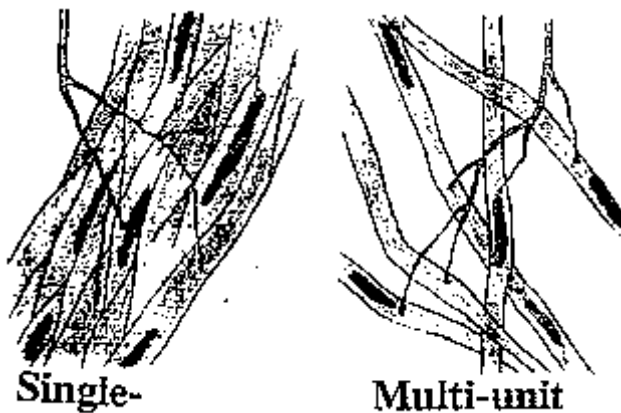


## SMOOTH MUSCLE

Smooth muscle is responsible for the contractility of hollow organs, such as blood vessels, the gastrointestinal tract, the bladder, or the uterus. Its structure differs greatly from that of skeletal muscle, although it can develop isometric force per cross-sectional area that is equal to that of skeletal muscle. However, the speed of smooth muscle contraction is only a small fraction of that of skeletal muscle.

**Structure:** The most striking feature of smooth muscle is the lack of visible cross striations (hence the name smooth). Smooth muscle fibers are much smaller (2-10  $\mu$  in diameter) than skeletal muscle fibers (10-100  $\mu$ ). It is customary to classify smooth muscle as single-unit and multi-unit smooth muscle (Fig. SM1). The fibers are assembled in different ways. The muscle fibers making up the single-unit muscle are gathered into dense sheets or bands. Though the fibers run roughly parallel, they are densely and irregularly packed together, most often so that the narrower portion of one fiber lies against the wider portion of its neighbor. These fibers have connections, the plasma membranes of two neighboring fibers form gap junctions that act as low resistance pathway for the rapid spread of electrical signals throughout the tissue. The multi-unit smooth muscle fibers have no interconnecting bridges. They are mingled with connective tissue fibers.



**Fig. SM1.** Single-unit and multi-unit smooth muscle.

Electron micrographs of smooth muscle reveal that the actin filaments are organized through attachment to the dense bodies that contain  $\alpha$ -actinin, a Z-band protein in skeletal muscle. Thus, it is assumed that the dense bodies function as Z-lines. The ratio of thin to thick filaments is much higher in smooth muscle (~15:1) than in skeletal muscle (~6:1). Smooth muscle is rich in intermediate filaments that contain two specific proteins, desmin and vimentin.

**Innervation and stimulation:** Smooth muscle is primarily under the control of autonomic nervous system, whereas skeletal muscle is under the control of the somatic nervous system. The single-unit smooth muscle has pacemaker regions where contractions are spontaneously and rhythmically generated. The fibers contract in unison, that is, the

single unit of smooth muscle is syncytial. The fibers of multi-unit smooth muscle are innervated by sympathetic and parasympathetic nerve fibers and respond independently from each other upon nerve stimulation.

Nerve stimulation in smooth muscle causes membrane depolarization, like in skeletal muscle. Excitation, the electrochemical event occurring at the membrane is followed by the mechanical event, contraction. In the case of smooth muscle, this excitation-contraction coupling is termed *electromechanical coupling*; the link for the coupling is  $\text{Ca}^{2+}$  that permeates from the extracellular space into the intracellular water of smooth muscle. There is another excitation mechanism in smooth muscle, which is independent of the membrane potential change; it is based on receptor activation by drugs or hormones followed by muscle contraction. This is termed *pharmacomechanical coupling*. The link is  $\text{Ca}^{2+}$  that is released from an internal source, the sarcoplasmic reticulum.

The role of mechanical events of smooth muscle in the wall of hollow organs is twofold: 1) Its tonic contraction maintains organ dimensions against imposed load. 2) Force development and muscle shortening, like in skeletal muscle.

Myofibril proteins: In general, smooth muscle contains much less protein (~110 mg/g muscle) than skeletal muscle (~200 mg/g). Notable is the decreased myosin content, ~20 mg/g in smooth muscle versus ~80 mg/g in skeletal muscle. On the other hand, the amounts of actin and tropomyosin are the same in both types of muscle. Smooth muscle does not contain troponin, instead of it there are two other thin filament proteins, caldesmon and calponin.

The amino acid sequence of smooth muscle actin is very similar to that of its skeletal muscle counterpart, and it seems likely that their three-dimensional structures are also similar. Smooth muscle actin combines with either smooth or skeletal muscle myosin. However, there is a major difference in the activation of myosin ATPase by actin, smooth muscle myosin has to be phosphorylated for actin-activation to occur.

The size and shape of the smooth muscle myosin molecule is similar to that of the skeletal muscle myosin (Fig. M1). There is a small difference in the light chain composition; out of the four light chains (LCs) of the smooth muscle myosin, two have molecular weight of 20,000 and two of 17,000. The 20,000 light chain is phosphorylatable. Upon phosphorylation of the light chain the actin-activated smooth muscle myosin ATPase increases about 50-fold, to about 0.16 mol ATP hydrolyzed per mol of myosin head per sec, at physiological ionic strength and temperature. (Under the same conditions, the actin-activated skeletal muscle myosin ATPase is 10-20 mol/mol/sec). The ionic strength dependence of smooth muscle myosin  $\text{Ca}^{2+}$ -activated ATPase also differs from that of skeletal muscle myosin (Fig. M5), increasing ionic strength increases the smooth muscle myosin ATPase but decreases the skeletal muscle myosin ATPase.

Four smooth muscle specific myosin heavy chain isoforms are known (described in Quevillon-Cheruel et al., 1999). Two isoforms (named SMB and SMA) are defined by

the presence or the absence of an insert of seven amino acids in the N-terminal globular head region. The absence of SMB myosin leads to decreased velocity of shortening and increased isometric force generation in various vascular muscles, indicating that SMB is a critical determinant of smooth muscle contraction (Babu et al., 2004).

The two other heavy chain isoforms (SM1 and SM2) differ at their C-termini by 43 versus 9 amino acids. To understand the role of the C-terminal extremities of SM1 and SM2 in smooth muscle thick filament assembly, various fragments of these myosins, such as the rod region, the rod with no tailpiece, or light meromyosins were prepared as recombinant proteins in bacterial cells (Rovner et al., 2002; Quevillon-Cheruel et al., 1999). The results showed that the smooth muscle myosin tailpieces differentially affect filament assembly and suggested that homogeneous thick filaments containing SM1 or SM2 myosin could serve distinct functions within smooth muscle cells.

Although the mechanism of thick filament assembly for purified smooth muscle myosins *in vitro* has been described, the regulation of thick filament formation in intact muscle is poorly understood. Cross-sectional density of the thick filaments measured electron microscopically in intact airway smooth muscle (Herrera et al., 2002) showed that the density increased substantially (144%) when the muscle was activated. In resting muscle, in the absence of  $\text{Ca}^{2+}$ , the filament density decreased by 35%. It appears that in smooth muscle filamentous myosin exists in equilibrium with monomeric myosin; activation favors filament formation.

Kathleen Trybus pioneered in expressing and purifying smooth muscle myosin subfragments using the baculovirus/insect cell expression system. This procedure and the methods needed to characterize the new proteins (gel assays, ATPase activity determinations, transient state kinetic parameters, and the *vitro* motility assay) are described in her review (Trybus, 2000). Studies on engineered smooth muscle myosin and heavy meromyosin showed: the interaction between the regulatory light chain domains on two heads is critical for regulation of smooth muscle myosin (Li et al., 2000; Sweeney et al., 2000), a long, weakly charged actin-binding loop is required for phosphorylation-dependent regulation of smooth muscle myosin (Rovner, 1998), and coiled-coil unwinding at the smooth muscle myosin head-rod junction is required for optimal mechanical performance (Lauzon et al., 2001). Work with myosin mutants demonstrated the actin-induced closure of the actin-binding cleft of smooth muscle myosin (Yengo et al., 2002). Both heads of native smooth muscle heavy meromyosin bind to actin, irrespective of regulatory light chain phosphorylation; one head binds rapidly and the second head binds more slowly (Ellison et al., 2003). Two-headed binding of the unphosphorylated non-muscle heavy meromyosin-ADP complex to actin was also reported (Kovács et al., 2004).

*In vitro*, both caldesmon and calponin are inhibiting the actin-activated ATPase activity of phosphorylated smooth muscle myosin. In case of calponin, this inhibitory activity is reversed by the binding of  $\text{Ca}^{2+}$ -calmodulin or by phosphorylation. Calponin is a 34-kDa protein containing binding sites for actin, tropomyosin and  $\text{Ca}^{2+}$ -calmodulin. Caldesmon is a long, flexible, 87-kDa protein containing binding sites for myosin, as well as actin, tropomyosin, and  $\text{Ca}^{2+}$ -calmodulin. Electron microscopy and three-dimensional image

reconstruction of isolated smooth muscle thin filaments revealed that calponin and caldesmon are located peripherally along the long-pitch actin helix (Hodgkinson et al., 1997; Lehman et al., 1997). The physiological role of caldesmon or calponin is not known.

#### Other proteins:

Calveolins: These are principal components of caveolar membranes, the invagination of the plasma membrane. Calveolins are scaffolding proteins that are involved in the signaling process. Signaling molecules that bind calveolins include G protein-coupled receptors, heterotrimeric G proteins, or receptor tyrosine kinases. Caveolin-1 regulates contractility in differentiated vascular smooth muscle (Je et al., 2004).

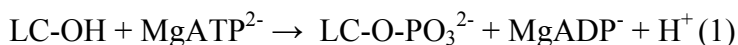
CHASM: is the abbreviation of Calponin Homology Associated Smooth Muscle. CHASM is a novel phosphoprotein that contains a calponin homology domain and shares sequence similarity with the smoothelin family of smooth muscle specific proteins. It modulates smooth muscle contractility (Borman et al., 2004).

Twitchin: This large protein (~530 kDa) regulates catch force maintenance in molluscan catch muscle. Two major phosphorylation sites, D1 and D2 were identified. Correlation of the phosphorylation state of twitchin, using antibodies specific for D1 and D2, with mechanical properties of the anterior byssus retractor muscle of the mussel *Mytilus* suggested that phosphorylation of both D1 and D2 is required for relaxation from the catch state (Funabara et al., 2003).

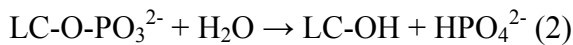
Sarcoplasmic reticulum (SR):  $\text{Ca}^{2+}$  release from SR in smooth muscle is initiated either via pharmaco-mechanical coupling due to the action of an agonist and involving  $\text{IP}_3$  receptors, or via E-C coupling, mostly involving L-type  $\text{Ca}^{2+}$  channels in the plasmalemma, (DHPRs), and ryanodine receptors (RyRs), or  $\text{Ca}^{2+}$  release channels of SR. Immunolabeling detected that two proteins of the SR: calsequestrin and RyR, and DHPR are colocalized with each other within peripherally sites in the calveolar domains (Moore et al., 2004).

## **Phosphorylation and Dephosphorylation of the 20-kDa Myosin Light Chain**

Myosin light chain kinase and myosin light chain phosphatase: Smooth muscle (as well as skeletal and cardiac muscle) contains myosin light chain kinase (MLCK), activated by  $\text{Ca}^{2+}$ -calmodulin, the enzyme which transfers the terminal phosphate group of ATP to serine (and/or threonine) hydroxyl groups of phosphorylatable light chain (LC) according to the following reaction:

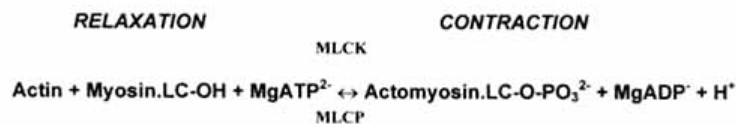


Dephosphorylation is brought about by smooth muscle myosin light chain phosphatase (MLCP) according to the following reaction:



The properties of MLCK are reviewed by Stull et al., (1996) and the properties of MLCP are reviewed by Hartshorne et al., (1998).

It is generally believed that LC phosphorylation-dephosphorylation controls the contraction-relaxation cycle of smooth muscle:



Protein kinases for myosin LC: (reviewed by Murthy, 2006; Ratz et al., 2005) The Ca<sup>2+</sup>- and calmodulin-dependent MLCK is the key enzyme for smooth muscle contraction. The Ca<sup>2+</sup>-independent MLCK is called ZIP kinase, which phosphorylates both .Ser<sup>19</sup> and Thr<sup>18</sup> residues in MLC. ZIP kinase cloned from rat aorta is homologous (49%) to smooth muscle Ca<sup>2+</sup>-dependent MLCK, but unlike MLCK, it has neither an autoinhibitory nor a calmodulin binding region. ROCK II is a member of the 20-kDa Rho-kinases, which belong to a family of serine/threonine protein kinases that includes myotonic dystrophy kinase, Cdc42-binding kinase, and citron kinase. All these kinases possess an NH<sub>2</sub>-terminal kinase domain, a coiled coil region, and other functional motifs at the carboxyl end.

Protein phosphatases for myosin LC: MLCP is composed of three subunits: a catalytic subunit of 37-38-kDa of the type 1 phosphatase, a subunit of about 20-kDa whose function is not known, and a larger 110-130-kDa subunit that targets MLCP to myosin. The phosphatase activity of the catalytic subunit is low and it is enhanced significantly by addition of the targeting subunit. Upon phosphorylation of serine and threonine residues in the targeting subunit, its activating effect on the catalytic subunit is lost, and thereby the MLCP holoenzyme is inhibited. Hartshorne et al., (2004) provided a comprehensive review of this complex field. Moreover, they added new information in their subsequent papers (Wooldridge et al., 2004; Murányi et al., 2005). Crystal structures about myosin phosphatase were reported by Terrak et al., (2004).

Several reports (Feng et al., 1999; Kaibuchi et al., 1999; Nagumo et al., 2000; Somlyo and Somlyo, 2000; Sward et al., 2000) indicate that in smooth muscle a Rho-regulated system of MLCP exists. Rho-kinase is the major player in this system; the enzyme phosphorylates the 130-kDa myosin binding subunit of MLCP and thereby inhibits MLCP activity. Due to the antagonism between MLCK and MLCP, inhibition of MLCP results in an increase in the phosphoryl content of LC with concomitant increase in muscle force. Under these conditions, submaximal Ca<sup>2+</sup>-levels are sufficient for maximal force, a phenomenon called increased Ca<sup>2+</sup>-sensitivity (Somlyo and Somlyo, 1994). Specific inhibitors for Rho-kinase Y-27632 (Feng et al., 1999; Kaibuchi et al., 1999), and HA-1077 (Nagumo et al., 2000; Sward et al., 2000) are available. Furthermore, a myosin phosphatase-rho interacting protein was described (Surks et al., 2003).

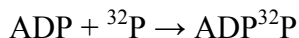
MLCP activity can also be inhibited by a 17-kDa myosin phosphatase inhibitor protein, called CPI-17, (Kitazawa et al., 2000), which inhibits the catalytic subunit of MLCP and the holoenzyme MLCP. Phosphorylation of CPI-17 at Thr38 increases its inhibitory potency 1000-fold. The solution NMR structure of CPI-17 has been determined (Ohki et al., 2001), it forms a novel four-helix. Phosphorylation of Thr38 induces a conformational change involving displacement of one helix without significant movement of the other three helices. Rho-kinases and PKC are responsible for the phosphorylation of CPI-17. Subsequent work by Kitazawa et al., (2004) delineated that CPI-17 is essential for G protein/PKC-mediated  $\text{Ca}^{2+}$  sensitization in smooth muscle. The novel observation was made that integrin-linked kinase phosphorylated CPI-17 (Ti Deng et al., 2002).

A rich array of second messengers regulate MLCP activity under physiological and pathological conditions (Solaro, 2000) through phosphorylation of either the targeting subunit of MLCP or CPI-17.

Regulation of  $\text{Ca}^{2+}$ -sensitivity by KCl: (Ratz et al., 2005). KCl is a classical agent to induce smooth muscle contraction. It activates the voltage-dependent  $\text{Ca}^{2+}$  channels that leads to increase in cytoplasmic  $\text{Ca}^{2+}$  concentration, followed by activation of the  $\text{Ca}^{2+}$ -calmodulin dependent MLCK, phosphorylation of MLC, and contraction. The mechanism of the KCl caused  $\text{Ca}^{2+}$ -sensitization is a complex one, involving multiple cell signalings such as translocation of proteins from cytoplasm to plasma membrane, and activating of enzymes such as RhoA kinase and protein kinase C.

“Dick Murphy: three decades as the touchstone of smooth muscle physiology”: (Dillon, 2004). As one of the dominant figures in smooth muscle physiology from 1974 – 2004, Professor Richard A. Murphy developed the latch theory, the slow detachment of dephosphorylated crossbridges, that has been a major focus in smooth muscle research. At the time of his retirement, he added to the latch hypothesis the cooperative activation of crossbridges and has built a model which required both cooperative activation and latch-bridge formation to predict force (Rembold, 2004).

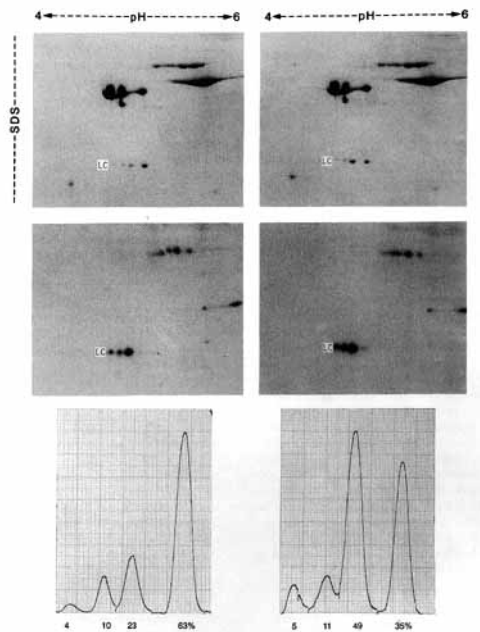
Myosin light chain phosphorylation followed by  $^{32}\text{P}$ -labeling in intact smooth muscle: When a dissected smooth muscle, e.g. artery or a uterine strip, is incubated at  $37^\circ\text{C}$  in physiological salt solution containing radioactive inorganic phosphate, the  $^{32}\text{P}$  permeates the plasma membrane and enters the cytoplasm of the muscle. Through the oxidative phosphorylation mechanism the  $^{32}\text{P}$  incorporates into the terminal P group of ATP:



Transfer of the terminal  $^{32}\text{P}$  of ATP to LC-OH by MLCK (equation 1) yields the radioactive LC-O- $^{32}\text{PO}_3^{2-}$  species that can be isolated and quantified. The isolation involves two-dimensional (2D) gel electrophoresis and the quantification requires measuring the specific radioactivity of the terminal  $^{32}\text{P}$  of ATP.

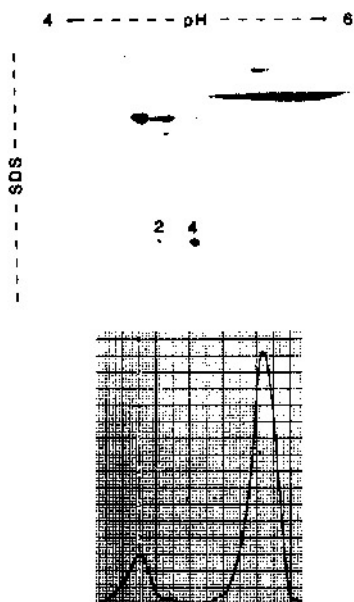
Smooth muscle contraction is correlated with LC phosphorylation (reviewed by Bárány and Bárány, 1996c). Fig. SM2 illustrates an experiment: Two carotid arteries were dissected from freshly killed pigs and labeled with  $^{32}\text{P}$ . One artery was contracted with KCl for 30 sec then frozen in liquid nitrogen, while the other artery was frozen in the resting state. The arteries were pulverized, washed with perchloric acid (PCA) to precipitate the muscle proteins and remove  $^{32}\text{P}$ -containing phosphate metabolites from the muscle. The washed residue was neutralized with a NaOH solution then dissolved in sodium dodecyl sulfate (SDS). After centrifugation at high speed to remove insoluble particles, the protein content of the supernatant was determined and aliquots of 360  $\mu\text{g}$  protein were subjected to 2D polyacrylamide gel electrophoresis. This procedure separates the proteins according to their charge (pH 4-6) in the first dimension and according to their size (SDS) in the second dimension. After staining, the profile of the arterial proteins appeared, shown in the upper row of Fig. SM2. LC is in the lower middle part of the gel, it contains multiple spots. The LC spots were scanned, the staining intensities are shown in the lower row of the Figure. The radioactive spots on the gel were detected by autoradiography, the middle row of Fig. SM2 shows the black spots on the film corresponding to the radioactive spots on the gel.

Visual inspection of the radioactive LC spots in the Figure shows much more radioactivity in LC from the contracting muscle (*right*) than from the resting muscle (*left*). One can calculate the incorporation of the  $^{32}\text{P}$ -phosphate into LC as follows. First one has to determine the specific radioactivity of the terminal  $^{32}\text{P}$  of ATP from the muscle. The  $\text{ATP}^{32}$  is in the PCA extract of the frozen and pulverized muscle (see previous paragraph), and its specific radioactivity is determined (Bárány and Bárány 1996c). The next step is the determination of the radioactivity in LC: the gel spots are excised, digested with  $\text{H}_2\text{O}_2$ , and after the gel is dissolved, radioactivity (counts per minute) is measured. The extent of LC phosphorylation can be calculated from the radioactivity in the LC spots and in the terminal phosphate of  $\text{ATP}^{32}$ , from the total protein applied onto the gel, and from the LC content of the total protein (Bárány and Bárány, 1996c). Such a calculation shows that under conditions of Fig. SM2, the LC of the resting muscle contained 0.25 mol  $^{32}\text{P}$ -phosphate/mol LC, whereas the LC of the contracting muscle contained 0.70 mol. Thus, 0.45 mol  $^{32}\text{P}$ -phosphate was transferred by MLCK from the terminal phosphate of  $\text{ADP}^{32}\text{P}$  to free LC-OH groups as the result of muscle contraction.



**Fig. SM2.** Light chain phosphorylation during smooth muscle contraction as studied by 2D gel electrophoresis (Bárány and Bárány, 1996a, with permission from *Biochemistry of Smooth Muscle Contraction*, 1996, Academic Press). *Left*,  $^{32}\text{P}$ -labeled porcine carotid arterial muscle was frozen at rest. *Right*,  $^{32}\text{P}$ -labeled porcine carotid arterial muscle was frozen 30 sec after 100 mM KCl challenge. Upper panel shows the Coomassie blue staining pattern of the arterial proteins; middle panel shows the corresponding autoradiograms; bottom panel shows the corresponding densitometric scans of LC.

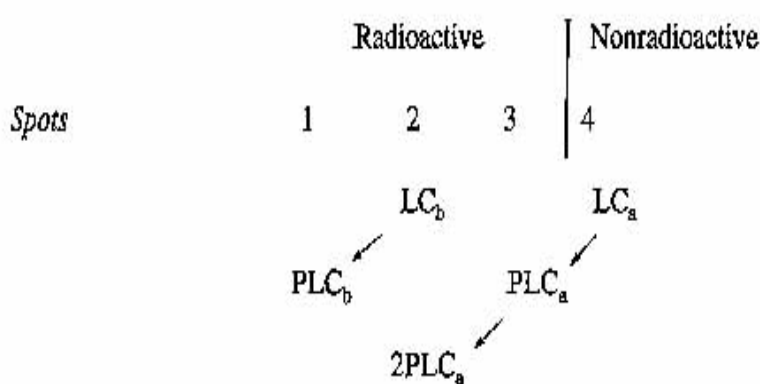
Isoforms of the 20-kDa myosin light chain: Protein isoforms have the same size but different charge. They are generated either by protein modification or genetic alteration. Protein phosphorylation is the physiological protein modification, because phosphorylation of a protein increases its negative charge. Thus, LC has at least two isoforms, a non-phosphorylated and a phosphorylated one. Genetic alteration changes the amino acid composition of a protein, thereby providing at least two isoforms. For instance, completely dephosphorylated LC exhibits two spots on 2D gels (Fig. SM3) with a percentage distribution of 85% and 15%, corresponding to the major and minor LC isoforms.



**Fig. SM3.** Myosin light chain isoforms as analyzed by 2D gel electrophoresis. LC was dephosphorylated by homogenizing porcine carotid arteries in 150 mM NaCl and 1 mM EGTA, followed by incubation at 25°C for 2 hours. *Top*, stained gel, LC spots are numbered as 2 and 4, corresponding to their isoform number. *Bottom*, densitometric tracing of the LC spots.

If both major and minor LC are phosphorylated, that results in four isoforms (two non-phosphorylated and two phosphorylated). In Fig. SM2 *on top level*, four LC isoforms are seen; *on middle level*, the autoradiograms reveal that three spots are radioactive. The non-radioactive, most basic spot, corresponds to the major isoform of LC (Fig. SM3).

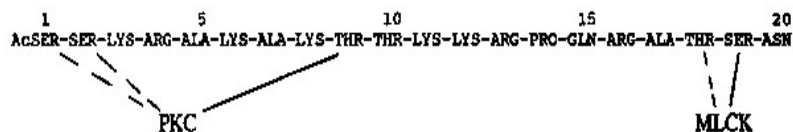
Figure SM4 illustrates the formation of LC isoforms as a result of phosphorylation. The major isoform ( $LC_a$ ) when mono-phosphorylated ( $PLC_a$ ) moves into Spot 3, and when it is di-phosphorylated ( $2PLC_a$ ) moves into Spot 2. The same Spot 2 also contains the non-phosphorylated minor isoform ( $LC_b$ ), thus the comigration of the di-phosphorylated LC isoform with the minor isoform makes Spot 2 radioactive. This explains why out of the four LC spots three are phosphorylated. The mono-phosphorylated minor isoform ( $PLC_b$ ) moves into Spot 1, which is the most acidic spot.



**Fig. SM4.** Scheme for the explanation of four stained and three radioactive LC spots, shown on Fig. SM2 (Bárány and Bárány, 1996a, with permission from Biochemistry of Smooth Muscle Contraction, 1996, Academic Press).

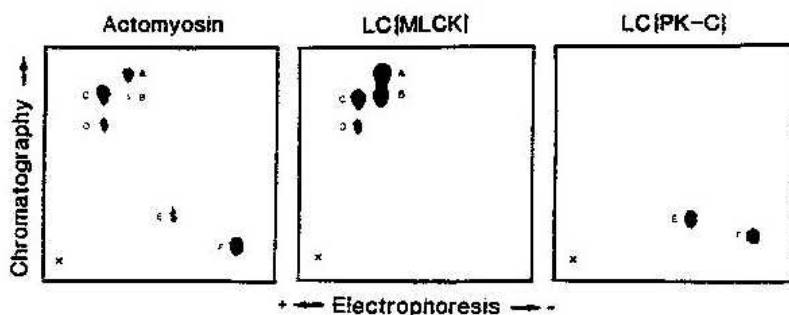
The last row of Fig. SM2 shows the muscle contraction-induced changes in the staining intensity of LC spots. In the resting muscle the last peak, the non-phosphorylated major isoform contains as much as 63% of the total intensity, which decreases to 35% in the contracting, muscle. At the same time, the 23% intensity in the preceding peak of the resting muscle increases to 49% upon contraction. This is an example of a simple (but not sensitive) method to follow changes in LC phosphorylation during contraction, without using  $^{32}P$ -labeling of the muscle.

**Phosphorylation site:** The amino acid sequence of LC exhibits a similarity among LCs from various smooth muscles. Such a conservative sequence suggests a functional significance for the protein. The phosphorylation sites are located at the amino terminal part of the LC molecule, shown in Fig. SM5. Ser<sup>19</sup> is the site that is phosphorylated by MLCK in the intact muscle. Thr<sup>18</sup> is phosphorylated by MLCK rarely, but ZIP kinase phosphorylates both Ser<sup>19</sup> and Thr<sup>18</sup>. Protein kinase C (PKC) phosphorylates LC at Ser<sup>1</sup>, Ser<sup>2</sup>, and Thr<sup>9</sup>.



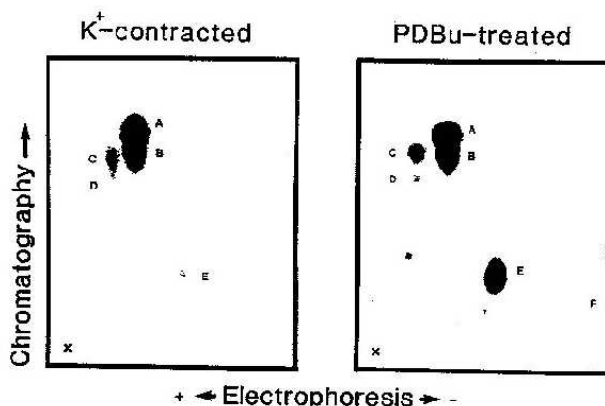
**Fig. SM5.** Phosphorylation sites of LC.

**Two-dimensional tryptic peptide mapping:** Phosphopeptide maps differentiate MLCK-catalyzed LC phosphorylation from that catalyzed by PKC (Erdodi et al., 1988). Fig. SM6 illustrates the experiment: With  $\text{ADP}^{32}\text{P}$  as a substrate, pure LC was phosphorylated either by MLCK (middle panel), or PKC (right panel). Actomyosin that contains endogenous LC, MLCK, and PKC, was also phosphorylated (left panel). The  $^{32}\text{P}$ -LC was isolated by 2D gel electrophoresis, digested by trypsin, and the peptides were separated by 2D peptide mapping. The map of LC phosphorylated by MLCK exhibits four peptides: A, B, both containing serine residues, corresponding to  $\text{Ser}^{19}$ , and C, D, both containing threonine, corresponding to the  $\text{Thr}^{18}$ . When LC is phosphorylated by PKC, the map exhibits two peptides: E, containing serine, corresponding to  $\text{Ser}^1$  or  $\text{Ser}^2$ , and F, containing threonine, corresponding to the  $\text{Thr}^9$ . When LC is phosphorylated in actomyosin, peptides characteristic for both MLCK and PKC phosphorylation are present.



**Fig. SM6.** Autoradiograms of 2D phosphopeptide maps of LC tryptic digests.

Based on two-dimensional peptide mapping, one can identify the enzymes that phosphorylate LC during smooth muscle contraction (Fig. SM7). The phosphopeptide pattern of LC from  $\text{K}^+$ -contracted artery (*left part*) is practically identical with that of LC {MLCK} pattern shown in Fig. SM6; thus, the conclusion is clear, MLCK is the main enzyme that phosphorylates LC during the  $\text{K}^+$ -contraction. In contrast, when the muscle is contracted with phorbol dibutyrate (PDBu), an activator of PKC, the LC pattern (*right part*) resembles that of Actomyosin shown in Fig. SM6, thus, both enzymes, MLCK and PKC phosphorylate LC during the PDBu-contraction. We determined the contribution of each enzyme to the phosphorylation, by densitometry of the spots, and by counting the radioactivity in the spots; the results showed about 35% contribution by PKC.



**Fig. SM7.** Phosphopeptide maps of LC from  $\text{K}^+$ -contracted muscle versus PDBu-treated muscle.

Need for  $\text{Ca}^{2+}$  and calmodulin for light chain phosphorylation in intact smooth muscle: In vitro the  $\text{Ca}^{2+}$  complexed to calmodulin is the activator of MLCK which phosphorylates MLC. Intact smooth muscle cease contracting when  $\text{Ca}^{2+}$  is omitted from the bathing solution, or when it is complexed with EGTA. Furthermore, inhibitors of calmodulin, such as trifluoperazine or chlorpromazine inhibit smooth muscle contraction.

In the resting muscle there is about  $0.1 \mu\text{M}$   $\text{Ca}^{2+}$ , upon stimulation the  $\text{Ca}^{2+}$  concentration increases about 100-fold through *electromechanical or pharmacomechanical coupling*. It is conventional to use fluorescent indicators to follow changes in the intracellular  $\text{Ca}^{2+}$  concentration immediately after the stimulation and during the plateau of the mechanical activity. Large variations are reported, depending on the nature of the smooth muscle, the tissue preparation, or the drug used. However, all investigators agree that in order to elicit relaxation the  $\text{Ca}^{2+}$  level in the sarcoplasm must be returned near to the resting value. Two mechanisms participate in decreasing the  $\text{Ca}^{2+}$  level: 1) The plasma membrane  $\text{Ca}^{2+}$  transporting ATPase pumps  $\text{Ca}^{2+}$  from the inside into the extracellular space. 2) The sarco(endo)plasmic reticulum  $\text{Ca}^{2+}$  transporting ATPase pumps  $\text{Ca}^{2+}$  into the SR.

Stretch-induced light chain phosphorylation: Smooth muscle can be stimulated electrically, by chemical agents, or by stretch, a mechanochemical activation. Stretching of arterial or uterine muscles induced light chain phosphorylation to the same extent as was observed in muscles contracted by  $\text{K}^+$  or norepinephrine (Bárány and Bárány, 1996c). Muscles which were stretched 1.6 times their resting length did not develop tension, but contracted normally when the stretch was released and the muscles were allowed to return to their rest length. Importantly, this contraction was spontaneous, indicating that the stretch-induced activation carries all the information necessary for normal contraction. Mobilization of  $\text{Ca}^{2+}$  was necessary for the stretch-induced light chain phosphorylation and contraction to occur. When EGTA (the strong  $\text{Ca}^{2+}$  complexing agent) was added to the muscle bath both the stretch-induced phosphorylation and the stretch-release-induced tension were inhibited; however, upon removal of EGTA by washings, both processes were fully restored. Treatment of the muscle with chlorpromazine also abolished both the stretch-induced LC phosphorylation and the stretch-release-induced tension development. These results suggest the presence of mechanosensitive receptors in smooth muscle that are interacting with  $\text{Ca}^{2+}$  release channels in SR.

## **Phosphorylation of Heat-Shock Proteins**

Low molecular weight heat shock proteins are phosphorylated in smooth muscle: A 27-28-kDa protein is phosphorylated in various intact smooth muscles and smooth muscle cells (reviewed in Bárány and Bárány, 1996c). Cyclic nucleotide-dependent vasorelaxation is associated with the phosphorylation of a 20-kDa heat shock protein, called HSP20 (Beal et al., 1997; Rembold et al., 2000). It was found that HSP20 is an actin-associated protein (Brophy et al., 1999; Rembold et al., 2000) suggesting that smooth muscle relaxation may be brought about by the binding of the phosphorylated HSP20 to the actin filaments. However, subsequent work did not support the idea that

phosphoryl-HSP20 suppresses force by inactivation of the thin filaments (Meeks et al., 2005).

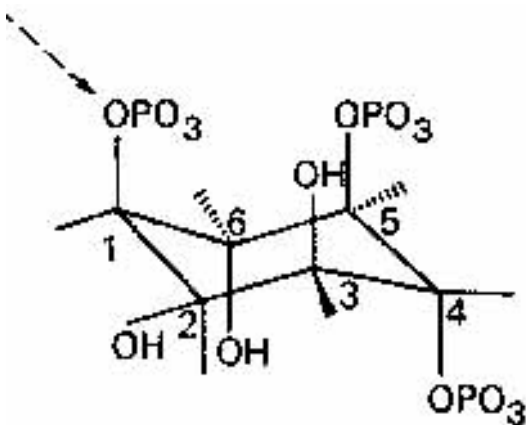
In agonist-induced contraction of smooth muscle, HSP27 undergoes a rapid phosphorylation that strengthens its interaction with tropomyosin. This raises the possibility that in smooth muscle the movement of TM in the groove of actin filaments is initiated by HSP27P (Somara and Bittar, 2004).

## Signal Transduction

The binding of an agonist (e.g. norepinephrine or oxytocin) to the surface receptor of smooth muscle induces a signal that spreads from the outside to the inside of the plasma membrane and activates several effectors that ultimately initiate contraction. There are three components of this system that we discuss: 1) Inositol 1,4,5-trisphosphate, 2) G-proteins, 3) Phosphoinositide-specific phospholipase C.

Inositol 1,4,5-trisphosphate: The inositol ring contains six hydroxyl residues; most of them can be phosphorylated by specific kinases. Inositol 1-monophosphate is the constituent of phosphatidylinositol (PI) one of the phospholipids in animal cell membranes. PI 4-kinase and PI (4) P 5-kinase generate PI (4) P and PI (4,5) P<sub>2</sub>, respectively, by sequentially phosphorylating PI. Inside the cell membrane resides a phosphoinositide specific phospholipase C, one of its hydrolytic product is inositol 1,4,5-trisphosphate (IP<sub>3</sub>), (see Fig. SM 8).

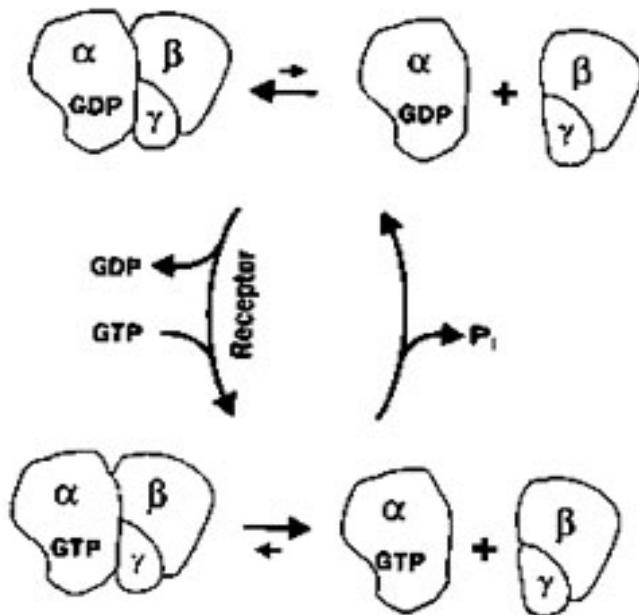
Inositol 1,4,5-trisphosphate receptor: The three dimensional structure of the inositol 1,4,5-trisphosphate receptor has been determined (Serysheva et al., 2003). The channel structure exhibits a 4-fold symmetry and comprises two morphologically distinct regions: a large pinwheel and a smaller square. The pinwheel region has four radial curved spokes interconnected by a central core.



**Fig. SM8.** *D-myoinositol* 1,4,5-trisphosphate (Bárány and Bárány, 1996b, with permission from *Biochemistry of Smooth Muscle Contraction*, 1996, Academic Press). The arrow indicates the site of the ester link with diacylglycerol in phosphatidylinositol. The negative charge of the phosphate group is not indicated.

G-proteins: The guanine nucleotide binding proteins (G-proteins) are heterotrimers consisting of  $\alpha$ -,  $\beta$ - and  $\gamma$ -subunits. The  $\alpha$ -subunits appear to be most diverse and are believed to be responsible for the specificity of the interaction of different G-proteins with their effectors. Fig. SM9 depicts a simple model for the activation of G-proteins. In

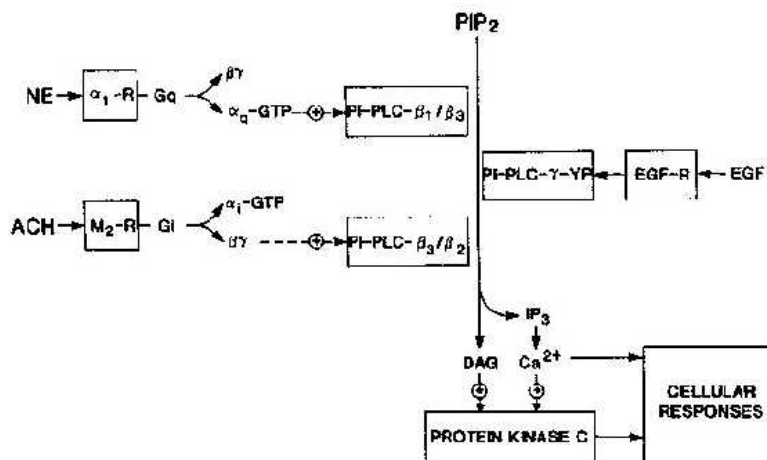
the basal state, the  $\alpha$ -subunit contains bound GDP and association of  $\alpha$ - and  $\beta$   $\gamma$ -subunits are highly favored, keeping the G-protein in the inactive form. Stimulation of the G-protein results when it binds GTP rather than GDP. Receptors interact most efficiently with the heterotrimeric form of the G-protein and accelerate activation by increasing the rate of dissociation of GDP and enhancing the association of GTP. Activation of G-protein coupled receptor results in the dissociation of heterotrimeric G-proteins into  $\alpha$ -subunits and  $\beta$   $\gamma$ -dimers. Finally, the G-protein  $\alpha$ -subunit has an intrinsic hydrolytic activity that slowly converts GTP to GDP and returns the G-protein to its inactive form.



**Fig. SM9.** Model for the activation of G-proteins (Bárány and Bárány, 1996b, with permission from *Biochemistry of Smooth Muscle Contraction*, 1996, Academic Press).

Phosphoinositide-specific phospholipase C: This term refers to a family of enzymes all specific for the phosphoinositide moiety of the phosphatidylinositol, but differing in their specificity depending on the number of the phosphoryl groups on the inositol ring. The  $\beta$ -,  $\gamma$ - and  $\delta$ -isoforms of PI-phospholipase C (PI-PLC) show the greatest specificity for the trisphosphorylated phospholipid (PIP<sub>2</sub>). There are two basic mechanisms by which agonists activate PIP<sub>2</sub> hydrolysis (Fig. SM10). In case of hormones, neurotransmitters, and certain other agonists, the signal is transduced to  $\beta$ -isozymes of PI-PLC. The upper left row of Fig. SM10 shows the most common pathway for PI-PLC  $\beta$ -isozyme activation, initiated by stimulation of a  $\alpha_1$ -adrenergic receptor ( $\alpha_1$ -R) with norepinephrine (NE), and involving G  $\alpha_q$ -proteins. The lower left row shows the activation of PI-PLC-  $\beta$  isoforms, initiated by acetylcholine (ACH) stimulation of M<sub>2</sub>-muscarinic receptor (M<sub>2</sub>-R), and mediated by the  $\beta$   $\gamma$ -subunit of the pertussis toxin-sensitive G-protein (G<sub>i</sub>). Concerning the other basic activating mechanism, e.g. in the case of growth factors, activation of their receptors results in enhanced tyrosine kinase activity. The right part of Fig. SM10 shows the activation of PI-PLC-  $\gamma$  isoforms, initiated by the binding of epidermal growth factor (EGF) to its receptors, and executed by the tyrosine phosphorylation (YP) of PI-PLC-  $\gamma$ . In all three examples, the activated PI-PLC hydrolyzes PIP<sub>2</sub> to form the messengers IP<sub>3</sub> and diacylglycerol (DAG). IP<sub>3</sub> releases Ca<sup>2+</sup> from the sarcoplasmic reticulum and

thereby initiates smooth muscle contraction. DAG activates protein kinase C, the exact result of this activation is not known at the cellular level.



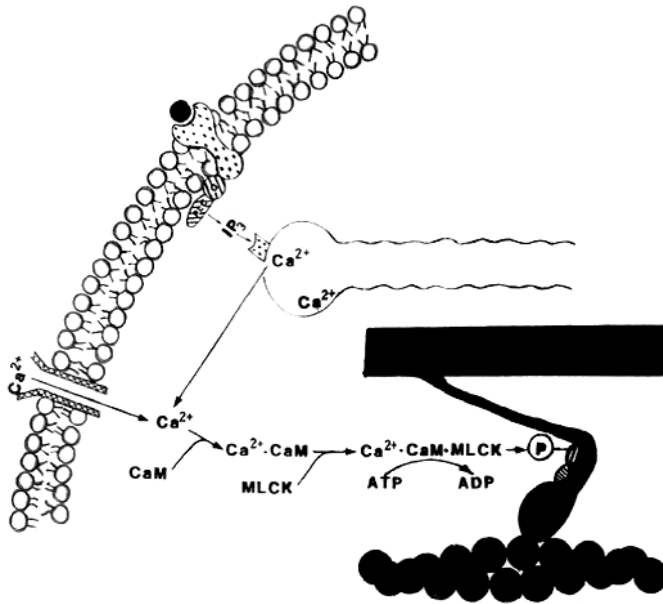
**Fig. SM10.** Pathways for activation of PI-PLC isoforms (Bárány and Bárány, 1996b, with permission from *Biochemistry of Smooth Muscle Contraction*, 1996, Academic Press).

Signaling for contraction in smooth muscle of the gut is reviewed by Murthy (2006).

## The Contractile Event of Smooth Muscle

A scheme for smooth muscle contraction is shown in Fig. SM11. Contraction is initiated by the increase of Ca<sup>2+</sup> in the myoplasm; this happens in the following ways:

1. Ca<sup>2+</sup> may enter from the extracellular fluid through channels in the plasmalemma. These channels open, when the muscle is electrically stimulated or the plasmalemma is depolarized by excess K<sup>+</sup>.
2. Due to agonist induced receptor activation, Ca<sup>2+</sup> may be released from the sarcoplasmic reticulum (SR). In this pathway, the activated receptor interacts with a G-protein (G) which in turn activates phospholipase C (PLC). The activated PLC hydrolyzes phosphatidyl inositol biphosphate; one product of the hydrolysis is inositol 1,4,5-trisphosphate (IP<sub>3</sub>). IP<sub>3</sub> binds to its receptor on the surface of SR, this opens Ca<sup>2+</sup> channels and Ca<sup>2+</sup> from SR is entering the myoplasm.
3. Ca<sup>2+</sup> combines with calmodulin (CaM) and the Ca<sup>2+</sup>-CaM complex activates MLCK, which in turn phosphorylates LC. The phosphorylated myosin filament combines with the actin filament and the muscle contracts.



**Fig. SM11.** A scheme for smooth muscle contraction (Bárány, 1996, with permission from *Biochemistry of Smooth Muscle Contraction*, 1996, Academic Press).

Two books (Bárány, 1996; Kao and Carsten, 1997) and a special journal issue (Murphy, 1999) are recommended for further studying the mechanism of smooth muscle contraction and relaxation.

## Mechanism of Smooth Muscle Contraction

### Activation of the Arp2/3 Complex by N-WASP during Smooth Muscle Contraction

Zhang et al., (2005) made the important discovery that the neuronal Wiskott-Aldrich syndrome protein (N-WASP)-mediated activation of the actin-related protein 2 and 3 (Arp2/3) complex regulates actin polymerization and tension development initiated by muscarinic stimulation in canine tracheal smooth muscle tissues. Plasmids encoding EGFP-tagged wild-type N-WASP, the N-WASP VCA and CA domains, or enhanced green fluorescent protein were introduced into tracheal smooth muscle strips by reversible permeabilization, and the tissues were incubated for two days to allow for expression of the proteins. Expression of the CA domain inhibited actin polymerization and tension development in response to ACh, whereas expression of the wild type N-WASP, the VCA domain, or EGFP did not. The increase in MLC phosphorylation in response to contractile stimulation was not affected under these conditions. This proves that the N-WASP-mediated activation of the Arp2/3 complex is for actin polymerization and tension development.

Simultaneously with these experiments, the Gunst's laboratory has also shown that the adapter protein CrkII regulates neuronal Wiskott-Aldrich syndrome protein, actin polymerization, and tension development during contractile stimulation of smooth muscle

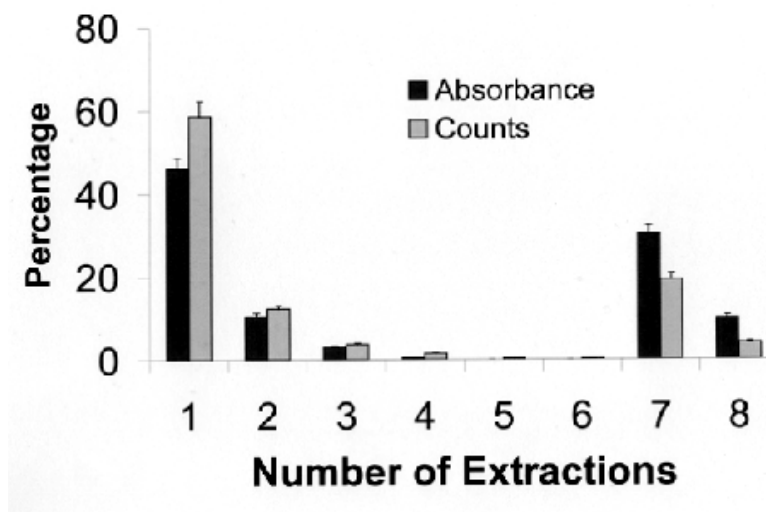
(Tang et al., 2005). Previously, the same physiological effect was found for the small GTPase, Cdc43 (Tang and Gunst, 2004).

In continuation of their creative work, Zhang and Gunst (2006) discovered a dynamic association between  $\alpha$ -actinin and  $\beta$ -integrin as a regulatory mechanism for contraction in canine tracheal smooth muscle. ACh stimulation of the tracheal muscle causes the rapid recruitment of  $\alpha$ -actinin to  $\beta$ -integrin complexes at the membrane, and these complexes are necessary for active tension development in the smooth muscle.

### Monomer (G) to Polymer (F) Transformation of Actin in Smooth Muscle

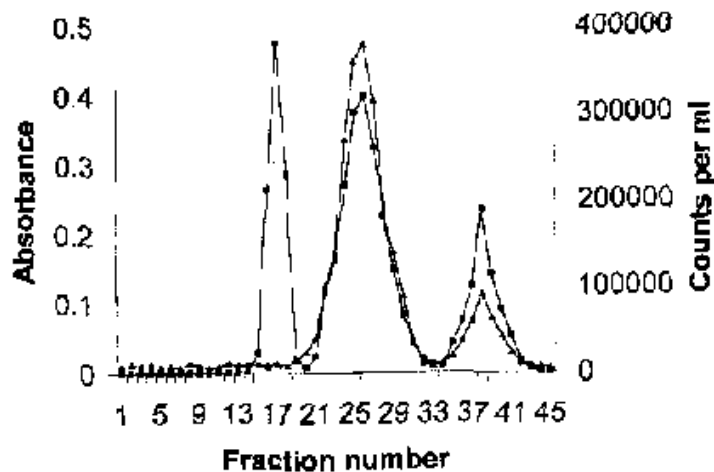
Mehta and Gunst (1999) and Jones et al., (1999) reported the existence of G-actin in smooth muscle, based on the method of DNase I inhibition and phalloidin staining, respectively. Subsequently, Bárány, et al., (2001) showed the exchange of the actin-bound nucleotide in intact smooth muscle. This was based on the separation of the actin bound nucleotides from the cytoplasmic nucleotides with 50% ethanol (Fig. SM12):

$^{32}\text{P}$ -labeled arteries were extracted in the refrigerator with 50% ethanol six times for elutions of the nucleotides (Absorbance at 260 nm) and the  $^{32}\text{P}$ -label (Counts). The absorbance and the counts are very high in the first extract and drop in the successive five extractions virtually to zero. In the 7th and 8th elution the muscles were extracted with 1.5% perchloric acid (PCA). New absorbance and radioactivity appeared, corresponding to the release of the bound-nucleotide and the associated  $^{32}\text{P}$ -label from actin.



**Fig. SM12.** Extraction of nucleotides and radioactivity from  $^{32}\text{P}$ -labeled arterial smooth muscles (From Bárány et al., 2001). The percentage of the total absorbance and counts eluted from the muscles in 8 extractions is shown on the ordinate.

The composition of the PCA extract is shown on Fig. SM13.



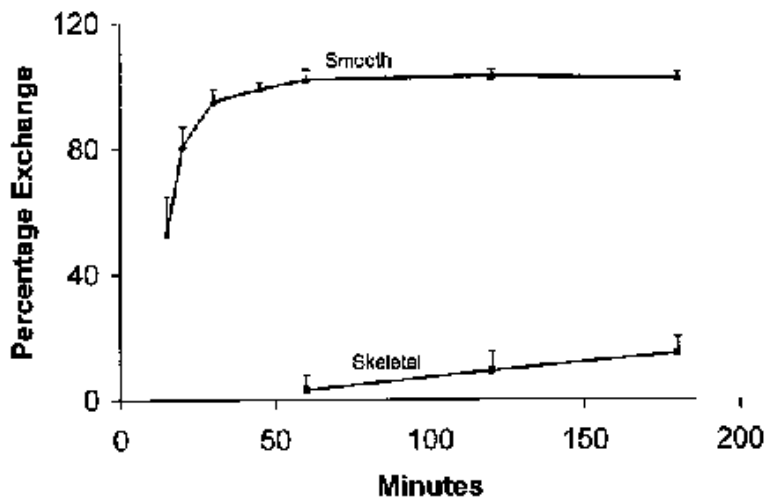
**Fig. SM13.** Dowex -1 chromatography of Extracts No. 7 and 8, shown in Fig. SM12 (From Bárány et al., 2001). Squares correspond to Counts per ml and triangles correspond to Absorbance.

Three radioactive peaks are seen, the first from  $P_i$  the second from ADP, and the third from ATP. For ADP and ATP the absorbance is also shown; 85% of the total absorbance is from ADP and 15% from ATP. Since F-actin contains bound-ADP and G-actin contains bound-ATP, the data indicate that 85% of the total actin in this muscle preparation was in the polymeric and 15% in the monomeric form. Determination of the  $P_i$  concentration by a colorimetric method showed that the molar ratio of  $P_i$  to ADP was close to one.

In order to quantify the extent of exchange of the actin-bound nucleotide and  $P_i$ , one has to determine their specific activity (counts/min/mol nucleotide or  $P_i$ ) and compare it with those of the specific activities (s.a.) of the gamma- and beta-phosphates of the cytoplasmic ATP and that of PCr (Bárány et al., 2001). With this knowledge one can calculate the percentage exchange for each of the actin components; for instance, the percentage exchange of the actin-bound- ADP is:

$$(\text{s.a. of actin-ADP} / \text{s.a. of beta-P of cytoplasmic ATP}) \times 100$$

Fig. SM14 compares the exchange of the actin-bound ADP between smooth and skeletal muscles. The exchange is rapid in smooth muscle, half-time about 15 min, whereas the exchange is slow in skeletal muscle, about 15% in three hours, in agreement with the studies of Martonosi et al., (1960), in live animals.



**Fig. SM14.** Time course of the exchange of the actin-bound ADP in smooth (porcine carotid artery) and skeletal (rat vastus lateralis) muscles (From Bárány et al., 2001).

#### Characteristics of the exchange of the actin-bound nucleotide in smooth muscle:

ATP is a prerequisite for the exchange to take place. If ATP synthesis is inhibited by azide or iodoacetamide the exchange is also inhibited. If ATP synthesis is reduced, by incubation of the muscles with deoxyglucose, instead of glucose, the exchange is also reduced.

$\text{Ca}^{2+}$  is not required for the exchange, i.e. full exchange is observed in the muscle in the presence of EGTA.

Several smooth muscles, arteries, uteri, urinary bladder, and stomach exhibited the exchange of the actin-bound nucleotide and phosphate, suggesting that the exchange is a property of every smooth muscle.

Upon contraction of smooth muscle, the exchange of the bound-nucleotide and phosphate decreased, and upon relaxation from the contracted state it increased, suggesting that polymerization-depolymerization of actin is a part of the contraction-relaxation cycle of smooth muscle.

Suggested readings: Murthy (2006); Ratz et al., (2005); Somlyo and Somlyo (2004).

### References

Babu, G.J., Pyne, G.J., Zhou, Y., Okwuchukwasanya, C., Brayden, J.E., Osol, G., Paul, R.J., Low, R.B., and Periasamy, M. (2004). Isoform switching from SM-B to SM-A myosin results in decreased contractility and altered expression of thin filament regulatory proteins. *Am J Physiol Cell Physiol.*, **287**, C723-C729.

Bárány, M. (1996). *Biochemistry of Smooth Muscle Contraction*. Academic Press.

- Bárány, K. and Bárány, M. (1996a). Myosin light chains. *In Biochemistry of Smooth Muscle Contraction* (M. Bárány, ed.), pp. 21-35, Academic Press.
- Bárány, M. and Bárány, K. (1996b). Inositol 1,4,5-trisphosphate production. *In Biochemistry of Smooth Muscle Contraction* (M. Bárány, ed.), pp. 269-282, Academic Press.
- Bárány, M. and Bárány, K. (1996c). Protein phosphorylation during contraction and relaxation. *In Biochemistry of Smooth Muscle Contraction* (M. Bárány, ed.), pp. 321-339, Academic Press.
- Bárány, M., Barron, J.T., Gu, L., and Bárány, K. (2001). Exchange of the actin-bound nucleotide in intact arterial smooth muscle. *J. Biol. Chem.*, **276**, 48398-48403.
- Beall, A.C., Kato, K., Goldenring, J.R., Rasmussen, R., and Brophy, C.M. (1997). Cyclic nucleotide-dependent vasorelaxation is associated with the phosphorylation of a small heat shock-related protein. *J. Biol. Chem.*, **272**, 11283-11287.
- Borman, M.A., MacDonald, J.A., and Haystead, T.A.J. (2004). Modulation of smooth muscle contractility by CHASM, a novel member of the smoothelin family of proteins. *FEBS Letters*, **573**, 207-213.
- Brophy, C.M., Lamb, S., and Graham, A. (1999). The small heat shock-related protein-20 is an actin-associated protein. *J. Vasc. Surg.*, **29**, 326-333.
- Dillon, P.F. (2004). Dick Murphy: three decades as the touchstone of smooth muscle physiology. "Focus on cooperative attachment of cross bridges predicts regulation of smooth muscle force by myosin phosphorylation". *Am. J. Physiol. Cell Physiol.*, **287**, C590-C591.
- Ellison, P.A., DePew, Z.S., and Cremo, C.R. (2003). Both heads of tissue-derived smooth muscle heavy meromyosin bind to actin in the presence of ADP. *J. Biol. Chem.*, **278**, 4410-4415.
- Erdödi, F., Rokolya, A., Bárány, M., and Bárány, K. (1988). Phosphorylation of the 20,000 dalton myosin light chain isoforms of arterial smooth muscle by myosin light chain kinase and protein kinase C. *Arch. Biochem. Biophys.*, **266**, 583-591.
- Feng, J., Ito, M., Ichikawa, K., Isaka, N., Nishikawa, M., Hartshorne, D.J., and Nakano, T. (1999). Inhibitory phosphorylation site for rho-associated kinase on smooth muscle myosin phosphatase. *J. Biol. Chem.*, **274**, 37385-37390.
- Funabara, D., Watabe, S., Mooers, S.U., Narayan, S., Dudas, C., Hartshorne, D.J., Siegman, M.J., and Butler, T.M. (2003). Twitchin from molluscan catch muscle: Primary structure and relationship between site-specific phosphorylation and mechanical function. *J. Biol. Chem.*, **278**, 29308-29316.

- Hartshorne, D.J., Ito, M., and Erdödi, F. (2004). Role of protein phosphatase type 1 in contractile functions: myosin phosphatase. *J. Biol. Chem.*, **279**, 37211-37214.
- Hartshorne, D.J., Ito, M., and Erdödi, F. (1998). Myosin light chain phosphatase: subunit composition, interactions and regulation. *J. Muscle Res. Cell Motil.*, **19**, 325-341.
- Herrera, A.M., Kuo, K-H., and Seow, C.Y. (2002). Influence of calcium on myosin thick filament formation in intact airway smooth muscle. *Am. J. Physiol. Cell Physiol.*, **282**, C310-C316.
- Hodgkinson, J.L., el-Mezgueldi, M., Craig, R., Vibert, P., Marston, S.B., and Lehman, W. (1997). 3-D image reconstruction of reconstituted smooth muscle thin filaments containing calponin: visualization of interactions between F-actin and calponin. *J. Mol. Biol.*, **273**, 150-159.
- Je, H.D., Gallant, C., Leavis, P.C., and Morgan, K.G. (2004). Caveolin-1 regulates contractility in differentiated vascular smooth muscle. *Am. J. Physiol. Heart Circ. Physiol.*, **286**, H91-H98.
- Jones, K.A., Perkins, W.J., Lorenz, R.R., Prakash, Y.S., Sieck, G.C., Warner, D.O. (1999). F-actin stabilization increases tension cost during contraction of permeabilized airway smooth muscles in dog. *J. Physiol.*, **519**, 527-538.
- Kaibuchi, K., Kuroda, S., and Amano, M. (1999). Regulation of the cytoskeleton and cell adhesion by the rho family GTPases in mammalian cells. *Annu. Rev. Biochem.*, **68**, 459-486.
- Kao, C.Y. and Carsten, M. E. (1997). *Cellular Aspects of Smooth Muscle Function*. Cambridge University Press.
- Kitazawa, T., Eto, M., Woodsome, T.P., and Brautigan, D.L. (2000). Agonists trigger G protein-mediated activation of the CPI-17 inhibitor phosphoprotein of myosin light chain phosphatase to enhance vascular smooth muscle contractility. *J. Biol. Chem.*, **275**, 9897-9900.
- Kitazawa, T., Polzin, A.N., and Eto, M. (2004). CPI-17-deficient smooth muscle of chicken. *J. Physiol.*, **557.2**, 515-528.
- Kovács, M., Tóth, J., Nyitrai, L., and Sellers, J.R. (2004). Two-headed binding of the unphosphorylated nonmuscle heavy meromyosin•ADP complex to actin. *Biochemistry*, **43**, 4219-4226.
- Lauzon, A-M., Fagnant, P.M., Warshaw, D.M., and Trybus, K.M. (2001) Coiled-coil unwinding at the smooth muscle myosin head-rod junction is required for optimal mechanical performance. *Biophys. J.*, **80**, 1900-1904.
- Lehman, W., Vibert, P., Craig, R. (1997). Visualization of caldesmon on smooth muscle thin filaments. *J. Mol. Biol.*, **274**, 310-317.

Li, X-D., Saito, J., Ikebe, R., Mabuchi, K., and Ikebe, M. (2000). The interaction between the regulatory light chain domains on two heads is critical for regulation of smooth muscle myosin. *Biochemistry*, **39**, 2254-2260.

Martonosi, A., Gouvea, M.A., and Gergely, J. (1960). Studies on actin. III. G-F transformation of actin and muscular contraction (experiments *in vivo*). *J. Biol. Chem.*, **235**, 1707-1710.

Meeks, M.K., Ripley, M.L., Jin, Z., and Rembold, C.M. (2005). Heat shock protein 20-mediated force suppression in forskolin-relaxed swine carotid artery. *Am. J. Physiol. Cell Physiol.*, **288**, C633-C639.

Mehta, D. and Gunst, S.J. (1999). Actin polymerization stimulated by contractile activation regulates force development in canine tracheal smooth muscle. *J. Physiol.*, **519**, 820-840.

Moore, E.D., Voigt, T., Kobayashi, Y.M., Isenberg, G., Fay, F.S., Gallitelli, M.F., and Franzini-Armstrong, C. (2004). Organization of Ca<sup>2+</sup> release units in excitable smooth muscle of the guinea-pig urinary bladder. *Biophys. J.*, **87**, 1836-1847.

Murányi, A., Derkach, D., Erdódi, F., Kiss, A., Ito, M., Hartshorne, D.J. (2005). Phosphorylation of Thr695 and Thr850 on the myosin phosphatase target subunit: Inhibitory effects and occurrence in A7r5 cells. *FEBS Letters*, **579**, 6611-6615.

Murphy, R.A. (1999). Signal transduction in smooth muscle. *Reviews of Physiology Biochemistry and Pharmacology*. vol.134

Murthy, K.S. (2006). Signaling for contraction and relaxation in smooth muscle of the gut. *Annu. Rev. Physiol.*, **68**, 345-374.

Nagumo, H., Sasaki, Y., Ono, Y., Okamoto, H., Seto, M., and Takuwa, Y. (2000). Rho-kinase inhibitor HA-1077 prevents rho-mediated myosin phosphatase inhibition in smooth muscle cells. *Am. J. Physiol.*, **278**, C57-C65.

Ohki, S-Y., Eto, M., Kariya, A., Hayano, T., Hayashi, Y., Yazawa, M., Brautigan, D., and Kainosho, M. (2001). Solution NMR structure of the myosin phosphatase inhibitor protein CPI-17 shows phosphorylation-induced conformational changes responsible for activation. *J. Mol. Biol.*, **314**, 839-849.

Quevillon-Cheruel, S., Foucault, G., Desmadril, M., Lompre, A.M., and Bechet, J.J. (1999). Role of the C-terminal extremities of the smooth muscle myosin heavy chains: implication for assembly properties. *FEBS Letters*, **454**, 303-306.

Ratz, P.H., Berg, K.M., Urban, N.H., and Miner, A.S. (2005). Regulation of smooth muscle calcium sensitivity: KCl as a calcium-sensitizing stimulus. *Am. J. Physiol. Cell Physiol.*, **288**, 769-783.

- Rembold, C.M., Foster, D.B., Strauss, J.D., Wingard, C.J., Van Eyk, J.E. (2000). cGMP-mediated phosphorylation of heat shock protein 20 may cause smooth muscle relaxation without myosin light chain dephosphorylation in swine carotid artery. *J. Physiol.*, **524**, 865-878.
- Rembold, C.M., Wardle, R.L., Wingard, C.J., Batts, T.W., Etter, E.F., and Murphy, R.A. (2004). Cooperative attachment of cross bridges predicts regulation of smooth muscle force by myosin phosphorylation. *Am. J. Physiol. Cell Physiol.*, **287**, C594-C602.
- Rovner, A.S., Fagnant, P.M., Lowey, S., and Trybus, K.M. (2002). The carboxyl-terminal isoforms of smooth muscle myosin heavy chain determine thick filament assembly properties. *J. Cell. Biol.*, **156**, 113-124.
- Rovner, A.S. (1998). A long, weakly charged actin-binding loop is required for phosphorylation dependent regulation of smooth muscle myosin. *J. Biol. Chem.*, **273**, 27939-27944.
- Serysheva, I.I., Bare, D.J., Ludtke, S.J., Kettlun, C.S., Chiu, W., and Mignery, G.A. (2003). Structure of the type 1 inositol 1,4,5-trisphosphate receptor revealed by electron cryomicroscopy. *J. Biol. Chem.*, **278**, 21319-21322.
- Solaro, R.J. (2000). Myosin light chain phosphatase a cinderella of cellular signaling. *Circ. Res.*, **87**, 173-175.
- Somara, S., and Bitar, K.N. (2004). Tropomyosin interacts with phosphorylated HSP27 in agonist-induced contraction of smooth muscle. *Am. J. Physiol. Cell Physiol.*, **286**, C1290-C1301.
- Somlyo, A.P. and Somlyo, A.V. (1994). Signal transduction and regulation in smooth muscle. *Nature*, **372**, 231-236.
- Somlyo, A.P. and Somlyo, A.V. (2000). Signal transduction by G-proteins, Rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J. Physiol.*, **522**, 177-185.
- Stull, J.T., Krueger, J.K., Kamm, K.E., Gao, Z-H., Zhi, G., and Padre, R. (1996). Myosin light chain kinase. *In Biochemistry of Smooth Muscle Contraction* (M. Bárány, ed.), pp. 119-130. Academic Press.
- Surks, H.K., Richards, C.T., and Mendelsohn, M.E. (2003). Myosin phosphatase-rho interacting protein. A new member of the myosin phosphatase complex that directly binds rhoA. *J. Biol. Chem.*, **278**, 51484-51493.
- Sward, K., Dreja, K., Susnjar, M., Hellstrand, P., Hartshorne, D.J., and Walsh, M.P. (2000). Inhibition of rho-associated kinase blocks agonist induced Ca<sup>2+</sup> sensitization of myosin phosphorylation and force in guinea pig ileum. *J. Physiol.*, **522 Pt 1**, 33-49.

- Sweeney, H.L., Chen, L-Q., and Trybus, K.M. (2000). Regulation of asymmetric smooth muscle myosin II molecules. *J. Biol. Chem.*, **275**, 41273-41277.
- Tang, D.D., and Gunst, S.J. (2004). The small GTPase Cdc42 regulates actin polymerization and tension development during contractile stimulation of smooth muscle. *J. Biol. Chem.*, **279**, 51722-51728.
- Tang, D.D., Zhang, W., and Gunst, S.J. (2005). The adapter protein CrkII regulates neuronal Wiskott-Aldrich syndrome protein, actin polymerization, and tension development during contractile stimulation of smooth muscle. *J. Biol. Chem.*, **280**, 23380-23389.
- Terrak, M., Kerff, F., Langsetmo, K., Tao, T., and Dominguez, R. (2004). Structural basis of protein phosphatase 1 regulation. *Nature*, **429**, 780-784.
- Ti Deng, J., Sutherland, C., Brautigan, D.L., Eto, M., and Walsh, M.P. (2002). Phosphorylation of the myosin phosphatase inhibitors, CPI-17 and PHI-1, by integrin-linked kinase. *Biochem. J.*, **367**, 517-524.
- Trybus, K. (2000). Biochemical studies of myosin. *METHODS*, **22**, 327-335.
- Wooldridge, A.A., MacDonald, J.A., Erdodi, F., Ma, C., Borman, M.A., Hartshorne, D.J., and Haystead, T.A.J. (2004). Smooth muscle phosphatase is regulated in vivo by exclusion of phosphorylation of threonine 696 of MYPT1 by phosphorylation of serine 695 in response to cyclic nucleotides. *J. Biol. Chem.*, **279**, 34496-34504.
- Yengo, C.M., De La Cruz, E.M., Chrin, L.R., Gaffney II, D.P., and Bergert, C.L. (2002). Actin-induced closure of the actin-binding cleft of smooth muscle myosin. *J. Biol. Chem.*, **277**, 24114-24119.
- Zhang, W., and Gunst, S.J. (2006). Dynamic association between  $\alpha$ -actinin and  $\beta$ -integrin regulates contraction of canine tracheal smooth muscle. *J. Physiol.*, **572.3**, 659-676.
- Zhang, W., Wu, Y., Du, L., Tang, D.D., and Gunst, S.J. (2005). Activation of the Arp2/3 complex by N-WASp is required for actin polymerization and contraction in smooth muscle. *Am. J. Physiol. Cell Physiol.*, **288**, 1145-1160.