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Cross-talk between Rho-associated Kinase and Cyclic Nucleotide-dependent Kinase Signaling Pathways in the Regulation of Smooth Muscle Myosin Light Chain Phosphatase
Michael E. Grassie, Cindy Sutherland, Annegret Ulke-Lemée, Mona Chappellaz, Enikö Kiss, Michael P. Walsh and Justin A. MacDonald
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SMOOTH MUSCLE CONTRACTION AND RELAXATION

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This brief review serves as a refresher on smooth muscle physiology for those educators who teach in medical and graduate courses of physiology. Additionally, those professionals who are in need of an update on smooth muscle physiology may find this review to be useful. Smooth muscle lacks the striations characteristic of cardiac and skeletal muscle. Layers of smooth muscle cells line the walls of various organs and tubes in the body, and the contractile function of smooth muscle is not under voluntary control. Contractile activity in smooth muscle is initiated by a Ca^{2+}-calmodulin interaction to stimulate phosphorylation of the light chain of myosin. Ca^{2+} sensitization of the contractile proteins is signaled by the RhoA/Rho kinase pathway to inhibit the dephosphorylation of the light chain by myosin phosphatase, thereby maintaining force generation. Removal of Ca^{2+} from the cytosol and stimulation of myosin phosphatase initiate the process of smooth muscle relaxation.

Sheets or layers of smooth muscle cells are contained in the walls of various organs and tubes in the body, including the blood vessels, stomach, intestines, bladder, airways, uterus, and the penile and clitoral cavernosal sinuses. When made to contract, the smooth muscle cells shorten, thereby propelling the luminal contents of the organ, or the cell shortening varies the diameter of a tube to regulate the flow of its contents. There are also bundles of smooth muscle cells attached to the hairs of the skin and to the iris and lens of the eye. When these bundles contract, the hairs become erect and the lens of the eye changes shape to focus light on the retina.

Smooth muscle cells lack the striated banding pattern found in cardiac and skeletal muscle, and they receive neural innervation from the autonomic nervous system. In addition, the contractile state of smooth muscle is controlled by hormones, autocrine/paracrine agents, and other local chemical signals. Smooth muscle cells also develop tonic and phasic contractions in response to changes in load or length. Regardless of the stimulus, smooth muscle cells use cross-bridge cycling between actin and myosin to develop force, and calcium ions (Ca^{2+}) serve to initiate contraction.

This brief review will serve as a refresher for those educators who teach in medical and graduate courses of physiology. Additionally, those professionals who are in need of an update on smooth muscle physiology may find this review to be useful. New concepts about regulatory mechanisms are presented to add depth to the understanding of the integrated responses of contraction and relaxation in smooth muscle. For those individuals desiring a more in-depth treatment of the subject, several recent reviews are recommended (1, 3, 5, 7, 9, 10, 18).

THE CONTRACTILE MECHANISM

In the intact body, the process of smooth muscle cell contraction is regulated principally by receptor and mechanical (stretch) activation of the contractile pro-

teins myosin and actin. A change in membrane potential, brought on by the firing of action potentials or by activation of stretch-dependent ion channels in the plasma membrane, can also trigger contraction. For contraction to occur, myosin light chain kinase (MLC kinase) must phosphorylate the 20-kDa light chain of myosin, enabling the molecular interaction of myosin with actin. Energy released from ATP by myosin ATPase activity results in the cycling of the myosin cross-bridges with actin for contraction. Thus contractile activity in smooth muscle is determined primarily by the phosphorylation state of the light chain of myosin—a highly regulated process. In some smooth muscle cells, the phosphorylation of the light chain of myosin is maintained at a low level in the absence of external stimuli (i.e., no receptor or mechanical activation). This activity results in what is known as smooth muscle tone and its intensity can be varied.

Ca^{2+}-DEPENDENT CONTRACTION OF SMOOTH MUSCLE

Contraction of smooth muscle is initiated by a Ca^{2+}-mediated change in the thick filaments, whereas in striated muscle Ca^{2+} mediates contraction by changes in the thin filaments. In response to specific stimuli in smooth muscle, the intracellular concentration of Ca^{2+} increases, and this activator Ca^{2+} combines with the acidic protein calmodulin. This complex activates MLC kinase to phosphorylate the light chain of myosin (Fig. 1). Cytosolic Ca^{2+} is increased through Ca^{2+} release from intracellular stores (sarcoplasmic reticulum) as well as entry from the extracellular space through Ca^{2+} channels (receptor-operated Ca^{2+} channels). Agonists (norepinephrine, angiotensin II, endothelin, etc.) binding to serpentine receptors, coupled to a heterotrimeric G protein, stimulate phospholipase C activity. This enzyme is specific for the membrane lipid phosphatidylinositol 4,5-bisphosphate to catalyze the formation of two potent second messengers: inositol trisphosphate (IP_3) and diacylglycerol (DG). The binding of IP_3 to receptors on the sarcoplasmic reticulum results in the release of Ca^{2+} into the cytosol. DG, along with Ca^{2+}, activates protein kinase C (PKC), which phosphorylates specific target proteins. There are several isozymes of PKC in smooth muscle, and each has a tissue-specific role (e.g., vascular, uterine, intestinal, etc.). In many cases,

PKC has contraction-promoting effects such as phosphorylation of L-type Ca^{2+} channels or other proteins that regulate cross-bridge cycling. Phorbol esters, a group of synthetic compounds known to activate PKC, mimic the action of DG and cause contraction of smooth muscle. Finally, L-type Ca^{2+} channels (voltage-operated Ca^{2+} channels) in the membrane also open in response to membrane depolarization brought on by stretch of the smooth muscle cell.

Ca^{2+} SENSITIZATION MECHANISM AND CONTRACTION OF SMOOTH MUSCLE

In addition to the Ca^{2+}-dependent activation of MLC kinase, the state of myosin light chain phosphorylation is further regulated by MLC phosphatase [aka myosin phosphatase (1, 4, 9, 11–16)], which removes the high-energy phosphate from the light chain of myosin to promote smooth muscle relaxation (Fig. 1). There are three subunits of MLC phosphatase: a 37-kDa catalytic subunit, a 20-kDa variable subunit, and a 110- to 130-kDa myosin-binding subunit. The myosin-binding subunit, when phosphorylated, inhibits the enzymatic activity of MLC phosphatase, allowing the light chain of myosin to remain phosphorylated, thereby promoting contraction. The small G protein RhoA and its downstream target Rho kinase play an important role in the regulation of MLC phosphatase activity. Rho kinase, a serine/threonine kinase, phosphorylates the myosin-binding subunit of MLC phosphatase, inhibiting its activity and thus promoting the phosphorylated state of the myosin light chain (Fig. 1). Pharmacological inhibitors of Rho kinase, such as fasudil and Y-27632, block its activity by competing with the ATP-binding site on the enzyme. Rho kinase inhibition induces relaxation of isolated segments of smooth muscle contracted to many different agonists. In the intact animal, the pharmacological inhibitors of Rho kinase have been shown to cause relaxation of smooth muscle in arteries, resulting in a blood pressure-lowering effect (2, 17).

An important question facing the smooth-muscle physiologist is: what is the link between receptor occupation and activation of the Ca^{2+}-sensitizing activity of the RhoA/Rho kinase-signaling cascade? Currently, it is thought that receptors activate a heterotrimeric G protein that is coupled to RhoA/Rho kinase signaling via guanine nucleotide exchange factors
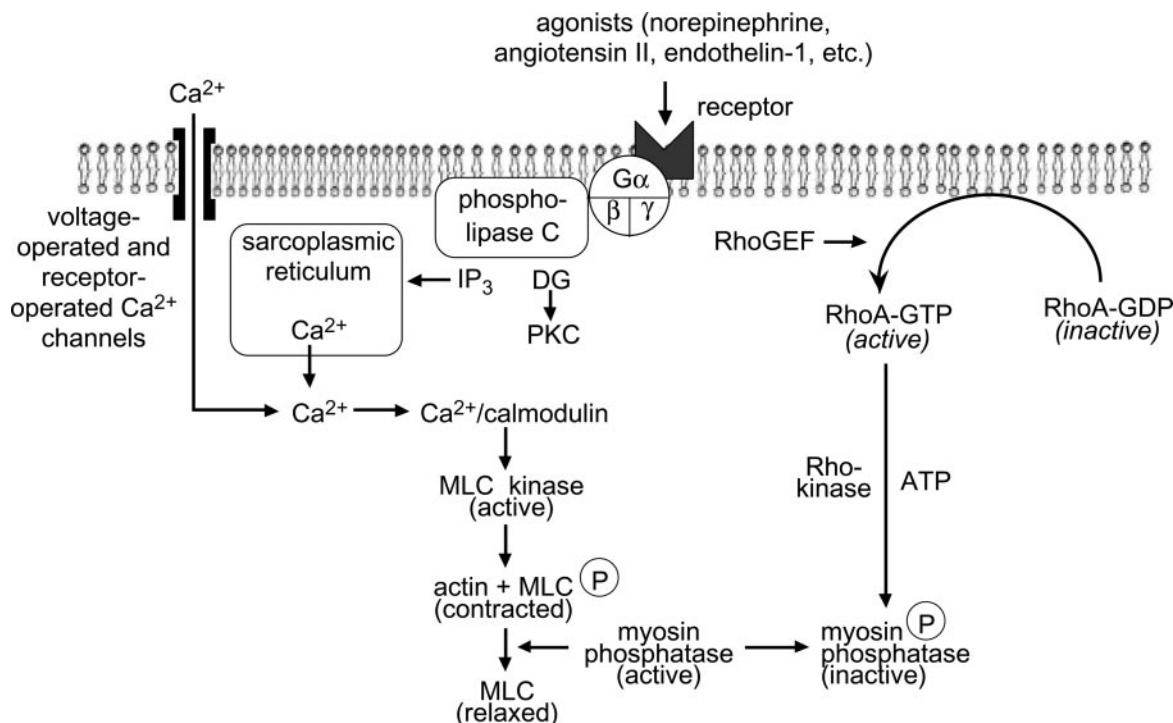
FIG. 1.

Regulation of smooth muscle contraction. Various agonists (neurotransmitters, hormones, etc.) bind to specific receptors to activate contraction in smooth muscle. Subsequent to this binding, the prototypical response of the cell is to increase phospholipase C activity via coupling through a G protein. Phospholipase C produces two potent second messengers from the membrane lipid phosphatidylinositol 4,5-bisphosphate: diacylglycerol (DG) and inositol 1,4,5-trisphosphate (IP_3). IP_3 binds to specific receptors on the sarcoplasmic reticulum, causing release of activator calcium (Ca^{2+}). DG along with Ca^{2+} activates PKC, which phosphorylates specific target proteins. In most smooth muscles, PKC has contraction-promoting effects such as phosphorylation of Ca^{2+} channels or other proteins that regulate cross-bridge cycling. Activator Ca^{2+} binds to calmodulin, leading to activation of myosin light chain kinase (MLC kinase). This kinase phosphorylates the light chain of myosin, and, in conjunction with actin, cross-bridge cycling occurs, initiating shortening of the smooth muscle cell. However, the elevation in Ca^{2+} concentration within the cell is transient, and the contractile response is maintained by a Ca^{2+}-sensitizing mechanism brought about by the inhibition of myosin phosphatase activity by Rho kinase. This Ca^{2+}-sensitizing mechanism is initiated at the same time that phospholipase C is activated, and it involves the activation of the small GTP-binding protein RhoA. The precise nature of the activation of RhoA by the G protein-coupled receptor is not entirely clear but involves a guanine nucleotide exchange factor (RhoGEF) and migration of RhoA to the plasma membrane. Upon activation, RhoA increases Rho kinase activity, leading to inhibition of myosin phosphatase. This promotes the contractile state, since the light chain of myosin cannot be dephosphorylated.

(RhoGEFs; Fig. 1). Because RhoGEFs facilitate activation of RhoA, they regulate the duration and intensity of signaling via heterotrimeric G protein receptor coupling. There are ~70 RhoGEFs in the human genome, and three RhoGEFs have been identified in smooth muscle: PDZ-RhoGEF, LARG (leukemia-associated RhoGEF), and p115-RhoGEF. Increased expression and/or activity of RhoGEF proteins could augment contractile activation of smooth muscle and therefore play a role in diseases where an augmented response contributes to the pathophysiology (hypertension, asthma, etc.).

Several recent studies suggest a role for additional regulators of MLC kinase and MLC phosphatase (13–16). Calmodulin-dependent protein kinase II promotes smooth muscle relaxation by decreasing the sensitivity of MLC kinase for Ca^{2+}. Additionally, MLC
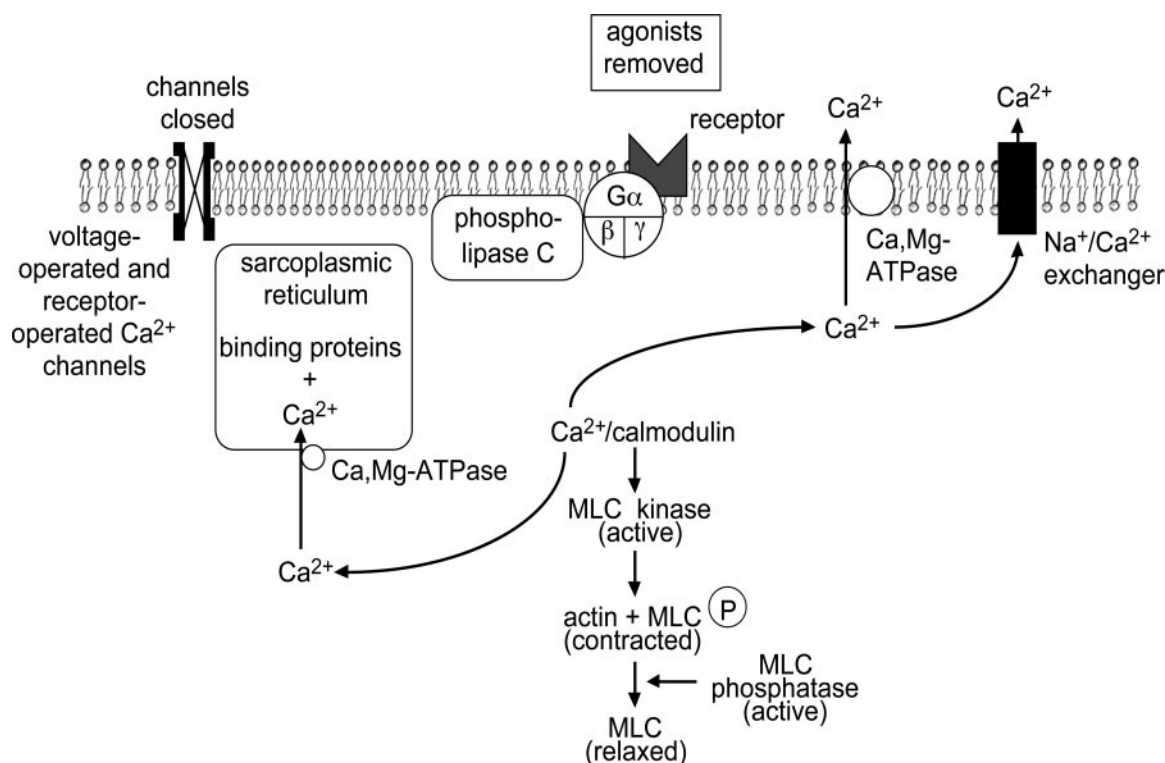
FIG. 2.

Relaxation of smooth muscle. Smooth muscle relaxation occurs either as a result of removal of the contractile stimulus or by the direct action of a substance that stimulates inhibition of the contractile mechanism. Regardless, the process of relaxation requires a decreased intracellular Ca^{2+} concentration and increased MLC phosphatase activity. The sarcoplasmic reticulum and the plasma membrane contain Ca,Mg-ATPases that remove Ca^{2+} from the cytosol. Na^+/Ca^{2+} exchangers are also located on the plasma membrane and aid in decreasing intracellular Ca^{2+}. During relaxation, receptor- and voltage-operated Ca^{2+} channels in the plasma membrane close resulting in a reduced Ca^{2+} entry into the cell.

phosphatase activity is stimulated by the 16-kDa protein telokin in phasic smooth muscle and is inhibited by a downstream mediator of DG/protein kinase C, CPI-17.

SMOOTH MUSCLE RELAXATION

Smooth muscle relaxation occurs either as a result of removal of the contractile stimulus or by the direct action of a substance that stimulates inhibition of the contractile mechanism (e.g., atrial natriuretic factor is a vasodilator). Regardless, the process of relaxation requires a decreased intracellular Ca^{2+} concentration and increased MLC phosphatase activity (Fig. 2) (10, 16). The mechanisms that sequester or remove intracellular Ca^{2+} and/or increase MLC phosphatase activ-

ity may become altered, contributing to abnormal smooth muscle responsiveness.

A decrease in the intracellular concentration of activator Ca^{2+} elicits smooth muscle cell relaxation. Several mechanisms are implicated in the removal of cytosolic Ca^{2+} and involve the sarcoplasmic reticulum and the plasma membrane. Ca^{2+} uptake into the sarcoplasmic reticulum is dependent on ATP hydrolysis. This sarcoplasmic reticular Ca,Mg-ATPase, when phosphorylated, binds two Ca^{2+} ions, which are then translocated to the luminal side of the sarcoplasmic reticulum and released. Mg^{2+} is necessary for the activity of the enzyme; it binds to the catalytic site of the ATPase to mediate the reaction. The sarcoplasmic

reticular Ca,Mg-ATPase is inhibited by several different pharmacological agents: vanadate, thapsigargin, and cyclopiazonic acid. Sarcoplasmic reticular Ca^{2+}-binding proteins also contribute to decreased intracellular Ca^{2+} levels. Recent studies have identified calsequestrin and calreticulin as sarcoplasmic reticular Ca^{2+}-binding proteins in smooth muscle.

The plasma membrane also contains Ca,Mg-ATPases, providing an additional mechanism for reducing the concentration of activator Ca^{2+} in the cell. This enzyme differs from the sarcoplasmic reticular protein in that it has an autoinhibitory domain that can be bound by calmodulin, causing stimulation of the plasma membrane Ca^{2+} pump.

Na^+/Ca^{2+} exchangers are also located on the plasma membrane and aid in decreasing intracellular Ca^{2+}. This low-affinity antiporter is closely coupled to intracellular Ca^{2+} levels and can be inhibited by amiloride and quinidine.

Receptor-operated and voltage-operated Ca^{2+} channels located in the plasma membrane are important in Ca^{2+} influx and smooth muscle contraction, as previously mentioned. Inhibition of these channels can elicit relaxation. Channel antagonists such as dihydropyridine, phenylalkylamines, and benzothiazepines bind to distinct receptors on the channel protein and inhibit Ca^{2+} entry in smooth muscle.

ABNORMAL CONTRACTILE REGULATION OF SMOOTH MUSCLE

Alterations in the regulatory processes maintaining intracellular Ca^{2+} and MLC phosphorylation have been proposed as possible sites contributing to the abnormal contractile events in smooth muscle cells of various organs and tissues (2, 5, 8, 9). In addition, alterations in upstream targets that impact Ca^{2+} and MLC phosphorylation have also been implicated. For example, changes in the affinity, number, or subtype of α-adrenergic receptors leading to enhanced vasoconstriction have been characterized in arterial smooth muscle cells in some types of hypertension. Increases in the activity of RhoA/Rho kinase signaling lead to increased contractile responses that may contribute to erectile dysfunction in the penis and clitoris. Increased activity of the RhoA/Rho kinase-signal-

ing pathway may also contribute to augmented contraction or spastic behavior of smooth muscle in disease states such as asthma or atherosclerosis.

Impaired function may occur as the result of a change in the direct action of a substance that stimulates inhibition of the contractile mechanism. For example, decreased relaxation responses can be due to a reduction in cyclic nucleotide-dependent signaling pathways coupled with reductions in receptor activation (β-adrenergic receptors and cyclic AMP) or agonist bioavailability (endothelium dysfunction, reduced nitric oxide and cyclic GMP). Importantly, it is the complexity and redundancy of these cell signaling pathways regulating intracellular Ca^{2+} and MLC phosphorylation in smooth muscle that provide therapeutic potential for dysfunction.

SUMMARY

Smooth muscle derives its name from the fact that it lacks the striations characteristic of cardiac and skeletal muscle. Layers of smooth muscle cells line the walls of various organs and tubes, and the contractile function of smooth muscle is not under voluntary control. Contractile activity in smooth muscle is initiated by a Ca^{2+}-calmodulin interaction to stimulate phosphorylation of the light chain of myosin. A Ca^{2+} sensitization of the contractile proteins is signaled by the RhoA/Rho kinase pathway to inhibit the dephosphorylation of the light chain by myosin phosphatase, maintaining force generation. Removal of Ca^{2+} from the cytosol and stimulation of myosin phosphatase initiate the process of smooth muscle relaxation.

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