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REVIEW

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Tuning the human heart molecular motors by myosin light chains

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Abstract Cardiac contraction is triggered by the cyclic interaction of the "molecular motor" protein myosin with the actin filament, consuming ATP as the energy source to produce tension or shortening. The myosin heavy chain (MHC) contains the actin- and ATP-binding sites and represents the molecular motor of muscle contraction. This review describes the various subunits of human heart myosin in health and disease and discusses their functions. Two different MHC genes (α and β) with distinct biochemical features are expressed in the human heart. α-MHC confers a higher ATPase activity and higher shortening velocity to the heart than β-MHC. Motor function is regulated by myosin light chain (MLC) isoforms. Expression of the atrial MLC-1 isoform in the hypertrophied human ventricle increases cross-bridge cycling and contractility. It is suggested that MLC-1 acts as a MHC/actin tether. Weakening of this tether increases myosin function. MLC-2 slows the rate of tension development of myosin. This relative inhibition is relieved upon phosphorylation of the MLC-2 perhaps caused by "swing-out" of cross-bridges from the myosin filament. Mutations in all ventricular myosin subunits have been found in patients with hypertrophic cardiomyopathy.

Key words Human heart · Myosin light chain · Myosin heavy chain · Hypertrophy · Cross-bridge cycling

Abbreviations *ALC* Atrial myosin light chain · bHLH Basic helix-loop-helix · *HAND* Heart-autonomic nervous system-neural crest derivatives · *HOCM* Hypertrophic obstructive cardiomyopathy · *MHC* Myosin heavy chain · *MLC* Myosin light chain · *MLCK* Myosin light chain kinase · *Pi* Inorganic phospohate · *S1* Subfragment 1 · *VLC* Ventricular myosin light chain

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Scope of this review

This review considers the molecular motor myosin of the human heart. I will describe the molecular mechanisms through which the motor function is fine-tuned by interaction with various light subunits.

Structure of the molecular motor of striated muscle

Under the electron microscope the sarcomeric motor protein myosin appears as an elongated 150-nm rod-shaped molecule with two N-terminal pear-shaped head domains. The globular head domain and the α-helical part which noncovalently binds two types of light chains are designated as subfragment 1 (S1). Limited proteolysis of S1 reveals three major segments, a 25-kDa N-terminal, a central 50-kDa, and a 20-kDa C-terminal domain (for review see [1]).

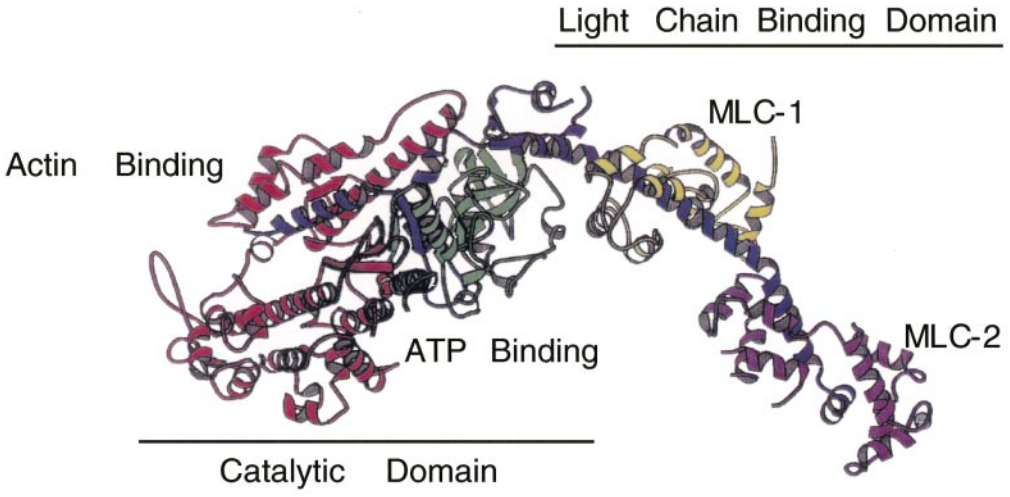

Fig. 1 Three-dimensional ribbon structure of myosin-S1 (see references in [2]). The MHC consists of a 25-K N-terminal (*green*), a 50-K (*red*), and a C-terminal joining 20-K (*blue*) domain. The pear-shaped head is called the catalytic domain. Essential myosin light chain MLC-1 (*yellow*) and regulatory MLC (*MLC-2*) are associated with the α-helical C-terminal part of the 20-K domain (light chain binding domain)

The three-dimensional structure of the S1 domain of the myosin molecule of chicken skeletal muscle has been elucidated by X-ray crystallographic analysis [2]. It consists of a heavy chain (MHC) which folds at the N-terminus into an asymmetric globular head domain (Fig. 1). This head is16.5 nm long, 6.5 nm wide, and 4 nm thick and comprises a seven-stranded β-sheet connected by flanking α-helices and/or loops which constitute the catalytic domain. The 50-kDa domain is split by a long narrow cleft with actin-binding sites located on both sites of the cleft. Also, the ATP-binding (active) site forms an open cleft and is located opposite from the actin binding site at the 25/50 kDa junction. The apex of the long cleft through the 50 kDa domain is very close to the nucleotide binding cleft, thus mediating signal transduction between the actin and nucleotide binding clefts. Amino acids 771–843 at the C-terminus form a 8.5-nm α-helical structure -the neck region – which binds two types of light chains. The essential myosin light chain binds between amino acids 783 and 806 and the regulatory light chains further downstream between amino acids 808 and 842. The catalytic and light chain binding domains are joined by a so-called converter domain (around amino acids 711–771). Further downstream, not represented by the three-dimensional structure [2], the 140-nm α-helical "rod" domain joins the S1 part which forms the myosin filament. Therefore the basic structure of the myosin molecule is a trimer. In vivo two trimers associate to the native double-headed myosin molecule with M_r approx. 470.

Function of the molecular motor

In the intact contractile structure the ATP- or ADP-P_i-loaded MHC binds as the "cross-bridge" to the N-terminus of actin. Myosin undergoes changes in actin affinity and structure, being strongly attached to actin (having high affinity) or weakly attached (having low affinity) [3, 4]. Force is generated upon the transition from the weakly to the strongly attached state. This transition is considered to be coupled to the P_i release step [5].

These structural changes in the cross-bridges are associated with the strain of an elastic component present in the cross-bridges which operates over a range of 8–10 nm [6]. In a contemporary model the α-helical neck domain is believed to function as a lever arm which swings relative to the catalytic domain: picometer changes in the active site of S1 are magnified into nanometers of motion by rotation of the converter domain [3, 7]. In fact, truncation and elongation of the light chain binding domains show that the sliding velocity of actin filaments is proportional to the length of the lever arm [8]. Furthermore, the light chain binding domain, rather than the catalytic domain, reveals tilting motions during length perturbations of an isometrically contracting muscle [9, 10, 11] (see Fig. 2). This is in contrast to text-book cartoons shich show tilting S1 portions. The elastic element of the cross-bridge may therefore reside in the lever arm [12].

Orientation of the myosin head in the isometric state does not seem to be perpendicular to the actin filament longitudinal axis, as revealed by X-ray diffraction studies with submillisecond time resolution [10] and fluorescence polarization studies [9]. The force-generating cross-bridges are arranged more parallel to the actin filament axis. They pass through the perpendicular during shortening. Hence the angle between the actin filament axis and the force-generating myosin cross-bridge appears to be less than 90° (Fig. 2).

Due to its elasticity the cross-bridge exhibits variable free energy profiles [13]. In addition to the chemical potential of the cross-bridge, either stretching or compressing the "spring" changes the free energy of the cross-bridge attached to actin. Assuming linear elasticity of the spring with a constant k, the free energy G of an attached state equals: $G_0+(kx^2)2$, where G_0 is the free energy in the unstrained position and x is the relative position of the actin filament. Hence under isometric conditions basic free energy of the unstrained actomyosin-ADP-P_i state equals that of the strained actomyosin-ADP state: the chemical energy is converted into mechanical energy. Upon detachment the cross-bridge recoils and the stored energy is dissipated as heat. Under isotonic conditions

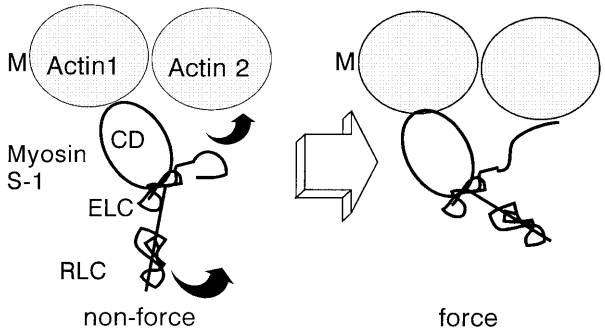

Fig. 2 Schematic synopsis of recent structural and biochemical studies of actin-myosin interaction. The catalytic domain (*CD*) of myosin S1 binds to actin in a non-force-generating state. Force generation is elicited by elastic bending of the light chain binding domain, the lever arm rather than by rotation of the catalytic domain. In addition to the CD, the N-terminus of the essential myosin light chain (*ELC*) binds to a neighboring actin monomer in the force (high Ca^{2+}) but not in the non-force-generating (low Ca^{2+}) state. M: Towards M-line

the actin filament is allowed to slide, the stretched spring becomes discharged, and the mechanical energy stored in the spring is liberated as work.

Myosin heavy chain expression in the human heart

In the human heart two sarcomeric myosin II genes are expressed generating two isoenzymes of the myosin heavy chain (designated α- and β-MHC). These are located in tandem on the long arm of human chromosome 14q11.2-q13 [14] (Table 1). In the normal heart both MHC isoenzymes are expressed in a tissue-specific manner: the α-MHC is preferentially expressed in the atrium, and the β-MHC is almost exclusively expressed in the ventricle (Table 2; for review see [15]). In the rodent β-MHC reveals a lower ATPase activity and is associated with a lower maximal shortening velocity of the cardiac fibers than α-MHC. It is still not clear to what extent the various myosin light chain subunit isoforms present in atrium and ventricle contribute to the higher cycling kinetics of human atrial myosin than in human ventricular myosin [13].

In the hypertrophied atrium considerable amounts of β-MHC are expressed [16, 17], which is associated with a decrease in maximal shortening velocity [18]. α-MHC, however, is either found to be a minor component of human ventricular myosin [16, 19, 20] or could not be detected at all [21]. Furthermore, there are no changes in α-MHC expression during hypertrophy of the human ventricle [16, 19, 20, 21]. Since α-MHC can be detected on the mRNA level [21, 22, 23] in the human ventricle, α-MHC mRNA translation may be either suppressed or may be too low to be recognized by the translational machinery [21].

Table 1 Chromosomal localization (*Chr*) and amounts of amino acid (*AS*) of myosin subunits in the human heart

Subunit	Chr	AS	Reference
α-MHC	14q11–13	1939	14
β-MHC	14q11.2–13	1935	14
ALC-2	nd	175	34
VLC-2	12q23	166	33
ALC-1	17q21	196	32
VLC-1	3p21	194	31

Table 2 Myosin subunits in the normal and hypertrophied human heart on the protein level: in the hypertrophied ventricle, ALC-1 becomes reexpressed in addition to the ventricular-specific myosin subunits

Normal	Hypertrophied
Atrium	
α-MHC	α -MHC, β-MHC
ALC-1	ALC-1, VLC-1
ALC-2	ALC-2, VLC-2a, VLC-2b
Ventricle	
β-MHC	β-MHC
VLC-1	VLC-1, ALC-1
VLC2a, VLC-2b	VLC-2a, VLC-2b

Myosin heavy chain mutations

Around 40 mutations – predominantly missense mutations – of the β-MHC gene, and an α-MHC chimeric gene have been described in some familiar forms of hypertrophic cardiomyopathy, an autosomal dominant inherited heart disease. Some of these mutations have already been characterized at the functional level using in vitro motility assays and demembrated fiber mechanics. Since it is not within the scope of this contribution to review myosin mutations (excellent articles include [24, 25]), only some are considered. Arg-403 Gln [26, 27] in the actin-binding site reveals a decreased function and coincides with a high incidence of sudden cardiac death and decreased life expectancy [28]. Normal myosin function and low incidence of sudden cardiac death are associated with Gly-256 Glu mutation [26].

Myosin light chain expression in the human heart

Two types of myosin light chains (MLC), essential and regulatory, are associated with the neck region of the MHC. The essential MLC is designated as the MLC-1 or alkali MLC. The regulatory MLC is designated as MLC-2 or phosphorylatable MLC or 5,5-dithio-bis-(2-nitrobenzoate)-MLC [21]. Together with calmodulin and troponin C, both types belong to the superfamily of EF-hand Ca^{2+} binding proteins [30].

In the human heart two different genes encode the essential myosin light chain isoforms, the ventricular-specific (VLC-1) and an atrial specific (ALC-1). VLC-1 is located on chromosome 3p21 [31], coding for a 194 amino acid protein, which is the same isoform as the MLC-

1s present in the adult slow skeletal muscle. ALC-1 is located on chromosome 17q21 [32] coding a 196 amino acid protein (Table 1).

Several MLC-2 isoforms exist in the human, preferentially expressed in the atrium (ALC-2) or in the ventricle (VLC-2). VLC-2 has been mapped to chromosome 12q23-q24 [33] in human encoding a 166 amino acid protein. ALC-2 is composed of 175 amino acids [34], but no data are available yet about the chromosomal localization. Biochemical experiments have revealed that the human ALC-2 is slightly more acidic than the VLC-2 [35]. Furthermore, sequence analysis suggests the existence of two VLC-2 isoforms in chicken [36], cow, and rabbit [37]. In addition, electrophoretic analysis suggests the existence of two different VLC-2 isoforms in the human heart having the same molecular weight but different isoelectric points (VLC-2a for the more acidic and VLC-2b for the more basic isoform) [35].

Essential MLC

Expression of ALC-1 is tissue specific and developmentally regulated. Human embryos express large amounts of ALC-1 both in the whole heart and in skeletal muscle (for review see [38]). ALC-1 protein levels decrease in the ventricle to undetectable levels during early postnatal development but persisted in the atrium throughout the whole life [39].

The situation differs in patients with congenital heart disease such as Tetralogy of Fallot. Tetralogy of Fallot is a complex congenital heart disease characterized by four components: right ventricular infundibular stenosis, ventricular septal defect, dextroposed aorta overriding the interventricular septal defect, and right ventricular hypertrophy [40]. The hypertrophied right ventricle of children with tetralogy of Fallot express large amounts of VLC-1 in the atrium [41] and ALC-1 in the ventricle, up to adulthood [40]. Similarly, the hypertrophied left ventricle of patients with ischemic, dilative, and hypertrophic cardiomyopathy express ALC-1 [42, 43, 44]. Surgical intervention and subsequent normalization of the hemodynamic state decrease ALC-1 expression in these patients [45].

Regulation of ALC-1 expression in the human heart is still not well understood. In the mouse two E boxes, which interact with muscle-specific basic helix-loop-helix (bHLH) regulatory proteins of the MyoD family, and a diverged CArG box, which binds to the serum response factor, exist within the first 630 bp of the ALC-1 promoter region [46]. E boxes have been shown to be sufficient for ALC-1 transcription regulation during skeletal muscle differentiation [46]. bHLH regulatory factors of the MyoD family regulate skeletal muscle differentiation by forming heterodimers with E12 bHLH factors that bind to E-box elements, thus increasing the transcription rate of target genes (for review see [47]). Recently two cardiac-specific bHLH proteins homologous to the MyoD family have been detected in the heart, designated as the e and d forms of heart–autonomic nervous system–neural crest derivative (HAND) [48, 49]. They are important for early development and looping of the embryonic heart [50]. Since both HAND transcription factors bind weakly to E-box as E12 heterodimers, the ALC-1 may be a target gene. Recently it has been reported that in the human hypertrophied ventricle there is upregulation of HAND gene expression and a positive correlation between HAND and ALC-1 mRNA [43]. Furthermore, ALC-1 expression may be regulated by endogenous antisense ALC-1 mRNA present the human ventricle [43].

The essential MLCs modulate myosin function

The functional role of the essential MLC in the heart has remained obscure for many years. Recently we described for the first time that maximal shortening velocity, rate of tension redevelopment, isometric force generation, and Ca^{2+} sensitivity of isometric force generation increase upon partial replacement of VLC-1 by ALC-1 in the human ventricles [44, 51]. Furthermore, there was a significant positive correlation between ALC-1 expression and dP/dt_{max} of patients with hypertrophic obstructive cardiomyopathy (HOCM) in vivo [21]. Thus cross-bridge cycling kinetics and tension generation per cross-bridge are modulated by differential expression of MLC-1 genes. These results demonstrated for the first time that there is a molecular mechanism which allows the ventricular cardiomyocyte to adjust to enhanced work load through modification of the structure of the molecular motor – the partial substitution of VLC-1 by ALC-1 – which increases power output of the sarcomeric motor macromolecules and improves cardiac contractility. This is in contrast to the rodent heart, which modulate myosin motor function by MHC gene variation. These results have been supported in a transgenic mouse model which overexpresses atrial essential MLC in the ventricle: working heart function increases upon atrial essential MLC expression in the ventricle of transgenic rats overexpressing ALC-1 [52]. In the same mouse model a rise in cardiac beating frequency caused by running exercise was significantly lower in the transgenic group, demonstrating a large contractile reserve in ventricles with ALC-1 [52].

ALC-1 expression in the hypertrophied and failing human ventricle is heterogeneous and varies between 0% and 35% of total MLC-1. However, mean amount of ALC-1 appears higher in hypertrophied ventricles of patients with HOCM than in the failing ventricles of patients with dilated cardiomyopathy. Patients with HOCM express mean ALC-1 levels of 13% (range 0–32%) [43] while those with dilated cardiomyopathy reveal mean ALC-1 levels of 4% (range: 0–10%) [44] in their ventricles.

Thus, ALC-1 expression can be considered as a molecular adaptation mechanism to compensate an increased work demand or impaired sarcomeric function. In dilated cardiomyopathy the expressed ALC-1 levels may be too

low to balance adequately decreased contractile function. Hence patients with dilated cardiomyopathy may be a target for therapeutic upregulation of ALC-1 expression either by genetic (viral) transfer of ALC-1 cDNA into the cardiomyocyte or by upregulation of ALC-1 gene transcription.

The essential MLC isoforms act as MHC/actin tether

MLC-1 binds not only to the neck domain of the MHC [2] but also with its N-terminus to the C-terminus domain of actin [53, 54, 55, 56]. Thus MLC-1 tethers the MHC to the actin filament (Fig. 2). It has been demonstrated that MHC and MLC-1 bind to different actin monomers [57]. Binding of MLC-1 to actin cannot be predicted from the crystal structure of myosin S1 [2]. This is due to the limited resolution of the three-dimensional structure of the N-terminus of MLC-1 [2]. In fact, around 40 N-terminal amino acid residues of MLC-1 are not seen in the crystal structure [2]. This missing part of MLC-1 contains ten Pro and ten Ala residues, which could form an antennalike structure long enough to bridge the gap to the actin filament.

Experimental evidence for the functional importance of the MHC/actin tether has been obtained by weakening the tether on the MLC-1/actin interface and/or MLC-1/MHC interface and simultaneous registration of cross-bridge function. Inhibition of the MLC-1/actin interaction by peptide competition using synthetic N-terminal MLC-1 peptides increases force production and shortening velocity of both demembrated (skinned) and of intact electrically driven human ventricular fibers [58] as well as myofibrillar ATPase activity [59]. We propose that tethering MHC to the actin filament via MLC-1 imposes a load on the myosin cross-bridge. Relieving or weakening the MHC/actin tether decreases this load and accelerates cross-bridge cycling kinetics and at the same time enhances tension output per cross-bridge, thus increasing contractility [58].

To explain the effects on contractility by partial VLC-1/ALC-1 replacement in the hypertrophied human ventricle [51, 44], we have suggested that binding of ALC-1 to actin is weaker than the binding of VLC-1 to actin, representing a weaker MHC/actin tether. Interestingly, ALC-1 and VLC-1 differ in the primary structure of the N-terminus [31] (Table 3). Indeed, the affinity for actin of the N-terminal peptide 5–14 derived from ALC-1 is significantly lower than the actin affinity of the corresponding N-terminal peptide of VLC-1 [56]. These results support the initial hypothesis [51]: because of its low actin affinity ALC-1 is a weaker MHC/actin tether than VLC-1 and has increased cross-bridge cycling kinetics and force generation.

There is further experimental evidence in favor of this hypothesis: reconstitution of demembraned skeletal muscle fibers with MLC-1, with charged residues substituted by uncharged Ala at the most N-terminus increased shortening velocity [60]. The N-terminus of ALC-1 contains seven charged amino acids while VLC-1 contains nine [31]. From these data it appears that cross-bridge kinetics can be regulated by charge interactions between the N-terminus of MLC-1 and the actin C-terminus. Decreasing the amount of charged amino acid – by Ala substitution or differential MLC-1 isoform expression – weakens the MHC/actin tether and increased cross-bridge function.

Interaction between the N-terminus of essential MLC and actin may depend on the ionic conditions. In the test tube the N-terminus of essential MLC is cleaved by proteases only in the presence of Ca^{2+} [61]. Thus, various conformational states of the N-terminus of essential MLC may exist: an extended state with Ca^{2+} and a bent state without Ca^{2+} [61]. In the cardiomyocyte this would require a fluctuation of the N-terminus from an extended antennalike form which could bind to actin during systole (high Ca^{2+}) and a bent form during diastole (low Ca^{2+}) dissociated from actin. The molecular mechanism proposed for force generation and essential MLC action are schematically demonstrated in Fig. 2.

There is an additional interpretation of the functional effects of essential MLC isoforms. Maximal force production of the myosin cross-bridge may depend on the stiffness of the lever arm in the sense that the less stiff it is, the less the force that would be generated per cross-bridge [12]. Since the light chains are associated with the lever arm, various MLC isoforms may affect the cross-bridge stiffness. It may be suggested that the β-MHC associated with the VLC-1 has a greater stiffness than β-MHC with the ALC-1 thus generating more force per motor molecule.

Another important feature in the regulation of the contractile cardiac system by essential MLC is the sensitivity. Only small changes in the VLC-1/ALC-1 ratio are required to produce strong effects in the human heart [44, 51]. Furthermore, peptide competition experiments with the N-terminal VLC-1 peptide reveal maximal inhibitory peptide concentrations of MLC-1/actin interaction in the nanomolar range [58]. This phenomenon could be explained by the cooperative interaction of myosin with actin: it is well known that binding of a cross-bridge to the actin filament facilitates binding of other cross-bridges [62, 63, 64]. Ca^{2+} binding to troponin C weakens the binding between actin and troponin I, but

Table 3 Primary structures of the N-terminal domains of human ventricular (*VLC-1*) and atrial (*ALC-1*) essential myosin light chain isoforms (*bold* positively charged residues, *italics* negatively charged residues)

Human VLC-1[31]	1-Ala-Pro-**Lys**-**Lys**-Pro-*Glu*-Pro-**Lys**-**Lys**-*Asp*-*Asp*-Ala-**Lys**-Ala-Ala-Pro-Ala-Pro-**Lys**-Ala-Ala-
Human ALC-1[32]	1-Ala-Pro-**Lys**-**Lys**-Pro-*Glu*-Pro-**Lys**-**Lys**-*Glu*-Ala-Ala-**Lys**-Pro-Ala-Pro-Ala-Pro-Ala-Pro-Ala-

strengthens the troponin I/troponin C interaction. At the same time changes in tropomyosin position along the actin filament are believed to allow transition of cross-bridges into the force-generating, strong binding state [65, 66]. Conversely, cross-bridge binding to actin may be able to modulate troponin C conformation and its Ca^{2+} affinity. Thus inhibition of the MLC-1/actin interaction would result in the force-generating state with enhanced tension output (of the cross-bridges) which in turn would facilitate recruitment of non-force-generating cross-bridges for tension production. In fact, MLC-1 peptides derived from the N-terminus are especially effective in those myofibrils with complete actin filament structure [59] and therefore intact reciprocal coupling.

What is the role of MLC-2?

The presence of MLC-2 in striated muscle has been shown to be important for both myosin structure and function. Selective removal of MLC-2 changed the structure of the cardiac myosin molecule [67]. Extraction of MLC-2 from demembrated skeletal muscle fibers decreases V_{max} [68] and actin filament sliding velocity [69].

The rate of force redevelopment of cross-bridges (k_{redev}) increases with the level of activating Ca^{2+} [70]. This phenomenon seems to depend on the presence of MLC-2, since removal of MLC-2 eliminates the Ca^{2+} dependency of k_{redev} [71, 72]. In these experiments extraction of MLC-2 increased k_{redev} at submaximal Ca^{2+} levels to the value normally observed at maximal Ca^{2+} activation. Therefore it is possible that MLC-2 selectively downregulates the attachment rate constant of cross-bridges. This hypothesis is supported by the increased stiffness and Ca^{2+} sensitivity of isometric tension generation observed upon MLC-2 extraction [73]. An increased attachment rate constant upon MLC-2 elimination leads to an increased number of force-generating cross-bridges at a given Ca^{2+} activation level and consequently to increased stiffness and Ca^{2+} sensitivity [70].

The molecular mechanism for the regulation of k_{redev} by MLC-2 probably lies in the different myosin filament structures with and without MLC-2. The highly ordered myosin filament structure with MLC-2 is lost upon MLC-2 extraction [74]. This suggests a swing-out of cross-bridges away from the myosin filament backbone closer to the actin filament. The relative proximity of the cross-bridge to the thin filament may increase the probability of attachment and subsequent force production. The same mechanism is also discussed for phosphorylation of the MLC-2 (see below).

As mentioned above, MLC-2 is a member of the superfamily of Ca^{2+}-binding proteins. MLC-2 has lost its Ca^{2+}-binding ability in subdomains 2, 3, and 4 [30]. Only the first subdomain in the N-terminus retains high-affinity divalent metal binding [75]. This binding site seems to be important for normal myosin function since a mutation in the cation binding site results in reduced tension generation and stiffness [76].

Some years ago it was reported that MLC-2 protein levels are reduced in dilated cardiomyopathy, suggesting that this is the cause of heart failure [77]. However, in our own studies we have never observed any changes in MLC-2 content in patients with dilated or ischemic cardiomyopathy [44, 78].

Towards understanding cardiac MLC-2 isoforms

The functional roles of the three different MLC-2 isoforms in the human heart – ALC-2, VLC-2a, VLC-2b – are still not clear. Transgenic approaches, which replace cardiac MLC-2 with skeletal muscle MLC-2 [79] or ALC-2 with VLC-2 in the atrium [80] of mice, have recently been introduced to explore the functional role of the various MLC-2 isoforms. Substitution of VLC-2 by skeletal MLC-2 depressed contractility and relaxation of the left ventricle, leaving V_{max} unchanged [79]. However, MLC kinase (MLCK) in the heart differs from that in skeletal muscle [81], and it is possible that the skeletal MLC-2 is not a suitable substrate for the cardiac MLCK in vivo. It remains to be elucidated whether the observed decreased contractility in skeletal MLC-2 transgenic animals is the result of an incomplete posttranslational modification of the MLC-2 transgene product in the heart. Substitution of ALC-2 by VLC-1 in cardiomyocytes of the mouse atrium causes a shift of contractile characteristics to the ventricular phenotype [80]. No information concerning the MLC-2 phosphorylation levels of the transgene products have been provided.

The existence of two different VLC-2 isoforms (VLC-2a and VLC-2b; see above) in the human ventricle suggests three different myosin isoenzymes: a VLC-2a-homodimer, VLC-2a/VLC-2b-heterodimer, and VLC-2b-homodimer. In the normal human heart mainly the VLC-2b isoform is expressed (LC-2b to LC-2a ratio of 2.3) [44, 51, 78]. In most patients with limited cardiac functions, this ratio remains at its normal level. However, recently we found that in patients with HOCM VLC-2a expression declines in favor of the VLC-2b form [43] The physiological function of different VLC-2 isoenzymes in the human heart is still not understood. It is puzzling that slow-contracting (soleus, ventricle) but not fast-contracting (vastus, atrium) muscle types showed a MLC-2 polymorphism [82, 83]. In addition, species with fast-contracting ventricular muscle fibers (rat) reveal no MLC-2 polymorphism [84]. Thus there may be a functional role of the MLC-2 isoforms in the regulation of cross-bridge cycling kinetics. It may be speculated that the predominant expression of VLC-2b in HOCM [43] is a molecular mechanism to increase contractility of the cardiomyocyte.

MLC-2 phosphorylation increased cross-bridge cycling kinetics

The MLC-2 of the heart and skeletal muscle can be reversibly phosphorylated by a specific enzyme system

Table 4 Primary structures of the N-terminal domains of ventricular MLC-2 (*VLC-2*) of human and chicken

Human VLC-2[121]	1-Ala-Pro-Lys-Lys-Ala-Lys-Lys-Arg-Ala-Gly-Gly-Ala-Asn-Ser-Asn-Val-Phe-
Human ALC-2[34]	1-Ala-Ser-Arg-Lys-Ala-Gly-Thr-Arg-Gly-Lys-Val-Ala-Ala-Thr-Lys-Gln-Ala-Gln-Arg-Gly-Ser-Ser-Asn-Val-Phe
Chicken VLC-2a[36]	1-Pro-Lys-Lys-Ala-Lys-Lys-Arg-Ile-Glu-Gly-Ala-Asn-Ser-Asn-VaL-Phe-
Chicken VLC-2b[36]	1-Pro-Lys-Lys-Ala-Lys-Lys-Lys-Val-Glu-Glu-Gly-Gly-Ser-Asn-Val-Phe-

MLCK recognizes specific sequences upstream (Lys-Lys-Arg or Lys-Arg-Arg) and downstream (Val-Phe) of the phosphorylated serine residue [120]. These are Ser-14 for human VLC-2, Ser-21and Ser-22 for human ALC-2, and Ser-13 for both chicken VLC-2a and VLC-2b

comprised of a MLC phosphatase and a Ca^{2+}-calmodulin-dependent kinase (MLCK) [81]. MLCK catalyzes the transfer of the γ-phosphoryl residue of ATP to Ser-14 of the N-terminus of the human VLC-2 [81]. Also, Ser-21 and Ser-22 of human ALC-2 are phosphorylated by MLCK [34] (Table 4). Thus, human VLC-2 isoforms are monophosphorylated, while human ALC-2 may be mono- or diphosphorylated [83]. Ser-14 is also phosphorylated by protein kinase C but not by protein kinase A [67].

The first evidence of a functional role of MLC-2 phosphorylation came from studies with demembraned (skinned) fiber. In these experiments MLC-2 was thiophosphorylated using ATPγS [85, 86, 87] instead of the physiological substrate ATP. Thiophosphorylated proteins are resistant to dephosphorylation by protein phosphatases, and thus high levels of MLC-2 (thio)phosphorylation were obtained. In these studies thiophosphorylation of the MLC-2 depressed V_{max} and ATP consumption while isometric force generation was barely affected [87]. However, when ATPγS was substituted by ATP as substrate for MLCK, there was an increase in isometric force production of demembrated cardiac [88, 89] and skeletal muscle fibers [90] which was very pronounced at submaximal Ca^{2+} activation levels. Experiments at the single cardiomyocyte level have verified the MLC-2 phosphorylation effect [91]. V_{max} of demembrated fibers with phosphorylated MLC-2 remains unchanged in both demembraned cardiac [92, 93] and skeletal [90] muscle preparations. Some years later the cause of the controversial results between thiophosphorylation and physiological phosphorylation were elaborated: With ATPγS, the essential light chain rather than the regulatory light chain – the physiological substrate – became predominantly phosphorylated [93].

MLC-2 phosphorylation changes cross-bridge function. Maximal shortening velocity – which is determined by the detachment rate constant of negatively strained cross-bridges [94] – does not change with MLC-2 phosphorylation [90, 92, 93]. Similarly, the ratio of ATPase/force under isometric steady state conditions, which equals the detachment rate of positively strained cross-bridges remains unchanged in the phosphorylated and unphosphorylated states [71]. A mechanical method [70] was than used to determine the effect of MLC-2 phosphorylation on the rate constant of force redevelopment (k_{redev}) of demembrated skeletal muscle fibers. In fact, k_{redev} rose upon increasing the MLC-2 phosphorylation at both low and (in a less pronounced manner) high Ca^{2+} activation levels [95, 96, 97]. This finding obtained with skinned skeletal muscle fibers has been reproduced with skinned cardiac muscle fibers using an alternative experimental approach [98].

These results allowed elucidation of the molecular basis of force increase by MLC-2 phosphorylation. Since k_{redev} equals the sum of attachment rate (f_{app}) and detachment rate (g_{app}) of cross-bridges under isometric conditions [70], and since the detachment rate remains unchanged [71], MLC-2 phosphorylation selectively enhances the attachment rate constant of myosin cross-bridges of striated muscle types. Isometric force in the steady state equals the proportion of cross-bridges in the force-generating state, which is given by $f_{app}/(f_{app}+g_{app})$ [94, 70]. Simulations of force-Ca^{2+} ratios at various values for f_{app} and/or g_{app} [84] demonstrate that the Ca^{2+} sensitivity of isometric tension production increases with increasing values of f_{app} – perfectly explaining the Ca^{2+}-sensitizing effect of MLC-2 phosphorylation in striated muscle fibers [70, 88, 89, 90].

MLC-2 phosphorylation-induced "swing out"

Based on the introduction of negative charges by phosphorylation it has been hypothesized that the cross-bridges move away from the filament backbone [99]. This "swing-out" positions the cross-bridge closer to the actin filament, thus increasing the probability of attachment and force generation. In fact, electron microscopic studies of myosin filaments reveal that MLC-2 phosphorylation increases the distance of the cross-bridge from the myosin filament backbone [99, 100].

MLC-2 phosphorylation modifies the inotropic state of the heart

In the human heart steady-state phosphorylation of MLC-2 isoforms is not uniform. The ALC-2 in quickly frozen atrial resections are mono- (35.7% of ALC-2) and biphosphorylated (20.3% of ALC-2) [83]. Both VLC-2 isoforms in quickly frozen biopsy specimens of human ventricles reveal phosphorylation levels of 0.39 mol P_i/mol VLC-2b and 0.26 mol P_i/mol VLC-2a [102a]. In 25% of patients with severe cardiac failure (NYHA III or IV) the two VLC-2 isoforms were completely dephosphorylated [102a], providing an attractive hypothesis to explain cardiac insufficiency. Similar to the human ventricle, we detected in vivo phosphorylation of the pig ventricular VLC-2 isoforms with values of 0.39 mol

P_i/mol for VLC-2b and 0.25 mol P_i/mol for VLC-2a [98]. In the rabbit ventricle the phosphorylation states VLC-2b and VLC-2a are 0.36 and 0.15 mol P_i/mol, respectively [82]. In the rat ventricle in vivo phosphorylation is 0.39\mol/P_i/mol VLC-2 [84, 102b]. This normal level decreases significantly to less than one-half in old spontaneously hypertensive rats but remains normal in age-matched Wistar-Kyoto rats [102b]. The onset of abnormal MLC-2 phosphorylation may be associated with the onset of myocardial insufficiency, which has been observed in old hypertensive rats [103]. Long-term running training of rats caused an increased MLC-2 phosphorylation level in the ventricle [104], a phenomenon which might be considered as an adaptive mechanism to improve cardiac performance. In the European hamster during summer activity we have observed a phosphorylation level of 0.45 mol P_i/mol VLC-2, which declines to 0.18 mol P_i/mol VLC-2 during hibernation [105]. Reduced in vivo VLC-2 phosphorylation levels could mean a reduced cross-bridge cycling and thus energy consumption of the heart in the hibernating state.

For decades it was not known whether MLC-2 phosphorylation of striated muscle types is of any functional significance in vivo. This was due to the fact that MLC-2 phosphorylation was not considered a prerequisite for striated muscle contraction [106, 107], as it is in the smooth muscle [81]. Furthermore, the involvement of MLC-2 phosphorylation in the beat-to-beat regulation of the heart was excluded [106, 107]. An exception may be the tortoise, kept at +4°C. Cardiac beating rate is approx. 5 times per minute: MLC-2 phosphorylation increases during systole and decreases during diastole [108].

MLC-2 phosphorylation levels do not change during systole and diastole due to a continuous activation of the MLCK during the heart cycles under physiological conditions. Assuming a similar kinetic behavior in skeletal and cardiac muscle, the half-time of inactivation of MLCK is 1.3 s in situ [109]. Taking a twitch-time of a cardiomyocyte of 300 ms, the diastolic periods of around 150 ms (i.e., low Ca^{2+} conditions) are too short to induce MLCK inactivation. The net phosphate content of the MLC-2 then reflects the steady-state equilibrium between phosphorylation and dephosphorylation catalyzed by the MLCK and the MLC phosphatase, respectively. In vivo around 40% of the MLC-2 are phosphorylated (see above), reflecting the low MLCK and MLC-phosphatase activity in the heart [110]. Decreasing steady-state MLC-2 phosphorylation to 50% of its initial value requires 30 min of cardioplegic arrest [109], most probably due to the low MLC-phosphatase activity in the heart. Maximal activation of MLCK even during diastole could explain the relative stability of steady-state phosphorylation of cardiac MLC-2 in vivo, which is hardly affected by pharmacological interventions: neither α- nor β-adrenergic stimulation of isolated perfused hearts changes the MLC-2 phosphorylation level [111, 112, 113, 114].

In addition to MLCK, protein kinase C phosphorylates MLC-2 in cultured cardiomyocytes [115]. However, it is not known whether the steady-state level of MLC-2 phosphorylation is affected by protein kinase C activators. Upon α-adrenergic stimulation of isolated perfused hearts, which activates protein kinase C, the steady-state MLC-2 phosphorylation level remains unchanged [111].

Synopsis of MLC-2 function: a hypothesis

Association of the MLC-2 with MHC increases the structural order of the myosin filament, moving the cross-bridge closer to the myosin filament backbone. This association reduces the probability of cross-bridge attachment to the actin filament and therefore the rate of force development at submaximal Ca^{2+} concentrations. As a consequence the number of cross-bridges in the force-generating states and thus force and stiffness at submaximal Ca^{2+} activation are low. Phosphorylation of MLC-2 relieves this relative inhibition: the cross-bridges swing out from the thick filament backbone towards the actin filament, increasing the probability of attachment and force generation, which is most pronounced at low Ca^{2+} activation levels. It is of interest that this mechanism is especially relevant for the heart, since Ca^{2+} concentrations during systole are submaximal [80].

Increased and decreased MLC-2 phosphorylation levels are associated with positive and negative inotropic states of the heart, respectively. A selective decrease in the endogenous MLC-2 phosphorylation level reduces cross-bridge cycling and induced hypertrophic cardiomyopathy.

Mutations shed new light on essential and regulatory MLC function

In patients with HOCM and middle left ventricular thickening, a Met-149 Val substitution in VLC-1 is observed [107]. Myosin with the mutated VLC-1 reveals an increased actin translocation velocity in the in vitro motility assay [117]. It is worth noting that Met-149 Val is at the binding interface with the MHC [2]. As suggested above, tethering the myosin head to actin via MLC-1 seems to control force generation and cycling kinetics of the cross-bridge. Therefore weakening the interaction between MHC and MLC-1 should produce the same enhancement of cross-bridge function as weakening the actin and MLC-1 interaction. It is not clear whether Met-149 Val mutation of VLC-1 really results in a weakening of MHC-MLC-1 interaction, but if it does, this would explain the improved function of myosin with Met-149 Val mutation in VLC-1 in the in vitro motility assay [117]. Changing the MHC/MLC-1 interaction on the MHC site may be represented by a missense mutation close to the MHC/MLC-1 interface (Arg-719 Gln) [118]. Again, this mutation close to the MHC/actin tether increases actin translocation velocity. These results suggest that any deterioration in the MHC/actin tether by mutation at the MHC/MLC-1 interface, increases myosin function.

In addition to VLC-1, there are also mutations in the VLC-2 (Ala-13 Thr, Glu-22 Lys, and Pro-94 Arg) associated with middle left ventricular HOCM [117]. Glu-22 Lys reveals an almost normal function in the in vitro motility assay [117] and confers a higher Ca^{2+} sensitivity of force generation in demembraned fibers [74]. Interestingly, Glu-22 Lys causes a loss of the relaxed order of purified myosin filaments associated with an increased Ca^{2+} sensitivity of force generation [74] similar to both MLC-2 extraction and VLC-2 phosphorylation. Additional VLC-2 mutations have recently been reported in French patients with HOCM, namely Phe-18 Leu and Arg-58 Gln [119]. Especially the mutation Phe-18 Leu modifies the recognition site for MLCK [120] and may change the substrate specificity for MLCK. Therefore it would be interesting to analyze the in vivo VLC-2 phosphorylation state in these hypertrophic patients, which may be reduced. It is interesting to note that a transgenic mouse model expressing a VLC-2 form that cannot be phosphorylated developed hypertrophic cardiomyopathy as a result of inefficient sarcomeric cross-bridge cycling [116].

Future prospects

The myosin cross-bridge drives cardiac contraction. A certain level of phosphorylation of its regulatory light chain is required for normal heart function. Increasing the phosphorylation level may represent an approach to improve cross-bridge cycling kinetics and contractility of the diseased human heart. This could perhaps be achieved by developing specific MLC phosphatase inhibitors. Another therapeutic approach to improving cardiac contractility could be the development of drugs resembling the N-terminus of essential MLC to inhibit the interaction between essential MLC and actin. Alternatively, gene constructs coding for the first 15 N-terminal peptides of VLC-1 could be transferred into cardiomyocytes for ectopic expression of the peptide which bind to actin, thus inhibiting its interaction with the essential MLC and eventually increasing the inotropic state of the heart.

Both pharmacological and genetic approaches could improve power output of cardiac contraction and can represent future therapeutic approaches for treatment of cardiac failure.

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