Channels 8:5, 402–413; September/October 2014; © 2014 Taylor & Francis Group, LLC

Regulation of calcium channels in smooth muscle: New insights into the role of myosin light chain kinase

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Keywords: Myosin light chain kinase, TRP, voltage-dependent calcium channels, vascular smooth muscle

Abbreviations: VSMC, vascular smooth muscle cell; VSM, vascular smooth muscle; SMC, smooth muscle cell; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; MLCK, myosin light chain kinase; VOC, voltage-operated Ca^{2+} (channel) ROC, receptor-operated Ca^{2+} (channel); SOC, store-operated Ca^{2+} (channel); CaM, calmodulin; TRP, transient receptor potential (channel); siRNA, small interfering RNA; $[Ca^{2+}]_{cyt}$, cytosolic Ca^{2+} concentration

Smooth muscle myosin light chain kinase (MLCK) plays a crucial role in artery contraction, which regulates blood pressure and blood flow distribution. In addition to this role, MLCK contributes to Ca^{2+} flux regulation in vascular smooth muscle (VSM) and in non-muscle cells, where cytoskeleton has been suggested to help Ca^{2+} channels trafficking. This conclusion is based on the use of pharmacological inhibitors of MLCK and molecular and cellular techniques developed to down-regulate the enzyme. Dissimilarities have been observed between cells and whole tissues, as well as between large conductance and small resistance arteries. A differential expression in MLCK and ion channels (either voltage-dependent Ca^{2+} channels or non-selective cationic channels) could account for these observations, and is in line with the functional properties of the arteries. A potential involvement of MLCK in the pathways modulating Ca^{2+} entry in VSM is described in the present review.

Introduction

Smooth muscle contractility is controlled by the phosphorylation status of myosin light chain (LC_{20}), which is determined by the balance between the activity of myosin light chain kinase (MLCK) and myosin light chain phosphatase. More recently, the contribution of cytoskeleton remodelling to facilitate the transmission or maintenance of force has been recognized.[1] The major role in the development of contraction is played by Ca^{2+} and results from the Ca^{2+} dependence of MLCK activity. An increase in Ca^{2+} and Ca^{2+}/Calmodulin (CaM) complex activates smooth muscle MLCK (smMLCK, referred to as MLCK; a product of 130 kDa from *mylk1* gene).[2,3] Without Ca^{2+} or CaM, MLCK cannot be activated.[4] In vascular smooth muscle cells (VSMC), cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) increases as a result of Ca^{2+} release from intracellular Ca^{2+} stores located in the sarcoplasmic reticulum (SR) and of Ca^{2+} entry from the extracellular space through selective voltage-operated Ca^{2+} (VOC) channels and non-selective cation channels.[5,6] Nevertheless, there are still several gaps in our understanding of the regulation of Ca^{2+} signaling in VSM. Particularly, the nature of the channels involved in Ca^{2+} entry and the mechanism of their activation remain unclear, disputed or not investigated. Interest in the involvement of MLCK and cytoskeleton in Ca^{2+} channels activation in smooth muscle and non-muscle cells has increased in recent years. This review provides an overview of the current state of knowledge on the contribution of MLCK to Ca^{2+} channels regulation mechanisms in VSM from large to small arteries.

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Submitted: 06/12/2014; Revised: 07/28/2014; Accepted: 07/29/2014
http://dx.doi.org/10.4161/19336950.2014.950537

Vascular smooth muscle: from large to small arteries

Mechanisms of $[Ca^{2+}]_{cyt}$ increase vary according to vessel types and excitatory stimuli, probably because the expression of contractile proteins differs from proximal to more distal arteries.[7] Time course of contractile response to a vasoconstrictor agonist differs in conduit versus resistance arteries. This correlates with either tonic smooth muscles that develop slower rates of force activation and relaxation, as observed in the aorta, or phasic smooth muscles that display faster rates of force activation and relaxation, as found in portal vein and in the microcirculation.[7,8] In addition, resistance arteries, which compose the microcirculation, exhibit myogenic tone, this is the ability to contract in response to change in intraluminal pressure and is closely related to resistance microarteries intrinsic role in blood supply and blood pressure regulation.[9] Significant differences in agonist-induced $[Ca^{2+}]_{cyt}$ increase in VSMC from large conductance vs. small resistance arteries stem from the smaller contribution of intracellular Ca^{2+} release from the SR[10] and the higher contribution of voltage-dependent Ca^{2+} entry[11,12] observed in small resistance artery compared to large conduit artery contraction.

Calcium channels expression in vascular smooth muscle

In response to vasoconstrictor agonist, Ca^{2+} entry from the extracellular space occurs through VOC channels activated by

membrane depolarization, and non-selective cation channels, most of them members of the transient receptor potential canonical (TRPC) channels family. TRPC channels are activated following receptor occupancy (and called receptor-operated cation channels or ROC) or by internal Ca^{2+} stores depletion inducing capacitative Ca^{2+} entry (store-operated cation channels or SOC). They simultaneously induce the entry of Na^+ and Ca^{2+} triggering cell membrane depolarization and $[Ca^{2+}]_{cyt}$ increase.[5,6]

All TRPC isoforms are found in VSM, with the exception of TRPC2 and TRPC7.[13,14] The expression level of TRPC members is varying depending on the vessel type.[15] Commonly, TRPC1 and TRPC6 are highly expressed.[16-21] In general TRPC4 is detected at a lower expression level than TRPC1 and TRPC6 as described in rat aorta,[17,20] resistance mesenteric artery,[16] cerebral artery,[19] renal artery[20] and is not detected in caudal artery.[18] TRPC3 level is higher in rat cerebral artery,[19,22] caudal artery[18,23] and renal artery[20] than in the conductance artery aorta.[17,20] TRPC3 is expressed in rat resistance mesenteric artery but its level of expression is disputed.[16,21] While TRPC5 is not detected in rat resistance mesenteric artery,[16,21] a slight signal is observed in aorta[17,20] as well as in renal artery.[17,20].

Voltage-dependent L-type ($Ca_V1.2$), P-/Q-type ($Ca_V2.1$) and T-type ($Ca_V3.1$ and $Ca_V3.2$) Ca^{2+} channels are expressed in VSMC. They are characterized by distinct pharmacological and electrophysiological properties.[24] However, their relative distribution varies along the vascular tree.[25] $Ca_V1.2$, $Ca_V2.1$, $Ca_V3.1$ and $Ca_V3.2$ are expressed in a quite similar manner in aorta, while in resistance mesenteric artery the expression of $Ca_V2.1$, $Ca_V3.1$ and $Ca_V3.2$ is higher than that of $Ca_V1.2$.[25-28] Similarly, although L-type and T-type Ca^{2+} channels are present in SMC from cerebral artery, T-type Ca^{2+} channels are predominantly expressed.[29] Gustafsson et al.[30] failed to detect L-type channels mRNA in rat mesenteric arterioles <40 μm in diameter while T-type channels are expressed, suggesting that T-type channels might be associated with the regulation of myogenic tone in resistance arteries.

Culture is reported to strongly affect TRPC and VOC channels expression. In cultured rat mesenteric myocytes, all TRPC proteins are up-regulated compared to freshly dissociated cells.[31] In addition, cultured cells lose their expression of L-type VOC channels, while the expression of T-type VOC channels is increased.[32] Conversely, in the aortic cell line A7r5, L-type, P-/Q-type and T-type VOC channels are expressed and mRNA content of TRPC1, TRPC4 and TRPC6 is detected at a high level, while the expression of TRPC2, TRPC5 and TRPC7 is controversial.[33-35]

TRPC and VOC channels are not static molecules. They operate in association with other intracellular molecules required for their trafficking and insertion in the plasma membrane and modulating their activity within signal transduction complexes. Interactions of channel proteins with regulatory proteins have already been considered in detail in several reviews.[13,15,36,37] In addition, modulation of Ca^{2+} channels activation by several protein kinases (PK) such as PKC, PKG, PKA, Src tyrosine kinase and Ca^{2+}/CaM-dependent protein kinase II (CaMKII) has also been described based on the effects of protein kinases inhibitors on TRPC and VOC channels activity.[38-40] How MLCK contributes to Ca^{2+} channel activation is the issue raised in the present review.

Calcium channels regulation by MLCK

Controversial contribution of MLCK to calcium channels regulation in cultured or freshly isolated cells

Growing evidence highlights a role of MLCK in $[Ca^{2+}]_{cyt}$ increase in several smooth muscle and non-muscle cellular models. Indeed, concurring studies suggest that MLCK is involved in the activation of Ca^{2+} channels (**Table 1**). They were performed in different cell types, measuring either cytosolic Ca^{2+} or cation current using pharmacological inhibitors to block MLCK activity (**Table 2** for IC_{50} or K_i values) or molecular techniques to depress the kinase expression.

Most of the data obtained in cultured or freshly isolated cells suggest MLCK implication in a ROC/SOC-dependent Ca^{2+} entry. Using the synthetic naphtalenesulphonyl derivatives, ML-9 and ML-7, Watanabe et al.[41] and Kim et al.[42] showed that MLCK contributes to agonist-induced Ca^{2+} influx in endothelial cells and murine ileal myocytes, respectively. This observation has been confirmed by using MLCK antisense oligonucleotide.[41] In rabbit portal vein myocyte, 5 μM of a substrate-specific peptide inhibitor of MLCK, $MLCK_{(11-19)}$amide, inhibits the cation current activated by noradrenaline by 80%, and 10 μM of AV25, a peptide of 25 amino acids targeting the auto-inhibitory site of MLCK, depresses the amplitude of the cation current by 72%.[43] MLCK is also reported to be involved in capacitative Ca^{2+} entry following thapsigargin or cyclopiazonic acid treatment in human monocytes/macrophages.[44] In addition, by using a MLCK dominant negative mutant and RNA interference for MLCK, Shimizu et al.[45] and Kim et al.,[46] respectively, suggested the involvement of MLCK in the activation of TRPC channels responsible for a non-capacitative Ca^{2+} entry in HEK293 cells activated by carbachol.

Other researchers reported either no effect of MLCK inhibitor on $[Ca^{2+}]_{cyt}$ increase, as observed in cultured aortic SMC (**Fig. 1A**)[47] or a MLCK independent effect[48,49] (**Table 1**). Smyth et al.[49] showed that ML-9 inhibits capacitative Ca^{2+} entry by preventing the localization of stromal interaction molecule 1 (STIM1) into punctae structures between the plasma and endoplasmic reticulum (ER) membranes where it can interact and activate Ca^{2+} channels. This observation could not be confirmed with a small interfering RNA (siRNA) directed against MLCK, which suggests that the effect of ML-9 is not related to MLCK inhibition.[49] Similarly, a direct effect of ML-9 on the channel protein, independently on change in MLCK activity, is suggested to explain the inhibition of TRPC6 current in transfected HEK293 cells.[48]

Only 2 reports suggest a role of MLCK in voltage-dependent Ca^{2+} entry.[10,50] We, recently, demonstrated that the decreased MLCK expression after anti-MLCK siRNA transfection in the aortic cell line A7r5 leads to the inhibition of L-type Ca^{2+} current and VOC-dependent Ca^{2+} entry (**Fig. 1A**).[10] However,

Table 1. Effect of MLCK inhibition on Ca^{2+} signaling in cultured or freshly isolated cells

Cell type	MLCK Inhibition	Stimulating agent	Effect on $[Ca^{2+}]_{cyt}$	References
Freshly isolated rabbit portal vein myocytes	ML-9 (5 μM), ML-7 (5 μM), $MLCK_{(11-19)}$amide (5 μM)	NA, GTPγS, OAG	↘I_{cat}	43
	AV25 (10 μM)		No effect on $I_{cat(ATP)}$	
Freshly isolated rabbit mesenteric artery myocytes	WT (20 μM)	AngII	↘I_{cat}	122
		OAG	No effect	
Freshly isolated guinea-pig gastric myocytes	ML-7 (3 μM)	CCh	↘I_{CCh}	50
		Voltage	↘I_{Ba} (L-type VOC)	
Freshly isolated human monocytes/ macrophages	ML-9 (10-100 μM), WT (1-100 μM), MLCK antisense	TG, CPA	↘CCE	44
Freshly isolated murine ileal myocytes	ML-7 (5 μM), ML-9 (10 μM)	CCh	↘I_{CCh}	42
Cultured bovine adrenal glomerulosa cells	WT (10 μM), ML9 (100 μM), KT5926 (100 μM), MS-347a (30 μM)	AngII	↘Ca^{2+} entry	123
		Iono, TG, KCl	No effect	
Cultured porcine aortic endothelial cells	ML-9 (1-100 μM)	BK, TG	↘CCE	124,125
	ML-7 (0.1-30 μM)			126
	MLCK antisense			41
Cultured rat pulmonary artery endothelial cells	ML-9 (100 μM)	TG	↘CCE	127
Cultured rat pulmonary artery smooth muscle cells	ML-9 (10-100 μM), ML-7 (1-100 μM)	Hypoxia	↘CCE	128
Cultured human HEK293 cells	ML-9 (100 μM)	TG	↘CCE; ↘I_{CRAC}	49
	MLCK siRNA, WT (20 μM)		No effect	
Cultured human HEK293 cells overexpressing TRPC5	ML-7 (3 μM), MLCK-siRNA	CCh	↘I_{CCh}	46
Cultured human HEK293 cells overexpressing TRPC5	ML-9 (3-50 μM), WT (1-10 μM),	CCh, ATP	↘Ca^{2+} entry (no effect on Ca^{2+} release)	45
	MLCK dominant negative		↘ I_{CRAC}	
Cultured human HEK293 cells overexpressing TRPC6	ML-9 (10 μM), ML-7 (10 μM)	CCh	↘I_{CCh}	48
	WT (3 μM), MLCK dominant negative inhibitory peptide		No effect	
Cultured rat aortic SMC	ML-7 (10 μM)	VP	No effect	47
Cultured rat aortic cell line A7r5	MLCK-siRNA	VP	↘Ca^{2+} entry	410
		Voltage	↘I_{Ba} (L- and T-type VOC)	

Abbreviations: NA: noradrenaline; VP: vasopressin; KCl: physiological solution enriched in K^+; TG: thapsigargin; Iono: ionomycin; AngII: angiotensinII; BK: bradykinin; CCh: carbachol; CPA: cyclopiazonic acid; WT: wortmannin; OAG: 1-oleoyl-2-acetyl-sn-glycerol ; ↘ decrease; VOC: voltage-dependent Ca^{2+} channels; I_{Ba}: barium current; I_{CRAC}: calcium-release activated calcium current; I_{cat}: cationic current; CCE: capacitative Ca^{2+} entry.

decreased Ca^{2+} flux is associated with decreased $Ca_V1.2$ expression in MLCK-depleted cells, preventing to confirm direct regulation of VOC channel activity by MLCK (**Fig. 2**).[10]

MLCK regulation of cytosolic calcium increase in agonist stimulated smooth muscle tissues

Few studies have investigated the involvement of MLCK in Ca^{2+} signaling in isolated artery or smooth muscle tissue (**Table 3**). The use of inhibitory peptides in whole artery is quite difficult due to their high molecular weight. In rat caudal artery, 75 μM of the inhibitory peptide AV25 is required to markedly inhibit the contractile tension without affecting the LC_{20} phosphorylation status.[51] Wortmannin, a more potent inhibitor of phosphoinositide 3-kinase (PI3K) than of MLCK, does not affect the cytosolic Ca^{2+} response to high KCl in rat aorta,[52] rabbit aorta,[53] guinea-pig ureter[54] or human myometrium,[55] where response to oxytocin is also not affected. This could be explained by the relatively low concentration in wortmannin used, whereas a higher concentration depresses the Ca^{2+} response to high KCl and vasoconstrictor agonist in porcine carotid artery,[56] this was confirmed when inhibiting MLCK with ML-9. Involvement of MLCK in Ca^{2+} channels activation in smooth muscle is also disputed by the observation that in fura-2-loaded guinea-pig tracheal smooth muscle, ML-9 inhibits the increase in $[Ca^{2+}]_{cyt}$ and the contraction induced by 60 mM KCl, methacholine or thapsigargin, while wortmannin inhibits the contraction elicited by these stimuli without affecting $[Ca^{2+}]_{cyt}$. Similarity between the effects of ML-9 and the cation channel blocker SKF-96365 suggests that ML-9 acts as a potent inhibitor of Ca^{2+}-permeable channels independently on MLCK inhibition in tracheal smooth muscle.[57]

Table 2. IC_{50} or Ki values of pharmacological inhibitors on MLCK activity

Pharmacological inhibitor	Target	IC_{50} or Ki (μM)
Wortmannin	acts at or near the catalytic domain of MLCK in a noncompetitive and irreversible manner; far more potent inhibitor of PI3K of Class 1 and Class 2[132,150]	IC_{50}: 1.9
AV25	targets the auto-inhibitory site of MLCK.[51]	IC_{50}: 0.2
MLCK(11-19)amide	binds to the substrate site of MLCK.[129]	Ki: 10
KT5926	targets the ATP-binding site of MLCK in a competitive mode and the substrate site of MLCK in a noncompetitive manner; far more potent inhibitor of CaMKII.[151,152]	Ki: 0.0044 - 0.018
K-252a	targets the ATP-binding site of MLCK; far more potent inhibitor of CaMKII.[152,153]	Ki: 0.0018 - 0.020
MS-347a	binds to the catalytic domain of MLCK in an irreversible manner.[130]	IC_{50}: 9.2
ML-9	targets the ATP-binding site of MLCK.[101,152]	Ki: 3.9 IC_{50} (on NA-evoked Icat): 2
ML-7	targets the ATP-binding site of MLCK.[101,152]	Ki: 0.3 IC_{50} (on NA-evoked Icat): 0.8

IC_{50} and Ki values with respect to MLCK activity, except for ML-7 and ML-9 where the IC_{50} values were obtained on noradrenaline (NA)-induced cationic current (Icat).

In opposition, in isolated rat aorta, the more potent MLCK inhibitor, ML-7, decreases the non-voltage-dependent and non-capacitative Ca^{2+} entry induced by vasopressin (**Fig. 1A**), and inhibits LC_{20} phosphorylation.[47] In rat resistance mesenteric artery, where contraction is mainly dependent on VOC activation, ML-7 depresses the voltage-dependent Ca^{2+} entry in response to noradrenaline (**Fig. 1A**) and the depolarization-activated L-type current.[10] These results were confirmed with a siRNA directed against MLCK, which was transfected by reversible permeabilization in resistance mesenteric artery.[10] After 3 days of artery culture, MLCK protein content was depressed by approximately 50%, which was associated with similar reduction of VOC-dependent Ca^{2+} signals in response to noradrenaline (**Fig. 1A**) or high KCl. As also observed in

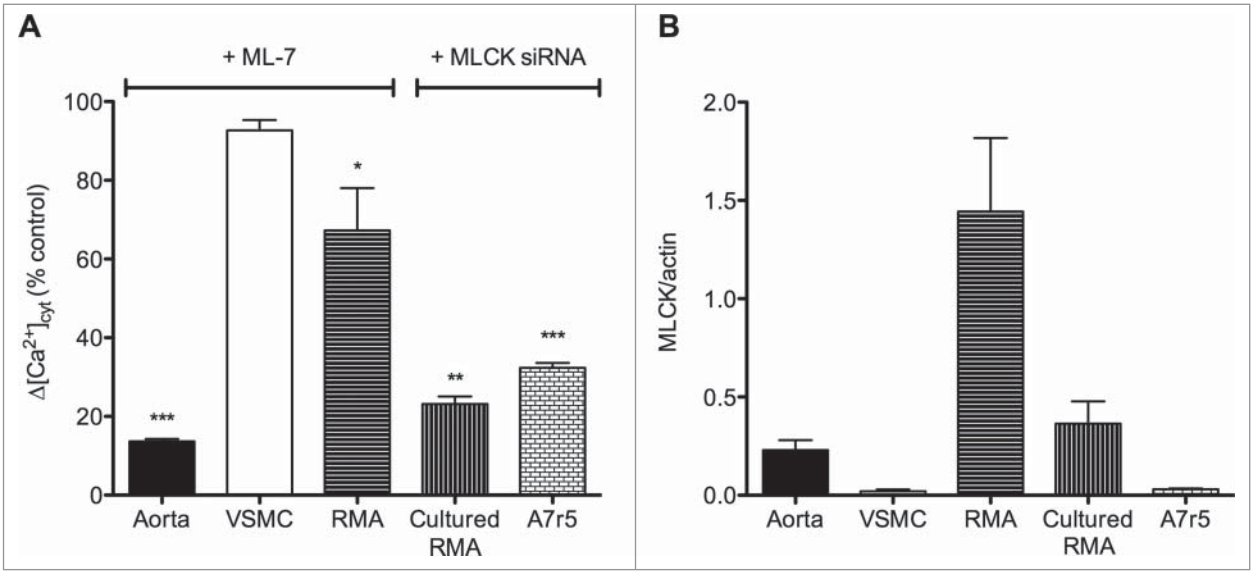

Figure 1. Effect of MLCK inhibition on agonist-induced cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) increase in comparison with MLCK expression level in aorta, cultured aortic smooth muscle cells (VSMC), resistance mesenteric artery (RMA), cultured RMA (organ-cultured for 4 days) and the aortic cell line A7r5. (**A**) $\Delta[Ca^{2+}]_{cyt}$ response after agonist stimulation (noradrenaline 10 μM in RMA and cultured RMA and vasopressin 10 nM in aorta, VSMC and A7r5 cells) in the presence of the MLCK inhibitor (ML-7 at 3 μM in aorta and RMA and 10 μM in VSMC) or after MLCK down-regulation with anti-MLCK-siRNA (in cultured RMA and A7r5) compared to control stimulated conditions. The VOC channel antagonist verapamil (1 μM) was used to block the voltage-dependent component of the $[Ca^{2+}]_{cyt}$ increase in response to vasopressin in aorta. (**B**) Mean values of Western blot data. MLCK expression was normalized to the actin content. Vertical bars represent the SEM (n = 3–7). *, **, *** Significant differences compared to control conditions. Results were extracted from published data.[10,47] The expression level of MLCK is lower in VSMC compared to whole vascular smooth muscle (aorta or RMA) but this does not explain the weaker contribution of MLCK to Ca^{2+} channel regulation in VSMC. Indeed larger inhibition of $\Delta[Ca^{2+}]_{cyt}$ is observed in aorta compared to RMA, while the expression level of MLCK is lower.

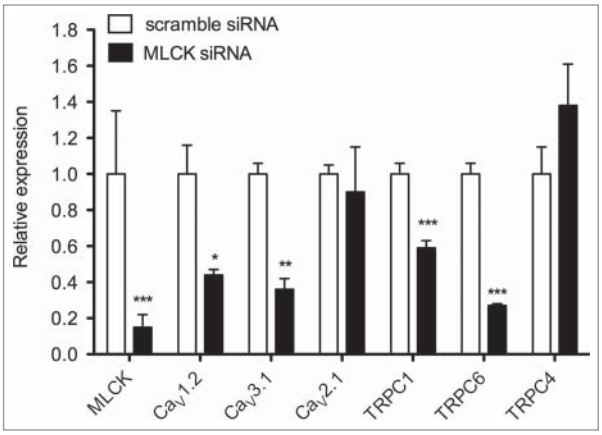

Figure 2. Effect of MLCK depletion on genes expression in A7r5 cells. Relative mRNA expression of L-type ($Ca_V1.2$), P/Q type ($Ca_V2.1$) and T-type ($Ca_V3.1$) voltage-dependent Ca^{2+} channels and TRPC1, TRPC6 and TRPC4 non-selective cation channels in anti-MLCK-siRNA-transfected A7r5 cells (black bars) and scramble-siRNA-transfected A7r5 cells (open bars) (n = 5-8). mRNA level of each gene was normalized to the level of RPL32 mRNA and compared to the value in scramble-siRNA-transfected cells. Results are expressed as mean ± SEM (vertical bars). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ vs. scramble-siRNA-transfected cells. Data on MLCK and voltage-dependent Ca^{2+} channels were extracted from published data.[10] MLCK downregulation in A7r5 cells is associated with a decrease in the mRNA expression of several Ca^{2+} channels.

A7r5 cells, the decrease in Ca^{2+} responses in MLCK-downregulated arteries is associated with a decrease in the mRNA expression of $Ca_V1.2$ channel, though this decrease was not statistically significant.[10]

Factors limiting the identification of calcium channels regulation by MLCK

Data summarized above indicate that the degree of MLCK contribution to the regulation of Ca^{2+} channels activity is variable among arteries and cell models and that no precise Ca^{2+} channel type can be identified as the target of MLCK activity.

The first difficulty lies in the lack of specific pharmacological inhibitors against MLCK. Selectivity of those inhibitors is controversial and should be viewed cautiously. Most of them are far more potent inhibitors of other kinases: wortmannin against PI3K,[58] KT5926 and K-252a against CaMKII.[59] The more efficient compound is the synthetic naphtalenesulphonyl derivative ML-7, which is approximatively 2.5 fold more potent than ML-9 to reduce noradrenaline-induced cation current.[43] However, effect of pharmacological inhibitor was not always confirmed in knockdown models and direct interaction with Ca^{2+} channel has been suggested in several studies.[48,57]

Down-regulation of MLCK protein is an alternative method to the use of chemical inhibitors. However, interpretation of changes in Ca^{2+} movements can be biased by the fact that depletion in MLCK protein is associated with a decrease in several Ca^{2+} channels expression, as described below.

The complexity of excitation-response coupling mechanisms and the different contribution of voltage-dependent and non-voltage-dependent Ca^{2+} channels to contraction according to the origin and the type of the vessel contribute to the difficulty of drawing a global model accounting for MLCK effect.[11,12] Identification of the channel targeted by MLCK is also hindered by the low selectivity of Ca^{2+} channels pharmacological inhibitors. The activity of TRPC is usually measured using compounds as SKF-96365, flufenamic acid or gadolinium, none of these being specific. The function of TRPC has then been highlighted by using downregulation techniques with oligonucleotide antisense or siRNA transfection methods or knockout (KO) animal models. However, KO models are reported to lead to compensatory overexpression as exemplified by the up-regulation of TRPC3 in aorta and cerebral artery from TRPC6 KO mice[60] or to conflicting results as observed for TRPC1, which does not appear to be an obligatory component of SOC or stretch-activated cation channels in cerebral artery from $TRPC1^{-/-}$ mice, but is required for ROC and SOC in siRNA transfected aortic cells[17] and in endothelial cells from $TRPC4^{-/-}$ mice or STIM1 mutant.[61] Selectivity of VOC channels blockers is also questionable.[27,62] Unfortunately the use of $Ca_V1.2$ KO mice is compromised as mice die before birth, probably due to heart defect.[63]

Table 3. Effect of MLCK inhibition on Ca^{2+} signaling in smooth muscle tissues

Tissue type	Inhibitor	Stimulating agent	Effect on $[Ca^{2+}]_{cyt}$	References
Rat aorta	WT (1 μM)	KCl, Phe	No effect	52
Rabbit aorta	WT (1 μM)	KCl, DPB	No effect	53
Rat aorta	ML-7 (3 μM)	NA	↘Ca^{2+} influx	47
Rat resistance mesenteric artery	ML-7 (3 μM), MLCK-siRNA	NA, KCl	↘Ca^{2+} influx; ↘I_{Ba} (L-type VOC)	10
Porcine carotid artery	ML-9 (10-100 μM), WT (10 μM)	KCl	↘Ca^{2+} influx	56
Porcine aortic endothelial cells (in valvular strips)	ML-9 (30-100 μM)	TG	↘CCE	131
	WT (10 μM)		No effect	
Guinea-pig tracheal smooth muscle	ML-9 (10-100μM)	KCl, MC, TG	↘Ca^{2+} influx	57
	WT (3 μM)		No effect	
Guinea-pig ureter	WT (4 μM)	KCl	No effect	54
Human myometrium	WT (4 μM)	Spontaneous contraction, oxytocin	No effect	55

Abbreviations: NA: noradrenaline; VP: vasopressin; KCl: physiological solution enriched in K^+; DPB: 12-deoxyphorbol 13-isobutyrate; MC: methacholine; TG: thapsigargin; Phe: phenylephrine; WT: wortmannin; ↘ decrease; I_{Ba}: barium current; CCE: capacitative Ca^{2+} entry.

The level of MLCK expression might also influence the degree of MLCK contribution to Ca^{2+} flux regulation. A comparison of MLCK protein expression in aorta versus small resistance mesenteric artery shows that the relative MLCK content is higher in resistance artery than in aorta (**Fig. 1B**).[64] The activity of MLCK is also different according to the vessel type, being 1.9-fold lower in tonic smooth muscle compared to phasic smooth muscle.[65] The higher activity of MLCK is likely to occur in phasic smooth muscle where faster contractile responses are observed.[65] As shown on **Figure 1** and as previously demonstrated in cultured tracheal SMCs,[66] the level of MLCK expression is lower in cultured aortic SMC compared to aorta and resistance mesenteric artery, and Ca^{2+} entry is also less affected by MLCK inhibition. This observation could suggest that a lower level of MLCK expression is associated with a weaker contribution of MLCK to Ca^{2+} channels regulation. However, this model does not fit with the larger inhibition of Ca^{2+} entry by ML-7 in aorta compared to resistance mesenteric artery (**Fig. 1A**).

Eventually, the intracellular organization of SMC from large and small artery could affect Ca^{2+} entry mechanisms by favoring interactions and cross-regulation between intracellular compartments. Intracellular distribution of different pools of MLCK as well as of Ca^{2+} ions might influence the contribution of MLCK to the regulation of the contractile machinery and the Ca^{2+} entry mechanisms. Further experiments would allow confirming this hypothesis.

The apparent absence of Ca^{2+} channel selectivity in the effect of MLCK could reflect an action of the kinase on a process in the activation pathway that might be shared by several types of channels. By regulating the translocation of the channel protein and its functional insertion in the membrane through a modulation of cytoskeleton filaments, MLCK could contribute to the activation of several types of Ca^{2+} channels, but also of other signaling molecules for which trafficking is an important step in the pathway leading to their activation.

Involvement of MLCK in calcium channels trafficking

Calcium channels translocation

Ca^{2+} channels activation requires their functional insertion in the plasma membrane. We currently know from studies essentially performed in non-VSMC models, that ion channels inserted in intracellular vesicles are translocated to plasma membrane following agonist or mechanical stimulation.[67-69]

Translocation of TRP channels to plasma membrane has been reported to occur within smooth muscle caveolae,[70] which are omega-shaped membrane invaginations containing caveolin-1, the main smooth muscle caveolin, involved in the clustering of signaling molecules and in the regulation of receptors/mediators trafficking.[71] Caveolae and caveolin-1 have been shown to spatially localize IP_3R1 and TRPC3 in cerebral myocytes.[72] Similarly, caveolin-1 is involved in potassium channels (BK_{Ca}) and L-type Ca^{2+} channels co-localization within caveolae in mesenteric artery SMC.[73] On the opposite, in conductance artery L-type Ca^{2+} channels translocation is independent of caveolae and KCl^-induced contraction is not affected in caveolin-1 KO models.[70,74,75] This observation can be related to the different intracellular organization of resistance and conductance arteries, the SR being preferentially localized in cell periphery in the former, while in conductance artery it is more centrally located.[76] It is worth mentioning that, in addition to their differential role in the regulation of Ca^{2+} flux, caveolae are reported to differentially contribute to RhoA activation and artery contraction in resistance artery[77] and in aorta.[74,75]

Scaffolding proteins such as the vesicle-associated membrane protein 2 (VAMP2) and the soluble N-ethylmaleimide-sensitive factor associated protein (αSNAP),[78] the protein Homer[79] and the PDZ motif [80,81] found in TRPC that have been identified in cultured HEK293 cells and neuronal cells could be involved Ca^{2+} channels trafficking in vascular smooth muscle. The only partner identified so far in A7r5 cells, primary cerebral artery myocytes and intact cerebral artery[82] is PKCδ, which mediates the trafficking of TRPM4-containing vesicles to plasma membrane in response to pressure increase.

Translocation of the voltage-dependent $Ca_v1.2$ channels to plasma membrane involves the auxiliary subunits of the L-type VOC channel. The β subunit in HEK293 cells[83-85] and the α2δ-1 subunit in cerebral artery SMC[86] are essential interacting partners for the α1C subunit translocation. Binding of intracellular galectin-1, which is a carbohydrate-binding protein, to the α1-interacting domain of the α1C subunit can reduce channel expression at the plasma membrane in mesenteric artery as well as in A7r5 cells.[87] But the relevance in small resistance artery where T-type channels instead of L-type channels are predominant and responsible for the myogenic tone remains elusive.

Recently, STIM1 activated by store depletion has been shown in HEK293 cells and A7r5 cells to bind to the C-terminal of $Ca_V1.2$ through its Ca^{2+}-release activated Ca^{2+} domain and to inhibit $Ca_V1.2$ expression at the plasma membrane.[88,89] There is an important discrepancy between this model and previous reports showing that store depletion-induced Ca^{2+} signal is depressed in the presence of the VOC Ca^{2+} channel blocker diltiazem in cultured aortic cells suggesting that $Ca_v1.2$ participates to capacitative Ca^{2+} entry.[90] Similarly, 2 reports demonstrate that VOC channels contribute to the refilling of Ca^{2+} stores in A7r5 cells[10] and guinea-pig intestinal smooth muscle.[91]

A potential role for MLCK in calcium channels trafficking

Although few data are available in whole vascular smooth muscle tissue, cytoskeleton elements appear to be ideal candidates to support Ca^{2+} channels trafficking in collaboration with scaffolding partners and regulatory proteins. In view of the role played by MLCK in cytoskeleton modulation, MLCK regulation of Ca^{2+} channel activity could implicate a contribution to channel protein trafficking.

In this line, MLCK through LC_{20} phosphorylation, and myosin motors are known to contribute to transport vesicles from the Golgi apparatus to the ER along actin filaments.[92,93] The issue is still poorly investigated in VSM but Dey et al.[94] demonstrated in $Ca_V3.1$ and $Ca_V3.2$ transfected HEK293 cells that brefeldin A,

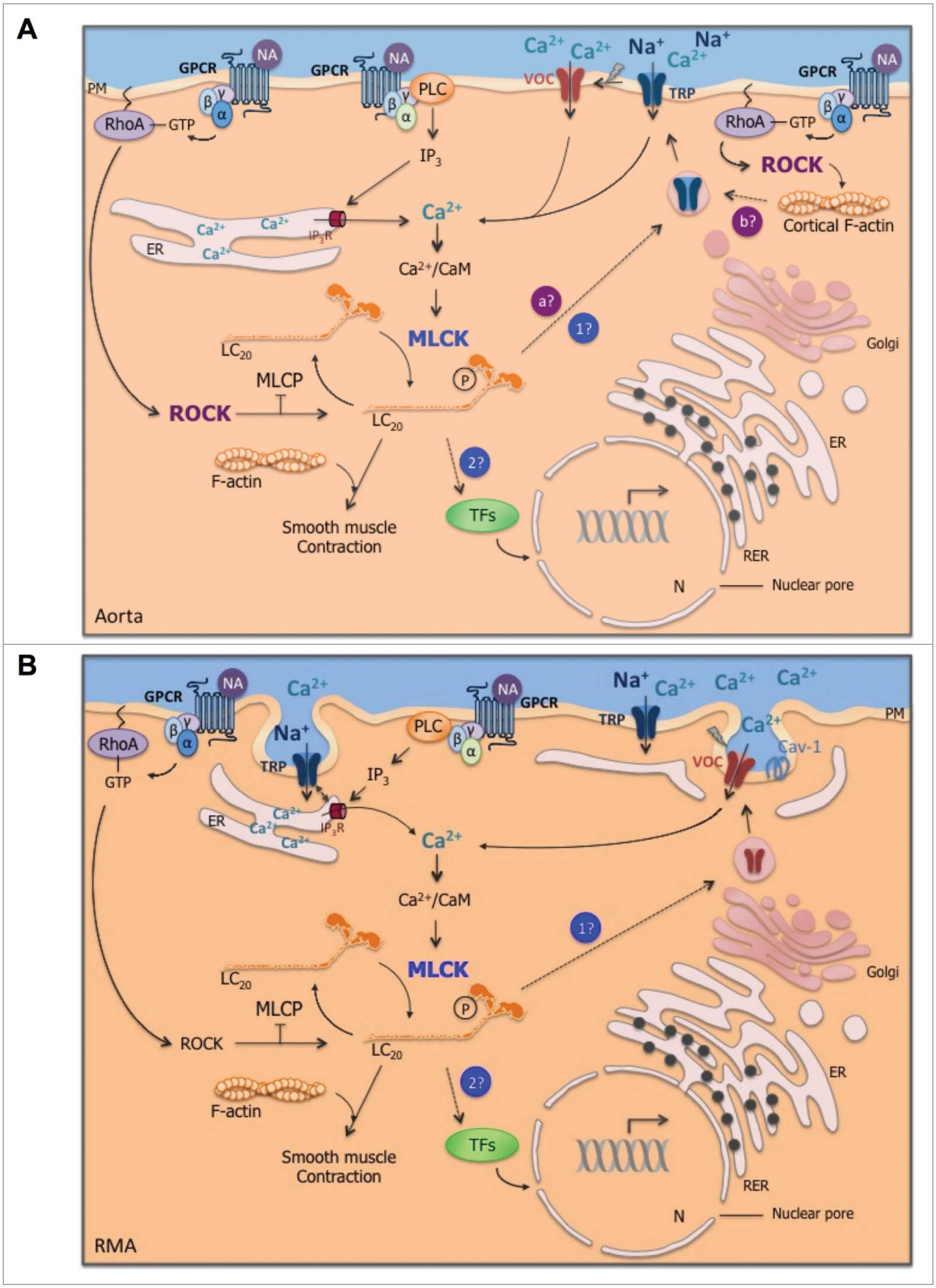

Figure 3. For figure legend, see page 409.

which disrupts the assembly of the Golgi apparatus, prevents the translocation of T-type Ca^{2+} channels to plasma membrane.

MLCK regulation of the actin cytoskeleton has been suggested in A7r5 cells, in which ML-7 partially blocks the disassembly of α-actin cables in response to $[Ca^{2+}]_{cyt}$ increase by A23187 or thapsigargin.[95,96] The role of the actin cytoskeleton in the regulation of smooth muscle contraction through the cross-bridge cycling process and in organizing and remodeling the submembranous cytoskeletal network[97] is not disputed. Although the point is still controversial, the actin cytoskeleton has also been suggested to contribute to cytosolic Ca^{2+} regulation through trafficking and conformational coupling of plasma membrane and ER Ca^{2+} channels. Several studies demonstrate that actin depolymerization using cytochalasin-D (which sequesters G-actin monomers[98]) completely blocks the contractile tension in rat aorta[12,99] and resistance mesenteric artery[12,100] in response to agonist or pressure, without affecting the Ca^{2+} response.[12] Conversely, in rat cerebral artery, pressure-induced $[Ca^{2+}]_{cyt}$ increase is depressed in the presence of cytochalasin D.[101] In cultured A7r5 cells[94,102] and HEK293 cells,[117] actin-depolymerizing agents also reduce L-type and T-type current, respectively. On the opposite, it has been shown that, in HEK293 cells,[103] depolymerized actin contributes to TRPC3 channel activation, and in isolated gallbladder myocytes[104] actin depolymerization with cytochalasin-D or latrunculin A (which blocks contractile tension by capping actin filaments[98]), enhances capacitative Ca^{2+} influx, while jasplakinolide, an activator of actin polymerization, decreases the influx of Ca^{2+}, but none of these inhibitors affects the L-type Ca^{2+} influx.

The contribution of the cytoskeleton to Ca^{2+} channel activation is further suggested by the observation that Rho kinase (ROCK), a known modulator of cytoskeleton regulating proteins,[105] is required for the non-voltage and non-capacitative Ca^{2+} entry in response to vasoconstrictor agonist in rat aorta and superior mesenteric artery,[106] although the Ca^{2+} signal and its sensitivity to ROCK inhibition are not affected by cytochalasin D. The same observation is reported in rat penile small artery,[107] but was not repeated in rat resistance mesenteric artery.[12]

In addition, in the mouse aorta cell line MOVAS, the non-selective cation channel TRPP2 has been shown to interact with filamin A, an actin-binding protein, to reduce stretch-activated cation (SAC) channels activity supporting the role of cortical F-actin cytoskeleton in the regulation of SAC channels in resistance arteries.[108]

From these elements, we can draw a hypothetical model describing the contribution of MLCK and myosin motors along with actin filaments and the Golgi-produced vesicles containing channels proteins, to Ca^{2+} channel activation (**Fig. 3**). In agreement with this model, myosin II activated by MLCK has been proposed to regulate the trafficking of aquaporin AQP2 vesicles to the apical plasma membrane in the rat renal collecting duct following vasopressin stimulation.[109] One year later, a multiprotein complex was identified as a motor for the water channel AQP2 trafficking, in which the channel is organized with actin proteins, smooth muscle myosin light chains and non-muscle myosin heavy chains.[110] In TRPC5 transfected HEK293 cells, MLCK inhibition by wortmannin impairs the translocation of TRPC5 to plasma membrane.[45] But this effect can be attributed to the inhibition of PI3K by wortmannin.[58] Indeed, in primary hippocampal neurons, PI3K is involved in TRPC5 homomeric channel translocation in collaboration with the Rho GTPase Rac1 and another phosphoinositide.[111] Nevertheless, the implication of MLCK and cytoskeleton proteins in Ca^{2+} channels translocation is further supported by Shimizu et al.[45] They showed, using ML-9 and a dominant-negative mutant of MLCK to inhibit MLCK, that MLCK inhibition in TRPC5 transfected HEK293 cells impairs the plasmalemmal localization of TRPC5.[45]

Involvement of MLCK in calcium channels gene expression

Experimental evidence indicates that the decrease in MLCK expression is associated with a decrease in the expression of several types of Ca^{2+} channels as demonstrated for $Ca_V1.2$ and $Ca_V3.1$ in A7r5 cells and rat resistance mesenteric artery (**Fig. 2**).[10] We also have observed in preliminary experiments, that TRPC1 and TRPC6 mRNA were decreased in MLCK-depleted A7r5 cells (**Fig. 2**), suggesting that different types of

Figure 3 (See previous page). Regulatory mechanisms of cytosolic Ca^{2+} concentration increase in response to agonist stimulation in aorta (**A**) and resistance mesenteric artery (RMA; **B**). In both representations, the stimulation of G protein-coupled receptor (GPCR) by vasoconstrictor agonist, for instance, noradrenaline (NA), activates phospholipase C (PLC), which produces inositol triphosphate (IP_3). IP_3 stimulates Ca^{2+} release from the endoplasmic reticulum (ER) and triggers cytosolic Ca^{2+} increase, which in association with calmodulin (CaM) activates myosin light chain kinase (MLCK). Phosphorylation of myosin light chains (LC_{20}) by MLCK triggers cross-bridge cycling between actin and myosin filaments. (**A**) In aorta, in addition to smooth muscle contraction, agonist-induced MLCK activation triggers Ca^{2+} influx. We hypothesize that MLCK could be involved in Ca^{2+} channels trafficking (point 1 in blue) and/or could be responsible for the trafficking of transcription factors (TFs) within the nucleus (N; point 2 in blue), which in turn regulate the transcription of Ca^{2+} channels mRNA. Furthermore, in aorta, Rho kinase (ROCK) activated by RhoA-GTP following $G\alpha_{12/13}$ activation contributes to non-voltage-dependent Ca^{2+} influx. ROCK could induce the trafficking of Ca^{2+} channels through myosin phosphorylation (point a in purple) as MLCK or through cortical F-actin polymerization (point b in purple). Caveolae do not appear to be directly involved in agonist-induced Ca^{2+} entry, while they contribute to contraction and ROCK activation. Further experiments are needed to validate or invalidate these potential Ca^{2+} regulation mechanisms (dotted lines) in conductance artery. (**B**) Conversely, in RMA, ROCK is not involved in Ca^{2+} entry, although ROCK contributes to smooth muscle contraction. MLCK is involved in voltage-dependent Ca^{2+} influx in response to a vasoconstrictor agonist. While caveolae are not required in agonist-induced Ca^{2+} entry in aorta, in RMA, the translocation of Ca^{2+} channels seems to involve the omega-shaped membrane invagination. We hypothesize that MLCK could contribute to voltage-dependent Ca^{2+} channels trafficking (point 1 in blue) and/or to the trafficking of transcription factors (TFs; point 2 in blue). The close proximity of the ER to the plasma membrane (PM) could make Ca^{2+} channel trafficking easier compared to aorta. Arrows are activating pathways; crossed out arrows are inhibiting processes; dotted lines are hypothetic pathways. RER: rough ER; Cav-1: caveolin-1; TRP: transient receptor potential channels; MLCP: myosin light chain phosphatase.

Ca^{2+} channels could be affected by MLCK down-regulation. A first explanation could be that alteration of Ca^{2+} influx in MLCK-depleted cells affects the pattern of gene expression, as gene transcription is highly dependent on a precise control of Ca^{2+} signaling.[112,113] Indeed, activation of Ca^{2+} influx via L-type Ca^{2+} channels is reported to increase mRNA and protein expression of several TRPC channels in the guinea-pig gallbladder smooth muscle and mechanisms that decrease cellular Ca^{2+} level, also induce a downregulation of TRPC channels expression.[114]

An alternative explanation for the control of gene expression by MLCK is that MLCK could be involved in the trafficking of transcription factors into the nucleus. Several transcription factors have been implicated in the regulation of $Ca_V1.2$ gene expression, such as the cAMP-response element binding protein (CREB) and the nuclear factor κB (NF-κB), CaMKII being a major regulator of their activation.[115] In endothelial cells, MLCK is reported to be involved in the nuclear translocation of NF-κB in response to tumor necrosis factor TNFα.[116] In mouse lung, TRPC6-mediated Ca^{2+} entry activates non-muscle MLCK, which in turn increases lung vascular permeability and serves as scaffolding partner to allow NF-κB activation.[117] The reciprocal modulation of MLCK and L-type Ca^{2+} channels expression is suggested by observations made in 2 separate studies showing that mRNA expression and density of L-type VOC channels and MLCK expression are upregulated in resistance mesenteric artery from hypertensive rats compared to normal Wistar Kyoto rats.[118,119] Whether there is a link between these observations should be further investigated but could be in the line with the involvement of the myosin cytoskeleton in the transcriptional mechanisms controlling genes expression, as suggested in **Figure 3**.

Conclusion

It is well recognized that dysregulation of Ca^{2+} homeostasis in VSMC has major pathological consequences. Although main partners in the control of cytosolic Ca^{2+} are identified, several gaps remain in the understanding of their regulation. Several processes modulating the activity of plasmalemmal Ca^{2+} channels have been identified, leading to the emergence of a dynamic model, in which the cytoskeleton could play an important role. Recent evidence suggests that, by mediating the phosphorylation of myosin, MLCK might contribute to the regulation of Ca^{2+} homeostasis in response to agonist stimulation. **Figure 3** summarizes the current knowledge about the role of MLCK in Ca^{2+} influx in stimulated conductance and resistance artery. An important problem in determining the role of MLCK in the regulation of Ca^{2+} channels is the discrepancy between studies performed in different cellular models or different arteries or tissues and between data obtained from patch-clamp recording of cation current and cytosolic Ca^{2+} measurement in cells or tissues loaded with fluorescent Ca^{2+} probes. Differences in cytoskeleton organization as reported in cultured cells, or in excitation-contraction coupling processes, as seen between conductance and resistance arteries, could be, at least partly, responsible for this variability.[120,121]

The potential interaction of MLCK with gene transcription suggested by the decreased expression of Ca^{2+} channels in MLCK-depleted cells should be further investigated. This action of MLCK should be considered when using MLCK knockdown models to study the involvement of MLCK in Ca^{2+} movements. As the specificity of pharmacological tools is always questionable, innovative approaches will be required to determine the role of MLCK and the cytoskeleton in the regulation of Ca^{2+} channels.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Funding

This work was supported by grants from the Ministère de l'Education et de la Recherche Scientifique of the Belgian French Community (Action Concertée no 06/11-339) and the Fonds pour la Recherche Scientifique Médicale. A.M. was supported by a fellowship of the Fonds Spéciaux de Recherche (UCL). C.D. is senior research associate of the Fonds National de la Recherche Scientifique (FNRS).

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