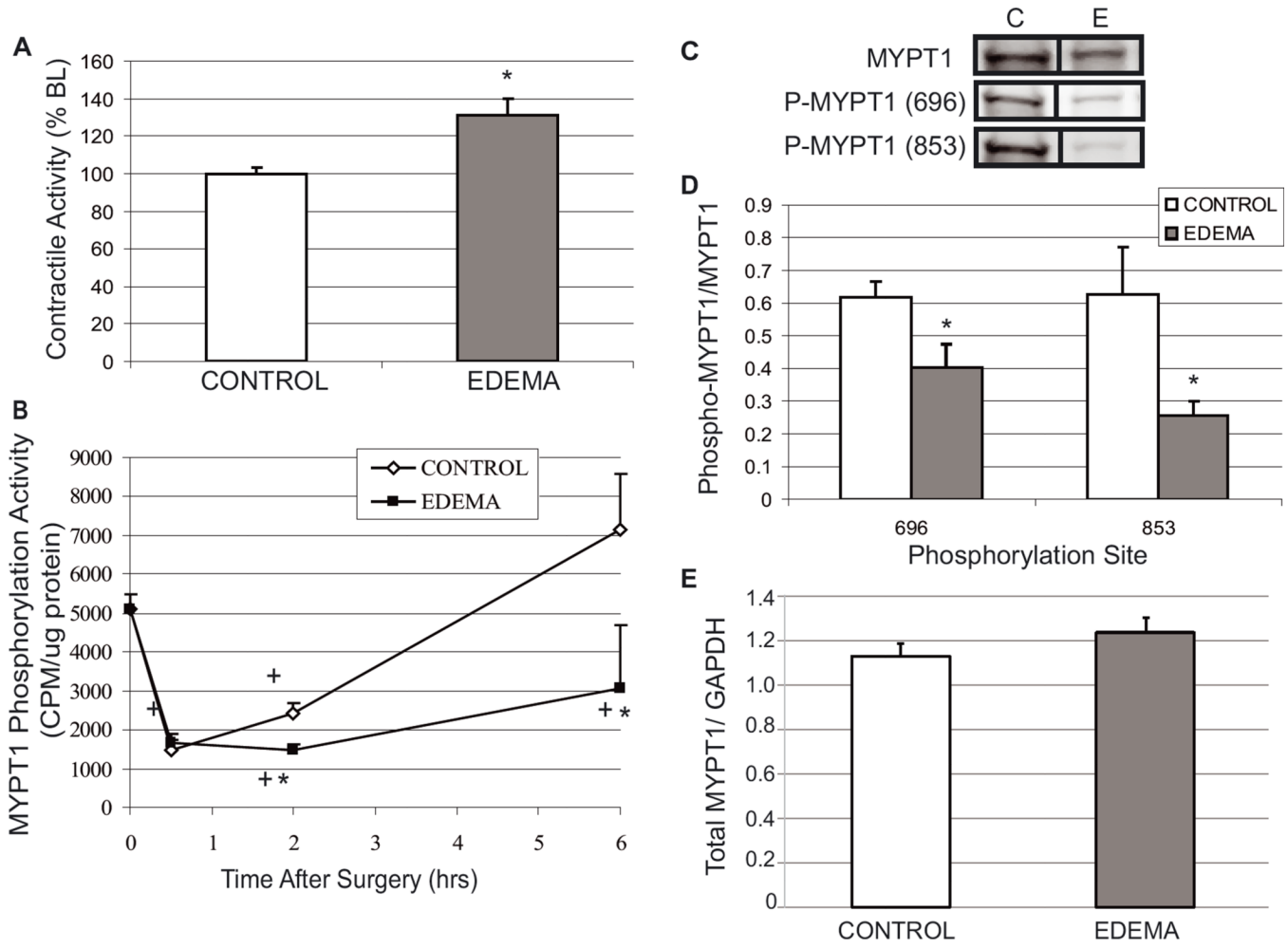

Figure 3.

The effects of edema on MYPT1 phosphorylation are shown. Panel A. Change in contractile activity from baseline after inhibition of the catalytic subunit of myosin light chain phosphatase with okadaic acid to (1 nM). Panel B. Phosphorylation of an MYPT1 peptide substrate measured *in vitro* after treatment with intestinal smooth muscle tissue lysates from CONTROL and EDEMA groups collected at 0, 0.5, 2 and 6 hour time points. Panel C. Representative Western blots showing phosphorylation of MYPT1 at thr696 and Thr853. Panel D. The ratio of phosphorylated to unphosphorylated MYPT1 in intestinal smooth muscle from CONTROL and EDEMA groups 6 hours after surgery. Panel E. The ratio of total MYPT1 (including phosphorylated and unphosphorylated MYPT1) to GAPDH in intestinal smooth muscle from CONTROL and EDEMA groups 6 hours after surgery. (Panel A, n=4 per group; Panel B, n=5–6 per group for 0–2 hour time points, n=9 per group for 6 hour time point; Panel D, n=11 per group for Thr(696), n=9 per group for Thr(853), Panel E, n=4 per group; *, $p<0.05$ vs. CONTROL; +, $p<0.05$ vs. 0 time point)

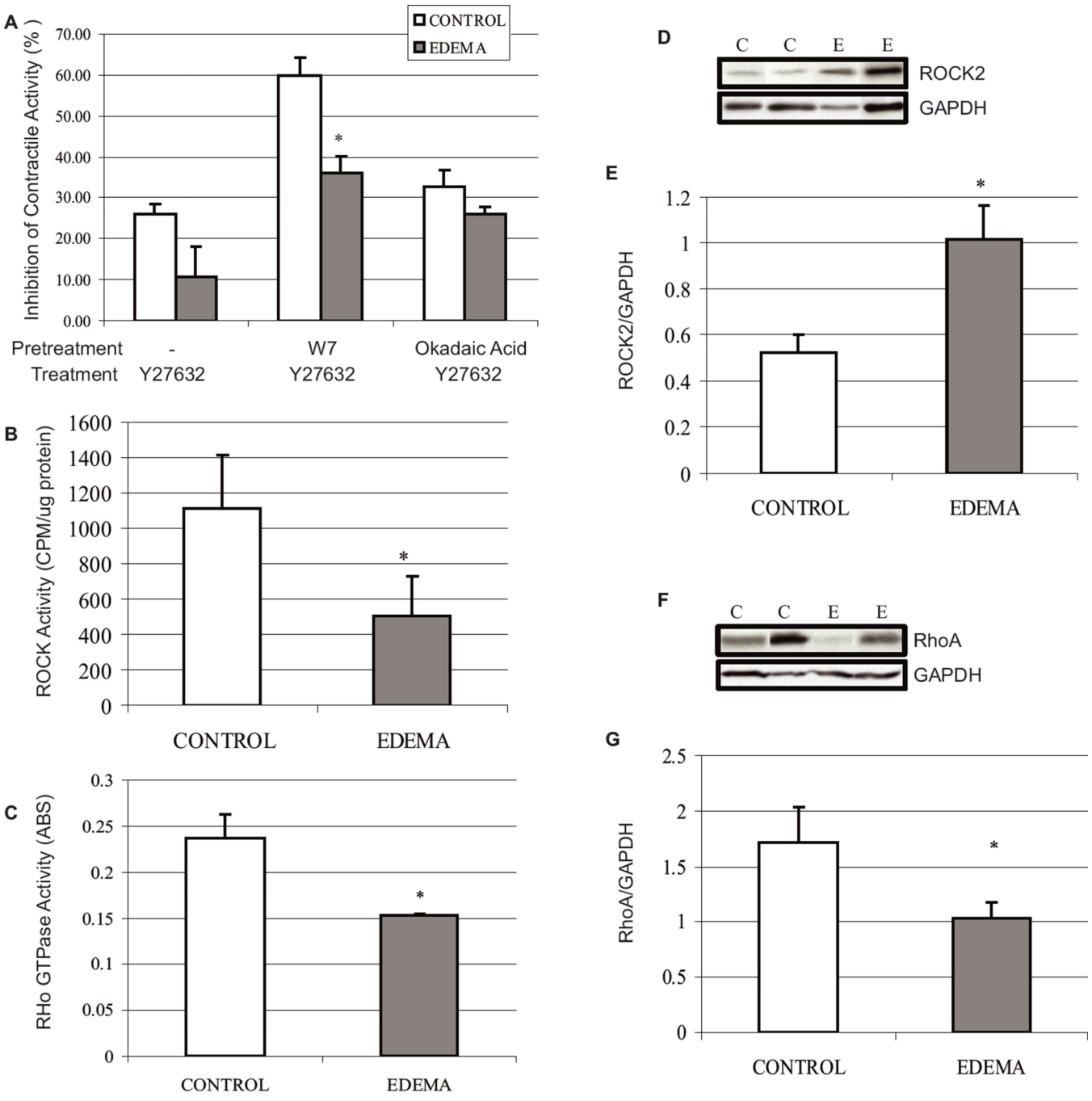

Figure 4.

The role of rho kinase (ROCK) in edema-induced suppression of intestinal contractile activity is demonstrated. Panel A. Inhibition of contractile activity after treatment with rho kinase inhibitor (Y-27632, 10 uM) alone, Y-27632 treatment after pretreatment with a myosin light chain kinase inhibitor (W-7, 50 uM), and Y-27632 treatment after pretreatment with a myosin light chain phosphatase inhibitor (Okadaic acid, 250 nM). Panel B. ROCK activity towards an MYPT1 peptide substrate in intestinal smooth muscle lysates from CONTROL and EDEMA groups collected 6 hours after surgery. Panel C. rhoA GTPase activity in intestinal smooth muscle tissue lysates from CONTROL and EDEMA groups measured as rhoA-GTP binding to an immobilized effector molecule. Panel D.

Representative Western blot of ROCK2 in CONTROL and EDEMA groups 6 hours after surgery. Panel E. Quantification of ROCK2 protein levels normalized to GAPDH in intestinal smooth muscle from CONTROL and EDEMA group at 6 hour time point (GAPDH, glyceraldehydes dehydrogenase). Panel F. Representative Western blot of rhoA in CONTROL and EDEMA groups 6 hours after surgery. Panel G. Quantification of rhoA protein levels normalized to GAPDH in intestinal smooth muscle from CONTROL and EDEMA group at 6 hour time point. (Panel A, n=5 per group for no pretreatment and okadaic acid pretreatment group, n=3 for W-7 pretreatment groups; Panel B, n=10 per group; Panel C, n=5 per group; Panel E, n=9 per group; Panel G, n=9 per group; *, $p<0.05$ vs. CONTROL)

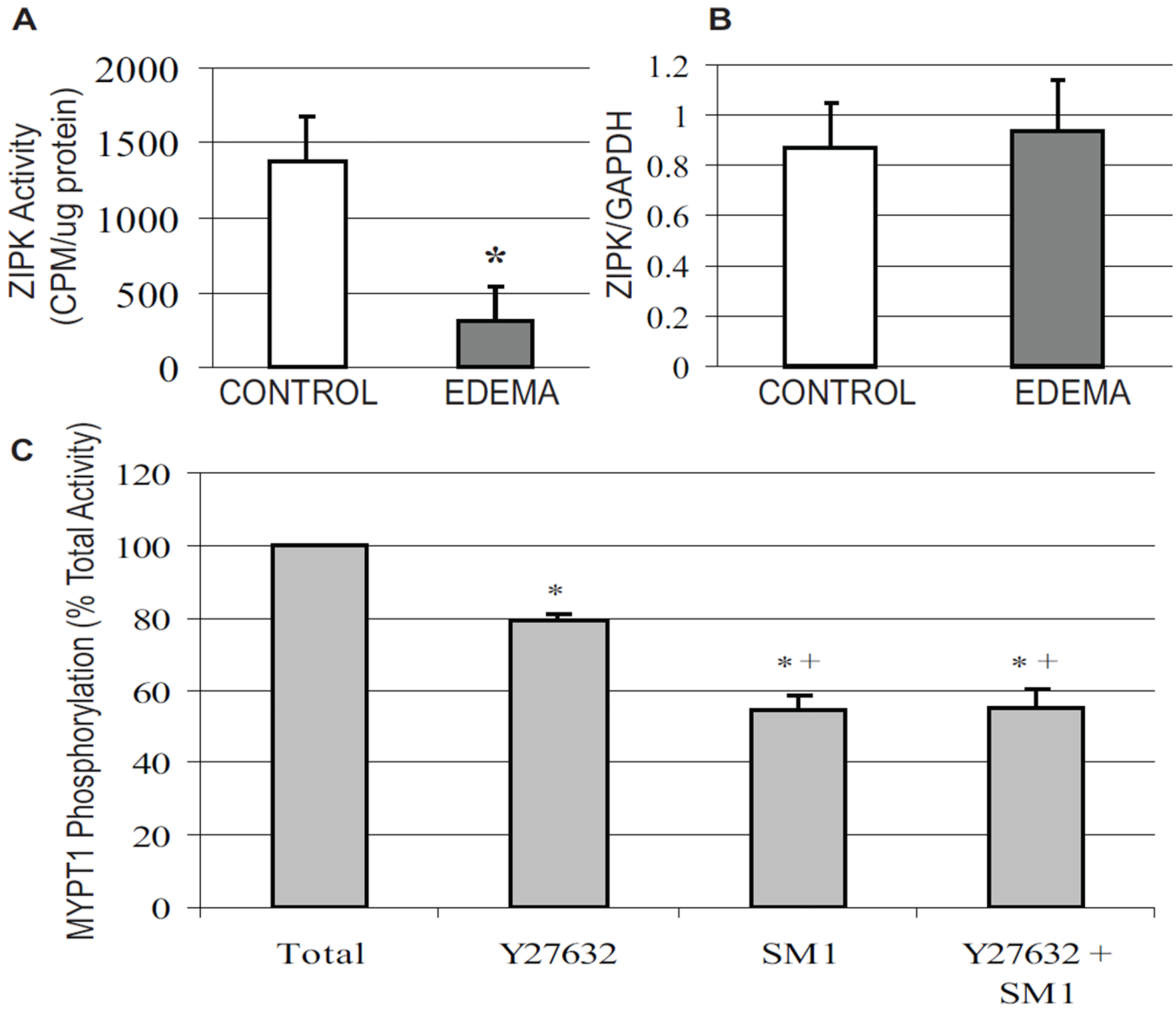

Figure 5.

The role of Zipper-interacting kinase (ZIPK) in edema-induced suppression of intestinal smooth muscle MYPT1 phosphorylation is shown. Panel A. ZIPK activity towards MYPT1 peptide substrate, as measured in the *in vitro* phosphate incorporation assay, in intestinal smooth muscle tissue lysates from CONTROL and EDEMA groups collected 6 hours after surgery. Panel B. ZIPK protein levels normalized to GAPDH in intestinal smooth muscle from CONTROL and EDEMA groups 6 hours after surgery. Panel C. MYPT1 phosphorylation activity in intestinal smooth muscle tissue lysates from CONTROL animals after treatment with Y-27632, SM1 or a combination of both inhibitors. (Panel A: n=6 in CONTROL and n=8 in EDEMA; Panel B: n=6 per group; Panel C: n=6 per group; Panel A: *, $p<0.05$ vs. CONTROL; Panel C: *, $p<0.05$ vs. Total; +, $p<0.05$ vs. Y-27632)

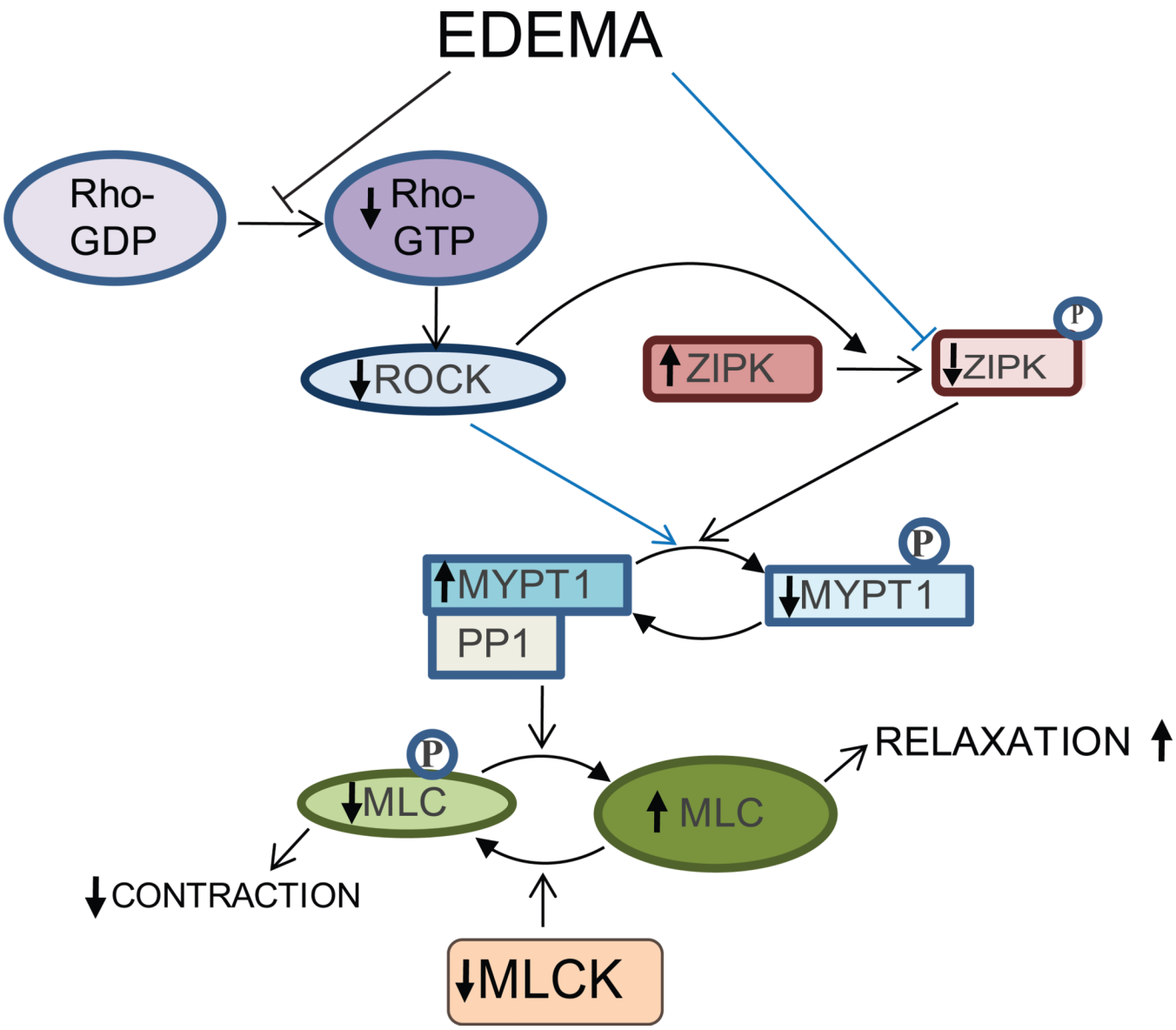

Figure 6.

Theoretical model of decreased intestinal smooth muscle myosin light chain phosphorylation after edema development. Edema induces decreased rhoA activation and thus less rho-associated protein kinase (ROCK) activity. Less ROCK activity results in less ZIPK activity and thus less phosphorylation of the myosin targeting subunit (MYPT1) of myosin light chain phosphatase. Alternatively (shown in blue), edema may independently inhibit both ROCK and ZIPK which then result in decreased MYPT1 phosphorylation. The catalytic subunit (PP1) of myosin light chain phosphatase is normally constitutively inhibited. However, decreases in MYPT1 phosphorylation induced by edema leads to less inhibition of the phosphatase and, therefore, more phosphatase activity. According to the functional studies, myosin light chain kinase (MLCK) activity is decreased. More myosin light chain phosphatase activity and less myosin light chain kinase activity leads to less myosin light chain (MLC) phosphorylation and therefore, less contractile activity.