

12-8-2016

Ca²⁺/calmodulin/MLCK pathway initiates, and RhoA/ROCK maintains, the internal anal sphincter smooth muscle tone.

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Recommended Citation

Rattan, Satish, "Ca²⁺/calmodulin/MLCK pathway initiates, and RhoA/ROCK maintains, the internal anal sphincter smooth muscle tone." (2016). *Division of Gastroenterology and Hepatology Faculty Papers*. Paper 39.

https://jdc.jefferson.edu/gastro_hepfp/39

1 **Ca²⁺/calmodulin/MLCK pathway initiates, and**
2 **RhoA/ROCK maintains the internal anal sphincter smooth**
3 **muscle tone**

4
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11 **Running Head:** Basal smooth muscle tone genesis and maintenance
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13 **Grant Support:** The work was supported by National Institutes of Diabetes and Digestive and
14 Kidney Diseases Grant RO1DK035385, and an institutional grant from Thomas Jefferson University.
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17 **Abbreviations used in the paper:**

18 IAS, internal anal sphincter; SMC, smooth muscle cells; LES, lower esophageal sphincter; RhoA/ROCK,
19 RhoA-associated kinase; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase;
20 I_{cl(ca)}, Ca²⁺-activated Cl current MYPT1, myosin phosphatase target subunit 1; PKC, protein kinase C;
21 CPI-17, protein kinase C-potentiate inhibitor
22

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27 **Disclosures:** The author has nothing to disclose
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30 It is well known that the smooth muscle contraction whether spontaneous or following
31 pharmacological stimulation, occurs in two phases, the initial phasic followed by the tonic phase (1, 2, 5,
32 6, 11, 12, 14, 16, 17, 22, 23, 25, 33, 37). Initial phasic contraction is critically dependent on an increase
33 in the intracellular levels of Ca^{2+} often caused by G protein-coupled receptor (GPCR) activation. The
34 increase in intracellular Ca^{2+} promotes the phosphorylation of the regulatory light chain of myosin
35 (MLC_{20}) by the Ca^{2+} /calmodulin-dependent myosin light chain kinase (MLCK) (Figure 1A, B).

36 The latter phase of tonic or sustained contraction has been described to be dependent on myosin light
37 chain phosphatase (MLCP) inhibition that maintains higher levels of phosphorylated MLC_{20} (p- MLC_{20}),
38 otherwise, the initiated contraction would cease and the smooth muscle would revert towards a more
39 relaxed state. Therefore, the state and nature of contractility, whether phasic, tonic, a mixture of phasic
40 and tonic, or a complete quiescence is determined by a balance between the Ca^{2+} /calmodulin/MLCK
41 stimulation and MLCP inhibition in different proportions of course a number of neurohumoral influences
42 may also play an important modulatory role in this regard. MLCP phosphorylation (which inhibits the
43 phosphatase) can be mediated through the RhoA-associated kinase (RhoA/ROCK) and protein kinase C
44 (PKC) pathways, as discussed below and illustrated in Figure 1A, B.

45 MLCP is a heterotrimeric enzyme consisting of a catalytic 38-kDa type 1 protein phosphatase δ
46 isoform (PP1c δ) and two regulatory subunits, a 110 kDa myosin phosphatase target subunit 1 (MYPT1)
47 and a 20 kDa small regulatory subunit (M20). RhoA/ROCK-mediated phosphorylation of MYPT1 (p-
48 MYPT1) at specific residues is associated with inhibition of MLCP leading to an increase in smooth
49 muscle contraction (18, 36). RhoA/ROCK can also increase p- MLC_{20} via an MLCK-like effect (29).
50 Additionally, ROCK inhibits catalytic subunit of MLCP via phosphorylation of protein kinase C-
51 potentiated inhibitor (CPI-17) (p-CPI-17). As such CPI-17 is known as an endogenous inhibitor of
52 MLCP. Phosphorylation of CPI-17 at threonine-38 (Thr³⁸) increases the inhibitory potency of CPI-17
53 ~7000 fold (8). Both ROCK and PKC can phosphorylate CPI-17 at Thr-38 residue (8, 19, 20).
54 RhoA/ROCK and PKC inhibit MLCP via phosphorylation of MYPT1 and CPI-17 leading to a sustained
55 increase in p- MLC_{20} thus maintaining the tone. Some of the common ways to assess MLCP activity are

56 to monitor phospho- levels of MYPT1 (at specific residues), CPI-17 and MLC₂₀ (21). In addition to
57 inhibition of MLCP, actin polymerization and actin cytoskeleton reorganization (either associated with or
58 independent of RhoA/ROCK (38)) play an important role in the sustained contraction. A number of
59 studies in different smooth muscles have shown that the myogenic contraction is associated with ~40%
60 reduction in the globular actin (G-actin) pool that constitutes ~10% of the total cellular actin, suggesting
61 an increased actin polymerization and filamentous actin (F-actin) formation. Dependence of such
62 contractions on increased actin polymerization was further shown by their sensitivity to the
63 polymerization inhibitors (7). Actin cytoskeleton reorganization may involve stimulation of G-protein-
64 coupled receptor, monomeric G-proteins, and macromolecular adhesion complex formation. The role of
65 actin polymerization and actin cytoskeleton reorganization however, in the IAS remains to be determined.

66 The sphincteric smooth muscles and the SMCs from humans and different animal species have been
67 shown to be characterized by the presence of higher levels of RhoA/ROCK, lower levels of MYPT1, and
68 higher levels of p-MYPT1, CPI-17, p-CPI-17 and p-MLC₂₀ (3, 26, 27, 29-31, 35, 39).

69 Acknowledging the fact that pharmacological stimulation may disturb and complicate underlying
70 molecular mechanisms for the original phasic or tonic states of the tissues, significant studies using purely
71 phasic and tonic tissues in the basal or unstimulated state have been performed. Examples of purely
72 phasic smooth muscles are esophageal body (EB) and anococcygeus (ASM), and those of tonic tissues are
73 the lower esophageal sphincter (LES) and internal anal sphincter (IAS) (14, 24, 26, 26, 27, 33, 41).
74 Working on purely tonic tissues, these and other investigators have shown that the initial phase of
75 development of the basal tone is critically dependent upon Ca²⁺/calmodulin/MLCK. In these studies, Ca²⁺
76 -free solutions and Ca²⁺-channel blockers maneuvers are routinely used to determine the levels of active
77 tone have been shown to produce near obliteration of the tone. Additionally, it has been reported that L-
78 type channel-mediated Ca²⁺ influx, and MLCK-mediated ryanodine receptor-induced spontaneous release
79 of Ca²⁺ leading to activation of Ca²⁺-activated Cl current (I_{cl(ca)}) (41), may play an important role in the
80 sphincteric smooth muscle tone. Conversely however, the later phase or the maintenance of tone is

81 primarily dependent upon the MLCP inhibitory factors especially via RhoA/ROCK with some element of
82 PKC (14, 31, 33, 35).

83 Collectively, above studies (14, 31, 33, 35) in animals and humans investigated the adjoining
84 phenotypic different tissues of purely tonic, phasic and mixed characteristics. These and additional studies
85 (4, 14, 26, 27, 30-35) revealed a tight correlation between the activities of RhoA/ROCK activity, MLCP,
86 and levels of p-MYPT1, p-CPI-17, and p-MLC₂₀, associated with distinctly higher levels of RhoA/ROCK
87 machinery in the IAS . These studies monitored basal IAS tone and its changes before and after selective
88 RhoA/ROCK activators/inhibitors and other molecular interventions, in the absence and presence of
89 GPCR activation. Additional data showed that in contrast to the tonic SM, the phasic smooth muscles
90 have lower levels of RhoA and ROCK signaling machinery that are relatively less responsive to upstream
91 activators, and direct manipulations of RhoA/ROCK. Studies using selective molecular intervention by
92 localized topical application of ROCKII-siRNA for transient silencing of ROCKII also demonstrated a
93 significant decrease in the IAS tone (4). Further evidence implicating the RhoA/ROCK pathway as
94 responsible for the basal tone has emerged from studies of bioengineered and reverse engineered IAS
95 reconstructs using human IAS SMCs (34). These reconstructs were shown to have functional and
96 molecular properties similar to the intact IAS, and demonstrated that the basal tone is dependent on
97 RhoA/ROCK. Altogether, these data suggest that the sphincteric tone is critically dependent upon
98 RhoA/ROCK that may be either constitutively active or involve GPCR activation via autocrine control (6,
99 32).

100 In support of these concepts, recent studies by Drs. Zhang et al., (40) have employed state-of-the art
101 methodologies involving conditional knock outs of MLCK and spontaneous transient inward currents
102 (STICs) in mouse IAS model. Data showed almost complete obliteration of the IAS tone by specific
103 conditional MLCK deletion and specific inhibition of Ca²⁺-channels, ryanodine receptors (RyRs), L-type
104 voltage-dependent Ca²⁺-channels (VDCCs) or TMEM16A Ca²⁺-activated Cl channels. MLCK deletion-
105 associated decrease in the IAS tone was shown to be without changes in RhoA/ROCK/PKC/CPI-17
106 suggesting independence of molecular mechanisms for the initial phase from those for the later phase of

107 maintenance of the basal tone. These data are in agreement with the above concept that the latter stage of
108 activation of RhoA/ROCK/PKC responsible for MLCP inhibition follows the initial phase, and does not
109 set in in the absence of initial development of tone. Additionally, it has been shown that Ca^{2+} activation
110 plays an important role in RhoA/ROCK activation (9). These data are consistent with the role of
111 Ca^{2+} /calmodulin/MLCK pathway in the initiation (10, 21, 36), and Ca^{2+} sensitization via RhoA/ROCK
112 activation for the maintenance of IAS tone. However, the role of actin polymerization and cytoskeleton
113 reorganization is likely and remains to be determined.

114 Based on data showing enhanced sustained contraction in the gastrointestinal and vascular smooth
115 muscles (15, 28), and characteristically lower levels of MYPT1 associated with the tone (26, 27), one
116 would expect an increase in the basal IAS tone following genetic manipulation for the decreased
117 expression of MYPT1. However, the mouse IAS studies (40) showed no such effect following
118 conditional knock out of MYPT1. Whether this is related to the morphological changes such as
119 hypertrophy following MYPT1 deletion (40), fibrosis, or other compensatory molecular changes in the
120 smooth muscle is not known. Noticeably, these studies did not monitor levels of p-MYPT1. It has been
121 reported that in spite of the lower levels of MYPT1, the sphincteric tissues have higher levels of p-MYPT
122 (26, 27). Such information could provide important clues for the molecular traffic in relation to the basal
123 tone before and after conditional knock outs. Additionally, in contrast with others, these studies (40)
124 monitored basal tone and its changes in ice-cold buffer; whether this accounts for certain unexpected
125 results remains unknown. It is also possible that not knowing the exact nature of unique sphincteric
126 smooth muscle-specific MYPT1 (13), the selected MYPT1 for deletion may not have been tissue and
127 species-specific.

128 In closing, there are presently substantial data to support the concept that Ca^{2+} /calmodulin/MLCK
129 activation are critical for the initial phasic stage of IAS tone development, whereas MLCP-inhibition
130 primarily by RhoA/ROCK pathway plays a crucial role in the tone maintenance (Figure 1A, B).
131 Molecular insights into the mechanisms underlying the spontaneous tone in the gastrointestinal smooth

132 muscles represented by the IAS and LES are crucial in the pathophysiology and therapeutic targeting of a
133 number of debilitating motility disorders such as fecal incontinence.

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138 **ACKNOWLEDGMENTS**

139 The author apologizes for not citing all the other relevant papers because of space limitations.

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270 **FIGURE LEGENDS**

271 **Fig. 1.**

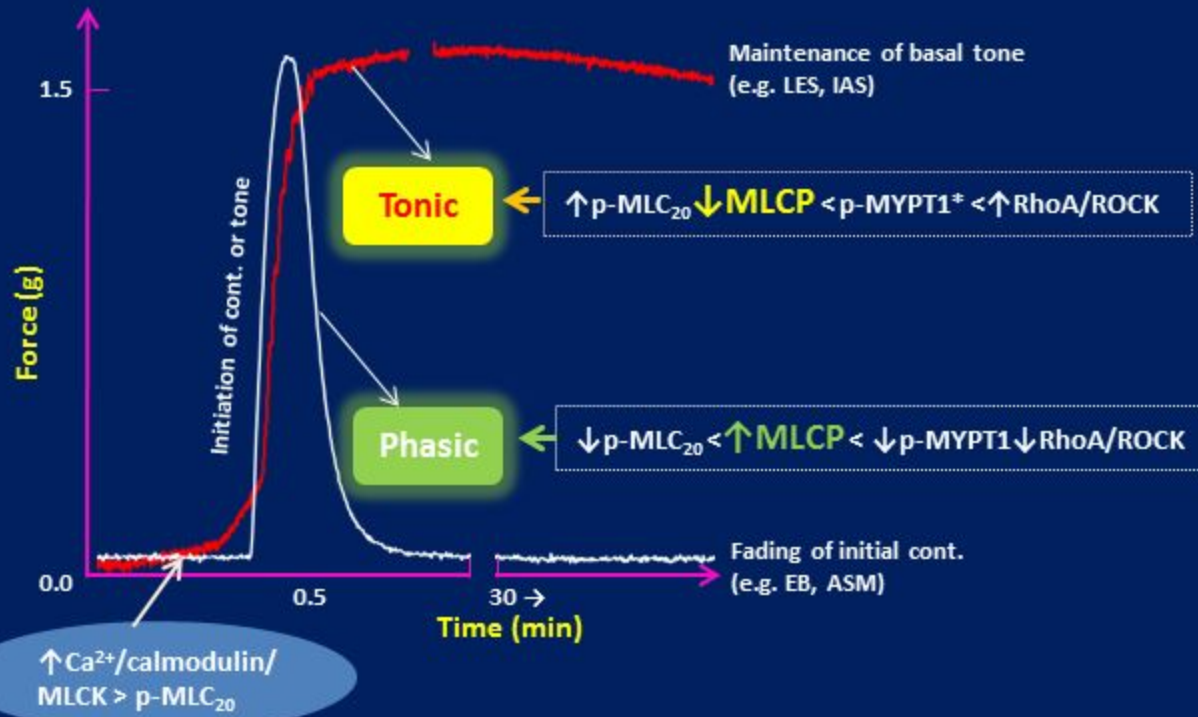
272 **A.** A simplified model showing basic differences in the myogenic molecular mechanisms responsible for
273 the initiation of contraction followed by its fade in the phasic (denoted by white tracing line) vs.
274 development of tone followed by its maintenance in the tonic (denoted by red tracing line) smooth
275 muscles. Typical examples of truly phasic smooth muscles are those of esophageal body (EB) and
276 anococcygeus and (ASM), while those of tonic smooth muscles are lower esophageal sphincter (LES) and
277 internal anal sphincter (IAS). In this illustration, smooth muscle contraction in rat ASM (induced by
278 electrical field stimulation) and spontaneous tone in the rat IAS (without any stimulus) represent phasic
279 and tonic activities, respectively. Initial events for the contractility both in the phasic and tonic smooth
280 muscles are similar as they are dependent upon increase in intracellular Ca^{2+} $[(\text{Ca}^{2+})_i]$, followed by
281 formation of Ca^{2+} /calmodulin complex and activation of MLCK leading to increase in p-MLC₂₀. The
282 triggers for the initial phasic contraction and tone maintenance have been discussed in the text. As
283 indicated by highlighted bold letters, myosin-light-chain phosphatase (MLCP) plays a critical role in the
284 characteristic fading of contraction in the phasic, and in the maintenance of developed tone in the tonic
285 smooth muscle. Once initiated, the phasic contraction quickly fades because of dephosphorization of p-
286 MLC₂₀ by active MLCP, and lack of other support mechanisms to maintain high levels of p-MLC₂₀.
287 However, in the tonic smooth muscles, the basal tone is sustained because higher levels of p-MLC₂₀ are
288 maintained primarily via inhibition of MLCP by RhoA/ROCK-mediated phosphorylation of regulatory
289 subunit of MLCP (p-MYPT1), and other effects as laid out in panel B. In the tonic smooth muscles,
290 RhoA/ROCK may be either constitutively active or GPCR-activated. This figure does not reveal the
291 source of increase in $[(\text{Ca}^{2+})_i]$, and the role of actin polymerization and cytoskeleton reorganization in the
292 smooth muscle contractility. These feature are however are discussed in the text.

293 $\uparrow\downarrow$, denote an increase or decrease respectively in the expression or activity; *, for simplicity only the
294 major target of RhoA/ROCK (MYPT1 which is phosphorylated by RhoA/ROCK) is shown here.
295 RhoA/ROCK does however have the additional ability to increase p-MLC₂₀ as shown in panel B.

296 **B.** This panel illustrates different mechanisms by which RhoA/ROCK can increase p-MLC₂₀ for the
297 sustained contraction initiated by Ca²⁺/calmodulin/MLCK as follows via: 1). inhibition of MLCP through
298 phosphorylation of its regulatory subunit MYPT1 (p-MYP1); 2). phosphorylation of protein kinase C-
299 potentiated inhibitor (CPI-17) (p-CPI-17) that causes subsequent inhibition of MLCP via its catalytic
300 subunit PP1c and via p-MYPT1; and 3). MLCK-like effect. In addition, this illustration suggests a partial
301 role of PKC in the mediation of basal smooth muscle tone by phosphorylation of CPI-17; and double
302 arrow between RhoA/ROCK and PKC suggests a cross-talk between the two pathways. An increase in p-
303 MLC₂₀ initiated by Ca²⁺/calmodulin/MLCK and sustained by RhoA/ROCK activation leads to smooth
304 muscle contraction, and its dephosphorylation via MLCP causes relaxation. For more details, consult
305 text.

306

A.



B.

