Phosphorylation of Myosin Regulatory Light Chain by Myosin Light Chain Kinase, and Muscle Contraction

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Not only muscle contraction, but also most cell movements depend on myosin–actin interaction using ATP. Many components of the contraction machinery are involved in the efficient coupling of energy source and force development. Among these, I have focused on myosin light chain kinase (MLCK) in this review. MLCK phosphorylates myosin regulatory light chain and controls all 3 types of muscle contraction: skeletal muscle, smooth muscle, and cardiac muscle. However, each muscle has specific MLCK and the role of MLCK in each muscle is different. This difference explains the specific role of each muscle in vivo and contributes to the activity of various force development in different ways in each tissue. Therefore, I also review the differences in the connection between each MLCK and muscle contraction in the 3 muscle types. (Circ J 2009; 73: 208–213)

Key Words: Muscle contraction; Myosin light chain kinase; Regulatory light chain

Biochemical analysis of the proteins involved in muscle contraction has a long history, since 1950, because many researchers have been interested in the physiologically dynamic output of muscle contraction. The large amount of protein involved in muscle contraction also helped the precise biochemical analysis of the proteins. In 1954, Huxley and Hanson showed that the force of a muscle was generated by slippage of the cross-bridges between actin filaments and myosin filaments. Soon after this, intracellular calcium ([Ca\(^{2+}\)]i) was discovered first that purified actin and myosin only are not enough for active movement of the cross-bridges and tried to purify missing protein for contraction. Next, they successfully identified troponin as a sensing protein of [Ca\(^{2+}\)]i. Researchers had already noticed that the response to [Ca\(^{2+}\)]i differed among the 3 types of muscles: smooth muscle, skeletal muscle, and cardiac muscle. Indeed, troponin was the sensor protein of [Ca\(^{2+}\)]i. The activity of MLCK is enhanced approximately 1,000-fold in response to increasing [Ca\(^{2+}\)]i. The most important regulator of MLCK activity is [Ca\(^{2+}\)]i and MLCK was maximally activated by about 1 μmol/L of [Ca\(^{2+}\)]i. Therefore, I will first summarize the [Ca\(^{2+}\)]i regulation of contraction in each muscle (Fig 1).

In smooth muscle cells, chemical mediators such as catecholamine induce a relatively slow Ca release from intracellular stores mediated by activation of the phosphoinositol pathway. Smooth muscle cells gradually contract according to this slow increase of [Ca\(^{2+}\)]i. In cardiac muscle cells, electro-stimulation from the sinus node activates the voltage-dependent Ca channel activated by terminal nerve stimuli directly opens the ryanodine receptor on the Ca store site, resulting in a rapid increase of [Ca\(^{2+}\)]i. In cardiac muscle cells, [Ca\(^{2+}\)]i increases from 0.1 μmol/L to rapidly recaptured in the store site. In cardiac muscle cells, after electro-stimulation [Ca\(^{2+}\)]i increases from 0.1 μmol/L to

The role of skMLCK or smMLCK in skeletal muscle where troponin works as the main [Ca\(^{2+}\)]i sensor for contraction is still controversial. Surprisingly, in 2007 a third MLCK was identified, which express only in the heart, called cardiac myosin light chain kinase (cMLCK).

In this review, I summarize the molecular mechanism of muscle contraction and compare the specific role of MLCKs in the different muscle tissues.

Intracellular Calcium Homeostasis and MLCK in Muscle Tissue

All 3 MLCK (smMLCK, skMLCK, cMLCK) are calmodulin-dependent protein kinases. The affinity for calmodulin is strongest among the calmodulin-dependent protein kinases and is estimated to be approximately 1 nmol/L. Indeed, it has been always co-purified. The activity of MLCK is enhanced approximately 1,000-fold in response to increasing [Ca\(^{2+}\)]i. The most important regulator of MLCK activity is [Ca\(^{2+}\)]i and MLCK was maximally activated by about 1 μmol/L of [Ca\(^{2+}\)]i. Therefore, I will first summarize the [Ca\(^{2+}\)]i regulation of contraction in each muscle (Fig 1).

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Function of Cardiac MCLK

Fig 1. Intracellular calcium and muscle contraction. In skeletal and cardiac muscle, electrical stimuli activate the voltage-dependent channel and rapidly increases \([\text{Ca}^{2+}]\). After 1s, however, \([\text{Ca}^{2+}]\) is rapidly absorbed into the intracellular Ca storage site. This transient increase of \([\text{Ca}^{2+}]\) activates myosin ATPase via troponin and muscle briefly contracts. On the other hand, in smooth muscle cells a vasoconstrictor induces a relatively longer period of increased \([\text{Ca}^{2+}]\). This slow increase of \([\text{Ca}^{2+}]\) activates MLCK by a calmodulin-dependent pathway. There is no troponin expressed in smooth muscle cells. For functional diversity, each muscle type has similar but distinct protein components, the genes for which are located on different loci. It is also evolutionarily interesting to suggest diversification of muscle cells to fit each purpose during development. Black arrow indicates increased \([\text{Ca}^{2+}]\) and red arrow indicates decreased \([\text{Ca}^{2+}]\). SR, sarcoplasmic reticulum; MLC, myosin light chain; MLCK, myosin light chain kinase.

1 \(\mu\)mol/L and back to the basal level in 0.1 s. Taken together, the 3 types of muscles, especially smooth muscle, have different molecular kinetics to increase \([\text{Ca}^{2+}]\) during muscle contraction. This specific \([\text{Ca}^{2+}]\) control seems to be necessary for the specific role of each muscle.

\([\text{Ca}^{2+}]\) Increase and Muscle Contraction

In smooth muscle cells, increased \([\text{Ca}^{2+}]\) leads to the activation of smMLCK by Ca–calmodulin pathway and myosin regulatory light chain (RLC) is phosphorylated by activated smMLCK. Phosphorylated RLC activates the ATPase of myosin and smooth muscle contracts. How the phosphorylated RLC activates the ATPase of myosin is still unknown; however, there is linear correlation between the degree of RLC phosphorylation and muscle contractility. In smooth muscle cells, the concentration of smMLCK and its substrate, RLC, is approximately 4 \(\mu\)mol/L and 30–40 \(\mu\)mol/L, respectively. \(K_m\) value and \(V_{\text{max}}\) of smMLCK is 3–5 \(\mu\)mol/L and 10–20/s, respectively. With these kinetics, RLC is totally phosphorylated within 1–2 s, if we hypothesize that smMLCK and RLC are located close enough to react in vivo. The affinity of Ca–calmodulin for smMLCK is very strong and the \([\text{Ca}^{2+}]\) increase after agonist stimulation is fast enough to negate the time of these reactions. This 1–2 s delay is well matched to the time taken from agonist stimulation to RLC phosphorylation. In contrast, both skeletal and cardiac muscles use troponin as a \([\text{Ca}^{2+}]\) sensor. For this reason, in these muscles there is not a linear correlation between RLC phosphorylation and contractility. Because rapid \([\text{Ca}^{2+}]\) transient occurs in these cells, slow kinase reactions such as MLCK are not suitable as a \([\text{Ca}^{2+}]\) sensor. Troponin directly binds to actin and structural change in troponin causes the release of troponin I, which inhibits the ATPase activity of myosin. Using this non-enzyme signal, increased \([\text{Ca}^{2+}]\) rapidly changes the contractility of these muscles. However, there are kinases that specifically phosphorylate RLC in both cardiac and skeletal muscles. It is hard to believe that phosphorylation of RLC, which is the main trigger for contraction of smooth muscle cells, does not affect contractility in these muscles. Different roles of RLC phosphorylation are implied in cardiac and skeletal muscles. How the rapid \([\text{Ca}^{2+}]\) transient activates MLCK and the degree to which RLC is phosphorylated are still controversial. Before approaching these questions, the biochemical character of MLCK will be summarized next.

Structure of MLCK

The myosin II molecule is a hexamer composed of 2 myosin heavy chains and 4 myosin light chains (MLCs). Of these 6 chains, MLCKs phosphorylate RLC and regulate the function of myosin. There are 3 homologues of MLCK that share similar catalytic domain and regulatory domain (Fig 2). The smMLCK is expressed ubiquitously in every tissue, whereas skMLCK or cMLCK is expressed specifical-
Cardiac Myosin Light Chain Kinase (cMLCK)

Smooth Muscle Myosin Light Chain Kinase (smMLCK)

Skeletal Muscle Myosin Light Chain Kinase (skMLCK)

MLCKs. When cloning cMLCK, all the fractions of tissue homogenate separated by fine-column chromatography were screened and only RLC was identified as a substrate of MLCK. Structural background contributed to this specificity and its analysis is expected.

Fig2. Three subtypes of myosin light chain kinase (MLCK) have a common conserved kinase domain near the C-terminal lesion. Ig, immunoglobulin domain; Fib, fibronectin domain.

smMLCK

Compared with the other 2 MLCKs, smMLCK is expressed ubiquitously in almost all tissues and is involved not only in contraction of smooth muscle cells but also many cellular activities. In non-muscle cells the rate of phosphorylation of RLC is small and other kinases can phosphorylate RLC. The role of smMLCK in non-muscle cells is reviewed in another article. In smooth muscle cells, the importance of smMLCK is obvious because there is a direct link between the activity of smMLCK and the contractility of the cell. Because inhibitors of smMLCK relax the smooth muscle, and are possible candidate antihypertensive drugs, physiological and biochemical analysis of smMLCK has been intensive. After phosphorylation of smooth muscle RLC by smMLCK, RLC is dephosphorylated by myosin phosphatase the activity of which is regulated by Rho kinase. Rho kinase phosphorylates myosin phosphatase and inhibits its activity. Therefore, activation of Rho kinase leads to muscle contraction. Other kinases, such as PKA, p21-activated kinase 1 (PAK1), p21-activated kinase 2 (PAK2), protein kinase C, mitogen-activated protein kinase, and calmodulin kinase II, are also reported to phosphorylate RLC and regulate muscle contraction; however, whether this activity exceeds the strong activation of smMLCK by Ca–calmodulin pathway is still questionable.

skMLCK

Compared with smMLCK, the investigation of skMLCK has been limited because in skeletal muscle contraction does not directly link to the phosphorylation of MLC. However, Perschini and Stull in Texas University have been intensively analyzing the specific role of skMLCK. The K_m

ly in skeletal muscle or cardiac muscle, respectively (Fig3). The regulatory mechanism of MLCK activity is also conserved in all MLCKs, which are all Ca–calmodulin-dependent kinases. Although the complete structure of MLCK has not been elucidated, the regulatory mechanism has been speculated from its similarity to the structure of protein kinase A (PKA). When [Ca^{2+}] is low, the regulatory domain can bind to the catalytic domain to inhibit kinase activity. Binding the Ca–calmodulin complex to this regulatory domain releases the catalytic domain and MLCK is activated. If the complete structure were available, the precise regulatory mechanism could be clarified. The intriguing thing is that MLKC has only 1 substrate: RLC. This specificity is common among the 3
value of skMLCK to skeletal muscle type MLC (skMLC) is approximately 5 μmol/L, which is far below the $K_m$ value of smMLCK to skMLC, which is approximately 100 μmol/L. Therefore, in skeletal muscle, skMLCK is the main kinase for the phosphorylation of skMLC.

In an experiment using skinned fibers, the phosphorylation of skMLC by skMLCK was reported to enhance contractility under the condition of submaximal $[\text{Ca}^{2+}]_i$. However, in skinned fibers it is difficult to evaluate the function of skMLCK, because the MLC phosphorylation rate of the initial preparation is rather different from that of the various following preparations. Also, assaying the level of phosphorylation by adding external MLCK cannot be done in real time. These experimental difficulties are the reason for the different reports on the physiological function of skMLCK. However, enhancement of contractility by phosphorylation of MLC is obvious under extreme experimental conditions, such as the comparison of completely unphosphorylated MLC by phosphatase and sufficiently phosphorylated MLC by skMLCK. These data suggest that the basic function of MLCK as enhancing contraction is still preserved in skMLCK.

In 2005, a knock-out mouse model of skMLCK was made. In skMLCK-deficient mice there is difference in contractility after 15 Hz tetanus stimulation; however, almost normal development and function of skeletal muscle are observed. These data suggest that the response to unphysiological stimulation is not important for the physiological function of skeletal muscle. Also, in these mice, some skMLCK phosphorylation is observed, which might be caused by redundancy between skMLCK and smMLCK, even though a compensatory increase in smMLC expression is not observed in skMLCK-deficient mice. Because skMLC is not a good substrate for smMLCK, this discrepancy may be explained by the specific localization of smMLC on the Z-band of the actomyosin fiber. However, the in vivo function of skMLCK is still unknown and only a complete in vitro assay system could clarify the function of skMLCK in skeletal muscle. Another important question is how much MLC is phosphorylated in skeletal muscle cells. Several reports calculate the phosphorylated ratio of skMLC as ranging from 5% to 40%. This wide range implies the difficulty of measuring the phosphorylation rate of MLC in vivo. Considering the dominancy of phosphatase in tissue, it is speculated that 30–40% of skMLC is phosphorylated. It is essential for a future study to determine the accurate ratio of phospho-MLC and clarify how the phosphorylation of skMLC affects the contractility of skeletal muscle.

cMLCK

cMLCK was first identified in 2007; however, before then several papers reported the importance of phosphorylation of cardiac MLC (cMLC) in cardiac structure and contractility. cMLC is also 1 of the genes causing hypertrophic cardiomyopathy. Almost all the causative genes of hypertrophic cardiomyopathy reported so far are coding proteins related to heart muscle contraction, such as the myosin heavy chain, troponin. These genetic findings imply the important role of cMLC in contraction of the heart. Also, the difference in the phosphorylation rate of cMLC in the outer and inner layers of the heart muscle contributes to the efficient contraction of the whole heart. As in the case of skeletal muscle, phosphorylation of cMLC in cardiac muscle also increases muscle contraction at submaximal levels of $[\text{Ca}^{2+}]_i$. To evaluate the role the cMLC phosphorylation, knowing the precise rate of phosphorylation of cMLC is also essential. In cardiac muscle, 20–40% of cMLC is reported to be phosphorylated. Repeated contraction might cause the high phosphorylation rate of cMLC induced by transient increase of $[\text{Ca}^{2+}]_i$ with each heart beat. On the other hand, the phosphorylation of cMLC is important for the establishment of the sarcomere structure of the heart. An inhibitor of MLCK, ML-7, impairs the establishment of sarcomere structure in the early embryo of Xenopus or the chicken. In mammalian cardiomyocytes, ML-7 also impairs the establishment of sarcomere structure. Moreover, mice lacking atrial or ventricular cMLC die in the early embryo stage because of poor development of the heart. Impaired cardiac development because of the lack of MLC is also verified in zebra fish, which can survive for 7 days without blood flow. One of the ENU mutant zebra fish, called tel mutant,
processes this phenotype. Cells transplanted from the tel mutant to the wild-type zebra fish also cannot establish the sarcomere structure, suggesting the important autonomous cell function of cMLC in heart muscle development! The cMLCK has been identified as a novel kinase, the expression of which is enhanced in severe human heart failure. Its substrate was purified, which turned out to be cMLC. Therefore, this kinase was named as cMLCK and it is expressed exclusively in the heart (Fig 3). The relationship between the establishment of sarcomere structure and the phosphorylation of cMLC was first analyzed in rat cultured cardiomyocytes. The sarcomere structure is disrupted by removing serum from the culture media and the phosphorylation rate of cMLC reduces. Treatment with reagents that increase [Ca\(^{2+}\)], such as catecholamines, re-establishes the sarcomere structure with an increased phosphorylation ratio of cMLC. This re-establishment of the sarcomere structure is blocked by RNAi pretreatment of cMLCK (Fig 4). Reduced cMLCK activation by increased [Ca\(^{2+}\)] is indispensable signaling for the establishment of the sarcomere structure under these conditions. In zebra fish, 3 MLCK orthologs (cardiac-, skeletal-, smooth-muscle-type MLCK), exist and have a similar gene location as the respective mammalian MLCKs. Zebra fish cMLCK also exclusively expresses in the heart from the early embryo (Fig 5). Reduced cMLCK expression by the antisense morpholino causes severely impaired heart development. After 72 h fertilization, reduced expression of cMLCK caused cardiac arrest following reduced contractility (Fig 6). Histologically, the structure of the sarcomere was poorly developed compared with control zebra fish. Taken together with the similar phenotype of the tel mutant (cMLC mutant), cMLC phosphorylation by cMLCK is an essential signaling pathway of the establishment of sarcomere structure. Compared with the mice lacking skMLCK, these phenotypes of the cMLCK knock-out gene are very severe, even though both are expressed specifically in each organ and smMLCK expresses almost equally in both organs. It is still unclear whether this difference is a species difference of MLCK or cMLC. Also, early lethality of cMLCK knock-out zebra fish made it difficult to assess the specific role of cMLCK in cardiac contractility. My group are now making Cre-lox mediated knock-out of cMLCK mice, which should help in the analysis of the role of cMLCK in the contractility of the developed heart. At which developmental stage cMLCK contributes to the sarcomere structure is also unknown. In avian heart development, the first structure of cardiac muscle starts from the Z-band, which includes only actin and fetal myosin. Next, fetal myosin is replaced by adult-type myosin and it interacts with actin to form mature muscle. This pathway is easily blocked by inhibitors of MLCK, suggesting that embedding of myosin in the actin structure may require phosphorylation of MLC.

In this review, I have focused on 3 MCLK homologs (smMLCK, skMLCK, cMLCK) and described the biochemical role of each in smooth muscle, skeletal muscle and heart muscle. Each muscle has similar components of the contraction machinery; however, every component of each muscle type has a specific subtype and those genes are located in different genome positions. Also, each muscle components seems to develop distinct and indispensable functions. These diversified processes must be necessary for each muscle to have its specific function. This precise adaptation of each component is mysterious and splendid. I hope the force–energy relationship hypothesized by Frank and Starling connects with the model of 1 molecule force development proposed by Yanagita et al for analyzing MLCKs.

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Fig 5. In situ hybridization of cardiac myosin light chain kinase in zebrafish.

Fig 6. Inhibition of cardiac myosin light chain kinase (cMLCK) impairs cardiac development. Injection of the antisense morpholino of cMLCK caused heart dilatation and reduced contractility at 48 h after fertilization.

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