Post N Med 2016; XXIX(12B): 34-38

Joanna Kołodziejczyk, Aleksandra Paterek, Michał Mączewski, Urszula Mackiewicz

Phosphorylation pattern of troponin I as a potential marker of the functional status of the failing myocardium

Status fosforylacji troponiny I jako potencjalny marker funkcji mięśnia sercowego w niewydolności serca

Department of Clinical Physiology, Centre of Postgraduate Medical Education, Warsaw
Head of Deppartment: Michał Mączewski, MD, PhD

Keywords

troponin I phosphorylation, hart failure, contraction-relaxation function

Słowa kluczowe

fosforylacja troponiny I, niewydolność serca, funkcja skurczowo-rozkurczowa serca

S u m m a r y

Sensitivity of troponin C to Ca^{2+} ions is an important parameter regulating strength of contraction and relaxation rate. It depends on the level of troponin I phosphorylation. Troponin I is phosphorylated in over a dozen amino acid positions. Currently, we know the functional significance of several of those locations only. Additionally, studies on troponin I phosphorylation in tissue of failing heart revealed that the pattern of troponin I phosphorylation in such tissue is completely different than in tissue sampled from healthy myocardia. Studies demonstrated that total troponin I phosphorylation in heart failure is reduced and this may cause an increase of troponin C sensitivity to Ca^{2+} ions in this pathology. This mechanism probably compensates the reduction of contractility in a failing heart. This increase of contractile function, however, may result in diastole disturbances. Additionally, changes in troponin I phosphorylation become more intensive as the disease progresses, and they can be used as a marker of disease progression. Studies on an animal model suggest that changes of troponin I phosphorylation regress when treatment is applied, so an analysis of troponin I phosphorylation could be used for evaluating treatment efficacy. It has been proposed that troponin I released to the circulation in the course of acute myocardial events should be the subject of studies on the level of troponin I phosphorylation in individual amino acid positions. Once the functional significance of phosphorylation in individual amino acid positions is comprehended, troponin I phosphorylation could be a useful marker of heart failure progression or a marker of treatment efficacy in this condition.

S t r e s z c z e n i e

Wrażliwość troponiny C na jony Ca^{2+} jest ważnym parametrem regulującym siłę skurczu i tempo rozkurczu. Jest ona regulowana stopniem fosforylacji troponiny I. Troponina I jest fosforylowana przynajmniej w kilkunastu pozycjach aminokwasowych. Dotychczas zbadano funkcjonalne znaczenie tylko kilku z tych miejsc. Dodatkowo, badania fosforylacji troponiny I w tkance pobranej z serc niewydolnych ujawniły, że wzór fosforylacji troponiny I w tej tkance jest całkowicie odmienny niż w tkance pobranej ze zdrowych serc. Badania wykazały, że całkowita fosforylacja troponiny I w niewydolności serca jest obniżona i to może być przyczyną wzrostu wrażliwości troponiny C na jony Ca^{2+} w tym stanie chorobowym. Prawdopodobnie jest to mechanizm kompensujący spadek kurczliwości w niewydolnym sercu. Ceną za wzmocnienie czynności skurczowej mogą być jednak zaburzenia rozkurczu. Dodatkowo zmiany w fosforylacji troponiny I nasilają się wraz z rozwojem choroby i mogą być markerem jej zaawansowania. Co więcej, badania w modelu zwierzęcym sugerują, że ma miejsce cofanie się zmian w fosforylacji troponiny I w wyniku zastosowanego leczenia, co mogłoby uczynić analizę fosforylacji troponiny narzędziem oceny skuteczności leczenia. Pojawiają się już propozycje, aby troponina I uwalniana do krążenia w przebiegu ostrych incydentów wieńcowych była poddawana badaniom pod kątem stopnia jej ufosforylowania w poszczególnych pozycjach aminokwasowych. Po zrozumieniu znaczenia funkcjonalnego fosforylacji poszczególnych pozycji aminokwasowych, fosforylacja troponiny I mogłaby się stać użytecznym markerem oceny stopnia zaawansowania niewydolności serca czy też markerem skuteczności leczenia tej choroby.

Conflict of interest
Konflikt interesów

None
Brak konfliktu interesów

Address/adres:

*Urszula Mackiewicz
Department of Clinical Physiology
Centre of Postgraduate Medical Education
Marymoncka 99/103, 01-813 Warszawa
tel.+48 (22) 569-38-42,
fax: +48 (22) 569-37-12
urszula.mackiewicz@cmkp.edu.pl

INTRODUCTION

Troponins are proteins found in the contractile apparatus of striated muscles, that is cardiac and skeletal muscles. There are three different troponins marked with the letters C, I, and T, with molecular weight of 18, 23.5, and 37 kDa, respectively. Troponin C (TnC)

in cardiac and skeletal muscles is coded by the same gene and has the same amino acid composition. Whereas troponins T (TnT) and I (TnI) are coded by different genes in the two types of muscles and have different structure. Hence, cardiac isoforms for these two troponins are distinguished (cTnI and cTnT).

Troponins C, I, and T form one protein complex and are mostly bound with proteins in the contractile apparatus. Small amounts of TnI and T (2-4% and 6-8%, respectively) are found in cytoplasm.

In clinical practice, troponins are a sensitive marker for myocardial damage. Even minor damage of cardiomyocyte cell membranes causes release of TnT and TnI unrelated to the contractile apparatus. Further ischaemic damage leads to the activation of proteolytic enzymes and to the release of troponins related to the contractile apparatus from cardiomyocytes.

Recent studies indicated that blood troponin levels provided information not only on the damage of the myocardium, buta, detailed examination of its phosphorylation pattern provided also information regarding the functional status of the myocardium, heart failure progression, or success of treatment in patients with heart failure. This is due to the fact that troponins are important modulators of systolic and diastolic cardiac function and the phosphorylation pattern of individual troponins changes as heart failure progresses.

LOCATION OF TROPONIN IN THE MYOCARDIUM

Cardiomyocyte contractile apparatus is composed of myofibriles and it comprises a major part of a cardiomyocyte. It is entwined with membranes of sarcoplasmic reticulum, which stores Ca^{2+} ions, and surrounded by numerous mitochondria, which produce ATP necessary for activation of contraction. A single myofibril is composed of a series of sarcomeres. A sarcomere, which is a morphological and functional unit of the contractile apparatus, is composed of thick filaments formed by myosin, thin filaments formed by actin, and multiprotein structures to which actin filaments are bound, called Z discs (fig. 1a).

During contraction, thin filaments slide along thick filaments, so the length of a sarcomere is reduced (while the length of filaments does not change) (fig. 1a). The sliding is possible due to a cyclical interaction of myosin heads with actin globules that form thin filaments. A thin filament is a helical structure comprised of two twisted globular actin chains. Complexes of so-called regulatory proteins are located on the actin helix. There is one regulatory complex for every seven actin globules (fig. 1b). Each complex is comprised of: tropomyosin (Tm) and a complex of three troponin subunits – C, I, and T. The function of each troponin in a muscle cell is strictly defined. TnC binds Ca^{2+} ions, which is a necessary element in activation of cardiomyocyte systole. TnI binds TnC, TnT, and actin; it also regulates the affinity of TnC to Ca^{2+} ions. TnT connects with TnI and Tm, anchoring the troponin complex to an actin filament (fig. 1c).

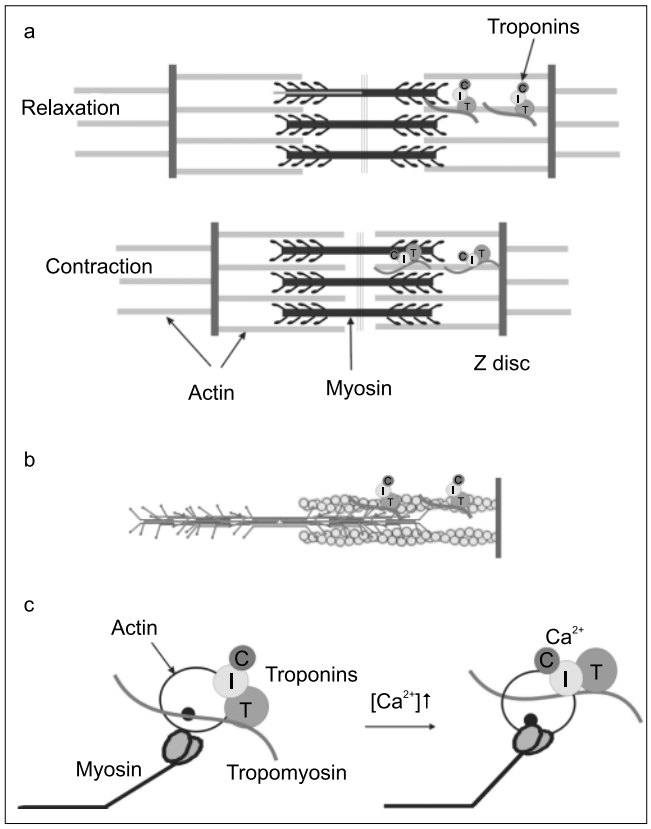

Fig. 1. The structure of contractile apparatus and mechanism of contraction activation (a), localization of troponin-tropomyosin complex (a and b) and its function in contraction-relaxation cycle (c)

ROLE OF TROPONIN COMPLEX IN ACTIVATION OF THE CONTRACTION-RELAXATION CYCLE.

For contraction to be activated in a cardiomyocyte, intracellular Ca^{2+} concentration must increase at least tenfold (over 1 μM). The increase is initiated by Ca^{2+} ions entering the cell through voltage-dependent L-type calcium channels. This influx then activates calcium channels of sarcoplasmic reticulum (SR), called ryanodine receptors (RyRs) (1). As Ca^{2+} ions are released from the SR, Ca^{2+} concentration in the cell increases to about 1 μM and it is sufficient for TnC to bind Ca^{2+}. Once it connects with a Ca^{2+} ion, TnC changes its conformation, causing the entire complex of regulatory proteins to move on the actin filament to a position in which sites of binding myosin heads to actin are exposed and the interaction of the two proteins and thus activation of contraction are possible (fig. 1c). After myocyte contraction, Ca^{2+} is transported from cytoplasm to the SR by sarcoplasmic reticulum calcium ATPase (SERCA) and out of the cell by the Na^{+}/Ca^{2+} exchanger. Reduction of intracellular Ca^{2+} level to below 1 μM causes Ca^{2+} to disconnect from troponin C and the tropomyosin-troponin complex to return to the resting position, in which sites of binding myosin on the actin filament are not exposed. Interaction of the two proteins is then impossible and mycyte relaxation is initiated (fig. 1c) (1).

DETAILED STRUCTURE OF THE TROPONIN COMPLEX AND CHANGES OF ITS CONFORMATION IN THE CONTRACTION-RELAXATION CYCLE.

TnC contains four Ca^{2+} binding domains (E-F hand motifs) located in pairs at the N- and C-terminal of the protein. Sites III and IV on the C-terminal domain have high affinity to Ca^{2+} ions and bind them even at diastolic Ca^{2+} concentration in a cell, which means that these sites do not participate in activation of contraction. Site I at the N-terminal domain is inactive and does not bind Ca^{2+} ions in physiological conditions. The site crucial for contraction activation is site II, with relatively low affinity to Ca^{2+} ions. This site is not occupied by Ca^{2+} ions at the resting concentration and it binds them only when ion concentration in a cell exceeds 1 μM. Site II is the only one of all four E-F hand motifs involved in myocyte contractile activity (fig. 2) (2).

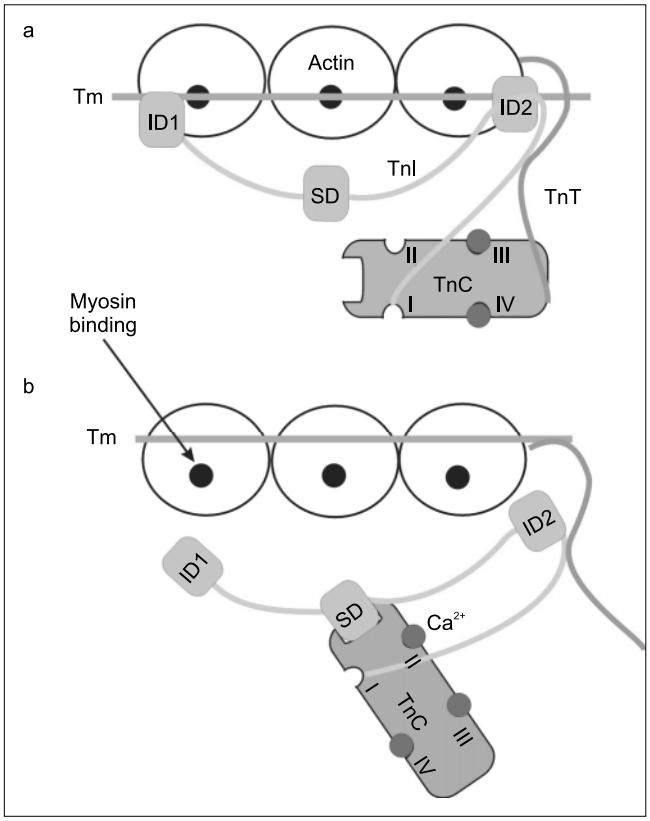

Fig. 2. The structure of the troponin-tropomyosin complex and its role in activation of cardiomyocyte relaxation (a) and contraction (b). TnC – troponin C, TnI – troponin I, TnT – troponin T, Tm – tropomyosin, SD – switching domain, ID1, ID2 – inhibitory domain 1 i 2.

Three functional domains can be identified in the structure of troponin I: the switching domain (SD) and two inhibitory domains (ID 1 and 2). The first inhibitory domain is located at the C-terminus of troponin I and it binds with actin and tropomyosin when Ca^{2+} is at resting concentration (during diastole). The ID 2 domain is located closer to the N-terminus and binds with actin. These links stabilise the position of tropomyosin on the actin filament in a position in which it covers sites of bonding myosin heads to actin (fig. 2a). The SD is located between the inhibitory domains; when Ca^{2+} concentration is low (diastole), it remains unbound with the remaining proteins of the complex.

When Ca^{2+} concentration increases prior to contraction, location II on TnC becomes occupied. TnC changes conformation and exposes the hydrophobic pocket to which the SD of TnI attaches. Relocation of the SD forces a movement of inhibitory domains, which must disconnect from the actin filament (fig. 2b). In effect, Tm is not stabilised in its position and moves on the actin filament, exposing binding sites for myosin heads and, thus, enabling contraction (3). This process is significantly regulated by TnI phosphorylation, which affects the process of binding Ca^{2+} ions to site II of TnC.

PHOSPHORYLATION OF TNI

So far, over a dozen amino acid positions of TnI have been discovered that undergo phosphorylation (4). The approximate location of these positions is presented in figure 3. Most of them are located near the N-terminus and C-terminus of TnI. The remaining positions are located near the inhibitory domains and the switching domain. These positions can be phosphorylated by several kinases: protein kinase A (PKA), protein kinase C (PKC), protein kinase G (PKG), and protein kinase D (PKD). These kinases are activated in cardiomyocytes when the several important signalling pathways are activated. PKA is activated due to the action of catecholamines on beta-adrenergic receptors, mainly on the beta-1 receptor coupled with Gs protein. Adenylate cyclase is then activated, cAMP level increases, and the kinase is activated. On the other hand, PKC in cardiomyocytes is activated by a number of hormones and neurotransmitters (e.g. angiotensin II, endotelin-1, and noradrenalin) binding with receptors coupled with Gq protein, which leads to phospholipase C activation and the formation of diacylglycerol (DAG), which activates PKC. PKD, in turn, initially considered an isoforms of protein kinase C, is located on the signalling pathway of protein kinase C and is activated by that kinase (a kinase kinase). PKG activation is a result of guanylyl cyclase activation by nitrogen oxide and natriuretic peptides (5). All the kinases listed above play an important role in regulating contraction-relaxation cycle in cardiomyocytes, as they phosphorylate a number of Ca^{2+} handling proteins.

The functional significance of phosphorylation of most amino acid positions of TnI is not known. So far, the effect on cardiomyocyte contraction and relaxation has been studied for only several of them: Ser 23, Ser 24, Ser 43 and 45, and Thr 144 (2, 4, 6). Most attention was given to Ser 23 and 24. Studies demonstrate that all the kinases listed above can phosphorylate Ser 23 and Ser 24 *in vitro*, but *in vivo* these locations are phosphorylated mainly by PKA, activated in a cell as a result of catecholamine stimulation.

Due to Ser 23/24 phosphorylation, N-terminus of TnI, which binds with the N-terminus of troponin C in the absence of phosphorylation (fig. 2), disconnects from this location and moves toward the inhibitory domain, stabilising it in this position and inhibiting

the access of Ca^{2+} to site II on TnC at the same time. In consequence, binding of Ca^{2+} by TnC is more difficult, as its sensitivity to Ca^{2+} ions is lower and, moreover, tropomyosine is stabilised in a position that inhibits activation of contraction. Such modification of troponin-tropomyosin complex conformation is very significant functionally, as relaxation is facilitated and contractile force is reduced. This contraction toning effect is substantially compensated by the effect of Ser 23/24 phosphorylation on the other mechanism regulating contractile force, that is the Frank-Starling mechanism. In this mechanism, force of contraction increases in proportion to sarcomere elongation before systole, which means that the longer a sarcomere is before contraction, the greater contractile force is activated (greater myocyte shortening). Ser 23/24 phosphorylation enhances the Frank-Starling mechanism, amplifying the increase of contractile amplitude for a given sarcomere elongation. Finally, phosphorylation of TnI in these amino acid positions affects two independent mechanisms (sensitivity of TnC to Ca^{2+} ions and Frank-Sterling mechanism), thus facilitating relaxation without impairing contractile force.

Other important locations of TnI phosphorylation are positions Ser 43 and Ser 45, located between the N-terminus and ID2, and Thr 144, located within this domain (fig. 3). Both these locations are probably phosphorylated mainly by PKC in vivo, with different effect of phosphorylation on individual positions. Thr 144 phosphorylation causes an increase of TnC sensitivity to Ca^{2+} ions, while Ser 43/45 phosphorylation causes a decrease. Therefore, it is difficult to foresee the resultant effect of PKC on TnC sensitivity to Ca^{2+} ions, which probably depends on the level to which individual amino acid positions are phosphorylated. Studies on transgenic mice with permanently phosphorylated selected amino acid positions (7) demonstrated that constant Ser 43/45 phosphorylation affected sensitivity to Ca^{2+} ions more than constant Thr 144 phosphorylation. It can be, therefore, concluded that the resulting effect of TnI phosphorylation by PKC, as well as by PKA reduces sensitivity of the contractile apparatus to Ca^{2+} ions and facilitates relaxation.

Recent studies using mass spectrometry enabled a comprehensive assessment of TnI phosphorylation and identification of all phosphorylation locations. These studies confirmed phosphorylation in the amino acid positions that were already known and lead to the discovery of over a dozen new locations. Of the newly discovered positions, Tyr 25, Ser 76, and Thr 180 are most strongly phosphorylated (fig. 3). Phosphorylation of these positions is even stronger than that found in locations believed so far to be crucial (Ser 23, 24, 43, and 45). It has not been studied yet, which kinases phosphorylated these locations of TnI in vivo and what the functional effect of these phosphorylations is. Studies using new techniques demonstrated that the status of TnI phosphorylation is very complicated and its resulting effect on contraction-relaxation cardiac function was difficult to determine at this point. Additionally, the status of TnI phosphorylation changed significantly in myocardial pathologies, which were nearly always accompanied by modified activation of individual intracellular signalling pathways, as well as changed kinase and phosphatase activation level.

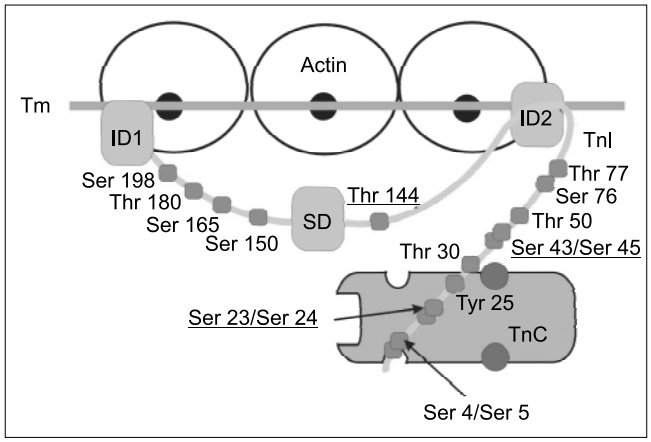

Fig. 3. The main phosphorylation of amino acid position in troponin I. Currently, we know the functional significance of only several of those locations (underlined)

CHANGED STATUS OF TNI PHOSPHORYLATION IN HEART FAILURE

Studies on the dependence of contractile force on intracellular Ca^{2+} ion concentration demonstrated that sensitivity of contractile apparatus to Ca^{2+} ions increases in filing heart muscle, which meant that the strength of an activated contraction was greater for a given Ca^{2+} concentration compared to healthy myocardium. In addition, incubation of tissue sampled from failing myocardium with PKA reduced the sensitivity of contractile apparatus to Ca^{2+}, which suggested the level of TnI phosphorylation by PKA reduced in heart failure (8).

It was also demonstrated that changes of contractile apparatus' sensitivity to Ca^{2+} ions correlated with the level of disease progression. Increased sensitivity to Ca^{2+} ions was observed only in patients with end-stage heart failure, qualified to group IV according *New York Heart Association* (NYHA) classification. In patients with less advanced disease symptoms (NYHA class I-III), sensitivity to Ca^{2+} ions did not differ from that measured in healthy myocardium (9).

Zhang et al. (10), in turn, studied the level of total phosphorylation of TnI and demonstrated that TnI phosphorylation in biopsy material sampled *post mortem* from the myocardium of patients with heart failure was lower than in material sampled from hearts without heart failure and this reduction is correlated with the level of disease progression. Also in myocardial tissue sampled during transplantation from donors and recipients, it was demonstrated that in terminally failing hearts (patients qualified for transplantation) TnI phosphorylation was significantly lower. This suggested that increased sensitivity to Ca^{2+} ions resulted from reduced TnI phosphorylation and that reduced phosphorylation found in patients with end-stage heart failure may have been an attempt to compensate the significant reduction of contractility of the failing myocardium.

Recently, Zhang et al. (11) used mass spectroscopy to compare phosphorylation in individual amino acid positions in healthy myocardia and in two groups of explanted failing heart muscle: with ischaemic and idiopathic aetiology. The level of TnI phosphorylation changed in most amino acid positions similarly in both groups of failing hearts compared to healthy myocardia. In general, phosphorylation reduction was noted in 5 amino acid positions, while increase was noted in 9 positions and no change – in one position. Although an increase of phosphorylation in heart failure was noted in more positions than reduction, the increase was smaller quantitatively and the total phosphorylation of TnI in heart failure was reduced. Studies using mass spectrometry confirmed the expected reduction of phosphorylation in Ser 23 and Ser 24, which caused an increase of TnC sensitivity to Ca^{2+} ions. They also demonstrated that TnI phosphorylation in three known positions phosphorylated by PKC (Ser 43/45 and Thr 144) was increased, especially in position Thr 144. Increased Thr 144 phosphorylation enhanced sensitivity to Ca^{2+} ions, but increased Ser 43/45 phosphorylation reduced the sensitivity.

An initial analysis of these results suggested that the increase of TnC sensitivity to Ca^{2+} ions in heart failure is certainly due to reduced Ser 23/24 phosphorylation and increased Thr 144 phosphorylation, which enhanced contractility of a terminally failing myocardium, but impeded relaxation. Counteracting these effects, increased Ser 43 and 45 phosphorylation can slightly alleviate diastole disturbances. Increased phosphorylation by PKC corresponds well with enhancement of signalling pathways leading to PKC activation in heart failure. PKA activation in heart failure is usually increased, but many proteins are underphosphorylated by this kinase due to strong action of phosphatases.

The functional effects of phosphorylation of the newly discovered locations (Ser 4/Ser 5), Tyr 25, Thr 50, Ser 76, Thr 77, Ser 165, Thr 180, Ser 198 are still unknown. It seems particularly important to study the significance of phosphorylation in position Tyr 25, in which phosphorylation in a healthy heart is relatively high and reduces significantly in heart failure. Phosphorylation of three locations near TnI C-terminus (Ser 165, Thr 180, Ser 198) can also be highly significant. It was recently demonstrated that phosphorylation significantly increased in these locations in a model of failing canine heart and significantly reduced due to resynchronization therapy that improved myocardial function (11).

CONCLUSION

Sensitivity of TnC to Ca^{2+} ions is an important parameter regulating contraction force and relaxation rate. It depends on the level of TnI phosphorylation. As innovative methods of testing proteins and their post-translation modifications appeared (mass spectrometry), knowledge about TnI phosphorylation increased significantly. It turned out that TnI is phosphorylated in over a dozen amino acid positions. Currently, we know the functional significance of only several of those locations. Additionally, studies on TnI phosphorylation in tissue sampled from failing hearts revealed that the pattern of TnI phosphorylation in such tissue was completely different than in tissue sampled from healthy myocardia. Studies demonstrated that total TnI phosphorylation in heart failure was reduced and this may cause an increase ofTnC sensitivity to Ca^{2+} ions in this pathology. This mechanism probably compensates the reduction of contractility in a failing heart. This increase of contractile function, however, may result in relaxation disturbances. Additionally, changes in TnI phosphorylation become more intensive as the disease progresses and they can be used as a marker of disease progression. Studies with animal models suggested that changes of TnI phosphorylation regressed when treatment was applied, so an analysis of TnI phosphorylation could be used for evaluating treatment efficacy. It has been proposed that TnI released to the circulation in the course of acute myocardial events or minor amounts of it appearing in blood in various cardiovascular diseases should be the subject of studies on the level of TnI phosphorylation in individual amino acid positions. Once the functional significance of phosphorylation in individual amino acid positions is comprehended, TnI phosphorylation could be a useful marker of heart failure progression or a marker of treatment efficacy in this condition.

BIBLIOGRAPHY

1. Bers DM: Cardiac excitation-contraction coupling. Nature 2002; 415: 198-205.
2. Solaro RJ, Rosevear P, Kobayashi T: The unique functions of cardiac troponin I in the control of cardiac muscle contraction and relaxation. Biochem Biophys Res Commun 2008; 369: 82-87.
3. Kobayashi T, Jin L, de Tombe PP: Cardiac thin filament regulation. Pflugers Arch 2008; 457: 37-46.
4. Solaro RJ, Henze M, Kobayashi T: Integration of troponin I phosphorylation with cardiac regulatory networks. Circ Res 2013; 112: 355-366.
5. Mackiewicz U, Klemensa E, Beręsewicz A: Receptory beta-adrenergiczne w zdrowym i niewydolnym sercu. Kardiol Pol 2007; 65: 294-302.
6. Solaro RJ, Kobayashi T: Protein phosphorylation and signal transduction in cardiac thin filaments. J. Biol. Chem 2011, 286: 9935-9940.
7. Wijnker PJ, Murphy AM, Stienen GJ et al.: Troponin I phosphorylation in human myocardium in health and disease. Neth Heart J 2014; 22: 463-469.
8. Wijnker PJ, Sequeira V, Witjas-Paalberends ER et al.: Phosphorylation of protein kinase C sites Ser42/44 decreases Ca^{2+}-sensitivity and blunts enhanced length-dependent activation in response to protein kinase A in human cardiomyocytes. Arch Biochem Biophys 2014; 554: 11-21.
9. van Der Velden J, Klein LJ, Zaremba R et al.: Effects of calcium, inorganic phosphate, and pH on isometric force in single skinned cardiomyocytes from donor and failing human hearts. Circulation 2001; 104: 1140-1146.
10. Zhang J, Guy MJ, Norman HS: Top-down quantitative proteomics identified phosphorylation of cardiac troponin I as a candidate biomarker for chronic heart failure. J Proteome Res 2011; 10: 4054-4065.
11. Zhang P, Kirk JA, Ji W et al.: Multiple reaction monitoring to identify site-specific troponin I phosphorylated residues in the failing human heart. Circulation 2012; 126: 1828-1837.

received/otrzymano: 10.11.2016
accepted/zaakceptowano: 29.11.2016