BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

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

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BACKGROUND & AIMS: The regulatory subunit of myosin light chain phosphatase, MYPT1, has been proposed to control smooth muscle contractility by regulating phosphorylation of the Ca^{2+}-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. 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 (PP1cδ), a regulatory subunit (MYPT1), and a 20-kilodalton subunit (M20) with unknown function.[5] MYPT1 is a central regulator of PP1cδ activity, acting through a combination of molecular and biochemical mechanisms.[2,6–8] First, MYPT1 enhances the catalytic activity of PP1cδ and directs its specificity to pRLC with its binding of both PP1cδ and myosin filaments. Second, PP1cδ activity is modulated by phosphorylation of MYPT1 itself. Phosphorylation of MYPT1 Thr696 or Thr850 inhibits PP1cδ 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

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Abbreviations used in this paper: ACh, acetylcholine; cGMP, guanosine 3′,5′-cyclic monophosphate; CTR, control; ICC, interstitial cells of Cajal; LC_{17}, 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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increases RLC phosphorylation and is described as Ca^{2+} sensitization. 3′,5′-Cyclic monophosphate (cGMP)-dependent protein kinase type I (PKGI) 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 PP1cδ 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 PP1cδ, 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 MTPT1 stimulates PP1c activity toward myosin RLC.

Materials and Methods

Generation of Smooth Muscle–Specific Mypt1 Knockout Mice (Mypt1^{SMKO})

The floxed *Mypt1* targeting vector of *lox*P 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 1*A* 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 1*C*).

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 1*D* 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 uroschesis 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 1*D*) but significant distention of the alimentary tract and retention of urine in the bladder (Supplementary Figure 1*A* 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 1*A–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 1*C*), 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 mm Hg; 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