Role of Protein Kinase Network in Excitation-Contraction Coupling in Smooth Muscle Cell

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1. Introduction

The aim of this chapter is to present a review of the main protein kinases involved in the signalling pathways between the stimulation of smooth muscle cell and the resulting dynamic contraction.

As in striated muscle cells, contraction in smooth muscle cells (SMC) is primarily triggered by intracytosolic Ca^{2+} ($[Ca^{2+}]_i$) increase. However, by contrast with striated muscle, in SMCs $[Ca^{2+}]_i$ increase generates contraction by activation of the myosin light chain kinase (MLCK) via the formation of the Ca^{2+}-calmodulin-MLCK complex. Activated MLCK phosphorylates the 20kDa regulatory light chain (MLC_{20}) of the thick filament. Phosphorylated MLC_{20} allows myosin to bind to actin, and this phosphorylation is critical for SMC contraction, since its inhibition generally abolishes agonist-dependent contraction, whereas relaxation is induced by MLC_{20} dephosphorylation by myosin light chain phosphatase (MLCP). Hence, excitation-contraction coupling in SMCs critically depends on Ca^{2+}-dependent MLCK activation and the balance of MLCK/MLCP activity. Moreover, it has been shown that these two major enzymes can be modulated by several protein kinases such as protein kinase A (PKA), protein kinase C (PKC) and Rho kinase (RhoK). As a consequence, these protein kinases indirectly modulate the activity of the thick filament of myosin. Additionally, actin-myosin interaction can be modulated by proteins associated to the thin filament of actin such as caldesmon and calponin, which modulation depends on their phosphorylation by several protein kinases. It appears then that in SMCs, the excitation-contraction coupling is determined by interacting signalling pathways involving various protein kinases, so that the canonical MLCK/MLCP enzymatic balance is embedded in a complex network of protein kinases acting both on the thick and thin filaments of the contractile apparatus. The chapter will present the functional structure of the contractile apparatus of SMC, and detail its activation by the Ca^{2+}-calmodulin-MLCK complex and down regulation by MLCP, and the action of the main PK that have been shown to modulate the sensitivity of the contractile

apparatus to Ca^{2+}. The resultant behaviour of the SMC stimulated by contractile agonists not only depends on the structure of the regulatory network that modulates the contractile apparatus but also on the dynamics of the reactions. Mathematical modelling of this signalling network is of great help to decipher how different protein kinases involved in this network participate to the time-dependent behaviour of the contractile system. Several theoretical models have been developed in this sense. The chapter will present the general principles of these models, their predictions and how they help in understanding the role of PK in the time course of the contractile response of SCMs.

In this chapter, examples are taken from airway smooth muscle cells, though this chapter is not limited to this tissue, and describes general mechanisms present in other SMC types.

2. Ca^{2+} signalling and the contractile apparatus

2.1 Ca^{2+} signalling in airway smooth muscle cells

In smooth muscle cells, as in heart and skeletal muscle, $[Ca^{2+}]_i$ is the primary intracellular messenger that generates contraction. Stimulation of smooth muscle cells by contractile agonists results in an increase in $[Ca^{2+}]_i$, which in turn activates the contractile apparatus. However, the mechanisms by which Ca^{2+} stimulates the formation of actin-myosin bridges critically differ in smooth muscle cells from striated muscle cells (see below). Though Ca^{2+} signalling is not primarily triggered via direct protein kinase activation by contractile agonists, several agents that contribute to the Ca^{2+} signal can be modulated by protein kinases and, on the other hand, activation of several protein kinases involved in the modulation of the contractile apparatus is Ca^{2+} sensitive. Hence, this section will present a general overview of the mechanisms of Ca^{2+} signalling in smooth muscle cells, including in airways.

$[Ca^{2+}]_i$ value in basal conditions is maintained around 100 nM, a low value compared to extracellular medium, around 1-2 mM, and intracellular organelles like the sarcoplasmic reticulum, by active mechanisms of Ca^{2+} efflux through the plasma membrane and Ca^{2+} pumping into intracellular Ca^{2+} stores. When cells are stimulated, $[Ca^{2+}]_i$ is increased via different mechanisms which relative importance depends on smooth muscle cell types and/or contractile agonists. Basically, the general mechanisms of $[Ca^{2+}]_i$ increase involve either intracellular Ca^{2+} influx or intracellular Ca^{2+} release from internal Ca^{2+} stores, or both (Sanders, 2001; Somlyo et al., 1994). Voltage-dependent Ca^{2+} occurs via L-type voltage-operated Ca^{2+} channel (VOC). Normal resting membrane potential in airway myocytes is around -60 mV (Roux et al., 2006), and is highly dependent on basal K^+ conductance. Some K^+ channels are active at rest, and contribute to the resting K^+ conductance and hence resting membrane potential. Closure of K^+ channels tends to depolarize the plasma membrane which in turn induces extracellular Ca^{2+} influx via VOC. By contrast, additional activation of K^+ channels tends to hyperpolarize the plasma membrane, to inhibit VOC-dependent Ca^{2+} influx and hence to induce relaxation. Voltage-dependent Ca^{2+} influx and subsequent contraction is called the electromechanical coupling. In parallel to voltage-operated Ca^{2+} entry, Ca^{2+} influx can be generated by voltage-independent membrane channels. Receptor-operated channels can be opened by direct binding of the agonist on the membrane receptor, like P2X purinergic receptors (Mounkaila et al., 2005). Another voltage-independent source of Ca^{2+} entry is Ca^{2+} influx through store-operated Ca^{2+} channels (SOC). These channels are activated by emptying of intracellular Ca^{2+} stores (Marthan, 2004).

The other origin of $[Ca^{2+}]_i$ increase is Ca^{2+} release from intracellular organelles (Sanders, 2001). The major one from which Ca^{2+} is released upon contractile stimulation is the sarcoplasmic reticulum (SR). Ca^{2+} release from the sarcoplasmic reticulum, with internal Ca^{2+} concentration in the millimolar range, occurs via two main types of sarcoplasmic receptors, the inositol-trisphosphate receptors (InsP$_3$Rs) and the ryanodine receptors (RyRs). InsP$_3$Rs are activated by InsP$_3$, which is produced from phosphatidylinositol phosphate by phospholipase C (PLC) upon stimulation by contractile agonists (Roux *et al.*, 1998). Activation of contraction via primary InsP$_3$ production and InsP$_3$R-operated Ca^{2+} release, a consequence of enzymatic activation of PLC, is called the pharmacomechanical coupling, in opposition with the electromechanical coupling described above. RyRs are physiologically activated by an increase in Ca^{2+} concentration on the cytosolic face of the RyR, or by direct mechanical coupling between L-type VOC, and it has been shown that cyclic ADP ribose is a co-agonist of RyR (Prakash *et al.*, 1998). RyR activation by Ca^{2+} self-amplifies $[Ca^{2+}]_i$ increase, whatever its initial mechanism. This Ca^{2+}-induced Ca^{2+} release (CICR), however, is not observed in all smooth muscle cells. In human bronchial smooth muscle, for example, RyR, though present and functional, do not participate in the acetylcholine-induced Ca^{2+} response (Hyvelin *et al.*, 2000a).

Basal maintenance of low $[Ca^{2+}]_i$ and Ca^{2+} removal form the cytosol upon and after stimulation is due to active mechanisms that either extrude Ca^{2+} in the extracellular medium or uptake Ca^{2+} into intracellular Ca^{2+} stores. Ca^{2+} extrusion is mainly due to the activity of the plasma membrane Ca^{2+} ATPase (PMCA), and the Na^+-Ca^{2+} exchanger (NCX) (Sanders, 2001).The main mechanisms of Ca^{2+} uptake from the cytosol are Ca^{2+} pumping back into the SR by sarcoendoplasmic Ca^{2+} ATPase (SERCA), Ca^{2+} uptake into the mitochondria. Also, several Ca^{2+}-binding proteins can buffer cytosolic Ca^{2+} and hence decrease $[Ca^{2+}]_i$ (Gunter *et al.*, 2000; Roux *et al.*, 2004).

The Ca^{2+} signal is not only the overall increase in the cellular Ca^{2+} pool and subsequent $[Ca^{2+}]_i$ but also, and most importantly, the pattern of the dynamic change in $[Ca^{2+}]_i$. Indeed, the time-dependent variation in the shape of $[Ca^{2+}]_i$ is the actual "Ca^{2+} signal", since Ca^{2+} binding to signalling protein, e. g., calmodulin, depends on cytosolic Ca^{2+} concentration. Overall $[Ca^{2+}]_i$ measurements generally show a transient increase in $[Ca^{2+}]_i$ (Ca^{2+} peak) followed by a progressive decay to a steady-state value (Ca^{2+} plateau) and/or by Ca^{2+} oscillations (Bergner *et al.*, 2002; Hyvelin *et al.*, 2000b; Kajita *et al.*, 1993; Liu *et al.*, 1996). Changes in $[Ca^{2+}]_i$ are not uniform within the cytosol, and studies have evidenced the role of local change in Ca^{2+} signalling (Prakash *et al.*, 2000). Ca^{2+} signalling should hence be defined as spatiotemporal changes in $[Ca^{2+}]_i$, from which depends the activity of the contractile apparatus, and other cell functions. The shape of this Ca^{2+} signal critically depends on the dynamics of Ca^{2+} fluxes between intra- and extracellular media and also between intracellular Ca^{2+} compartments. The dynamic relationship between the calcium signal and MLCK activity and its consequence on force development will be discussed in more detail in the section "Theoretical modelling of PK and ASMC contraction".

2.2 Smooth muscle contractile apparatus

2.2.1 Components and general organization

The contractile apparatus of smooth muscle is basically constituted of thick filaments of myosin II and thin filament of actin and associated proteins, the thin/thick filament ratio

being about 20/1 to 30/1 (Kuo *et al.*, 2003; Somlyo *et al.*, 1983). These filaments are not organized in sarcomeres and do not form well individualized myofibrils. Thick filaments are anchored on dense bodies in the cell and dense area on the plasma membrane and actin filaments are positioned between thick filaments. Dense bodies and filaments are connected by non-contractile intermediate filaments that constitute an intracellular network.

Thick filaments are 1.5 µm long and 12-14 nm in diameter, and are composed of polymerized monomers of myosin. Each monomer of myosin is formed by the association of 2 identical heavy chains (MHC) complexed to 2 pairs of light chains (MLC). Each of the 2 heavy chains has a C-terminal extended α-helix twisted to form a single tail and an N-terminal head. 4 isoforms of MHC have been described in smooth muscle cells, corresponding to 4 alternatively-spliced variants derived from the single smooth muscle myosin heavy chain gene MYH11, differing in their amino-terminal and carboxy-terminal portions (Hong *et al.*, 2011). Polymerized and monomeric myosins are in equilibrium and the stimulation of the myocyte increases myosin polymerization. The distal portion of the myosin head has the ATPase enzymatic activity required for its motor function, and a binding site for actin. A pair of light chains, a 17 kDa one and a 20 kDa one, is complexed with each of the head of the myosin heavy chain. The role of MLC_{17} is unclear, and it is thought to contribute to the stability of the molecule of myosin. Phosphorylation of the so-called regulatory MLC_{20} is required for actin-myosin binding, and hence phosphorylation/dephosphorylation of MLC_{20} regulates actin-myosin cross bridge and contraction. 2 residues, located in the amino-terminal portion of the protein, threonine 18 and serine 19, can be phosphorylated by MLCK. The major site of phosphorylation by MLCK in intact myocyte is ser19, though thr18 can also be phosphorylated by MLCK. It has been shown that Rho kinase can also directly phosphorylate MLC_{20}, but, at least in airway smooth muscle, Rho kinase regulates phosphorylated/dephosphorylated MLC_{20} ratio by acting on MLCP (Mbikou *et al.*, 2011). It has been shown that PKC may also phosphorylate MLC_{20} but on different residues, serine 1, serine 2 and threonine 9 (Barany *et al.*, 1996). However, it is generally admitted that PKC contributes to smooth muscle contraction mainly via MLCP inhibition.

Smooth muscle thin filaments are formed by a double helix of F-actin complexed with tropomyosin, caldesmon and calponin. Globular smooth muscle α-actin molecules assemble into filamentous polymers of F-actin, and 2 parallel strands of F-actin rotate on each other to form a double helix structure. F-actin filaments are about 1 µm long and 5-7 nmn in diameter. As in striated muscle, tropomyosin binds to F-actin in the furrow formed by the double helix of actin, and contributes to actin filament stabilization (Shah *et al.*, 2001). In contrast with striated muscle, here is no troponin on the thin filament of actin in smooth muscle, and other proteins, caldesmon and calponin, are associated with actin. Caldesmon and calponin are regulatory proteins that can be phosphorylated by several kinases and their phosphorylation modulates the formation of the actin-myosin bridge. Regulation of contraction by caldesmon and calponin is detailed below.

2.2.2 Actin-mysosin bridge cycling

Basically, muscle contraction is based on cycling attachment and detachment between thick filaments of myosin and thin filaments of actin. Interaction between actin and myosin, which corresponds to the formation of the actin-myosin crossbridge, is triggered by the

head of myosin. As seen above, in smooth muscle, phosphorylation of MLC_{20} is the key event that allows the formation of the actin-myosin bridge, a step required for contraction to occur (Harnett *et al.*, 2003; Wingard *et al.*, 2001). MLC_{20} phosphorylation induces a conformational change of the neck of myosin so that the head of myosin can bind to actin. This allows the rotation of the head of myosin responsible for the "sliding" of actin and myosin filaments. MLC_{20} phosphorylation also activates the ATPase activity of the myosin head, followed by actin and myosin detachment. When MLC_{20} remains phosphorylated all along the crossbridge cycle, crossbridge cycling is rapid, and associated with high ATP consumption. However, sustained contraction can occur even if $[Ca^{2+}]_i$ and subsequent MLC_{20} phosphorylation decrease (Mbikou *et al.*, 2006), and this is generally attributed to the fact that if dephosphorylation of MLC_{20} occurs after the attachment of myosin on actin, crossbridge cycle goes on but at a slower rate, in particular in the stage where dephosphorylated myosin detaches actin. These maintained dephosphorylated crossbridges that cycle at a slow rate are termed latch-bridges.

Hence, 2 types of crossbridges cycling can occur in smooth muscles, a fast, phosphorylated one (during which MLC_{20} remains phosphorylated) and a slow, partially dephosphorylated one (during which MLC_{20} is phosphorylated after actin-myosin attachment), and, accordingly, a 4-state model of the contractile apparatus has been proposed, initially by Hai and Murphy (Hai *et al.*, 1988a). This model is presented in Figure 1. The first state corresponds to unbound actin and unphosphorylated MLC_{20}, the second one to unbound actin and phosphorylated MLC_{20}, the third one to actin bond to phosphorylated MLC_{20}, and the fourth one to actin bond to dephosphorylated MLC_{20}. Transition between state 1 and 2, and 3 and 4, is reversible and depends on MLC_{20} phosphorylation/dephosphorylation. Transition between state 2 and 3 is reversible and corresponds to the phosphorylated crossbridge cycling. Rate constant from state 1 to 4 is very slow, and it can be considered that transition from state 4 to 1 is almost irreversible. State 1-2-3-4-1 cycling corresponds to the latch-bridge cycling.
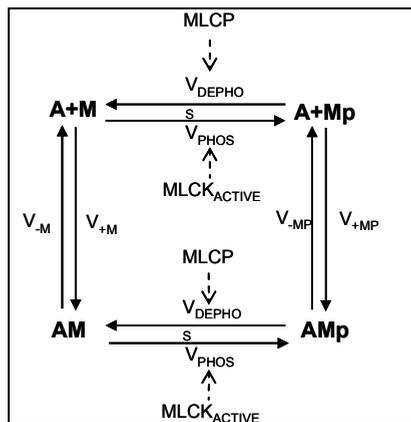

Fig. 1. **4 state model of smooth muscle contractile apparatus.** A, actin; M, unphosphorylated myosin; Mp, phosphorylated myosin; AM, unphosphorylated actin-myosin bridge; AMp, phosphorylated actin-myosin bridge; MLCK, myosin light chain kinase; MLCK, myosin light chain phosphatase; CaM, calmodulin. V, rate constants of the reactions.

The main enzyme by which MLC_{20} is phosphorylated is MLCK. Dephosphorylation of phosphorylated MLC_{20} is catalysed by MLCP. Though other enzymes can contribute to smooth muscle contraction, muscle contraction critically depends on the balance between MLCK and MLCP activity.

2.3 Modulation of MLC_{20} phosphorylation: The MLCK/MLCP balance

2.3.1 MLCK

MLCK has a ubiquitous distribution and is present in non-muscular cells as well as cardiac, skeletal and smooth muscle cells. Several genes encode for distinct isoforms of MLCK. In humans, MYLK2 and MYLK3 genes encode for the MLCK isoforms expressed in skeletal and cardiac myocytes, respectively, and the smooth muscle isoform is encoded by the MYLK1 gene. This gene has 2 initiation sites generating a short isoform corresponding to the smooth muscle MLCK and a long isoform corresponding to nonmuscle MLCK. The domain structure of smooth muscle mammalian MLCK is shown in Figure 2. MLCK has 2 CaM-binding domains, one at its aminoterminal portion, composed of 3 DFRxxL motifs, and the other at its carboxyterminal portion, before the IgT domain located at the N-terminus. The DFrxxL domain is also a binding site to actin, and Ca^{2+}-CaM binding to this domain results in weakened actin binding. Some results suggest that Ig1/Ig2 domains bind to actin. IgT domain is a binding site to myosin. The kinase domain is the catalytic portion of MLCK and includes a binding site for ATP and for MLC_{20}. The role of the other domains is unknown (Hong *et al.*, 2011). Hence, MLCK interacts with myosin on two sites. The catalytic core of MLCK interacts with the aminoterminal portion of MLC_{20}, which allows phosphorylation MLC_{20}. The IgT domain of MLCK may also interact with myosin at the head-neck junction. It is thought that IgT binding to myosin near the MLC_{20} increases the catalytic activity of MLCK. The concentration of MLCK is likely to vary from muscle to muscle, but typical values range from 1 to 8 µM, which is low compared with the typical concentration of myosin, classically ranging from 50 to 100 µM.
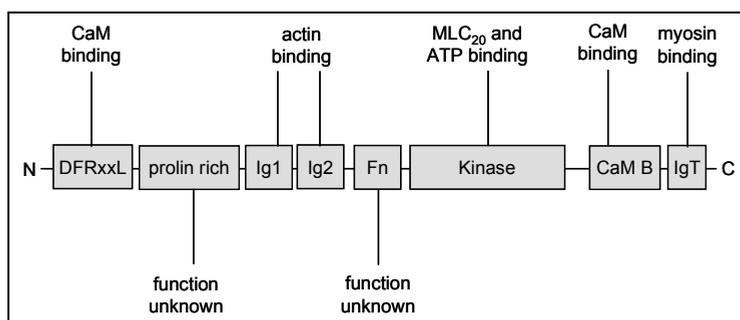

Fig. 2. **Structural scheme of myosin light chain kinase (MLCK) and its different domains.** In the absence of calmodulin (AcM) binding, the catalytic site (Kinase) is masked by the N-terminal CaM binding and IgT domains and hence inactive. Binding of Ca2+-CaM complex induces a conformational change and unmasks the kinase domain hence active.

The major substrate of MLCK in smooth muscle is the smooth muscle myosin, but recent results showed that non muscle myosin, which is also expressed in smooth muscle myosin,

can be a substrate for MLCK (Yuen *et al.*, 2009). MLCK phosphorylates MLC_{20} on serine 19 and, with lower enzymatic activity, on threonine 18 (Deng *et al.*, 2001). MLCK activity is essential for airway smooth muscle contraction, as it has been evidenced by pharmacological inhibition of MLCK in rat trachea (Mbikou *et al.*, 2006) and knockout mice (Zhang *et al.*, 2010).

The most important regulator of MLCK activity is the Ca^{2+}-CaM complex. MLCK unbound to Ca^{2+}-CaM is in an auto-inhibitory state. The IgT domain and the adjacent CaM binding domain constitute an auto-inhibitory sequence that masks the catalytic core. Ca^{2+}-CaM binding to the auto-inhibitory site induces a conformational change. This displacement of the auto-inhibitory sequence unmasks the catalytic core and thereby reveals the enzymatic activity of MLCK. On the other hand, phosphorylation of MLCK on serine 512 by several kinases such as protein kinase A in the C-terminal CaM binding domain decreases MLCK affinity for the Ca^{2+}CaM complex, thereby inactivating MLCK even in the presence of Ca^{2+}, a mechanism responsible for MLCK desensitization (Stull *et al.*, 1993). CaM is a rather small protein (16700 Da) consisting of a single polypeptidic chain of 148 to 149 aminoacids. 3 distinct domains can be identified: an N-terminal domain, a central helix domain, and a C-terminal domain. Both amino- and carboxy-terminal domain possesses 2 binding sites for Ca^{2+}. The 4 sites have a high affinity for Ca^{2+}, though the affinity of the C-terminal sites is higher. These sites have an EF-hand structure. Ca^{2+} binding induces a conformational change of the F-helix of the EF-hand motif. In this conformation CaM binds to several proteins and modulates their activity. Amongst almost 40 different proteins that have been shown to be regulated by CaM, the most important in smooth muscle are MLCK and CaM-kinase II.

2.3.2 MLCP

MLCP is a holoenzyme composed of three subunits: the 38 kDa phosphatase catalytic subunit PP1cδ, the 110 kDa regulatory subunit MYPT1, and a 20 kDa noncatalytic subunit, M-20, which role remains unclear (Hartshorne *et al.*, 1999; Hartshorne *et al.*, 1998). PP1cδ is a serine/threonine phosphatase that dephosphorylates MLC_{20} on serine 19 and threonine 18. Disruption of the quaternary structure of MLCP, either by dissociation of the regulatory subunit MYPT1 to PP1c or by disruption of the interactions between the three subunits, decreases MLCP activity toward MLC_{20} dephosphorylation.

PP1c alone is capable of dephosphorylating the MLC_{20}, but its activity is potentiated when complexed to MYPT1. Indeed, MYPT1s targets PP1c to its substrate, MLC_{20}, and confers substrate specificity to the phosphatase. A recent study by crystallography has revealed a basic structure which potentiates the catalytic activity of the holoenzyme (Terrak *et al.*, 2004). Structurally, MYPT1 subunit possesses a C-terminal leucine-zipper domain and a binding site of protein kinase G. Four N-terminal amino acids, KVKF, give a strong affinity for PP1c. MYPT1 also has six repeated domains of ankyrin, which serve as anchoring sites for protein-protein interactions. Several kinases are able to phosphorylate MYPT1 at various sites: Rho kinase, MYPT kinase (also known as Zip-like kinase), integrin-like kinase, myotonic dystrophy kinase (DMPK). PKC also has been shown to phosphorylate ankyrin repeated domains and inhibit the interaction between MYPT1 and PP1c (Toph *et al.*, 2000). In addition to this negative regulation of MLCP, MYPT1 phosphorylation is also associated with an upregulation. Recent studies have reported that protein kinases dependent on cyclic

nucleotides such as PKA and PKG are able to phosphorylate MYPT1 on serine 692, 695 and 852. The S695 and S852 residues are adjacent to two residues, T696 and T853, that are phosphorylation sites of MYPT1 by RhoK. Phosphorylation at S695 by PKG or PKA prevents RhoK phosphorylation at S696 and vice versa (Wooldridge *et al.*, 2004), and it is supposed that phosphorylation at S852 has a similar effect on T853 phosphorylation. Thus the phosphorylation of MYPT1 by PKA and PKG protein antagonizes the inhibition of MLCP induced by RhoK (Grassie *et al.*, 2011).

CPI-17 is another regulation pathway of MLCP activity (Ito *et al.*, 2004). CPI-17 is a 17 kDa protein composed of 147 aminoacids that can inhibit the activity of the MLCP holoenzyme as well as that of isolated PP1c. Phosphorylation of CPI-17 at threonine 38 enhances its inhibitory potency about 1000 fold. The proposed mechanism is that CPI-17 binds at the PP1c active site, resulting in formation of a complex of MYPT1/PP1C/CPI-17 (Eto *et al.*, 2004). The major kinase responsible for in *vivo* CPI-17 phosphorylation is PKC, especially PKCα and PKCδ. *In vitro* studies have shown that other kinases can phosphorylate CPI-17 at T38, including Rho kinase, protein kinase N (PKN), MYPT-kinase, integrin-like kinase and PKA (Ito *et al.*, 2004). The scheme of the regulation of MLCP by kinase activity is given in Figure 3.
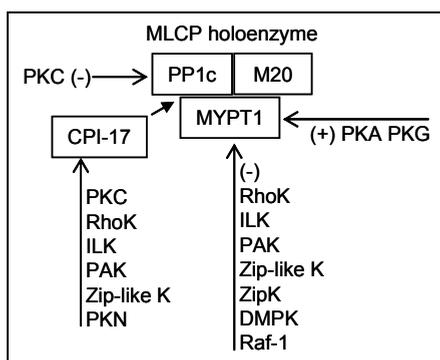

Fig. 3. **Regulation of MLCP activity by protein kinases.** DAP kinase, death-associated protein kinase;; DMPK, myotonic dystrophy protein kinase; ILK, integrin-linked kinase; MLCP, myosin light chain phosphatase; MYPT1, myosin phosphatase target subunit 1 of MLCP; M20, 20 kDa non-catalytic subunit of MLCP; PAK, p21-activated protein kinase; PKA, protein kianse A; PKC, protein kinase C; PKG, protein kinase G; PKN, protein kinase N; PLA2, phospholipase A2; PP1c, catalytic subunit of type1 protein phosphatase; RhoK, Rhokinase; ZipK; zipper-interacting protein kinase (adapted from (Hirano *et al.*, 2003)).

2.4 Thin filament-associated regulation of AM bridge cycling

2.4.1 Caldesmon

Caldesmon is a small protein of 87 kDa associated with the thin filaments of actin and involved in the regulation of smooth muscle contraction. Structurally, caldesmon possesses 4 distinct domains and can bind to actin, tropomyosin, myosin and calmodulin. The fourth domain located at the C-terminus is the most important since it is the binding domain to actin, to myosin and to Ca^{2+}-binding proteins. Caldesmon interacts stoichiometrically with

tropomyosin to consolidate the actin filaments (Gusev, 2001), and contributes to the sensitivity of the myofilament to Ca^{2+}. In the absence of Ca^{2+}, the fourth domain interacts closely with actin and inhibits the ATPase activity. $[Ca^{2+}]_i$ increase induces a conformational change of the actin-tropomyosin-caldesmon complex, allowing the ATPase activity of myosin. In addition, it allows an interaction between the thin and thick filaments as it is able to bind to both actin and myosin and this property seems to be involved in the sustained phase of the contractile response (Marston *et al.*, 1991). In vitro studies have shown that caldesmon can be a substrate for several kinases (Kordowska *et al.*, 2006).

2.4.2 Calponin

Calponin is a molecule of approximately 34 kDa related to the skeletal muscle troponin T. Calponin is present in smooth muscle at a concentration almost equivalent to that of tropomyosin. Calponin binding to F-actin disrupts actin-myosin interaction and hence negatively regulates the contraction of smooth muscle. Calponin inhibits the sliding of actin filament on myosin and the ATPase activity of myosin. The inhibitory effect of calponin is suppressed by high $[Ca^{2+}]_i$ values, but only in the presence of calmodulin. Phosphorylation and dephosphorylation of calponin plays a key role in the regulation of smooth muscle contraction. Indeed, the inhibitory effect of calponin is abolished when calponin is phosphorylated by CaM kinase II or PKC, and is restored when calponin is dephosphorylated by phosphatase 2A (Winder *et al.*, 1998; Winder *et al.*, 1990). Based on these properties, 3 physiological roles have been identified for calponin: (1) maintenance of relaxation at rest when $[Ca^{2+}]_i$ is low; (2) Ca^{2+}-dependent contribution, involving calmodulin, to contraction when $[Ca^{2+}]_i$ is increased and, (3) Ca^{2+}-independent contribution to contraction via PKC-dependent phosphorylation.

3. Role of protein kinases in the modulation of excitation-contraction coupling

3.1 General overview

3.1.1 Modulation of Ca^{2+} signalling

Though protein kinases modulate smooth muscle contraction by acting mainly downstream of the Ca^{2+} signal, some of them have been shown to act upstream of the Ca^{2+} signal and regulate the activity of structures involved in Ca^{2+} signal encoding. It has been shown by *in vitro* studies that PKA can phosphorylate $InsP_3$ receptors on serine 1755 and serine 1589 (Ferris *et al.*, 1991a). Studies using purified receptor protein reconstituted in liposomes have also evidenced that PKC and CaM kinase II can phosphorylate $InsP_3$ receptors (Ferris *et al.*, 1991b). Ryanodine receptors also can be phosphorylated by PKA, PKC and CaM kinase II (Coronado *et al.*, 1994). These phosphorylations may play an important role in smooth muscle physiology since Ca^{2+} release from SR plays a critical role in Ca^{2+} homeodynamics in airway myocyte (Ay *et al.*, 2004; Roux *et al.*, 2004). Indeed, contractile agonists such as acetylcholine act via $InsP_3$ production but also activate PKC (Mbikou *et al.*, 2006). On the other hand, PKA stimulation is a important pathway involved in bronchorelaxation since β_2-adrenergic stimulation, a major pharmacological treatment of asthma, acts mainly via Gs protein stimulation, adenylate cyclase activation, cAMP production and PKA activation (Anderson, 2006; Johnson, 2006). Extracellular ATP has also been shown to induce airway

relaxation via PKA activation (Mounkaila *et al.*, 2005). Protein kinase may also modulate Ca^{2+} homeostasis by acting on voltage-dependent Ca^{2+} entry. As explained above, K^+ channels critically contribute to the maintenance of myocyte membrane voltage, and it has been shown in vascular smooth muscle that PKA and PKC can phosphorylate several voltage-dependent K^+ channels, Ca^{2+}-activated K^+ channels and ATP-dependent K^+ channels, thus regulating the electromechanical coupling (Ko *et al.*, 2008). Phosphorylation by PKA activates these K^+ channels and hence tends to hyperpolarize the plasma membrane and limit extracellular Ca^{2+} entry, whereas phosphorylation by PKC has an opposite effect. The effective contribution of these mechanisms in airway in unclear, since it has been shown that in airways activation of Ca^{2+}-activated K^+ channels does not seem to have a significant effect on β-adrenergic relaxation (Corompt *et al.*, 1998).

3.1.2 Modulation of the Ca2+ sensitivity of the contractile apparatus

The signalling pathways capable of modulating the contraction of a given Ca^{2+} signal constitute the modulation of the sensitivity of the contractile apparatus to Ca^{2+}. As detailed previously, the importance of contraction depends on the balance between MLCK and MLCP activity, which is under control of several signalling pathways. In parallel with the canonical MLCK-dependent MLC_{20} phosphorylation, in several smooth muscles, MLC can be directly phosphorylated by Ca^{2+}-independent kinases like ZIP kinases and Integrin-linked kinase (ILK), which can also inhibit MLCP (Deng *et al.*, 2002; Huang *et al.*, 2006; Murthy, 2006; Niiro *et al.*, 2001). Rho kinase, activated by small GTPase (Rho), which seems to be activated both by Ca^{2+}-dependent and Ca^{2+}-independent pathways, can phosphorylate directly MLC and, by inhibition of MLCP, indirectly increase MLC phosphorylation and hence regulate smooth muscle contraction (Bai *et al.*, 2006; Murthy, 2006; Schaafsma *et al.*, 2006). Also, contractile agonists may, in parallel to MLCK activation, inhibit MLCP activity. PKC can inhibit MLCP, and hence modulate contraction (Bai *et al.*, 2006). By contrast, relaxant agonists, such as $β_2$-agonists in airways, may act via MLCP activation and/or MLCK inhibition (Janssen *et al.*, 2004; Johnson, 1998). So, in addition with the Ca^{2+}-calmodulin-MLCK pathway, other Ca^{2+}-dependent and independent enzymatic pathways regulate the contractile apparatus in smooth muscles, which status depends on the balance between MLCP/MLCK phosphorylation and dephosphorylation. Additionally, smooth muscle contraction can be modulated independently from MLC_{20} phosphorylation via caldesmon and calponin. The major kinases involved in smooth muscle contraction are listed below.

3.2 RhoK

Rho-associated kinase (Rho-kinase), originally identified as an effector of the small GTPase Rho, has been shown to play a major role in many processes including cell migration, neuronal polarisation, cytokinesis and cell contraction. It is a serine/threonine kinase structurally related to myotonic dystrophy kinase as there is 72% homology in the kinase domains. Different studies support the idea that Rho-kinase exists as a dimer resulting from parallel association through the coil-coil domain (Shimizu *et al.*, 2003). There are two Rho-kinase members, Rho-kinase I/ROKβ/p160ROCK and Rho-kinase II /ROKα/Rho kinase, which share 65% sequence identity and 95% sequence similarity at the amino acid level (Riento *et al.*, 2003b). The kinase domain is highly conserved between these two proteins (83% identical), suggesting that they may have similar substrate specificity. The consensus

phosphorylation sequence for Rho-kinase is R/KXS/T or R/KXXS/T (X is any amino acid). The Rho-kinase protein is composed of three domains: a N-terminal kinase domain, a central coiled-coil domain containing a Rho binding site (RhoBD), and a C-terminal pleckstrin homology-like domain (PH-like domain) containing a Cys-rich region similar to the C1 domain of protein kinase C. The C-terminal region including the RhoBD plus the PH domain has been shown to directly interact with the kinase domain to inhibit its activity (Amano *et al.*, 1999). The interaction between Rho•GTP and the RhoBD releases this autoinhibition and thus activates the kinase (Ishizaki *et al.*, 1996). In addition to Rho other small as GTPases such Rnd3/RhoE, Gem and Rad can bind Rho-kinase outside of the Rho-binding region and inhibit its function (Komander *et al.*, 2008; Riento *et al.*, 2003a). Arachidonic acid has been also shown to activate Rho-kinase via its PH domain (Araki *et al.*, 2001). Although both Rho-kinase I and Rho-kinase II proteins are ubiquitously expressed in most tissues, higher levels of Rho-kinase II are found in brain and muscles whereas higher levels of Rho-kinase I are found in non-neuronal tissues such as liver, lung and testis (Leung *et al.*, 1996; Nakagawa *et al.*, 1996). Functional differences have been reported between Rho-kinase I and Rho-kinase II. For instance Rho-kinase I seems to be essential for the formation of stress fibres, whereas Rho-kinase II seems important for phagocytosis and cell contraction, both being dependent on MLC phosphorylation (Wang *et al.*, 2009; Yoneda *et al.*, 2005). Binding tests revealed that RhoE preferentially binds Rho-kinase I, but not Rho-kinase II, whereas MYPT1 binds only Rho-kinase II (Komander *et al.*, 2008; Wang *et al.*, 2009).

There has been a great deal of interest in the involvement of the Rho/ROCK signalling pathway in excitation–contraction coupling (Iizuka *et al.*, 1999; Mbikou *et al.*, 2011; Yoshii *et al.*, 1999). Activation of the Rho/Rho-kinase pathways is likely due to the stimulation of the G-coupled protein receptor. Stimulation of M_3 muscarinic receptors agonist for instance activates the Gq, G_{12} and G_{13} α-subunits which cause the cascade activation of GEFs, RhoA and Rho-kinase leading to Ca^{2+} sensitization (Hirano *et al.*, 2004; Somlyo *et al.*, 2003). It is well-known that Rho/Rho-Kinase signalling modulates Ca^{2+} sensitivity of the smooth muscle likely either by inhibition of the MLCP activity or by direct phosphorylation of the MLC_{20}. Precisely, Rho-kinase has been shown to phosphorylate the MYPT1 subunit of the MLCP at two inhibitory sites, Thr696 and Thr853, by that causing the dissociation between the MLCP and MLC_{20} (Feng *et al.*, 1999; Kawano *et al.*, 1999; Kimura *et al.*, 1996; Velasco *et al.*, 2002). Phosphorylation of MYPT1 by Rho-kinase alters the MYPT1-PP1c interaction, which decreases MLCP activity toward MLC_{20}. Rho-kinase is also able to directly phosphorylate Ser19 and Thr18 in non-muscle cells (Ueda *et al.*, 2002), but the direct contribution of Rho-kinase to phospho-MLC_{20} levels *in vivo* is not yet proven. Moreover, Rho kinase exerts an inhibitory activity toward PP1c subunit of MLCP upon the phosphorylation of the latter (Eto *et al.*, 1995; Eto *et al.*, 1997). Taken together, the mechanisms triggered by Rho-kinase activity enhance the level of phosphorylated MLC_{20} and, consequently, myosin ATPase activity and therefore contraction. *In vitro* tests have shown that Rho-kinase is also able to phosphorylate CPI17 (MLCP inhibitor protein), thus inducing the inhibition of MLCP and thereby raising the level of phosphor MLC20 (Amano *et al.*, 2010).

3.3 PKC

The protein kinase C (PKC) family is the largest serine/threonine-specific kinase family known. It embraces a large family of enzymes that differ in structure, cofactor requirements

and function (Nishizuka, 1995). Depending on their cofactor requirements, the homologous group of PKC can be divided into three groups as follows: the group of conventional (c)PKC isoforms (α, β_I, β_{II} and γ), that require Ca^{2+} and diacylglycerol (DAG) to become activated; the group of novel (n)PKC isoforms (δ, ϵ, ζ, θ and μ) that require only DAG; and group of the atypical (a)PKC isoforms, namely ζ, ι and λ (the mouse homologue of human PKCι), that require neither Ca^{2+} nor DAG. The general structure of a PKC molecule consists of a catalytic domain in N-terminal and a regulatory pseudosubstrate in C-terminal, both framing 3 distinct sites able to bind specifically ATP, Ca^{2+} or phosphatidyl serine. The pseudosubstrate region is a small sequence of amino acids that binds the substrate-binding cavity in the catalytic domain, thus keeping the enzyme inactive. The activity of PKC is controlled by its compartmentalization within the cell. All PKC family members possess a phosphatidylserine binding domain for membrane interaction. The expression and distribution of PKC isoforms is tissue- and species–specific. Some isoforms (e.g. PKCα) are ubiquitously expressed in tissues whereas others seem to be restricted to certain tissues (Webb *et al.*, 2000). In ASM, the protein and mRNA expression of PKC isoforms differs depending on the specie. In human trachealis for instance, there is expression of the conventional α, β_I, and β_{II} PKC isoforms as well as novel (δ, ϵ, ζ, θ) and atypical (ζ) (Webb *et al.*, 1997) while canine trachealis does not express the α isoform and bovine ASM expresses the θ-PKC variants (Webb *et al.*, 2000).

It is well known that the stimulation of a Gq protein-coupled receptor, for instance by cholinergic agonist, induces the elevation of the cytosolic Ca^{2+} concentration and the production of DAG by phospholipase C. Both Ca^{2+} and DAG bind to the C2 and C1 domain, respectively, and recruit PKC to the membrane (Bell *et al.*, 1991; Huang, 1989). This interaction with the membrane results in release of the pseudosubstrate from the catalytic site and activation of the enzyme(Lester *et al.*, 1990).

PKC may modulate the sensitivity of the contractile apparatus to Ca^{2+}, since it can inhibit MLCP activity. However, the effectiveness of PKC contribution to airway contraction remains controversial. PKC has been shown to be involved in force maintenance in human airways (Rossetti *et al.*, 1995). However, other studies do not implicate PKC in agonist-induced Ca^{2+} sensitization and point to other effectors such as the small GTP-binding protein p21rho (Akopov *et al.*, 1998; Itoh *et al.*, 1994; Otto *et al.*, 1996; Yoshii *et al.*, 1999). Our recent studies have shown that PKC contributes to the sustained phase, but not to the initial phase, of cholinergic-induced contraction in rat airways (Mbikou *et al.*, 2006).

3.4 PKA

Cyclic-AMP-dependent protein kinase (PKA) is an ubiquitous mammalian enzyme which catalyzes Ser/Thr phosphorylation in protein substrates that in turn control a wide range of cellular functions including gene regulation, cell cycle, metabolism and cell death (Shabb, 2001). This tetrameric holoenzyme comprises two catalytic (C) subunits that possess kinase activity and two inhibitory regulatory (R) subunits, each including two tandem cAMP binding domains, *i.e.* CBD-A and CBD-B (Johnson *et al.*, 2001). cAMP is the essential second messenger that activates PKA (Berman *et al.*, 2005; McNicholl *et al.*). In the absence of cAMP, the R-subunit and the C-subunit create a complex that blocks substrate access and thus prevents the kinase activity. cAMP binding to the R-subunits releases these inhibitory interactions and unleashes the C-subunit, allowing substrate phosphorylation. There are

three isoforms of C (Cα, Cβ, and Cγ) and two major isoforms of R (R_I and R_{II}) that are further distinguished into subforms (α and β) (Zhao *et al.*, 1998). The physiological importance of these isozyme variations is not fully understood, but anchoring proteins (AKAPs) for R_{II} give it a unique cellular distribution (Scott *et al.*, 1994) R_I and R_{II} show sequence homology in their cAMP-binding and pseudosubstrate domains but differ extensively in their dimerization domains as well as in the sequence connecting the dimerization and pseudosubstrate domains. All known R-subunit isoforms share a common organization that consists of a dimerization domain at the NH_2 terminus followed by an autoinhibitor site and two-tandem cAMP-binding domains noted CBD-A and B (Taylor *et al.*, 2005). While the portion of the R subunit COOH-terminal to the inhibition site is responsible for high affinity binding of the C-subunit and cAMP, the remaining NH_2 terminus serves as an adaptor for binding to kinase anchoring proteins (Scott *et al.*, 1994) and is responsible for *in vivo* subcellular localization and targeting of PKA.

The ordered sequential mechanism of PKA activation is described as follows: cAMP binds first to CBD-B, making site CBD-A accessible to a second molecule of cAMP, which in turn causes the release of the active C-subunit (Kim *et al.*, 2007; Su *et al.*, 1995). In other words CBD-B functions as a gatekeeper for CBD-A, whereas the latter acts as the central controlling unit of the PKA system and provides the primary interfaces with the C-subunit (McNicholl *et al.*). The structures of the R-subunit in its active and inhibited states have also demonstrated that although CBD-A and CBD-B play clearly distinct roles in the activation of PKA, they both share similar allosteric features.

PKA activation is closely dependent on the cytosolic cAMP level which is itself regulated by G proteins via an enzyme named adenylate cyclase. Stimulation of GPCRs, by muscarinic receptor agonist for instance, can rise up or drop off AMPC production depending on the type of GPCRs. Indeed, GPCRs that activate the Gαi subunits inhibit cAMP production whereas GPCRs that activate the Gαs subunits activate cAMP production through this specific sequence: 1) activated Gαs subunit interacts with the adenylate cyclase 2) adenylate cyclase quickly converts ATP into cAMP, 3) AMPc molecule activate the PKA. Activation of PKA is involved in airway smooth muscle relaxation (Zhou *et al.*, 1992), which may involve phosphorylation of a number of effector proteins that cause either reduction of $[Ca^{2+}]_i$ and/or reduction of MLCK sensitivity to Ca^{2+}-calmodulin (de Lanerolle *et al.*, 1991).

 Studies have demonstrated that cAMP-dependent signaling pathway activation prevents or reverses the ASM contraction indirectly, via the inhibition of $InsP_3$ receptor (InsP3R) of the sarcoplasmic reticulum, reducing the $[Ca^{2+}]_i$. The InsP3R are responsible for mobilizing Ca^{2+} from sarcoplasmic reticulum in response to agonist binding. PKA has been shown to mediate the phosphorylation of the InsP3R which consequently reduces the ability of Inositol(l,4,5)triphosphate to release Ca^{2+} from membrane vesicles (Schramm *et al.*, 1995). The mechanism by which PKA-induced phosphorylation decreases insP3-induced Ca^{2+} release has not been determined, but known consequences of receptor/channel phosphorylation include altered agonist affinity as well as altered function.

3.5 CaMkinase II

Calcium-calmodulin-dependent protein kinase II (CaMKII) is an oligomeric serine/threonine-specific protein kinase which belongs to a family of enzymes regulated by

the calcium-calmodulin complex similarly to the MLCK. Increases in the cytosolic Ca^{2+} concentration following the stimulation modulate the function of many intracellular proteins (Zhou *et al.*, 1994). One of the most important intracellular acceptors of the Ca^{2+} signal is calmodulin (CaM), which exerts a modulating influence on the function of Ca^{2+}/CaM-dependent protein kinases. Among them, the CaMKII shows a broad substrate specificity and has been thought to be a multifunctional protein kinase (Cohen *et al.*, 1992; Colbran *et al.*, 1989a; Colbran *et al.*, 1989b). Four genes encode related but distinct isoforms of CaM kinase II (α, β, γ, and δ). It was originally isolated from brain tissues (Fukunaga *et al.*, 1982) (Goldenring *et al.*, 1983; Kuret *et al.*, 1984) and preparations of CaM kinase II purified from rat forebrain consist of the α (50 kDa) and β (60 kDa) subunits whose cDNAs have been cloned and sequenced (Kolb *et al.*, 1998). Non neuronal tissues express mostly the isoform γ, and δ but in such a low level that it makes difficult the purification of the enzyme; so most biochemical and physical data on the enzyme have been established with CaMkinase II-α and -β/β' from mammalian brain. In smooth muscle, CaM kinase II was first isolated as caldesmon kinase (Ikebe *et al.*, 1990b) with a molecular mass of the major subunit of 56 kDa. Isolated smooth-muscle CaM kinase II has enzymological properties similar to that of brain; however, smooth-muscle CaMKII is a tetramer according to its native molecular mass rather than a decamer or octamer as are the brain enzymes (Zhou *et al.*, 1994).

The CaMKII subunits are thought to assemble to holoenzyme through their C-terminal association domains (Fahrmann *et al.*, 1998). The linear structure of the CaMKII core consists of a catalytic/autoregulatory domain (A) containing a variable region V1, a conserved linker (B), and an association domain that contains two highly conserved sequences (C and D) as well as multiple variable regions (V2-V4). Function of each variable region has been identified as follows: V1 contains insert implicated in SR-membrane targeting; V2 possesses a functional nuclear localization signal, as well as a site of autophosphorylation; Insert X within V3 is rich in proline residues and conforms to a SH_3-binding sequence. The variable regions are diversely expressed in the different subunits. For instance the α and $δ_G$ isoforms are the smallest catalytically competent CaMKII products because they contain the A–D core sequences, but no inserts (Hudmon *et al.*, 2002). The most prominent proteins phosphorylated by the Type II CaM kinase are its own subunits (Bennett *et al.*, 1983; Kennedy *et al.*, 1983; Miller *et al.*, 1985; Miller *et al.*, 1986). Some studies suggest that kinase activity decrease after autophosphorylation (Kuret *et al.*, 1984; LeVine *et al.*, 1985; Yamauchi *et al.*, 1985) while others suggest that it increases (Shields *et al.*, 1984) or becomes autonomous (Saitoh *et al.*, 1985). Despite these discrepancies, the most accepted theory is that the autophosphorylation allows for the activation of the catalytic domain (Hanley *et al.*, 1987). The Ca2+-CaM complex interacts with and promotes autophosphorylation of each subunit of the CaMKII.

The implication of the CAMKII in the artery smooth muscle reactivity has been extensively investigated whereas little is known regarding its role in the airways smooth muscle. In contracted cultured ASM cells from bovine, studies demonstrated that CaMKII is responsible for the phosphorylation of the MLCK (Stull *et al.*, 1993). Biochemistry data showed that MLCK is phosphorylated by CaMKII (Hashimoto *et al.*, 1990; Ikebe *et al.*, 1990a) at a specific serine near the calmodulin-binding domain; and this phosphorylation brings about the reduction of the affinity of MLCK for the Ca^{2+}•CaM complex (Stull *et al.*, 1990). In another hand the phosphorylation of MLCK by CaMKII have been shown to decrease the

Ca^{2+} sensitivity of MLC_{20} phosphorylation (Tansey *et al.*, 1994; Tansey *et al.*, 1992). Taken together, these findings would suggest that the net effect of the CaMKII is the relaxation of the ASM. However, enzymatic tests revealed two key elements which exclude this hypothesis: 1) the rate of phosphorylation of MLCK likely by the CaMKII is first of all slower than the rates of increase in cytosolic Ca^{2+} concentrations, 2) and also slower than the rate of phosphorylation of the MLC in intact tracheal smooth muscle cells in culture (Tansey *et al.*, 1994). Therefore, the CaMKII activity is thought not to affect significantly the reactivity of the bovine ASM. Moreover, other studies showed that the CAMKII do not play a role in the profile of the contractile response upon stimulation in intact ASM from rat or cow (Liu *et al.*, 2005; Mbikou *et al.*, 2011; Sakurada *et al.*, 2003).

3.6 PI3K

The phosphatidy inositol 3-kinases (PI3K) superfamily draws together all the enzymes capable of phosphorylating specifically the hydroxyl group of a membrane phospholipid called phosphatidyl inositol (PtdIns). Cloning approaches revealed the existence of eight distinct PI3K genes expressing eight isoforms in human and mouse genomes. Based on their domain structure, lipid substrate specificity and associated regulatory subunits, these isoforms have been divided into three main classes as follows: class I including p110α, p110β, p110δ and p110γ; class II including PI3K-C2α, PI3K-C2β and PI3K-C2γ, and the class III consisting of the sole enzyme Vps34. The PI3K phosphorylates the PtdIns into four possible products, PtdIns(3)phosphate, PtdIns(3,4)biphosphate, PtdIns(3,5) biphosphate and PtdIns(3,4,5)triphosphate, which are involved in a wide range of cellular functions, including cell growth, proliferation, motility, differentiation, survival and intracellular trafficking (Fry, 2001; Fry, 1994; Katso *et al.*, 2001; Rameh *et al.*, 1999).

Purified PI3K is a heterodimer of 85 and 110 kDa subunits (Carpenter *et al.*, 1990; Fry *et al.*, 1992; Morgan *et al.*, 1990; Shibasaki *et al.*, 1991). Analysis of the primary sequence of p85 reveals a multidomain protein which contains a number of non-catalytic domains, a Src homology region 3(SH3), and a region with significant sequence similarity to the product of the breakpoint cluster region gene BCR (Otsu *et al.*, 1991). In vascular smooth muscle, PI3K appears to play a role in the regulation of contraction as experiments showed that the specific isoform PI3K-C2α is necessary for Rho/RhoKinase-dependent MLCP inhibition and consequently for the MLC20 phosphorylation and the contraction (Yoshioka *et al.*, 2007). However, in the ASM, the PI3K does not regulate the agonist-induced contractile response (Mbikou *et al.*, 2006).

4. Theoretical modelling of PK and ASMC contraction

4.1 Interest and general priniples

Since the contractile pattern of SMC in response to contractile agonists not only depends on the structure of the regulatory network that modulates the contractile apparatus but also on the dynamics of the reactions, understanding of the mechanisms underlying this contractile profile is quite impossible by a non-mathematical intuitive approach. In the following sections, we will present the concepts of our recent mathematical modelling of isometric contraction and force development in airway smooth muscle cells based on the 4-state latch bridge model (Hai *et al.*, 1988a) and upgraded by the unique description of MLCK and

MLCP regulatory pathways (Fajmut *et al.*, 2008; Fajmut *et al.*, 2005a; Fajmut *et al.*, 2005b; Fajmut *et al.*, 2005c; Mbikou *et al.*, 2011; Mbikou *et al.*, 2006).

The latch state was first described by the mathematical model by Hai and Murphy in 1988 (Hai *et al.*, 1988a). They introduced the model of isometric contraction based on the 4-state kinetic scheme of actomyosin crossbridges, in which to actin bound and unbound to myosin (phosphorylated and dephosphorylated) represent four different states. Even nowadays, that model represents the reference in modelling of smooth muscle contraction.

Huxley (Huxley, 1957) pioneered the modelling of smooth muscle contraction. His model from 1957 was based on the sliding filament theory. Until the occurrence of Hai and Murphy's model in 1988 (Hai *et al.*, 1988b), relatively small number of models of smooth muscle contraction was developed compared to striated muscles. In 1986 Gestrelius and Börgström (Gestrelius *et al.*, 1986) introduced new concepts in modelling. They first considered viscoelastic properties of the filaments and the cytoskeleton. The later model and Huxley's model enabled the study of nonisometric contraction and took into account mostly the dynamics and mechanics of the filaments and myosin crossbridges. Although the original Hai and Murphy's model (Hai *et al.*, 1988a) enabled only the prediction of isometric force its advantage was in considering the regulatory mechanisms that drive smooth muscle contraction. Several authors have later upgraded it to enable the prediction of nonisometric contraction. In 1997 Yu et al. (Yu *et al.*, 1997) expanded it to simulate the nonisometric contractions of smooth muscles, added length dynamics and assumed length-dependent bonding and unbonding rates to be distributed according to the Gaussian distribution. In 1999 Fredberg et al. (Fredberg *et al.*, 1999) and Mijailovich et al. (Mijailovich *et al.*, 2000) integrated the latch regulation scheme of Hai and Murphy with Huxley's sliding filament model of muscle contraction for the studies of the effects of length fluctuations on the dynamically evolving cross-bridge distributions, simulating those that occur in airway smooth muscle during breathing. The later model has been recently upgraded by simple description of Ca^{2+}-dependent regulation of MLCK activity (Bates *et al.*, 2009; Wang *et al.*, 2008). Our approach in the modelling of smooth muscle contraction is to upgrade the 4-state kinetic description of Hai and Murphy (Hai *et al.*, 1988a) with the signalling pathways that regulate the MLC phosphorylation and dephosphorylation. This was for a long time the missing part in the modelling of smooth muscle contraction.

Development of force in smooth muscles is achieved by interactions between myosin cross-bridges and actin filaments. To describe theses interactions Hai and Murphy proposed a kinetic scheme shown in Figure 1 (Hai *et al.*, 1988a). The scheme was later upgraded by Rembold and Murphy (Rembold *et al.*, 1990) with the consideration of the attachment of dephosphorylated myosin to actin with a very slow rate. Many authors ignore this interaction, since the rate of attachment of dephosphorylated myosin is 150-fold lower than that of phosphorylated myosin (0.002 s^{-1} and 0.3 s^{-1}, respectively) (Trybus, 1996). In our studies we consider it in the modelling (Fajmut *et al.*, 2008; Fajmut *et al.*, 2005a; Mbikou *et al.*, 2011; Mbikou *et al.*, 2006).

The four different states of the myosin cross-bridges are presented in Figure 1. : A+M - detached, dephosphorylated, A+MP - detached, phosphorylated, AMP - attached, phosphorylated, and AM - attached, dephosphorylated, the last one termed also a latch bridge. The corresponding reaction velocities of phosphorylation/dephosphorylation and attachment/detachment of myosin cross bridges, the relevant variables of mathematical

modelling, are indicated together with sites of action of enzymes MLCK and MLCP responsible for phosphorylation and dephosphorylation of MLCs, respectively. In the model, the magnitude of stress in smooth muscles is proportional to the concentration of myosin cross-bridges associated with actin filaments (AMP and AM), whereby myosin cross-bridges in the state AMP generate stress and cross-bridges in the state AM maintain stress in smooth muscles. v are either the velocities of crossbridge attachment/detachment or the velocities of phosphorylation/ dephosphorylation, which are regulated by MLCK and MLCP.

Hai and Murphy's model considered a very simple semi-theoretical description of the Ca^{2+}-dependent MLCK activation, and thus first coupled Ca^{2+} signalling pathway with the contraction. In this chapter we will present an upgrade from that model in the sense of purely theoretical and more detailed description of Ca^{2+}-dependent MLCK activation and functioning as well as MLCP regulation of force development. Our new concepts will be compared with the old ones. As a basic model scheme, we take the four-state model, in which we incorporate explicitly the description of both enzymatic reactions. The activity of MLCK is under the influence of transmitting Ca^{2+} signal and, hence, Ca^{2+} directly affects the force development. On the other hand, the action of MLCP is considered either independent of other signalling pathways or being under influence of Rho-kinase (RhoK), which phosphorylates MLCP and regulates its activity and catalytic properties.

4.2 Modelling of MLCK activity

According to the generally accepted view, MLCK is activated by the Ca^{2+}-CaM complex (Kamm *et al.*, 2001). In general, an increase in $[Ca^{2+}]_i$ is considered to initiate the binding of four Ca^{2+} ions to CaM, which is finally followed by association of complex Ca_4CaM with MLCK (Dabrowska *et al.*, 1982; Smith *et al.*, 2000). In this chapter we will present three different approaches to deal with interactions between Ca^{2+}, CaM and MLCK: a three-state model proposed by Kato et al. in 1984 (Kato *et al.*, 1984), our eight-state model proposed by Fajmut et al. in 2005 (Fajmut *et al.*, 2005b) and a semi-theoretical approach proposed by Rembold and Murphy in 1990 (Rembold *et al.*, 1990). The kinetic scheme of a three-state model is presented in Figure 4.
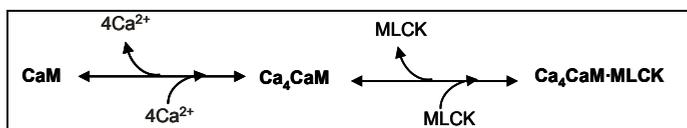

Fig. 4. **The three-state kinetic scheme of MLCK activation proposed by Kato *et al.*** (Kato *et al.*, 1984).

It assumes four independent and equivalent Ca^{2+} binding sites on CaM and considers a minimum number of possible states for CaM in the activation of MLCK. According to the kinetic scheme, MLCK is activated in two steps. The first step assumes simultaneous binding of four Ca^{2+} ions to CaM. In the second step, the intermediate complex of CaM with four Ca^{2+} ions bound (Ca_4CaM) is associated with MLCK. The final product Ca_4CaM-MLCK represents the active form of MLCK in the sense that it is able to phosphorylate MLCs. It should be noted, that Kato's kinetic scheme (Kato *et al.*, 1984) (Figure 4) reflects a somewhat

older and simplified view on the interactions between Ca^{2+}, CaM and MLCK. Newer experimental studies show that not only Ca_4CaM complex but also Ca_2CaM complexes and Ca^{2+}-free CaM interact with MLCK (Bayley *et al.*, 1996; Johnson *et al.*, 1996). It has been also shown that CaM and MLCK can interact without the presence of Ca^{2+} (Wilson *et al.*, 2002) and that the velocity of Ca^{2+} binding to CaM is different with respect to N and C terminals on CaM (Brown *et al.*, 1997; Johnson *et al.*, 1996; Minowa *et al.*, 1984; Persechini *et al.*, 2000).
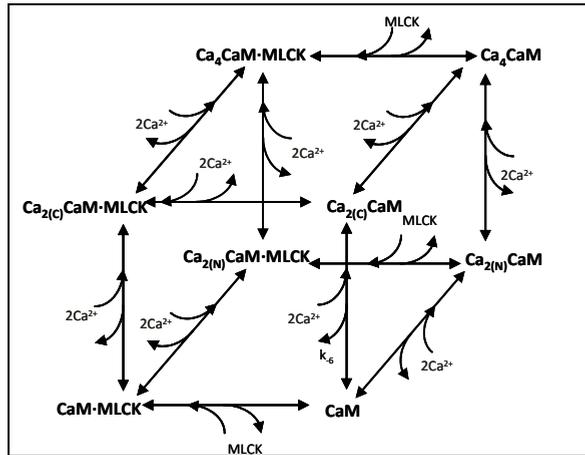
Fig. 5. **The eight-state kinetic scheme of MLCK activation proposed by Fajmut et al.** (Fajmut *et al.*, 2005a).

On the basis of these experimental findings we proposed more detailed and profound kinetic model of MLCK activation that considers either six (Fajmut *et al.*, 2005c) or eight (Fajmut *et al.*, 2005a) different states of CaM with respect to binding sites for Ca^{2+} on C and N terminals and a binding site for MLCK. The corresponding eight-state kinetic scheme is presented in Figure 5.

Binding reactions and reaction components involved can easily be recognised from the scheme. The complex $Ca_4CaM \cdot MLCK$ represents the active form of MLCK and can be applied in the 4-state kinetic scheme of force development as indicated in Figure 1.

In contrast to theoretical models of MLCK activation described here, a semi-theoretical approach was proposed by Hai and Murphy (Hai *et al.*, 1988a). This approach was based on the expression fitted to the experimentally determined dependence of MLC phosphorylation on $[Ca^{2+}]_i$. The corresponding expression was built into the mathematical model of force development proposed by them (Hai *et al.*, 1988a). The predictions of all abovementioned models for the steady state relative amount of the active MLCK (*A*), i.e., the ratio between $Ca_4CaM \cdot MLCK$ and total MLCK versus $[Ca^{2+}]_i$, are presented in Figure 6.

From the comparison of the model results (lines on the left panel) with the measurements (open circles) (Geguchadze *et al.*, 2004) in Figure 6 one can conclude that predictions of the eight-state model (Fajmut *et al.*, 2005a) (full line), most properly describes the process of MLCK activation. The other two models give either too sensitive (dotted line) (Rembold *et al.*, 1990) or too insensitive (dashed line) (Kato *et al.*, 1984) responses to $[Ca^{2+}]$ and they also
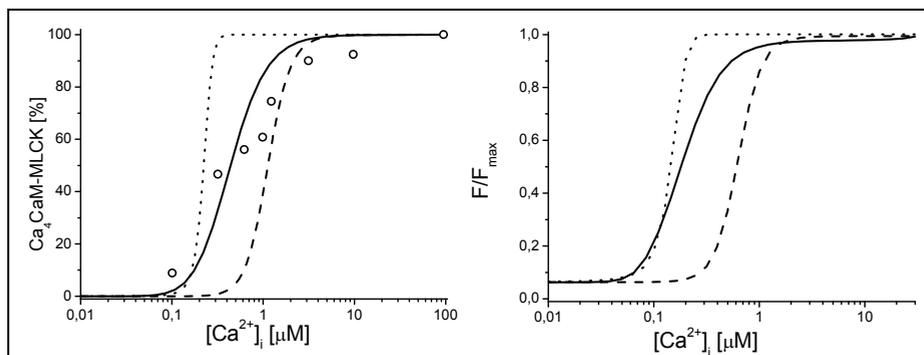
Fig. 6. **Model prediction of MLCK activation.** *Left panel:* Relative amount of the active MLCK (*A*) in dependence on [Ca²⁺] according to: semi-theoretical approach by Rembold and Murphy (Rembold *et al.*, 1990) (dotted line); eight-state model by Fajmut et al. (Fajmut *et al.*, 2005a) (full line); three-state model by Kato et al. (Kato *et al.*, 1984) (dashed line). Experimental results of Geguchadze et al. (Geguchadze *et al.*, 2004) (open circles). *Right panel:* The corresponding steady state force dependencies on [Ca²⁺] according to: Rembold and Murphy (Rembold *et al.*, 1990) (curve 1); Fajmut et al. (Fajmut *et al.*, 2005a) (curve 2); Kato et al. (Kato *et al.*, 1984) (curve 3).

do not have strong support by other experimental evidence (Gallagher *et al.*, 1993; Geguchadze *et al.*, 2004). The corresponding steady state force dependency on [Ca²⁺] (right panel) shows similar behaviour. Rembold and Murphy's model gives sensitive and Kato's model gives insensitive response to [Ca²⁺] and again don't have a strong support by experiments (Sieck *et al.*, 1998). These findings speak in favour of the necessity for more complex description of interactions between Ca²⁺, CaM and MLCK in Ca²⁺-signal transduction pathway. Another important property, which has not been implicated in this steady state analysis, is time dependency. In the past the interactions between Ca²⁺, CaM and MLCK were considered as very fast (Kasturi *et al.*, 1993; Torok *et al.*, 1994) and thus modelled as being in the steady state. The reason for that were the results obtained in *in vitro* experiments, which exhibited very fast kinetics between Ca²⁺, CaM and MLCK. However, experiments from 2001 performed by Wilson et al. (Wilson *et al.*, 2002) revealed that this might not be the case *in vivo*. Their experiments showed that at low [Ca²⁺]ᵢ MLCK is present in complexes with Ca²⁺-free CaM as well as with Ca_2CaM, in which Ca²⁺ is bound to the C-terminal of CaM (Wilson *et al.*, 2002). It has been suggested that this is a consequence of the increased affinity of CaM for Ca²⁺ by the presence of MLCK in the complex (Wilson *et al.*, 2002). In accordance with these findings the Ca²⁺-CaM activation of MLCK appears at sufficiently high Ca²⁺ levels, when in addition to the C-terminal binding sites, the N-terminal binding sites for Ca²⁺ on CaM are saturated. However, the transition from CaM-MLCK and $Ca_2CaM \cdot MLCK$ complexes is much slower compared to the transition from Ca²⁺- and MLCK- free CaM state. Our model simulations show for typical physiological total amounts of CaM and MLCK (10 μM and 2 μM, respectively) in smooth muscle cells that for [Ca²⁺]ᵢ = 0.3 μM approximately one third of MLCK is in complexes with Ca_2CaM, one third is Ca²⁺-CaM-free and one third is in active form. Moreover, the half saturation time for achieving the final active MLCK form for [Ca²⁺]ᵢ = 0.1 μM is 1.5 s and for [Ca²⁺]ᵢ = 0.5 μM it is 0.5 s. These results of the eight-state model show that the processes of

activation/deactivation of MLCK are not as fast as proposed by earlier models (Kato *et al.*, 1984) and some *in vitro* experiments on isolated CaM and MLCK (Kasturi *et al.*, 1993; Torok *et al.*, 1994). Moreover, the half-saturation time of MLCK activation/deactivation is of the same order of magnitude as the typical periods of oscillatory Ca^{2+} signals in smooth muscle cells (Mbikou *et al.*, 2006; Perez *et al.*, 2005), thus the processes of MLCK activation significantly contribute to decoding of oscillatory Ca^{2+} signal into a rather steady developed force already at the cellular level (Fajmut *et al.*, 2008; Fajmut *et al.*, 2005b; Mbikou *et al.*, 2011; Mbikou *et al.*, 2006) and add a small delay in force development after $[Ca^{2+}]_i$ increase.

4.3 Modelling of the MLCK/MLP balance and Ca^{2+}-contraction coupling

In our models (Fajmut *et al.*, 2008; Fajmut *et al.*, 2005b; Mbikou *et al.*, 2011; Mbikou *et al.*, 2006) we showed that the transduction of the Ca^{2+} signal from its appearance in the cytosol as a time-dependent variation of concentration to the development of force in smooth muscle cells is decoded mainly by the interactions between Ca^{2+}, CaM and MLCK, and is further translated to force by the balance between the phosphorylation and dephosphorylation of MLC, whereby both processes are regulated by MLCK and MLCP, respectively. The abundance of actomyosin crossbridges either phosphorylated or in the latch state is reflected in the magnitude of developed force. Our model results point out that a complete description of MLCK activation by Ca^{2+}-CaM is necessary for the relevant prediction of Ca^{2+}-contraction coupling and that 4-state latch bridge model upgraded with Ca^{2+}-CaM-dependent MLCK activation well describes the fast phase (first few minutes) of force development in the isometric contraction of rat tracheal rings (Fajmut *et al.*, 2008; Mbikou *et al.*, 2011; Mbikou *et al.*, 2006).

Essentially, the last and the most elaborated version of the model describing Ca^{2+}-contraction coupling consists of three parts (Mbikou *et al.*, 2011). The first one describes the activation of MLCK by Ca^{2+}-CaM complexes, considers binding of MLCK to Ca^{2+}-free CaM as well as to various Ca^{2+}-CaM complexes, predicts the concentration of the active form of MLCK, i.e. the complex $Ca_4CaM \cdot MLCK$, in dependence of $[Ca^{2+}]_i$. The kinetic scheme of these interactions is presented in Figure 5.

The second part describes the regulation of MLCP activity and represents an essential upgrade from the original description of Hai and Murphy (Hai *et al.*, 1988a), in which dephosphorylation velocity was taken as a linear function of AMP and MP. In our first models (Fajmut *et al.*, 2008; Fajmut *et al.*, 2005b; Mbikou *et al.*, 2006) we treated dephosphorylation process with two parallel enzymatic reactions of Michaelis-Menten type with AMP and MP as the substrates for MLCP and with $MLCP \cdot MP$ and $MLCP \cdot AMP$ as the intermediate complexes. All these models considered constant total amount of the enzyme and one Ca^{2+} independent catalytic activity of MLCP. In accordance with recent experimental results indicating that the activity of MLCP is under the influence of Rho-Kinase (RhoK) (Somlyo *et al.*, 2000), we modelled RhoK-dependent MLCP regulation (Mbikou *et al.*, 2011). RhoK phosphorylates MLCP and thus modifies its enzymatic properties. It decreases the rate constant of enzyme-substrate breakdown (k_{cat}) and increases the Michaelis constant (K_M) (Feng *et al.*, 1999; Ichikawa *et al.*, 1996; Lukas, 2004). The mechanisms by which RhoK is itself activated have not yet been fully determined. However, according to our present experimental results, RhoK is likely to be activated by the Ca^{2+} signal (Mbikou *et al.*, 2011). Thus, we consider the transition of RhoK from inactive state into

active state to be Ca²⁺-dependent. On these statements, RhoK activity has been modelled according to the kinetic scheme, represented in Figure 7 (Mbikou *et al.*, 2011).
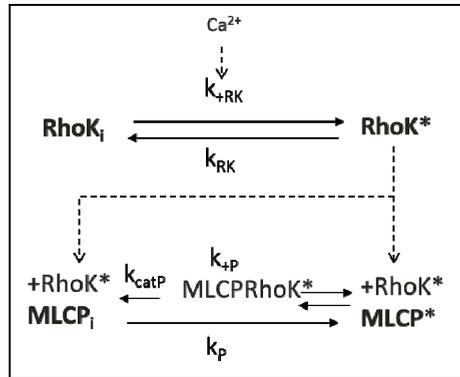

Fig. 7. **Kinetic scheme of RhoK dependent MLCP phosphorylation and partial inhibition proposed by Mbikou et al. (Mbikou *et al.*, 2011)**. RhoK$_i$, inactive RhoK; RhoK*, active Rhok. MLCP$_i$, inactive MLCP; MLCP*, active MLCP.

RhoK can be either in an inactive (RhoK$_i$) or active (RhoK*) state. k_{+RK} and k_{RK} are the corresponding on- and off- rate constants. k_{+RK} depends on the Ca²⁺ response, that is the [Ca²⁺]$_i$ above baseline. Modelling of MLCP phosphorylation by RhoK* is based on the steady-state Michaelis-Menten enzyme kinetics, whereby MLCP*RhoK* is the intermediate complex and k_{catP} is the rate constant for the breakdown of this intermediate complex into product, i.e. MLCP$_i$. k_{+P} and k_{-P} are the corresponding overall rate constants for MLCP phosphorylation and dephosphorylation, respectively. The values of the rate constant of MLCP-substrate breakdown (k_{cat}) and the Michaelis constant of MLCP (K_M) depend on the net effective form of MLCP, which is dependent on the fraction of unphosphorylated and inhibited MLCP ([MLCP]*/[MLCP]$_i$).

The third part of the modelling represents the well-known 4-state actomyosin latch bridge model (Hai *et al.*, 1988a). Links between all three parts of the model are the active form of MLCK and the net effective form of MLCP, which both modulate the rate of MLC phosphorylation and dephosphorylation.

In our experimental and theoretical study (Mbikou *et al.*, 2006), the version of the model without RhoK-dependent regulation of MLCP was first developed and analysed. The model was applied to the studies of the effect of different calcium signals to the amplitude and the velocity of force developed in airway smooth muscles. It was shown that the velocity and magnitude of the force that develops in several seconds after cholinergic stimulation are determined by the following signal parameters: the amplitude and the frequency of the oscillating Ca²⁺ signal as well as the plateau - but not the peak - in the biphasic Ca²⁺ signal, which comprises a peak followed by a decline to a plateau phase (Mbikou *et al.*, 2006). On the other hand, the increased frequency of oscillating Ca²⁺ signal is translated into the increase of force magnitude (Fajmut *et al.*, 2008; Mbikou *et al.*, 2006). One main physiological implication of that model (Mbikou *et al.*, 2006) was the prediction of the temporal delay of force generation with respect to Ca²⁺ transient. Figure 8 (left panel) presents the time

dependent relative MLC phosphorylation (*p*) (full line) and relative MLCK activity (*A*) (dotted line) after biphasic $[Ca^{2+}]_i$ with a peak and a plateau with the characteristic values (peak: 0.6 µM, plateau: 0.2 µM, baseline: 0.15 µM) as well as the time dependent relative force development (*F*) as predicted by the model (Mbikou *et al.*, 2006) (right panel).
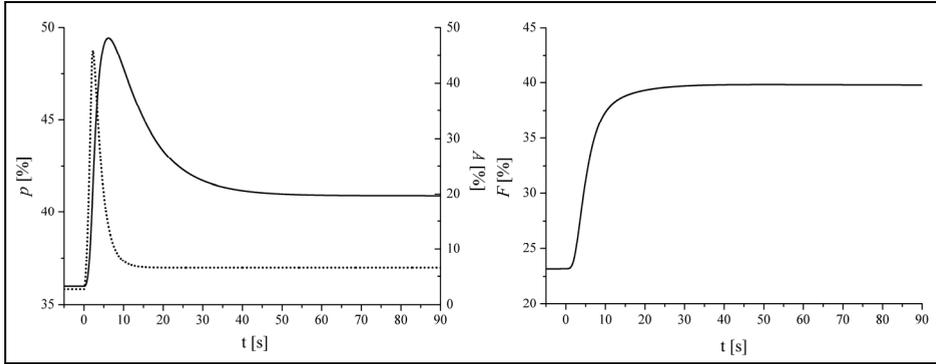

Fig. 8. **Predicted MLCK activity and MLC20 phosphorylation.** *Left panel:* Time dependent relative MLC phosphorylation (*p*) (full line) and relative MLCK activity (*A*) (dotted line) after biphasic $[Ca^{2+}]_i$ with a peak and plateau as predicted by the model (Mbikou *et al.*, 2006). *Right panel:* Time dependent relative force development (*F*) as predicted by the model (Mbikou *et al.*, 2006). *F* is defined relatively to the force obtained at supramaximal steady $[Ca^{2+}]_i$.

This time-delay originates also from the process of MLCK activation/inactivation. Namely, the process of Ca^{2+}-CaM-dependent MLCK activation contributes significantly to the time delay, in contrast with other studies (Kasturi *et al.*, 1993; Torok *et al.*, 1994), hypothesizing that the process of MLCK activation is extremely fast and is not likely to contribute more than a few milliseconds to the overall delay in force development (Sieck *et al.*, 1998). Slow force generation in our model is also a consequence of the slow-rate of MLCK activation/inactivation kinetics. Additionally, the kinetics of formation of actomyosin cross bridges explains the delay between MLC phosphorylation and force development, and the Hill-shaped time course of isometric contraction.

In another purely theoretical study (Fajmut *et al.*, 2008) we confirmed that upon biphasic Ca^{2+}-signal transduction through the system, MLCK controls amplitude more than duration, whereas MLCP tends to control both. These general characteristic regulatory properties of kinases and phosphatases were previously described by Heinrich et al. and Hornberg et al. (Heinrich *et al.*, 2002; Hornberg *et al.*, 2005) in other signalling processes.

In our most recent work (Mbikou *et al.*, 2011), the model was applied to the studies of RhoK contribution to the early phase of the Ca^{2+}-contraction coupling in airway smooth muscle. For this purpose, the simulation of RhoK inhibitor Y27632 was simulated by the model. Theoretical results of early stress development agreed with experimental results, which showed an evident drop in the stress development after RhoK inhibition in the early phase of contraction, whereby the shape and the characteristic time of stress development did not change significantly. The model further predicted that maximal RhoK activation and subsequent MLCP inactivation occur in less than 10 s, i.e., before the short time maximal contraction is achieved (Mbikou *et al.*, 2011).

An essential property of our models coupling Ca^{2+} and contraction is the explicit consideration of MLC phosphorylation and dephosphorylation steps described by Michaelis-Menten kinetics. This permits simulations of the effects that variations in enzyme contents and their catalytic properties exert on their velocities of phosphorylation/dephosphorylation, signal transduction and development of force. In this sense our models provide an upgrade with respect to the original Hai and Murphy's model as well as other models describing smooth muscle contraction.

4.4 Modelling of thin filament-associated regulation of contraction

An additional upgrade to the original Hai and Murphy's model has been proposed by Hai and Kim in 2005 (Fajmut *et al.*, 2005c) to address some experimental data that could not be explained by the four-state model. Because in phorbol ester-induced force development constant myosin phosphorylation could not be explained by the original 4-state model, the authors proposed and postulated a thin-filament-regulated latch-bridge model that includes two latch-bridge 4-state cycles, one of which is identical to the original Hai and Murphy's model, and the other one is the ultraslow 4-state cycle with lower cross-bridge cycling rates (Hai *et al.*, 2005). The model is able to fit phorbol ester-induced contractions at constant myosin phosphorylation. This was achieved by shifting cross bridges from the regular to the ultraslow cross-bridge cycle. It was also proposed that PKC activation leads to the thin-filament-based inhibition of actomyosin ATPase activity in ultraslow cycle, however, authors did not specify the target, the signalling pathway and the mechanism of this regulation. They hypothesized about calponin and caldesmon – the thin-filament-based regulatory proteins – being the candidates for the inhibition of actomyosin ATPase activity caused by PKC, because both proteins can exist in the unphosphorylated and phosphorylated form (Gerthoffer *et al.*, 1994).

In the early 1990s it was believed that the actin bound proteins, calponin and caldesmon, have large modulatory role in the latch state, however, in the late 1990s, the discovery of the significant regulatory role of myosin light chain phosphatase in smooth muscle contraction draw attention away from the thin-filament-based regulatory proteins (Paul, 2009). There is evidence that regulation of the response to a given $[Ca^{2+}]_i$, that is, regulation of the 'Ca^{2+} sensitivity' via modulating phosphatase activity, is as important as regulation of $[Ca^{2+}]_i$ in the control of contractility (Paul, 2009). PKC-mediated phosphorylation of CPI-17 has been postulated as a mechanism of PKC-mediated inhibition of MLCP (Hirano *et al.*, 2003). However, according to Hai and Kim (Hai *et al.*, 2005) this mechanism can potentially explain only the initial increase in the MLC phosphorylation but not the continued force development after myosin phosphorylation has already reached steady state.

In our experimental studies (Mbikou *et al.*, 2011; Mbikou *et al.*, 2006) we also observed the biphasic force development similar to that observed by phorbol ester-induced contraction (Hai *et al.*, 2005). The analysis of the time course of isometric contraction and MLC phosphorylation showed that the contractile response of rat tracheal rings to cholinergic stimulation developed in two distinct phases (Mbikou *et al.*, 2006). The first, short-time contractile response, which represents 70 % of the total contraction, was associated with a fast $[Ca^{2+}]_i$ peak followed by a plateau with, in some cases, with superimposed $[Ca^{2+}]_i$ oscillations, in correlation with fast and transient MLC phosphorylation, and Hill-shaped force development (Mbikou *et al.*, 2006) as shown in our model simulations presented in

Figure 8. The first fast phase was then followed by the second slow phase in which progressive increase of force reached plateau after 30 minutes (Mbikou *et al.*, 2006). Our model (Mbikou *et al.*, 2006) of force development generated by Ca^{2+}-dependent MLCK activation properly predicted the short-time contractile response. Simulations with the existing model showed that the long-time contractile response might be explained either by a long-term increase in oscillation frequency or $[Ca^{2+}]_i$ plateau. However, recordings of $[Ca^{2+}]_i$ responses to cholinergic stimulation for several minutes did not support such a hypothesis (Perez *et al.*, 2005). A possible explanation was, in parallel with the activation of MLCK, the inactivation of MLCP, which may be due to the action of PKC or RhoK (Mizuno *et al.*, 2008). For RhoK we have recently shown that it is implicated only in the fast phase of force development (Mbikou *et al.*, 2011). However, incorporation of progressive slow inactivation of MLCP in our modelling predicts a time course of isometric contraction similar to the experimental one, and explains the second increase in MLC phosphorylation and a slow phase in force development (Mbikou *et al.*, 2006). But, it does not explain a decrease in MLC20 phosphorylation associated with maximal force observed after 30 minutes (Mbikou *et al.*, 2006).

5. Conclusion

In airway smooth muscle as in other smooth muscles, actin-myosin cross bridge cycling critically depends on the phosphorylation of MLC_{20} and hence on MLCK/MLCP balance. MLCK, which activity is modulated by the Ca^{2+} signal through the formation of the Ca^{2+}-calmodulin-MLCK complex, is the most important kinase in airway myocyte contraction and the contractile properties of airway smooth muscle cells are lost when MLCK is inactivated or deleted. However, though activation of MLCK is indispensable, contraction of airway smooth muscle, both in its amplitude and time-course, is modulated by a network of kinases that can act upstream the Ca^{2+} signal, modulating the Ca^{2+} signal itself, or downstream, modulating the sensitivity of the contractile apparatus to Ca^{2+}. The main targets of the protein kinases acting on the decoding of the Ca^{2+} signal are MLCP and MLCK, though direct MLC_{20} phosphorylation, in parallel to MLCK, may be possible. Indeed, MLCP and MLCK have several sites of phosphorylation and their enzymatic activity depends on whether these sites are phosphorylated or not. These phosphorylations may up- or downregulate MLCP and MLCK activity. Stimulation of airways by contractile agonists such as acetylcholine activates kinases such as Rho kinase and PKC that inhibit MLCP activity and hence increase the sensitivity of the contractile apparatus to Ca^{2+}. By contrast, β_2-adrenergic stimulation, a major relaxant pathway, activates PKA which inhibits MLCK and favours MLCP activity. Additionally, contraction may be modulated by phosphorylation of caldesmon and calponin, proteins associated with the thin filament of actin. The MLCK/MLCP balance is hence embedded in a network of protein kinases. The resultant contractile behaviour of the SMC depends on the dynamics of the reaction of this regulatory network, and mathematical modelling is essential to decipher how the different protein kinases determine the time-dependent variation of the contractile status of the airway smooth muscle cell.

6. Acknowledgements

7. References

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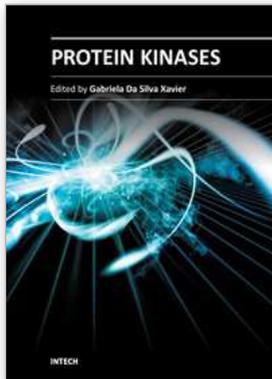
Protein Kinases

Edited by Dr. Gabriela Da Silva Xavier

Proteins are the work horses of the cell. As regulators of protein function, protein kinases are involved in the control of cellular functions via intricate signalling pathways, allowing for fine tuning of physiological functions. This book is a collaborative effort, with contribution from experts in their respective fields, reflecting the spirit of collaboration - across disciplines and borders - that exists in modern science. Here, we review the existing literature and, on occasions, provide novel data on the function of protein kinases in various systems. We also discuss the implications of these findings in the context of disease, treatment, and drug development.

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