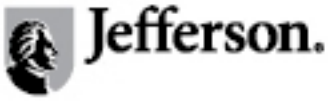

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Ca2+/calmodulin/MLCK pathway initiates, and RhoA/ROCK maintains, the internal anal sphincter smooth muscle tone.

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Running Head: Basal smooth muscle tone genesis and maintenance

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Abbreviations used in the paper:

IAS, internal anal sphincter; SMC, smooth muscle cells; LES, lower esophageal sphincter; RhoA/ROCK, RhoA-associated kinase; MLCK, myosin light chain kinase; MLCP, myosin light chain phosphatase; $I_{cl(ca)}$, Ca^{2+}-activated Cl current MYPT1, myosin phosphatase target subunit 1; PKC, protein kinase C; CPI-17, protein kinase C-potentiate inhibitor

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It is well known that the smooth muscle contraction whether spontaneous or following pharmacological stimulation, occurs in two phases, the initial phasic followed by the tonic phase (1, 2, 5, 6, 11, 12, 14, 16, 17, 22, 23, 25, 33, 37). Initial phasic contraction is critically dependent on an increase in the intracellular levels of Ca^{2+} often caused by G protein-coupled receptor (GPCR) activation. The increase in intracellular Ca^{2+} promotes the phosphorylation of the regulatory light chain of myosin (MLC_{20}) by the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK) (Figure 1A, B).

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

MLCP is a heterotrimeric enzyme consisting of a catalytic 38-kDa type 1 protein phosphataseδ isoform (PP1cδ) and two regulatory subunits, a 110 kDa myosin phosphatase target subunit 1 (MYPT1) and a 20 kDa small regulatory subunit (M20). RhoA/ROCK-mediated phosphorylation of MYPT1 (p-MYPT1) at specific residues is associated with inhibition of MLCP leading to an increase in smooth muscle contraction (18, 36). RhoA/ROCK can also increase p-MLC_{20} via an MLCK-like effect (29). Additionally, ROCK inhibits catalytic subunit of MLCP via phosphorylation of protein kinase C-potentiated inhibitor (CPI-17) (p-CPI-17). As such CPI-17 is known as an endogenous inhibitor of MLCP. Phosphorylation of CPI-17 at threonine-38 (Thr^{38}) increases the inhibitory potency of CPI-17 ~7000 fold (8). Both ROCK and PKC can phosphorylate CPI-17 at Thr-38 residue (8, 19, 20).

RhoA/ROCK and PKC inhibit MLCP via phosphorylation of MYPT1 and CPI-17 leading to a sustained increase in p-MLC_{20} thus maintaining the tone. Some of the common ways to assess MLCP activity are

to monitor phospho- levels of MYPT1 (at specific residues), CPI-17 and MLC_{20} (21). In addition to inhibition of MLCP, actin polymerization and actin cytoskeleton reorganization (either associated with or independent of RhoA/ROCK (38)) play an important role in the sustained contraction. A number of studies in different smooth muscles have shown that the myogenic contraction is associated with ~40% reduction in the globular actin (G-actin) pool that constitutes ~10% of the total cellular actin, suggesting an increased actin polymerization and filamentous actin (F-actin) formation. Dependence of such contractions on increased actin polymerization was further shown by their sensitivity to the polymerization inhibitors (7). Actin cytoskeleton reorganization may involve stimulation of G-protein-coupled receptor, monomeric G-proteins, and macromolecular adhesion complex formation. The role of actin polymerization and actin cytoskeleton reorganization however, in the IAS remains to be determined.

The sphincteric smooth muscles and the SMCs from humans and different animal species have been shown to be characterized by the presence of higher levels of RhoA/ROCK, lower levels of MYPT1, and higher levels of p-MYPT1, CPI-17, p-CPI-17 and p-MLC_{20} (3, 26, 27, 29-31, 35, 39).

Acknowledging the fact that pharmacological stimulation may disturb and complicate underlying molecular mechanisms for the original phasic or tonic states of the tissues, significant studies using purely phasic and tonic tissues in the basal or unstimulated state have been performed. Examples of purely phasic smooth muscles are esophageal body (EB) and anococcygeus (ASM), and those of tonic tissues are the lower esophageal sphincter (LES) and internal anal sphincter (IAS) (14, 24, 26, 26, 27, 33, 41). Working on purely tonic tissues, these and other investigators have shown that the initial phase of development of the basal tone is critically dependent upon Ca^{2+}/calmodulin/MLCK. In these studies, Ca^{2+} -free solutions and Ca^{2+}-channel blockers maneuvers are routinely used to determine the levels of active tone have been shown to produce near obliteration of the tone. Additionally, it has been reported that L-type channel-mediated Ca^{2+} influx, and MLCK-mediated ryanodine receptor-induced spontaneous release of Ca^{2+} leading to activation of Ca^{2+}-activated Cl current ($I_{cl(ca)}$) (41), may play an important role in the sphincteric smooth muscle tone. Conversely however, the later phase or the maintenance of tone is

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

Collectively, above studies (14, 31, 33, 35) in animals and humans investigated the adjoining phenotypic different tissues of purely tonic, phasic and mixed characteristics. These and additional studies (4, 14, 26, 27, 30-35) revealed a tight correlation between the activities of RhoA/ROCK activity, MLCP, and levels of p-MYPT1, p-CPI-17, and p-MLC_{20}, associated with distinctly higher levels of RhoA/ROCK machinery in the IAS . These studies monitored basal IAS tone and its changes before and after selective RhoA/ROCK activators/inhibitors and other molecular interventions, in the absence and presence of GPCR activation. Additional data showed that in contrast to the tonic SM, the phasic smooth muscles have lower levels of RhoA and ROCK signaling machinery that are relatively less responsive to upstream activators, and direct manipulations of RhoA/ROCK. Studies using selective molecular intervention by localized topical application of ROCKII-siRNA for transient silencing of ROCKII also demonstrated a significant decrease in the IAS tone (4). Further evidence implicating the RhoA/ROCK pathway as responsible for the basal tone has emerged from studies of bioengineered and reverse engineered IAS reconstructs using human IAS SMCs (34). These reconstructs were shown to have functional and molecular properties similar to the intact IAS, and demonstrated that the basal tone is dependent on RhoA/ROCK. Altogether, these data suggest that the sphincteric tone is critically dependent upon RhoA/ROCK that may be either constitutively active or involve GPCR activation via autocrine control (6, 32).

In support of these concepts, recent studies by Drs. Zhang et al., (40) have employed state-of-the art methodologies involving conditional knock outs of MLCK and spontaneous transient inward currents (STICs) in mouse IAS model. Data showed almost complete obliteration of the IAS tone by specific conditional MLCK deletion and specific inhibition of Ca^{2+}-channels, ryanodine receptors (RyRs), L-type voltage-dependent Ca^{2+}-channels (VDCCs) or TMEM16A Ca^{2+}-activated Cl channels. MLCK deletion-associated decrease in the IAS tone was shown to be without changes in RhoA/ROCK/PKC/CPI-17 suggesting independence of molecular mechanisms for the initial phase from those for the later phase of

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

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

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

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

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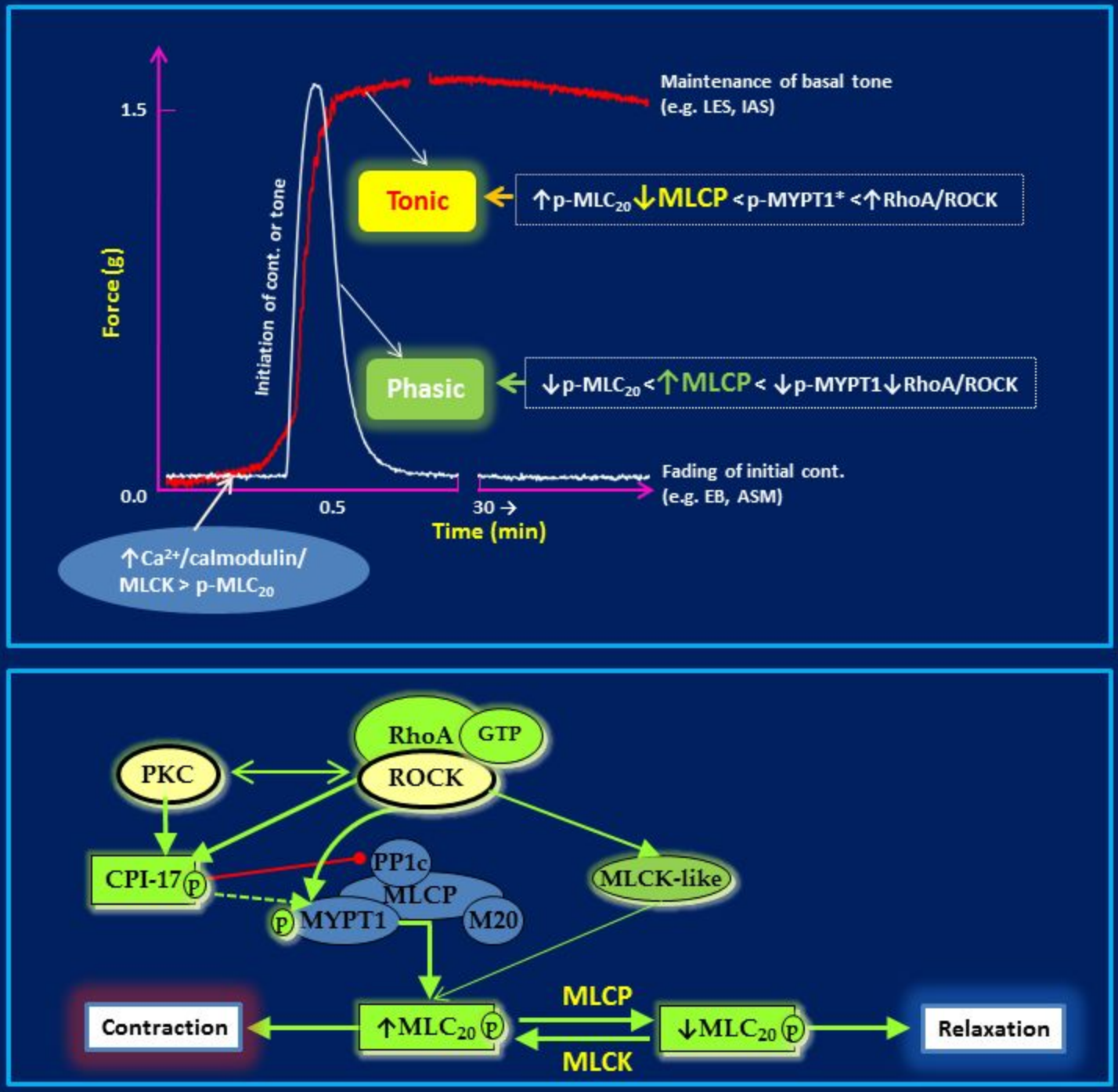
FIGURE LEGENDS

Fig. 1.

A. A simplified model showing basic differences in the myogenic molecular mechanisms responsible for the initation of contraction followed by its fade in the phasic (denoted by white tracing line) vs. development of tone followed by its maintenance in the tonic (denoted by red tracing line) smooth muscles. Typical examples of truly phasic smooth muscles are those of esophageal body (EB) and anococcygeus and (ASM), while those of tonic smooth muscles are lower esophageal sphincter (LES) and internal anal sphincter (IAS). In this illustration, smooth muscle contraction in rat ASM (induced by electrical field stimulation) and spontaneous tone in the rat IAS (without any stimulus) represent phasic and tonic activities, respectively. Initial events for the contractility both in the phasic and tonic smooth muscles are similar as they are dependent upon increase in intracellular Ca^{2+} [$(Ca^{2+})_i$], followed by formation of Ca^{2+}/calmodulin complex and activation of MLCK leading to increase in p-MLC_{20}. The triggers for the initial phasic contraction and tone maintenance have been discussed in the text. As indicated by highlighted bold letters, myosin-light-chain phosphatase (MLCP) plays a critical role in the characteristic fading of contraction in the phasic, and in the maintenance of developed tone in the tonic smooth muscle. Once initiated, the phasic contraction quickly fades because of dephosphorization of p-MLC_{20} by active MLCP, and lack of other support mechanisms to maintain high levels of p-MLC_{20}. However, in the tonic smooth muscles, the basal tone is sustained because higher levels of p-MLC_{20} are maintained primarily via inhibition of MLCP by RhoA/ROCK-mediated phosphorylation of regulatory subunit of MLCP (p-MYPT1), and other effects as laid out in panel B. In the tonic smooth muscles, RhoA/ROCK may be either constitutively active or GPCR-activated. This figure does not reveal the source of increase in [$(Ca^{2+})_i$], and the role of actin polymerization and cytoskeleton reorganization in the smooth muscle contractility. These feature are however are discussed in the text.

↑↓, denote an increase or decrease respectively in the expression or activity; *, for simplicity only the major target of RhoA/ROCK (MYPT1 which is phosphorylated by RhoA/ROCK) is shown here. RhoA/ROCK does however have the additional ability to increase p-MLC_{20} as shown in panel B.

B. This panel illustrates different mechanisms by which RhoA/ROCK can increase p-MLC_{20} for the sustained contraction initiated by Ca^{2+}/calmodulin/MLCK as follows via: 1). inhibition of MLCP through phosphorylation of its regulatory subunit MYPT1 (p-MYP1); 2). phosphorylation of protein kinase C-potentiated inhibitor (CPI-17) (p-CPI-17) that causes subsequent inhibition of MLCP via its catalytic subunit PP1c and via p-MYPT1; and 3). MLCK-like effect. In addition, this illustration suggests a partial role of PKC in the mediation of basal smooth muscle tone by phosphorylation of CPI-17; and double arrow between RhoA/ROCK and PKC suggests a cross-talk between the two pathways. An increase in p-MLC_{20} initiated by $Ca^{2+/}$calmodulin/MLCK and sustained by RhoA/ROCK activation leads to smooth muscle contraction, and its dephosphorylation via MLCP causes relaxation. For more details, consult text.


A.
1.5
0.0
Force (g)
Initiation of cont. or tone
0.5
30 →
Time (min)
Maintenance of basal tone
(e.g. LES, IAS)
Tonic
↑p-MLC20 ↓MLCP < p-MYPT1* < ↑RhoA/ROCK
Phasic
↓p-MLC20 < ↑MLCP < ↓p-MYPT1 ↓RhoA/ROCK
Fading of initial cont.
(e.g. EB, ASM)
↑Ca2+/calmodulin/
MLCK > p-MLC20
B.
RhoA
GTP
ROCK
PKC
CPI-17
P
PP1c
MLCP
M20
P
MYPT1
MLCK-like
Contraction
↑MLC20 P
MLCP
MLCK
↓MLC20 P
Relaxation