Enhancement of calcium uptake via the sarcoplasmic reticulum is a potent therapeutic strategy for dilated cardiomyopathy and heart failure

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Dilated cardiomyopathy (DCM), a distinct form of cardiomyopathy, is a myocardial disorder characterised by heart chamber dilation with severe contractile dysfunction and frequent association with heart failure. Analysing this subset of heart failure has provided mechanistic insights of intrinsic pathways for myocyte adaptation and survival. Despite the heterogeneous aetiologies, a calcium cycling defect is common in DCM. A growing body of evidence has shown that calcium homeostasis and calcium-dependent signalling pathways play a pivotal role in cardiac hypertrophy and heart failure. In this regard, recent studies demonstrate that a cardiac calcium cycling defect is identified as a critical regulator for the progression of heart failure in DCM and that enhancement of calcium uptake into the cardiac sarcoplasmic reticulum (SR) may have potential therapeutic value for cardiac dysfunction. This article will focus on the cardiac SR calcium ATPase (SERCA2a) and its regulatory protein, phospholamban (PLB), as new therapeutic targets for DCM and heart failure.

Keywords: calcium ATPase, calcium homeostasis, dilated cardiomyopathy, heart failure, phospholamban, sarcoplasmic reticulum


1. Introduction

Cardiomyopathy is defined as a disease of the myocardium associated with cardiac dysfunction by either extrinsic specific events or intrinsic/genetic disorders of the myocardium. DCM is one distinct form of cardiomyopathy, its aetiology is heterogeneous since DCM occurs secondary to various extrinsic biomechanical stresses like ischaemia, pressure and volume overloads, abnormal metabolism, inflammation or toxic agents, or is primarily caused by intrinsic disorders of the myocardium itself such as genetic abnormalities in the cytoskeletal and nuclear envelope proteins [1,2]. Despite the diverse aetiologies, the physiological hallmark of all forms of DCM is the severe loss of cardiac contractile function and most of them eventually result in heart failure, suggesting that there is a common signalling pathway leading to cardiac dysfunction and heart failure. Identifying the primary common pathways that provoke a vicious circle is particularly important for finding new therapeutic strategies which are based on
2. Basis of the regulation of calcium uptake into the sarcoplasmic reticulum

The SR, an extensive intracellular membrane system consisting of a lipid bilayer that surrounds each myofibril, plays a fundamental role in the co-ordination of the movement of cytosolic calcium during each cycle of cardiac contraction and relaxation. The SR releases calcium via the ryanodine receptor into the cytosol to activate contraction of cardiac muscle and re-accumulates calcium via SERCA2a into the SR lumen to initiate relaxation.

SERCA2a, a cardiac and slow-twitch skeletal muscle isoform of the SERCA family which belongs to P-type ATPases, is the primary regulator of the rate of calcium re-uptake during relaxation in the heart. The structure and function of SERCA are extensively reviewed elsewhere [9]. Briefly, SERCA2a is an integral membrane protein with ten transmembrane helices which pumps calcium into the SR lumen at the expense of ATP hydrolysis. The activity of SERCA2a is predominantly regulated by its endogenous inhibitor, PLB. Although SERCA2a is known to be phosphorylated by calcium/calmodulin-dependent protein kinase, the biophysiological significance of phosphorylated SERCA2a for calcium transport has yet to be established.

PLB, a 52 amino acid SR transmembrane phosphoprotein, principally controls the activity of SERCA2a in cardiac and slow-twitch muscle. The structure and function of PLB are extensively reviewed elsewhere [10,11]. SERCA2a and PLB interact at their cytoplasmic and transmembrane domains. PLB inhibits SERCA2a activity through direct interaction in its unphosphorylated form, whereas the phosphorylated form of PLB dissociates from SERCA2a (Figure 1). A dramatic change in charge is observed in PLB from pl = 10 in the dephosphorylated state to pl = 6.7 in the phosphorylated state [10]. In this manner, the phosphorylation of PLB relieves its inhibition on SERCA2a activity following phosphorylation at Ser16 and Thr17 by cAMP dependent protein kinase (PKA) and calcium/calmodulin-dependent protein kinase, respectively. Although PLB at Ser10 is phosphorylated by protein kinase C (PKC), the physiological relevance has been doubted. Under physiological conditions, phosphorylation at Ser16 by PKA is the predominant event leading to a proportional increase in the rate of calcium uptake into the SR and accelerates ventricular relaxation. PLB is considered to be mostly responsible for the lucitropic effect on the heart by β-adrenergic stimulation since PLB-deficient mice display maximal cardiac contractility and relaxation in the absence of any catecholamine stimulation [12]. The further stimulation with isoproterenol could not alter the elevated contractile parameters in PLB-deficient hearts. These findings demonstrated that PLB also plays a pivotal role in force development and cardiac contractility by β-adrenergic stimulation. In addition to PLB phosphorylation by protein kinases, the myocardial SR possesses phosphatase activity capable of dephosphorylating PLB by phosphatase Type 1 and Type 2A [13]. The activity of phosphatase Type 1 is decreased by phosphatase inhibitor-1, which can be phosphorylated by PKA. Thus, PKA also contributes to an increase in PLB...
phosphorylation by decreasing the activity of phosphatase Type 1 through the phosphorylation of phosphatase inhibitor-1 [14].

PLB forms a pentamer of five identical subunits. It has been debated whether PLB monomers preferentially bind to SERCA2a and predominantly inhibit SERCA2a activity when compared with PLB pentamers [10]. However, myocytes overexpressing some PLB mutants preferentially expressed pentameric PLB and have high inhibitory effects on SERCA2a in vivo [15]. There is no data to show how the ratio of monomer/pentamer PLB is regulated in physiological and pathophysiological condition. Even though the physiological significance of monomer/pentamer formation is equivocal, certain PLB mutants appear to interact less with SERCA2a when compared with wild type PLB. In this regard, to elucidate which PLB residues interact with SERCA2a, MacLennan and co-workers have generated extensive site-directed mutations of PLB to identify the mutants harbouring loss or gain of function [16].

3. The rationale for enhancement of calcium uptake is a potent therapeutic strategy for dilated cardiomyopathy and heart failure

The rationale for augmentation of calcium re-uptake as a novel therapeutic strategy for heart failure is based on a considerable number of findings. Impaired calcium handling and a decrease in calcium uptake are central physiological hallmarks of a number of animal models of heart failure, as well as in human failing hearts [17,18]. The protein expression levels of SERCA2a and PLB modulate myocardial contractility and relaxation by altering calcium handling. Transgenic mice overexpressing SERCA2a resulted in enhanced calcium transients, myocardial contractility and relaxation [19,20]. Heterozygous SERCA2a knockout mice displayed impaired cardiac performance [21]. The relative PLB expression level closely correlates with cardiac performance [22]. Therefore, the relative SERCA/PLB ratio appears to be a critical regulator of cardiac contractility and relaxation [23]. However, it has been debated whether the protein expression levels of SERCA2a are decreased [24] or unchanged in heart failure [25]. Attenuated PLB phosphorylation by PKA has been detected in DCM and heart failure, which may contribute to the depression of SERCA2a activity. It is reasonable to conclude that this attenuation could be the consequence of downregulation of β-adrenergic receptors in heart failure. In addition, the enhanced activity of phosphatases in human failing myocardium may contribute to the decreased responsiveness to inotropic agents [26]. However, until recently, it has been difficult to distinguish whether these abnormalities are the primary event that drives the progressive decline in contractility or a secondary phenomenon. In this regard, a recent genetic complementation study has identified that calcium cycling defects are critical in the progression of DCM [6]. In addition, using an adenoviral vector, a simple increase in SERCA2a expression restored muscle contractility and relaxation in an...
animal model of pressure overload [8] as well as in cardiomyocytes from patients with heart failure [27]. Thus, the evidence supports that one can consider augmentation of calcium uptake by increasing SERCA2a activity as a novel therapeutic strategy for DCM and heart failure [28].

The current prime goal is to increase SERCA2a activity. Genetic approaches and pharmacological interventions, designed to increase SERCA2a activity, may prove valuable in preventing or reversing the adverse physiological impairment in DCM and heart failure. There are potentially several options to achieve it: 1) by increasing total SERCA2a levels in the heart, 2) by modulating SERCA2a itself to increase the calcium transport, 3) by decreasing total PLB levels in the heart, and 4) by disruption of interaction between SERCA2a and PLB.

4. Strategies to increase SERCA2a protein in heart failure

It may be a fundamental approach to increase SERCA2a activity by simply increasing the expression levels of SERCA2a. This can be achieved through SERCA2a gene transfer and upregulation of SERCA2a gene at transcriptional level. As described above, SERCA2a expression is often decreased in DCM. In these cases, complementation of this depletion could be efficient. The results from the transgenic mice overexpressing SERCA2a revealed that SERCA2a expression could be feasibly increased in vivo [19,20]. However, it may be difficult to achieve the high levels of SERCA2a protein expression by SERCA2a gene transfer, since protein expression was only increased by 1.2- to 1.5-fold in transgenic mice despite high levels of SERCA2a mRNA [19,20]. Nevertheless, fairly encouraging data have come from Dr Hajjar's group, indicating that muscle contractility and relaxation were restored by adenovirus-mediated SERCA2a gene transfer in an animal model of pressure overload [8] as well as in cardiomyocytes from patients with heart failure [27]. The technique of efficient adenovirus-mediated gene transfer into the myocardium is claimed by the same group [102]. In these studies, SERCA2a expression levels had been decreased prior to gene transfer. It is an intriguing question whether SERCA2a gene transfer can be efficiently achieved in cases of heart failure, with unchanged SERCA2a expression, and improve cardiac performance.

When considering gene transfer into the heart when the targeted gene is expressed in broad types of cell lineage, it is not only high efficiency, but also heart specificity of gene delivery that is particularly important. Since SERCA2a lacks the tissue specificity and SERCA2b, an alternative spliced isoform of SERCA2a, is expressed in all types of non-muscle cells, it remains unclear whether unexpected biological outcomes may occur after wide-spread gene delivery of SERCA2a.

Transcriptional regulation of SERCA has not been extensively explored. Thyroid hormone has been known to increase SERCA1 and SERCA2 expression at transcriptional level [29,30]. However, in addition to the chronotropic effect, thyroid hormone displays a lack of tissue specificity which makes it an unsuitable therapeutic agent for heart failure. A more recent study demonstrated that Egr-1 is a transcriptional inhibitor of the SERCA2 gene in doxorubicin-induced cardiomyopathy and Egr-1 antisense oligonucleotides blocked the doxorubicin-induced reduction in SERCA2 mRNA [31]. Although further investigation will be required, the strategy to upregulate SERCA2a transcription may not be so efficacious because complex post-transcriptional regulation may determine the SERCA2a protein level [19,20].

5. Strategies to modulate SERCA2a to increase calcium transport

Pharmacological interventions, designed to modulate SERCA2a itself in order to increase SERCA2a activity and calcium uptake, may be another option. As described in the previous section, SERCA2a can be phosphorylated by calcium/calmodulin-dependent protein kinase. However, the biophysiological effect of phosphorylated SERCA2a on calcium transport is equivocal. No other kinase or phosphatase has been known to modulate SERCA2a activity. Gingerols [32,33], 1-(3,4-dimethoxyphenyl)-3-dodecanone [34], plakortones [35] and nitric oxide [36] have been known to directly increase SERCA2a activity with mAbs against PLB, indicating that their action is, at least in part, independent from PLB. On the other hand, thapsigargin and cyclosporin acid are specific SERCA inhibitors. These compounds will provide the opportunity to investigate the pharmacological mechanism of SERCA activation.
independent of PLB interaction. However, the validity of the application for human use remains to be elucidated since these SERCA activators have yet to be tested in vivo in animals.

6. Strategies to decrease PLB protein in heart failure

There are several reasons to consider PLB as a prime target to increase SERCA2a activity:

1) PLB is the strongest (probably only) endogenous inhibitor of SERCA2a. When PLB function is completely diminished, SERCA2a can be fully activated.

2) PLB is a terminal effector of β-adrenergic signalling pathways. The inhibitory effect of PLB is almost abolished when PLB is phosphorylated by PKA. PKA has various biological effects and some of them may have adverse effects on failing hearts. Therefore, targeting PLB may restrict the adverse effects of PKA.

3) PLB expression is highly cardiac specific and is much higher in ventricles than atria. This tissue specificity is a great advantage for designing interventions that interact with PLB. One report demonstrated that small amounts of PLB are expressed in vascular endothelial cells and affect vascular relaxation [37], although the biological significance remains unclear.

4) PLB inhibition does not affect chronotropic responses, which is beneficial for patients with heart failure.

5) PLB is a small protein consisting of 52 amino acids. It should be easy to manipulate genetic modification on PLB gene.

6) PLB-deficient mice have not displayed any adverse events so far and can survive normal lifespan [38]. In heart failure, ameliorated effects of PLB gene ablation last for at least a year without increased mortality [6]. Therefore, diminishing PLB function is unlikely to provoke a detrimental event. This will be discussed more in the section of Expert opinion.

7) PLB is remarkably conserved between species and only a single type of PLB molecule has been found. Therefore, functional redundancy by other isoforms can be excluded.

Strategies for decreasing PLB protein expression could be designed to decrease PLB transcription, or disrupt PLB mRNA stability because PLB gene dosage correlates with the level of protein and cardiac performance [22]. The promoter sequence of PLB has been identified in various species [39,40] and potential consensus cis-promoter elements similar to known muscle-specific promoters have been found. A GATA-4 motif is proposed to be important for cardiac specific expression of the PLB gene [39]. Although the cis-element sequence predicts the transcriptional factors known to bind the sites, no study has identified a transcriptional factor that decreases the PLB expression. It is possible to design antisense oligomers of PLB promoter elements when the cis-elements required for PLB gene expression have been identified. Adenovirus-mediated antisense expression of PLB coding lesion resulted in the successful depression of PLB mRNA and protein and increased calcium uptake in neonatal rat myocytes [41,42]. However, one study demonstrated that adenovirus-mediated antisense failed to significantly alter the endogenous PLB level in adult rabbit myocytes [41]. This strategy using antisense RNA has not been tested for in vivo hearts nor myocytes from failing hearts. Although further investigation will be required, inhibition of PLB expression seems to have promising value for pharmaceutical interventions to improve cardiac performance.

7. Strategies to disrupt the interaction between SERCA2a and PLB

To disrupt the interaction between SERCA2a and PLB, interventions could be designed to increase the phosphorylated state of PLB, or to interrupt PLB to associate with SERCA2a. Increasing PLB phosphorylation can be achieved by enhancing PKA activity or decreasing phosphatase activity. A recent study demonstrated that enhanced contractility mediated by βARKct (a truncated form of β-adrenergic receptor kinase which works in a dominant negative manner) transgene rescued the phenotypes of an animal model of DCM [43]. Augmented PLB phosphorylation is supposed to contribute, at least in part, to these rescue effects. As mentioned earlier, in the setting of heart failure, β-adrenergic signals are downregulated and phosphatase activity is increased. When pharmaceutical interventions are designed to modify the activity of PKA or phosphatases, it could be problematic that neither has specificity for PLB. This appears to be the limitation of its therapeutic application. The novel system will be required to specifically recruit PKA to PLB or to keep PLB from phosphatase Type 1 or Type
2a. In this regard, recruitment of GM (a regulatory subunit of phosphatase 1), which specifically interacts with PLB at the transmembrane lesion and does not have catalytic activity of phosphatase 1, may provide a specific inactivation of PLB in heart [44].

To disrupt the interaction between SERCA2a and PLB is probably an ideal strategy to increase the SERCA2a activity since ablation of endogenous PLB can fully activate SERCA2a. However, there are fundamental difficulties in disrupting the interaction between SERCA2a and PLB. Disruption of protein-protein interaction is generally difficult. In addition, compounds have to be delivered into the intracellular membrane system. Therefore, only peptides, small-molecular-weight compounds or gene transfer can be available to date. Moreover, binding affinity and critical binding sites of SERCA2a and PLB are yet to be elucidated, although PLB is known to interact with SERCA2a at both cytosolic and transmembrane domains. Several nucleotide mimetics such as quercitin [45,46], tannin [47] and ellagic acid [48] have been reported to increase SERCA2a activity at low concentration through the inhibition of interaction between SERCA2a and PLB. High dose of these compounds inhibits SERCA2a activity. These compounds interact with the nucleotide binding site of SERCA2a, not directly with PLB, suggesting that the conformational change of this site can affect the binding affinity of SERCA2a and PLB. Orion claims bisethers of 1-oxa, aza and thianaphthalen-2-ones as binding affinity of SERCA2a and PLB. Disruption of protein-protein interaction is generally difficult. In addition, compounds have to be delivered into the intracellular membrane system. Therefore, only peptides, small-molecular-weight compounds or gene transfer can be available to date. Moreover, binding affinity and critical binding sites of SERCA2a and PLB are yet to be elucidated, although PLB is known to interact with SERCA2a at both cytosolic and transmembrane domains. Several nucleotide mimetics such as quercitin [45,46], tannin [47] and ellagic acid [48] have been reported to increase SERCA2a activity at low concentration through the inhibition of interaction between SERCA2a and PLB. High dose of these compounds inhibits SERCA2a activity. These compounds interact with the nucleotide binding site of SERCA2a, not directly with PLB, suggesting that the conformational change of this site can affect the binding affinity of SERCA2a and PLB. Orion claims bisethers of 1-oxa, aza and thianaphthalen-2-ones as PLB inhibitors [103]. The compounds are stated to increase calcium uptake into vesicles prepared from guinea-pig ventricular myocardium and be useful for preventing heart failure and stunned myocardium. Orion also claims a different PLB inhibitor for increasing coronary flow [104]. However, none have been tested for in vivo in animals yet. The validity for the application for in vivo use remains to be elucidated. A recent study unveiled the high-resolution structure of SERCA1a, a fast-twitch skeletal muscle isoform of SERCA family, providing in-depth information to understand the fundamental SERCA function for calcium transport [49]. Since SERCA1a has high homology with SERCA2a and has been known to similarly interact with PLB [50], the high-resolution structure of SERCA1a may provide new insights into the design of small molecular weight compounds or peptides which interact directly with SERCA2a or disrupt the interaction between PLB and SERCA2a.

As described earlier, previous studies by MacLennan and co-workers have identified the PLB residues which affect SERCA2a activity and calcium uptake by extensive site-directed mutagenesis [16]. Taking advantage of using these pioneer data, one may design specific PLB mutations to increase SERCA2a activity. Recent in vitro studies demonstrated that certain PLB mutants transferred into myocytes using adenovirus vector increased the contractility and relaxation of myocytes [6,41]. This may be the encouraging first step, although there are still many steps to be overcome before these mutant PLB genes can be used to treat heart failure and DCM. The molecular mechanism by which the PLB mutants disrupt the interaction between SERCA2a and endogenous PLB remains unknown. It has not been elucidated whether a mutant PLB increases SERCA2a activity in vivo. The most effective gene delivery system, and PLB mutants to increase SERCA2a activity and calcium uptake, also need to be elucidated. Although further investigation will be required, selective disruption of the interaction between SERCA2a and PLB is a novel promising strategy for genetic and pharmacological interventions to prevent or reverse cardiac performance in DCM and heart failure.

8. Expert opinion

It has been suggested that enhancement of SR function has therapeutic value for heart failure. This concept has not been tested in failing hearts until several recent studies [6,8,27]. These studies demonstrated that increasing SERCA2a activity could prevent the progression of heart failure or restore myocardial contractility and cardiac performance in heart failure. Thus, the development of specific SERCA2a activators or PLB inhibitors is of particular interest, although there aren't very many claimed patents regarding this specific issue yet. One probable reason is that the development and design of the intervention is so difficult. So far only adenovirus mediated gene transfer has been successfully achieved to improve in vivo contractility and relaxation. The SERCA2a gene, mutant PLB genes and PLB antisense RNA could be good candidates for gene transfer, provided the genes have are efficiently transferred into the heart. Adenovirus vectors are the most popular for gene transfer to date. Protein expression by adenovirus mediated gene transfer is only temporary since transfected genes are dramatically diminished after several weeks. For clinical
application, gene therapy to enhance SERCA2a activity may be a short-term alternative to other inotropic agents for the treatment of acute cardiac dysfunction. In such an instance, temporal gene expression could be advantageous. Other vectors such as a recombinant adeno-associated virus vector may achieve longer gene expression. In this regard, efficient and stable in vivo gene transfer to cardiomyocytes using recombinant adeno-associated virus vectors is claimed [105].

Compounds that increase SERCA2a activity and calcium uptake will be potent inotropic and lusitropic agents. However, compounds have to be delivered into the intracellular membrane system, which is a hurdle in the development of a new compound. High-throughput screening on a large scale will be required to find peptides or small-molecular-weight compounds that activate SERCA2a and increase calcium uptake. Several drugs have been identified as SERCA2a activators in vitro. However, it is important to elucidate whether these compounds can improve in vivo contractility and relaxation without any serious adverse effects.

Although the present review mainly describes the promising possibility of achieving enhanced SERCA2a activity for DCM and heart failure, it should be noted here that this strategy may involve several disadvantages as well. When increasing SERCA2a activity in failing hearts, there are two major potential concerns for human patients. One is that energy consumption in the heart could be increased because the process of calcium uptake into the SR requires ATP hydrolysis. Energy production is often impaired in heart failure and DCM. However, compounds have to be delivered into the intracellular membrane system, which is a hurdle in the development of a new compound. High-throughput screening on a large scale will be required to find peptides or small-molecular-weight compounds that activate SERCA2a and increase calcium uptake. Several drugs have been identified as SERCA2a activators in vitro. However, it is important to elucidate whether these compounds can improve in vivo contractility and relaxation without any serious adverse effects.

In conclusion, enhancement of calcium uptake into the cardiac SR, by activation of SERCA2a or inhibition of PLB, will provide potential therapeutic value for heart failure and DCM. However, further exploration will be required to effectively achieve the enhancement of SERCA2a activity, especially to find a specific compound or gene that disrupts the interaction between SERCA2a and PLB. In addition, the potential adverse effects of augmenting SERCA2a activity need to be determined.

Bibliography

Papers of special note have been highlighted as:

- of interest
- of considerable interest

1700 Enhancement of calcium uptake via the sarcoplasmic reticulum

• A thorough review of SERCA.

• A most systematic and comprehensive review.


•• A comprehensive review of SERCA2a activators and PLB inhibitors.


Minamisawa 1701
50. - High-resolution structural analysis of SERCA1.

Patents

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