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Altered Contractile Phenotypes of Intestinal Smooth Muscle in Mice Deficient in Myosin Phosphatase Target Subunit 1

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Abstract

BACKGROUND & AIMS—The regulatory subunit of myosin light chain phosphatase, MYPT1, has been proposed to control smooth muscle contractility by regulating phosphorylation of the Ca²⁺-dependent myosin regulatory light chain. We generated mice with a smooth muscle-specific deletion of MYPT1 to investigate its physiologic role in intestinal smooth muscle contraction.

METHODS—We used the Cre/loxP system to establish *Mypt1*-floxed mice, with the promoter region and exon 1 of *Mypt1* flanked by 2 loxP sites. These mice were crossed with SMA-Cre transgenic mice to generate mice with smooth muscle-specific deletion of MYPT1 (*Mypt1*^{SMKO} mice). The phenotype was assessed by histologic, biochemical, molecular, and physiologic analyses.

RESULTS—Young adult *Mypt1*^{SMKO} mice had normal intestinal motility in vivo, with no histologic abnormalities. On stimulation with KCl or acetylcholine, intestinal smooth muscles isolated from *Mypt1*^{SMKO} mice produced robust and increased sustained force due to increased phosphorylation of the myosin regulatory light chain compared with muscle from control mice. Additional analyses of contractile properties showed reduced rates of force development and relaxation, and decreased shortening velocity, compared with muscle from control mice.

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Supplementary Material

To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at <http://10.1053/j.gastro.2013.02.045>.

Conflicts of interest

The authors disclose no conflicts.

Permeable smooth muscle fibers from *Mypt1^{SMKO}* mice had increased sensitivity and contraction in response to Ca^{2+} .

CONCLUSIONS—MYPT1 is not essential for smooth muscle function in mice but regulates the Ca^{2+} sensitivity of force development and contributes to intestinal phasic contractile phenotype. Altered contractile responses in isolated tissues could be compensated by adaptive physiologic responses in vivo, where gut motility is affected by lower intensities of smooth muscle stimulation for myosin phosphorylation and force development.

Keywords

Mouse Model; Development; Calcium Signaling; Phosphorylation

Smooth muscle contractions are initiated by Ca^{2+} binding to calmodulin, which then activates myosin light chain kinase (MLCK).^{1–4} The activated kinase phosphorylates the regulatory light chain (RLC) of myosin, allowing myosin to cross bridges to bind to actin filaments and thereby develop force or shorten the smooth muscle cell. When the kinase is inactivated by decreases in cytosolic Ca^{2+} concentrations that dissociate calmodulin from MLCK, RLC is dephosphorylated by myosin light chain phosphatase (MLCP), thereby initiating relaxation by returning myosin to an inhibited state. Thus, the ratio of MLCK/MLCP activities determines the extent of RLC phosphorylation and contractile force.

MLCP activity is regulated by distinct biochemical mechanisms. MLCP is a heterotrimer consisting of a catalytic type 1 phosphatase subunit (PP1c), a regulatory subunit (MYPT1), and a 20-kilodalton subunit (M20) with unknown function.⁵ MYPT1 is a central regulator of PP1c activity, acting through a combination of molecular and biochemical mechanisms.^{2,6–8} First, MYPT1 enhances the catalytic activity of PP1c and directs its specificity to pRLC with its binding of both PP1c and myosin filaments. Second, PP1c activity is modulated by phosphorylation of MYPT1 itself. Phosphorylation of MYPT1 Thr696 or Thr850 inhibits PP1c activity by an intramolecular mechanism.⁹ Phosphorylation of both sites is catalyzed by a RhoA-associated kinase (ROCK), while other Ca^{2+} -independent kinases phosphorylate Thr696. Thus, at a fixed cytosolic Ca^{2+} concentration, inhibition of MLCP activity by phosphorylation of Thr696 or Thr850 increases RLC phosphorylation and is described as Ca^{2+} sensitization. 3,5 -Cyclic monophosphate (cGMP)-dependent protein kinase type I (PKG) activated by cGMP phosphorylates Ser695 and blocks phosphorylation of Thr696 and its inhibitory effect on MLCP activity, thereby causing Ca^{2+} desensitization. Third, MYPT1 changes the conformation of PP1c to increase its sensitivity for binding CPI-17 phosphorylated by protein kinase C, thereby inhibiting MLCP activity.⁸ Fourth, MYPT1 acts as a scaffold for other proteins, including Par-4, HSP27, and M-RIP.⁷ Collectively, these published reports emphasize the central importance of MYPT1 in regulating MLCP activity through direct interactions with PP1c, its phosphorylation by different kinases, and its actions as a scaffold for proteins that may affect RLC phosphorylation.^{2,5–7}

Smooth muscles are heterogeneous and can be broadly classified into phasic and tonic subtypes based on their electrophysiological and mechanical properties.^{10–14} The phasic smooth muscles found in digestive and urogenital systems display rhythmic contractile activity related to the generation of action potentials. Tonic smooth muscles found in the large blood vessels, trachea, and sphincter muscles contract continuously and do not normally generate action potentials. In response to continuous stimulation, phasic muscles show rapid force development that then decreases to a lower steady-state level, whereas tonic muscles exhibit a slower rate of developed contractile force that is maintained at a high level.^{12,15,16} These unique contractile properties serve diverse physiologic functions and are

based on many cellular processes, including differences in signaling modules converging on RLC phosphorylation.

Genetic approaches to understand the contributions of MYPT1 to smooth muscle regulation have been limited. The conventional knockout of MYPT1 causes embryonic lethality, which could be due to its essential functions in non-muscle cells as well as smooth muscle cells.¹⁷ Therefore, we knocked out MYPT1 specifically in smooth muscle tissues to determine if it was essential for developmental programming and a prominent component of the RLC phosphorylation signaling module in phasic intestinal smooth muscle. Surprisingly, the smooth muscle-specific knockout of MYPT1 resulted in mice surviving to adulthood with modest phenotypic changes in vivo. However, examination of the contractile properties of MYPT1-deficient intestinal smooth muscle showed (1) a pronounced increase in force maintenance and (2) an increase in Ca^{2+} sensitivity for force development. These results are consistent with biochemical reports that MYPT1 stimulates PP1c activity toward myosin RLC.

Materials and Methods

Generation of Smooth Muscle-Specific *Mypt1*^{SMKO} Mice

The floxed *Mypt1* targeting vector of *loxP* sites was constructed by bacterial artificial chromosome retrieval methods. Chimeric mice were generated by injecting ES cells into C57BL/6 blastocysts, followed by transfer to pseudo-pregnant mice. The chimeric mice were crossed with SMA-Cre (tg) mice to ablate *Mypt1* specifically in smooth muscle.

Additional details on the generation of *Mypt1*^{SMKO} mice, as well as details on genotyping, histologic analysis, gut transit test, myoelectrical activities, immunostaining, Western blotting, reverse-transcription polymerase chain reaction, smooth muscle contractility analysis, live imaging, and data analysis are provided in Supplementary Materials and Methods.

Results

Characterization of *Mypt1*^{SMKO} Mice

To ablate *Mypt1* expression specifically in smooth muscle, we crossed the *Mypt1* floxed mice with SMA-Cre (tg) mice (Figure 1A and B). We used the mice with a single *Mypt1*^{+/flox} allele and *SMA-Cre* (*Mypt1*^{+/flox}; *SMA-Cre*) as controls (CTR) and the mice with 2 floxed *Mypt1* alleles and *SMA-Cre* (*Mypt1*^{flox/flox}; *SMA-Cre*) as knockout mice (*Mypt1*^{SMKO}). Western blots confirmed no detectable MYPT1 protein expression in the muscle of the ileum, bladder, aorta, or mesenteric artery from *Mypt1*^{SMKO} mice (Figure 1C).

Mypt1^{SMKO} mice were viable, had normal body size, and reached adulthood. At necropsy, the whole digestive tract from 4- and 16-week-old *Mypt1*^{SMKO} mice appeared normal, including the small intestine. Histologic examination revealed no abnormalities in the jejunal or ileal sections of 4- and 8-week-old mice (Figure 1D and E). Similar results were found with 16-week-old *Mypt1*^{SMKO} mice. Male *Mypt1*^{SMKO} mice showed a protuberant lower abdomen at 6 months of age, and some of them died from uroschisis at approximately 12 to 15 months of age. Necropsy of 8-month-old *Mypt1*^{SMKO} mice revealed a normal appearance of the esophagus (Supplementary Figure 1D) but significant distention of the alimentary tract and retention of urine in the bladder (Supplementary Figure 1A and E). Histologic examination showed an enlargement of the small intestine with varied thickness of the muscle layer in 8-month-old *Mypt1*^{SMKO} mice (Supplementary Figure 1A–C), in contrast to results obtained with younger animals. The cross-sectional thicknesses of the

duodenum and jejunum were comparable between 8-month-old CTR and Mypt1^{SMKO} mice, but the thickness of the MYPT1-deficient ileal muscle layer was significantly greater compared with CTR. Additionally, an enhanced infiltration of inflammatory cells was observed in the mucosal lamina propria and the subglandular area of the intestine of 8-month-old Mypt1^{SMKO} mice (Supplementary Figure 1C), implying that immunologic responses might be additional compensatory effects of MYPT1 deletion. To avoid the potential influence of inflammatory processes on intestinal smooth muscle properties, we used tissues from 16-week-old mice or younger for ex vivo studies described in the following text.

Female Mypt1^{SMKO} mice were fertile and had normal-sized bladders up to 8 months of age. In contrast to CTR mice, male and female Mypt1^{SMKO} mice also had higher systolic blood pressure (140 ± 3 mmHg vs 115 ± 3 mmHg; $n = 10$; $P < .01$).

Changes in bowel motility in vivo were assessed by intestinal propulsion by a charcoal transit test. The distance traveled by delivered test material showed no significant difference between 16-week-old CTR and Mypt1^{SMKO} mice (percentage of the total length of the small intestine: CTR, $59.0\% \pm 4.0\%$; Mypt1^{SMKO}, $49.9\% \pm 13.9\%$; $n = 5$, $P > .05$). The normal bowel motility of Mypt1^{SMKO} mice was also reflected by normal eating and defecation functions (Figure 2A and B).

Progressive Impairment of Rhythmic Contraction, Networks of Interstitial Cells of Cajal, and Myoelectrical Activity in the Absence of MYPT1

We measured rhythmic contractions of the ileum at different postnatal ages. At 2 weeks after birth, both the MYPT1-deficient and CTR intestinal tracts displayed similar contractions with comparable frequencies, waveforms, and tension amplitudes (Figure 2C). However, peristalsis of the knockout mice was gradually impaired. At 4 weeks of age, the ileum from the knockout mice showed reduced and variable waveform and tension amplitudes (Figure 2D). The average tension of spontaneous contractions in the adult Mypt1^{SMKO} mice (16 weeks of age) decreased significantly from 0.22 ± 0.05 g in CTR to 0.11 ± 0.02 g ($n = 4$; $P < .01$; Figure 2E). Despite this finding, the MYPT1-deficient ileum appeared to have a similar frequency of contractions compared with CTR ileum (Figure 2E). To test whether the MYPT1-deficient ileum responded to mechanical stimuli, 50 μ L of HEPES-Tyrod buffer was infused into the lumen of ligated ileal tissue segments to increase hydrostatic pressure. The waveform and tension amplitudes of the spontaneous contractions were partially restored (Figure 2E).

To determine whether impaired peristalsis was caused by dysfunction of pacemaker cells, we stained the interstitial cells of Cajal (ICC) in the myenteric plexus region with anti-c-Kit antibody. At 2 weeks of age, intact but low-density ICC networks were observed in both CTR and MYPT1-deficient ileum in parallel with small amplitude and rhythmic contractions (Figure 2F). However, ICC networks were gradually disrupted in the MYPT1-deficient ileum with aging. Only low-density ICC staining was observed at 4 weeks of age, and staining became weak at 16 weeks of age in the MYPT1-deficient ileum (Figure 2F). We then monitored the electrophysiological activities of intestinal smooth muscle. In CTR mice (16 weeks old), the small intestine had slow waves of normal rhythm with spike potentials. In contrast, the intestine from knockout mice displayed arrhythmic activities, and typical spike potentials were not observed (Supplementary Figure 2). These changes in ICC networks preceded development of inflammation in 8-month-old animals.

Developed Forces of Jejunum Smooth Muscle From Mypt1^{SMKO} Mice Are Enhanced

To determine the contractile properties of MYPT1-deficient smooth muscle, we measured the force developed by isolated jejunal muscle strips from adult mice in response to KCl or the muscarinic agonist acetylcholine (ACh) (Figure 3A–D). The maximal contractile forces developed with both KCl and ACh treatments were comparable between tissues from CTR and Mypt1^{SMKO} mice. Strikingly, the jejunal segments from Mypt1^{SMKO} mice developed a significantly increased sustained phase in response to KCl ($25.7\% \pm 4.1\%$ vs $73.4\% \pm 6.6\%$ of the peak value at 1 minute; $n = 5$; $P < .01$) (Figure 3A and C). The MYPT1-deficient jejunum also showed significantly enhanced sustained contractions ($42.4\% \pm 2.9\%$ vs $78.5\% \pm 9.0\%$ of the peak value at 1 minute; $n = 5$; $P < .01$) in response to ACh (Figure 3B and D). The ileal muscle of the adult Mypt1^{SMKO} mice showed similar responses to KCl and ACh (Supplementary Figure 3A and B). Interestingly, bladder, portal vein, and small arteries from the mesentery also displayed enhanced sustained contractions in response to KCl (Supplementary Figure 4A–C). Similar to responses from phasic smooth muscles from adult mice, the MYPT1-deficient ileum from both 2- and 4-week-old mice displayed enhanced sustained contractions in response to either KCl or ACh (Supplementary Figure 4D and E).

We also measured spontaneous tone development in ileal tissues from Mypt1^{SMKO} mice. After applying an initial stretch of 0.5 g, both control and MYPT1-deficient ileal strips did not develop spontaneous tone, while the internal anal sphincter, a typical tonic smooth muscle, showed clear spontaneous tone formation (data not shown).

Contractile Properties of Permeable MYPT1-Deficient Smooth Muscles Are Modified

The contractile properties of α -toxin permeabilized muscle strips from MYPT1-deficient mice were measured (Figure 3E and F). Following activation by increasing the Ca^{2+} concentration to pCa 4.5, permeable MYPT1-deficient muscle strips showed significantly reduced rates of force development compared with tissues from CTR mice. The time for a half-maximal increase in force ($t_{1/2}$) for Mypt1^{SMKO} tissues (40 ± 5 seconds) was slower than for tissues from CTR mice (10 ± 5 seconds; $n = 3$; $P < .01$). Similarly, the times to peak force after stimulation by KCl or ACh of both intact jejunum and ileum muscle strips from CTR mice were 2- to 2.5-fold faster than those from MYPT1-deficient strips (Supplementary Figure 3C). As an index of shortening velocity, the rate of ileal force regeneration was measured by a step-shortening method at the maximal force responses to ACh. The time to reach $t_{1/2}$ for MYPT1-deficient muscle strips was approximately 4-fold longer than that for force regeneration of CTR muscle strips ($n = 5$; $P < .01$) (Figure 4A and B). Thus, MYPT1-deficient muscles had an apparent reduced shortening velocity during the initial phase of contraction.

To examine the relaxation rate in permeable fibers, the pCa 4.5 solution was replaced after maximal tissue force was reached with a pCa 9.0 solution also containing $10 \mu\text{mol/L}$ ML-7 and $50 \mu\text{mol/L}$ 8-Br-cGMP. The rate of relaxation of CTR strips was significantly faster than that of the strips from Mypt1^{SMKO} mice ($t_{1/2}$ for Mypt1^{SMKO}, 254 ± 38 seconds; CTR, 77 ± 12 seconds; $n = 3$; $P < .01$).

The Absence of MYPT1 Changed Contractile Properties in Isolated Smooth Muscle Cells

We extended the investigations of intact and permeable intestinal smooth muscle strips to isolated smooth muscle cells from the ileum. Live cell imaging showed no differences in cell lengths under resting conditions (CTR, $122.9 \pm 6.2 \mu\text{m}$; Mypt1^{SMKO}, $133.7 \pm 6.3 \mu\text{m}$; $n = 45$ for each group). There was a greater extent of shortening of ileal smooth muscle cells from Mypt1^{SMKO} mice in response to ACh compared with cells from CTR mice (Figure 5A and B). The time required for half-maximal responses in cell length, however, was increased in

Mypt1^{SMKO} smooth muscle cells (CTR, 260 ± 42 seconds; Mypt1^{SMKO}, 383 ± 55 seconds; $n = 15$; $P < .05$) (Figure 5C).

Expression of Proteins That Could Modify Rates of Contraction and Relaxation Are Not Changed in Smooth Muscle Tissues From Mypt1^{SMKO} Mice

Recent studies implicated myosin heavy chain (MHC) and the 17-kilodalton essential myosin light chain (LC₁₇) isoforms CaP and CaD in the regulation of actin-activated myosin Mg²⁺-adenosine triphosphatase activity and shortening velocity.^{11,18,19} We tested whether there were alterations in the expression of these proteins with the MYPT1 deletion. Sodium dodecyl sulfate/polyacrylamide gel electrophoresis analyses showed that MYPT1-deficient ileal smooth muscle contained similar amounts of MHC, tropomyosin, actin, CaD, CaP, and RLC compared with CTR muscles (Figure 4C). Because the ratios of expression of C-terminal SMB to SMA and N-terminal SM2 to SM1 potentially affect shortening velocity,¹¹ we examined the relative expression of MHC isoforms by reverse-transcription polymerase chain reaction. The ratios of both SMB/SMA and SM2/SM1 were not changed after MYPT1 deletion (Figure 4D).

Deletion of MYPT1 Increased RLC Phosphorylation During the Sustained Phase

The extent of RLC phosphorylation was measured in MYPT1-deficient muscle in response to KCl. The amount of RLC Ser19 phosphorylation (pRLC) in resting ileum muscle for smooth muscle strips from CTR and MYPT1-deficient mice was low and similar (Figure 6A and B). The pRLC in CTR ileum muscle increased to $34\% \pm 4\%$ with KCl treatment for 10 seconds (initial phase) and then declined during the sustained phase ($18\% \pm 6\%$ by 30 seconds and $6\% \pm 2\%$ by 60 seconds; Figure 6A and B). RLC phosphorylation was slower in Mypt1^{SMKO} ileal smooth muscle with a lower value than that observed in CTR muscle at 10 seconds after treatment with KCl ($23\% \pm 4\%$; $P > .05$). The maximal extent of RLC phosphorylation at 30 seconds was similar to the maximal extent obtained with CTR muscles at 10 seconds. However, RLC phosphorylation in MYPT1-deficient smooth muscles remained elevated during the sustained phase of contraction at 30 seconds ($41\% \pm 6\%$) and 60 seconds ($32\% \pm 5\%$) in contrast to the results obtained with tissues from CTR mice.

MYPT1-Deficient Smooth Muscle Displayed Increased Ca²⁺ Sensitivity

Steady-state force development was measured in α -toxin permeable smooth muscle with a cumulative increase in Ca²⁺ concentration, and the pCa median effective concentration was calculated to evaluate Ca²⁺ dependence of force development (Figure 6C). The pCa median effective concentration of the MYPT1-deleted smooth muscle was 6.6 ± 0.1 , which was significantly greater than that of CTR muscle (6.1 ± 0.1) ($n = 3$; $P < .05$), showing a shift of 0.5 pCa units. The pRLC response was also greater in permeable MYPT1-deficient fibers at pCa 6.5 and pCa 4.5 (Figure 6C).

MYPT1 as a Platform for the Signals That Regulate Ca²⁺ Sensitivity

Signaling cascades converging at MYPT1 were characterized in MYPT1-deficient smooth muscle. In both CTR and Mypt1^{SMKO} ileal segments, pretreatment with 10 μ Mol/L Y27632, a ROCK inhibitor, had no effect on the initial robust ACh-induced contraction (Figure 7B). In CTR ileal muscle, pretreatment with Y27632 led to significantly reduced sustained contraction ($11.6\% \pm 0.9\%$ of the initial peak force; $n = 3$; $P < .01$; 60 seconds after ACh stimulation). However, in Mypt1^{SMKO} ileal muscle, Y27632 did not reduce the enhanced sustained contraction (Figure 7A and B). After pretreatment with 100 μ Mol/L 8-Br-cGMP (an analogue of cGMP), the initial robust contraction of CTR ileal muscle in response to ACh was reduced to $27.4\% \pm 19.9\%$ relative to that without 8-Br-cGMP, while the sustained

contraction was reduced to $12.7\% \pm 3.3\%$ of the peak force ($n = 3$; Figure 7C). In Mypt1^{SMKO} ileal muscle, 8-Br-cGMP partially inhibited the initial peak ($74.7\% \pm 12.8\%$ of the peak force without 8-Br-cGMP) and partially relaxed the sustained contraction ($55.0\% \pm 1.2\%$ of the peak force without 8-Br-cGMP) ($n = 3$; Figure 7C). To rule out interference by the compensatory expression of signaling modules in RhoA/ROCK and cGMP/PKGI signaling pathways, we examined expression of proteins in these signaling modules. Western blots showed no difference in the expression levels of proteins, including PKGI, Rho-associated kinase II, and protein kinase C. MYPT1-deficient and CTR muscle also showed similar amounts of expression of CPI-17 and phosphorylated CPI-17 on treatment with ACh. Expression of MLCK and PP1c was also comparable between CTR and MYPT1-deficient muscle (Figure 7D–F).

Discussion

MYPT1 is identified as a centrally important protein in the smooth muscle myosin phosphorylation module.^{2,7,8,20} MYPT1 binds PP1c and myosin biochemically to enhance phosphatase activity toward pRLC. Phosphorylation of MYPT1 by Ca²⁺-independent protein kinases at Thr696 and Thr850 inhibits this activity intramolecularly.⁹ MYPT1 is phosphorylated under resting conditions, which increases in response to a variety of agents that initiate smooth muscle contraction to enhance RLC phosphorylation in a Ca²⁺-independent manner. Thus, it was surprising that the knockout of MYPT1 in smooth muscle cells had no significant effect on mouse development and birth in contrast to the conventional MYPT1 knockout, which is embryonically lethal.¹⁷ However, expression of MYPT1 is not restricted to smooth muscle cells, and it plays important roles in multiple and distinct cellular processes, including cell migration, cell division, and therefore morphogenesis, by not only regulating myosin function but also by interactions with other types of proteins.⁵ The full development and maturation of mice to adulthood with smooth muscle-specific MYPT1 knockout and the presence of PP1c indicate a less essential role of MYPT1 in smooth muscles.

Considering the defined biochemical and cellular functions for MYPT1 in smooth muscle cells,^{2,7,20,21} the phenotypic changes associated with deficiency of MYPT1 in adult mice were surprisingly modest. The MYPT1-deficient intestinal smooth muscle exhibited enhanced contractile force during the sustained phase of contraction, reduced rates of force development and relaxation, and slower shortening velocity. Collectively, these contractile properties are characteristic of a tonic smooth muscle, and thus MYPT1 appears to contribute significantly to phasic/tonic contractile phenotype. This proposal is supported by MLCP activity, expression level, and phosphorylation status of MYPT1 in adult and developing tonic smooth muscle.^{22–25} However, we note that deletion of MYPT1 does not completely alter the phasic to tonic phenotype, because cellular differences involve many different processes that are not regulated by a single gene.

A slower shortening velocity as a significant property of tonic smooth muscle has been implicated as due to expression of specific myosin II isoforms and thin filament-associated proteins.^{11,18,19} However, the MYPT1-deficient smooth muscle showed a comparable expression profile of LC1_{7a/b}, SMA/B, and SM1/2 relative to CTR muscle. The expression of CaD and CaP was also not changed after MYPT1 deficiency. The reduced rate of force development in MYPT1-deficient muscle is probably due to the slower rate of RLC phosphorylation, implying a reduction in MLCK activity. MLCK activity could be reduced by partial attenuation of increases in cytosolic Ca²⁺ concentration by unidentified mechanisms, which may contribute to responses in intact, but not permeable, fibers. Reduced MLCK activity could also occur by enhanced phosphorylation of MLCK at the site that desensitizes the kinase to activation by Ca²⁺/calmodulin.^{1,26} Evidence has been

presented that smooth muscle PP1c may dephosphorylate MLCK in addition to RLC.²⁷ Thus, the reduction of phosphatase activity in MYPT1-deficient smooth muscle would potentiate MLCK phosphorylation occurring during contraction and reduce its activation. Another possible cause for the reduction of RLC phosphorylation during the initial contractile phase may be the lack of inhibition of PP1c by the rapidly phosphorylated CPI-17 in MYPT1-deficient muscles, where MYPT1 is not present to facilitate binding of phosphorylated CPI-17 to PP1c.^{8,26,28,29}

During the sustained contraction in phasic smooth muscle, cytosolic Ca^{2+} concentration, MLCK activation, and RLC phosphorylation and force are reduced compared with the initial maximal responses.^{2,20,26,29} RLC phosphorylation and force are maintained under these steady-state conditions by a constant ratio of MLCK to MLCP activities. Under these conditions, the enhanced sustained RLC phosphorylation and contractile force in MYPT1-deficient smooth muscle are predicted from the lack of MYPT1, which normally stimulates PP1c activity.^{2,8} Additionally, relaxation occurs when cytosolic Ca^{2+} concentrations are reduced to inactivate MLCK with RLC dephosphorylated by MLCP. The rate of relaxation is reduced in MYPT1-deficient muscles, probably because of the reduced rate of RLC dephosphorylation. The amount of PP1c expressed does not change in intestinal smooth muscles from Mypt1^{SMKO} mice, but its specific activity would be reduced, not absent, in these muscles.²¹ Although PP1c itself can dephosphorylate RLC on myosin, it is also possible that PP1c binds to another member of the MYPT1 family in MYPT1-deficient smooth muscle to direct its activity toward myosin.⁷ These potential cellular mechanisms need to be explored.

The enhanced sustained force following the initial phase of contraction is normally due to Ca^{2+} -sensitization mechanisms involving phosphorylation of MYPT1 and CPI-17.^{2,6,12} The intact MYPT1-deficient ileum muscle displayed a significantly enhanced sustained force associated with an increased extent of RLC phosphorylation. In permeable MYPT1-deficient smooth muscle strips, there was a leftward shift of the pCa-force relationship, showing increased sensitivity to the effects of Ca^{2+} to initiate the force response. This apparent increase in Ca^{2+} sensitivity may result from the loss of the enhanced phosphatase activity with PP1c catalytic subunit binding to MYPT1.^{2,21} Thus, the response mimics the inhibition of MLCP activity due to MYPT1 and CPI-17 phosphorylation.

Major cellular mechanisms for inhibition of MLCP activity are mediated by GPCR/RhoA/ROCK/protein kinase C signaling modules, which result in MYPT1 and CPI-17 phosphorylation.^{2,7,8,20,21} The addition of a ROCK inhibitor did not affect the sustained contraction of the MYPT1-deficient smooth muscle, indicating that MYPT1 is required for ROCK-mediated Ca^{2+} sensitization and that MYPT1 serves as an important downstream target of ROCK in intestinal smooth muscle. Published reports indicate that ROCK phosphorylation of CPI-17 is minimal.²⁵ In addition, MYPT1 enhances the ability of phosphorylated CPI-17 to inhibit PP1c activity,³⁰ so the loss of MYPT1 reduces the ability of phosphorylated CPI-17 to inhibit the PP1c present in the knockout tissues, even if there were some phosphorylation by ROCK. Thus, Y27632 is expected to inhibit ROCK but not inhibit the sustained contraction of MYPT1-deficient smooth muscle.

The nitric oxide/cGMP/PKGI signaling cascade mediates smooth muscle relaxation through interactions with multiple cellular targets.^{12,20} PKGI may act on cell targets that decrease cytosolic Ca^{2+} concentrations by multiple mechanisms involving the inositol 1,4,5-trisphosphate receptor-mediated Ca^{2+} release and the $\text{BK}_{\text{Ca}2+}$ channel affecting membrane potential.³¹ cGMP also activates MLCP activity in smooth muscles, including the ileum, where PKGI phosphorylation of MYPT1 Ser695 reduces the inhibitory phosphorylation of Thr696.⁹ The loss of MYPT1 partially blocked the effect of 8-Br-cGMP to reduce force,

suggesting that MYPT1, as a target of PKGI, contributes partially but significantly to relaxation mediated by the nitric oxide/cGMP/PKGI signaling module.

A surprising observation with the Mypt1^{SMKO} mice was the slow diminution of the ICC network. ICC networks act as pacemakers of the intestinal smooth muscle and trigger electrical slow waves and peristalsis and thus are believed to be essential for gastrointestinal motility.¹⁴ In this study, we found that the ICC network was reduced with MYPT1 deletion, resulting in abnormal myoelectrical activity and impaired peristalsis of intestinal smooth muscle. The reason for the reduction is not clear and deserves additional study. Young knockout mice displayed undetectable MYPT1 protein in intestinal segments showing a comparable intact ICC network as well as normal histology. The subsequent reduction in the ICC network might be due to stresses caused by the loss of MYPT1.^{32,33}

Interestingly, bowel motility in vivo showed no significant difference between control and Mypt1^{SMKO} mice as assessed by the charcoal transit test or eating and defecation functions. Unlike the responses to high K⁺ and ACh in vitro, normal gut motility operates at much lower intensities of smooth muscle stimulation for myosin phosphorylation and force development. Because RLC phosphorylation and force reflect the balance between MLCK and myosin light chain phosphatase activities, it is likely that there is lower MLCK activity to balance the diminution of PP1C activity in intestinal smooth muscle to approximate normal motility function in vivo. The impairment of the peristalsis observed herein was improved with pressure, suggesting that induction of a mechanical response by food may partially restore motility. However, there are likely adaptive responses, including neurologic and unknown cellular signaling changes. Additional investigations may provide insights into these potential mechanisms and shed light on novel regulatory processes affecting intestinal motility. Importantly, the relevance of assessing signaling mechanisms in isolated tissues with elicitation of maximal responses to various stimuli may not provide sufficient perspectives on how identified pathways operate physiologically in different smooth muscle tissues.

In summary, the loss of MYPT1 had modest effects on intestinal smooth muscle function in vivo. Changes in contractile properties of isolated intestinal smooth muscle were generally consistent with the role of MYPT1 as an enhancer of PP1c phosphatase activity toward pRLC and were similar to properties of tonic smooth muscles. The results provide loss-of-function evidence that MYPT1 acts as a transduction integrator for the regulation of Ca²⁺ sensitivity by distinct signaling modules for intestinal smooth muscle contraction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations used in this paper

ACh acetylcholine

cGMP	guanosine 3',5'-cyclic monophosphate
CTR	control
ICC	interstitial cells of Cajal
LC₁₇	17-kilodalton myosin light chain
MHC	myosin heavy chain
MLCK	myosin light chain kinase
MLCP	myosin light chain phosphatase
MYPT1	myosin phosphatase target subunit 1
PKGI	3',5'-cyclic monophosphate-dependent protein kinase type I
pRLC	phosphorylated myosin regulatory light chain
RLC	myosin regulatory light chain
ROCK	Rho-associated kinase; $t_{1/2}$, The time for half-maximal increase in force

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Figure 1.

Ablation of the *Mypt1* gene in smooth muscle resulted in the loss of MYPT1 expression. (A) Schematic representation of the *Mypt1* smooth muscle–specific knockout strategy. (B) DNA isolated from chimeric (+/flox) and wild-type (+/+) mice tails was digested with *NcoI* (N) and analyzed by Southern blot. The wild-type and floxed alleles yield 12.7-kilobase (Kb) and 9.5-Kb fragments, respectively. (C) Western blot analysis of MYPT1 protein expression in the ileum, bladder, aorta, and small arteries of the mesentery from CTR and *Mypt1*^{SMKO} (KO) mice (D and E). H&E staining of transverse sections showed normal jejunum and ileum in the *Mypt1*^{SMKO} mice at (D) 4 weeks and (E) 16 weeks of age. Scale bars = 100 μm (short) and 50 μm (long).

Figure 2.

Normal food movement but progressive impairment of rhythmic contraction, ICC networks, and myoelectrical activity in Mypt1^{SMKO} intestine. The 16-week-old mice were singly housed in cages with a stainless steel grid on the bottom. (A) The amount of food consumed and (B) fecal boli (B) were weighed daily for 5 consecutive days and showed no significant differences between the CTR and Mypt1^{SMKO} mice (n = 11, analysis of variance). (C–E) Rhythmic contractions of ileum from CTR and Mypt1^{SMKO} mice at the age of 2, 4, and 16 weeks. (E) Mechanical stimuli partially restored peristalsis of ileum segments from 16-week-old Mypt1^{SMKO} mice. (F) ICC networks from CTR and Mypt1^{SMKO} mice at the age of 2, 4, and 16 weeks. Scale bars = 50 μ m.

Figure 3.

MYPT-deficient intestinal smooth muscles showed altered contractile properties. (*A* and *B*) Representative tracings of jejunum from 16-week-old CTR and Mypt1^{SMKO} mice elicited by 87 mmol/L KCl or 100 μmol/L ACh. (*C* and *D*) Quantification of the sustained force responses to treatment with KCl or ACh. The values represent 5 independent experiments and are expressed as percent of the peak force. (*E*) Representative force tracings of permeable ileal strips after Ca²⁺-mediated force development and relaxation. (*F*) Quantification of the $t_{1/2}$ values of force development and relaxation, respectively. $n = 3$, ** $P < .01$.

Figure 4.

Although the rate of force redevelopment was affected in Mypt1^{SMKO} ileum, contractile protein expression was not altered. (A) Representative force tracings of CTR and KO ileum at their respective maximal force responses to ACh after a quick reduction of 5% of the resting length. (B) Quantification of $t_{1/2}$ after quick releases. $n = 5$, $**P < .01$. (C) Sodium dodecyl sulfate/polyacrylamide gel electrophoresis with Coomassie blue staining of ileal protein samples from 16-week-old CTR and Mypt1^{SMKO} mice. (D) Reverse-transcription polymerase chain reaction analysis of expressed MHC or LC17 isoforms between CTR and KO ileums at indicated ages. The aorta from CTR mice was used as a reference for SMA and LC17b bands.

Figure 5.

Shortening properties of isolated smooth muscle cells. (A) Smooth muscle cells were isolated from the ileal smooth muscle layer from 12-week-old mice and stimulated with 100 $\mu\text{mol/L}$ ACh. Images were captured by a live cell imaging system at indicated time points. Scale bar = 50 μm . (B) Time-dependent changes in cell length of isolated smooth muscle cells. (C) Quantification of the time to reach half-maximal decrease in cell length ($t_{1/2}$). $n = 15$, * $P < .05$, ** $P < .01$.

Figure 6.

Altered RLC phosphorylation and increased sensitivity to Ca^{2+} for contraction of Mypt1^{SMKO} ileum. (A) After stimulation with 87 mmol/L KCl, ileal muscles from 16-week-old mice were quickly frozen at the indicated times. (B) pRLC level was expressed as the percentage of the total RLC. (n = 4, **P* < .05 compared with CTR values at the indicated times). (C) To obtain the pCa-force relationships, ileal muscle strips were made permeable with -toxin and subsequently activated with pCa 8.0 to pCa 4.5 solutions. pRLC was measured at indicated pCa solutions (n = 3).

Figure 7. Signaling pathways converging on MYPT1 were ineffective in Mypt1^{SMKO} ileum. (A–C) Representative tracings showed the effects of 10 μ mol/L Y27632 and 100 μ mol/L 8-Br-cGMP on 100 μ mol/L ACh-induced contractions of ileal strips from 16-week-old CTR and Mypt1^{SMKO} mice. 8-Br-cGMP or Y27632 was applied 15 minutes before the second stimulation. (D–F) Western blots of proteins from CTR and KO ileum involved in the relevant signaling pathways. Tissues were stimulated with 100 μ mol/L ACh for 60 seconds to measure the extent of phosphorylated Thr38-CPI-17. The amount of loaded protein was normalized by total actin control.