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Review

New perspectives on the role of SERCA2's Ca^{2+} affinity in cardiac function

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Abstract

Cardiomyocyte relaxation and contraction are tightly controlled by the activity of the cardiac sarco(endo)plasmic reticulum (SR) Ca^{2+} transport ATPase (SERCA2a). The SR Ca^{2+}-uptake activity not only determines the speed of Ca^{2+} removal during relaxation, but also the SR Ca^{2+} content and therefore the amount of Ca^{2+} released for cardiomyocyte contraction. The Ca^{2+} affinity is the major determinant of the pump's activity in the physiological Ca^{2+} concentration range. In the heart, the affinity of the pump for Ca^{2+} needs to be controlled between narrow borders, since an imbalanced affinity may evoke hypertrophic cardiomyopathy. Several small proteins (phospholamban, sarcolipin) adjust the Ca^{2+} affinity of the pump to the physiological needs of the cardiomyocyte. It is generally accepted that a chronically reduced Ca^{2+} affinity of the pump contributes to depressed SR Ca^{2+} handling in heart failure. Moreover, a persistently lower Ca^{2+} affinity is sufficient to impair cardiomyocyte SR Ca^{2+} handling and contractility inducing dilated cardiomyopathy in mice and humans. Conversely, the expression of SERCA2a, a pump with a lower Ca^{2+} affinity than the housekeeping isoform SERCA2b, is crucial to maintain normal cardiac function and growth. Novel findings demonstrated that a chronically increased Ca^{2+} affinity also may trigger cardiac hypertrophy in mice and humans. In addition, recent studies suggest that some models of heart failure are marked by a higher affinity of the pump for Ca^{2+}, and hence by improved cardiomyocyte relaxation and contraction. Depressed cardiomyocyte SR Ca^{2+} uptake activity may therefore not be a universal hallmark of heart failure.

Keywords: SR Ca^{2+}ATPase; Phospholamban; Sarcolipin; Cardiac hypertrophy; Heart failure; SERCA2a

1. Introduction

In eukaryotic cells, cytosolic calcium (Ca^{2+}) represents an almost universal second messenger controlling a multitude of cell functions, amongst which cardiac muscle contraction was one of the first to be recognized [1,2]. How a second messenger that cannot be formed nor destroyed, but can only be shifted from one location to another, has acquired the ability to independently and simultaneously control so many cellular activities remains incompletely understood. This short overview focuses on the role of the SERCA2 Ca^{2+}-transport ATPases which help to shape the complex spatio-temporal subcellular Ca^{2+} patterns in the cardiomyocyte.

Cardiomyocyte depolarization triggers Ca^{2+} entry into the cell via depolarization-activated L-type Ca^{2+} channels. Ca^{2+} that enters the cell binds to and opens specialized Ca^{2+}-release channels (ryanodine receptor type 2 or RYR2) in the nearby sarcoplasmic reticulum (SR), inducing the release of a much larger quantity of Ca^{2+} from this intracellular Ca^{2+} store. The concomitant transient rise in cytosolic Ca^{2+} leads to binding of Ca^{2+} to troponin C (TnC) allowing the thin (actin) and thick (myosin) filaments to interact and develop force [3]. For relaxation to occur, Ca^{2+} must be removed from the cytosol to dissociate Ca^{2+} from TnC. At least three processes coordinate this removal of Ca^{2+}: (i) the SR Ca^{2+} ATP-ase type 2a (SERCA2a) which pumps Ca^{2+} back into the SR using the hydrolysis of ATP as a source of energy, (ii) the Na^+/Ca^{2+} exchanger (NCX) which uses the energy of the Na^+ and Ca^{2+} gradients across the plasma membrane to exchange 3 extracellular Na^+ ions for 1 intracellular Ca^{2+} ion and (iii) the plasma-membrane Ca^{2+} ATPase (PMCA), which extrudes Ca^{2+} from the cell using the energy liberated by ATP hydrolysis [3].

In general, Ca^{2+} re-uptake into the SR is quantitatively more important than Ca^{2+} removal to the external medium. However, this relative importance varies between species [4]. In rabbit and

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humans, the SR takes up about 70% of the cytosolic Ca^{2+}, while the rest is extruded from the cell by NCX (28%) and PMCA (2%). Rodents cycle 90% of Ca^{2+} through the SR and only 10% via the extracellular space [3,5]. As Ca^{2+} release from the SR is the major source of Ca^{2+}, the amount of Ca^{2+} available for release and thus the SR Ca^{2+} content controls cardiac contractility. Therefore, Ca^{2+} uptake into the SR via SERCA2a is a major determinant of both cardiac relaxation and contraction [6].

Given this central role of SERCA2a in cardiac function, it is not surprising that alterations in its Ca^{2+} affinity strongly affect cardiac function. As will be discussed in this review, controlling the affinity of the pump for Ca^{2+} is not only crucial for normal cardiac contractile control, but it is also important for the regulation of cardiomyocyte growth. Modulators of the Ca^{2+} affinity are therefore essential tools to adjust the Ca^{2+} affinity of the pump to the physiological needs of the cardiomyocyte. Moreover, the choice of a SERCA isoform with a proper affinity for Ca^{2+} is of great physiological importance.

2. Cardiac splice variants of SERCA2 show differences in Ca^{2+} affinity

Cardiac muscle exploits splice variant SERCA2a, which presents a lower affinity for Ca^{2+} compared to the house-keeping SERCA2b, to control cellular Ca^{2+} movements [7]. The SERCA2a isoform is identical to SERCA2b except for its carboxyl terminus (Fig. 1A). The four COOH-terminal amino acids of SERCA2a are replaced by an extended hydrophobic sequence of 49 amino acids in SERCA2b, comprising an eleventh transmembrane and a short luminal segment. Consequently, the extreme C-termini of SERCA2a and SERCA2b are situated at opposite sides of the ER membrane (with the SERCA2b tail protruding into the lumen) [7]. Studies mainly using the COS or HEK-293 cell expression systems showed that the extended tail of SERCA2b alters the functional properties of the pump. These in vitro studies revealed that SERCA2b shows a two-fold higher affinity for Ca^{2+} and two-fold lower catalytic turnover rate for Ca^{2+} uptake and ATP hydrolysis. [8–11]. Recent studies on the isoform properties in vivo confirmed the higher Ca^{2+} affinity of SERCA2b compared to SERCA2a, but differences in the maximal pumping rate were not observed [12,13]. The isoforms share the same sensitivity to phospholamban (the endogenous SERCA2 regulator) and to thapsigargin (a specific inhibitor of the SERCA pumps) [9].

It is remarkable that a number of post-mitotic cells that make heavily use of Ca^{2+} signaling rely on SERCA2a to control either contraction (as in cardiac-, slow-twitch skeletal and differentiated contractile smooth muscle) [14] or neuro-transmitter release (as in some, mostly GABAergic neurons) [15]. While each of these cell types also express (usually lower levels of [16]) SERCA2b for house-keeping functions, the majority of the transcripts of the ATP2A2 gene in these cells are alternatively processed to generate a SERCA2a-encoding mRNA [17]. The alternative transcript processing that leads to the production of SERCA2a mRNAs involves choices that have to be made concerning the use of two alternative splice donor sites and two polyadenylation sites [18]. How exactly this process is controlled in these muscles and neuronal cells remains largely unexplored, but a detailed study of the 3′ end of the ATP2A2 gene described the minimal cis-acting structural requirements of the ATP2A2 transcript needed to allow regulated processing [18]. Clearly, the activation of an otherwise inefficient splicing mechanism is part of the differentiation pathway of muscle and neuronal cells and this is responsible for the switch from SERCA2b expression in undifferentiated cells to expression of SERCA2a in the differentiated state [18]. It is noteworthy that invertebrates (*Caenorhabditis*, Artemia), which, in contrast to vertebrates, do not have three but only a single SERCA gene, already show the expression of two different spliced SERCA isoforms that structurally and functionally correspond to the vertebrate SERCA2a and SERCA2b isozymes [19] [20]. The duality of the SERCA(2) isoforms is therefore an evolutionary old feature. In this review, we will focus on the SERCA2 isoforms and will not address the SERCA3 splice variants. All SERCA3 isoforms are characterized by a low (supra micromolar) Ca^{2+} affinity and are not expressed in myocardial cells [8,21].

3. Modulators of the Ca^{2+} affinity: phospholamban and sarcolipin

The need for an accurate regulation of SERCA2's Ca^{2+} affinity is underscored by the existence of two membrane-inserted regulator proteins phospholamban (PLB) and sarcolipin (SLN) (Fig. 1). The only known physiological function of these small proteins is to act in essence as Ca^{2+}-affinity modulators of SERCA2a, SERCA2b or occasionally of the skeletal-muscle variant SERCA1a. Note that the low-Ca^{2+}-affinity pump SERCA3 appears to be insensitive to these modulators.

3.1. Molecular properties and function of PLB

The main regulator of SERCA2a is PLB, a small transmembrane protein (52 amino acids) present in the ventricles and to a lower extent in the atria of the heart [22]. PLB monomers interact with SERCA2a and reversibly inhibit the Ca^{2+}-transport activity of the pump. PLB also forms homopentamers, which are considered as an inactive pool of PLB (Fig. 1B) [23]. The principal effect of the association of PLB with SERCA2a is to lower the apparent affinity of SERCA2a for Ca^{2+}, with little or no effect on the maximal pumping rate at saturating Ca^{2+} and ATP concentrations [24,25] (although a stimulatory effect on the maximal pumping activity has been suggested [26,27]).

The inhibition of the pump by PLB is reversible. At non-resting Ca^{2+} concentrations, the binding of Ca^{2+} to the pump promotes the dissociation of the PLB/SERCA2a complex [28]. Also phosphorylation of PLB relieves the functional inhibition of SERCA by PLB and increases the pump's affinity for Ca^{2+} (Fig. 1B) [28], without changing the maximal pumping rate

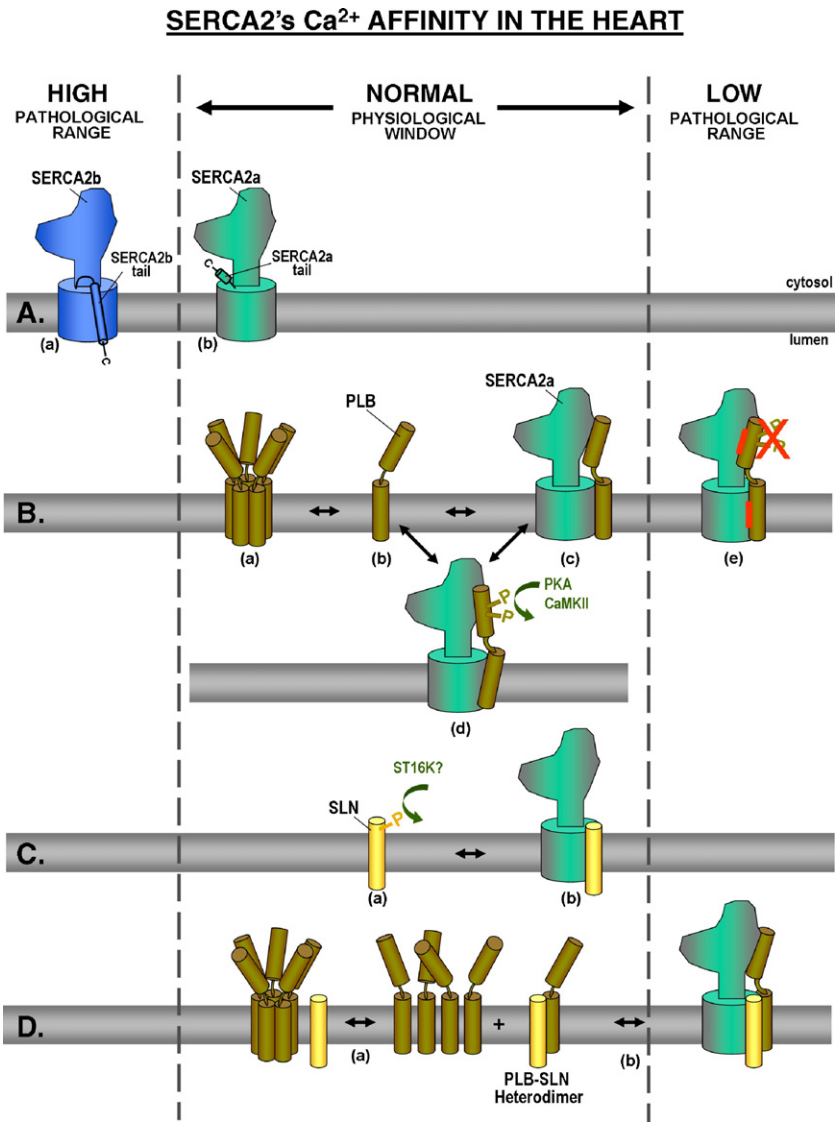

Fig. 1. A physiological window for the Ca^{2+} affinity of the SERCA2 pump. From left to right, modulations which gradually reduce the Ca^{2+} affinity of the SERCA2 pump. In the heart, the Ca^{2+} affinity needs to be critically controlled to remain in the physiological window of the Ca^{2+} affinity in order to ensure proper cardiac function. Perturbations which lead to excessive (extreme left) or depressed (extreme right) Ca^{2+} affinity induce cardiac hypertrophic remodeling. See main text for more details. (A) The cardiac-specific SERCA2a (b) and the house-keeping SERCA2b (a) isoforms differ in their *carboxyl*-terminus. The extra 49 amino acid tail of SERCA2b protrudes in the SR membrane and is responsible for the higher affinity of the pump for Ca^{2+}. An excessive affinity of the pump for Ca^{2+} in the heart may trigger cardiomyopathy (a). (B) PLB forms an equilibrium between the monomeric (inhibitory, b) and pentameric (inactive pool, a) state. PLB monomers inhibit SERCA2a by direct interaction (c). This functional inhibition is reversed by phosphorylation (d) by protein kinase A (PKA) or Ca^{2+}-calmodulin kinase II (CaMKII). (e) Superinhibition of the pump (e.g. by more PLB monomers, stronger PLB–SERCA2 interaction, defective phosphorylation) leads to chronically depressed cardiomyocyte contractility and cardiac hypertrophy and heart failure. (C) SLN monomers (a) interact with SERCA2a resulting in inhibition of the pump (b). Putative phosphorylation of SLN by serine/threonine 16 kinase (STK16) may be important in the regulation of SLN function, e.g. during β-adrenergic stimulation. (D) Superinhibition by PLB–SLN heterodimers. SLN disrupts PLB pentamers by direct interaction with PLB (a). SLN–PLB heterodimers bind with high affinity to SERCA2a (b). Superinhibition by SLN/PLB may trigger cardiac hypertrophy (b).

[27]. The extent of PLB phosphorylation critically determines its inhibitory properties and is controlled by the relative activity levels of different kinases and phosphatases. PLB is phosphorylated by cAMP-dependent protein kinase A (PKA) at Ser16 and by Ca^{2+}-calmodulin dependent protein kinase II (CaMKII) at Thr17 [29,30]. The major phosphatase responsible for dephosphorylation of PLB is protein phosphatase type 1 (PP1, 70%) whereas protein phosphatase type 2A (PP2A) and type 2C (PP2C) together account for about 20% of the phosphate removal [31].

3.2. Physiological role of PLB in cardiac function

Given the central position of SERCA2a in determining cardiac relaxation and contraction, it is not surprising that the phosphorylation-dependent regulation of SERCA2a by PLB controls cardiac performance [32]. PLB is considered as a physiological brake of the heart and is an important constituent of the β-adrenergic cascade. β-adrenergic activation triggers cAMP- and Ca^{2+}-dependent phosphorylation of PLB which reverses the inhibition of SERCA2a. Consequently, the

increased Ca^{2+} affinity of the pump promotes SR Ca^{2+} uptake and increases SR Ca^{2+} content and release. Together, strong stimulatory effects on cardiac relaxation (lusitropic effects) and contractility (inotropic effects) are observed (reviewed in [32]).

The expression level of PLB critically determines cardiac relaxation and contraction in a gene-dosage-dependent manner. Loss of PLB was associated with an increased Ca^{2+} affinity of SERCA2a, which promoted cardiac SR Ca^{2+} uptake, content and release, resulting in a hyperdynamic state of the heart [25]. Similar observations were done in the heart of heterozygous $PLB^{+/-}$ mice, although the effect of reduced PLB levels on cardiomyocyte and cardiac relaxation and contraction is smaller than in homozygous $PLB^{-/-}$ [33].

The associated hyperdynamic state of the heart is well tolerated in mice, without pathological consequences [34] or reducing the exercise capacity [35]. Conversely, transgenic overexpression of PLB in the murine heart reduced the apparent Ca^{2+} affinity of SERCA2a, resulting in diminished cardiomyocyte relaxation and contractility at baseline [36,37]. Stimulation with β-agonists could reverse the increased inhibition of SERCA2a via phosphorylation of PLB [36]. Interestingly, transgenic mice overexpressing PLB at four-fold normal levels displayed a spontaneous compensatory rise in (nor)adrenaline levels in the blood which enhanced phosphorylation of PLB [37]. In the long term, this compensatory response became maladaptive, resulting in heart failure (HF), a syndrome in which progressive deterioration of cardiomyocyte contractility and cardiac pump function leads to an insufficient blood supply in the body.

Chronic inhibition of the cardiac SR Ca^{2+} ATPase was also observed upon overexpression of gain-of-function PLB mutants in the heart [36–40]. These mutants greatly reduce the affinity of the pump for Ca^{2+}, decreasing cardiomyocyte Ca^{2+} kinetics and mechanics. When the inhibition by PLB can be reversed by endogenous β-agonists, life is not threatened, although significant left-ventricular hypertrophy was detected [36,38]. However, heart failure may develop upon chronic inhibition of SERCA2a, i.e. if the strong inhibition is not reversible by endogenous β-agonists [37,39,40] (Fig. 1B).

Many studies addressed the physiological relevance of the dual-site phosphorylation of PLB (more extensively reviewed in [41]). During β-adrenergic stimulation, Ser16 is the dominant phosphorylation site and a full β-effect can be mediated by single-site phosphorylation at Ser16 [42]. Phosphorylation at Thr17 may only occur after a sufficient rise in cytosolic Ca^{2+}, i.e. at the highest levels of β-adrenergic stimulation [41,43,44]. Both phosphorylations then contribute to the total PLB phosphorylation and have additive effects on cardiomyocyte relaxation [45]. Besides a role in the β-adrenergic response of the heart, a number of studies indicated that CaMKII-dependent phosphorylation of PLB at Thr17 may play an important role in the frequency-dependent acceleration of cardiac relaxation (FDAR) [46,47]. The rate of cardiac relaxation increases with frequency, which is a fundamental physiological modulator of myocardial performance. FDAR is thought to be mediated by CaMKII [48], which is supported by the unique behavior of CaMKII, acting as a frequency decoder by increasing its activity with the frequency of Ca^{2+} spikes [49]. However, phosphorylation of PLB may not be the only target of CaMKII in this process [50,51].

3.3. Molecular properties and function of SLN

Recent findings shed new light on the putative physiological role of SLN, a homologue of PLB, in the heart (Fig. 1C) [52]. Like PLB, SLN interacts with and inhibits the SR Ca^{2+} ATPase by lowering its apparent Ca^{2+} affinity without pronounced effects on the maximal pumping rate [53,54]. SLN has a transmembrane sequence similar to that of PLB, but differs at its C and N termini. It lacks the regulatory cytosolic domain (comprising Ser16 and Thr17 in PLB) and has a small luminal domain that is entirely absent in PLB, which is important for targeting SLN to the SR membrane [55] and anchoring SLN to the SERCA pump [55,56].

A remarkable synergistic inhibitory effect was observed when SLN and PLB were co-expressed with SERCA2a or SERCA1a in HEK-293 cells (Fig. 1D) [53]. SLN has a higher affinity for PLB monomers than PLB monomers have for other PLB monomers. Therefore, and because SLN only has a weak ability to form SLN oligomers [57], SLN can convert inactive PLB pentamers into a larger number of active inhibitory species, i.e. PLB monomers and PLB–SLN dimers. Modeling studies illustrate that not only PLB or SLN alone can fit into the hydrophobic PLB/SLN binding site of SERCA, but that a PLB–SLN heterodimer would fit even tighter, exerting a stronger inhibitory effect by blocking the conformational changes of the Ca^{2+} pump [56]. Immunoprecipitation experiments confirmed the formation of the stable ternary PLB–SLN–SERCA complex [53,54,58]. These studies of the PLB–SLN complex hint at another level of regulating the Ca^{2+} affinity of the SR Ca^{2+} ATPase.

3.4. Physiological role of SLN in cardiac function

Several observations point to a possible physiological role of SLN in the heart. First, based on overexpression in HEK-293 cells, SLN has the ability to interact not only with SERCA1a but also with SERCA2a affecting their Ca^{2+} affinity to a similar extent [53]. Second, several observations now indicate that SLN expression may be prominent in the heart [59]. Previously, SLN was considered to be the regulator of SERCA1a and hence the fast skeletal muscle counterpart of the SERCA2a inhibitor PLB in the heart. However, in humans, SLN mRNA is also found in the heart [52]. SLN in mouse and rat is abundantly expressed at the mRNA and protein level in the atria of the heart (but is absent in the ventricles) and is found to a lower extent in skeletal muscle [58,60,61]. The superinhibitory effect of the PLB/SLN complex on SR Ca^{2+} uptake activity may therefore be prominent in the atria of the heart, where both regulators are endogenously found [61].

SLN-mediated superinhibition in the atria appears counter-intuitive and in apparent contradiction with the fact that the atrial rate of Ca^{2+} removal is faster than that of the ventricles, an effect that previously was ascribed to the higher SERCA levels

and the lower PLB expression in atria versus ventricle [62]. SLN might however be important to fine-tune the β-adrenergic response in the atria. This idea is corroborated by recent reports describing a regulatory effect of β-stimulation mediated by SLN [63,64]. In addition, the atrium and ventricle respond differently to β-adrenergic stimulation. Koss et al. [22] and Freestone et al. [65] both reported a smaller β-effect on atrial than on ventricular relaxation, and attributed this to the lower PLB content in the atria. However, in some conditions a stronger [66] or more sensitive β-adrenergic effect was observed in atria compared to ventricle [22]. This might require the relief of combined SLN/PLB inhibition on SERCA, whereas the relief of PLB inhibition alone would be insufficient.

The absence of SLN protein in the normal ventricle [61] does not exclude developmental or disease related SLN expression in this chamber. To better define the role of SLN in cardiac physiology, adenoviral or transgenic overexpression of SLN was studied in ventricular cardiomyocytes. When SLN is introduced in ventricular cardiomyocytes, it appears to be targeted to the same SR subcompartments as PLB [58] where it has the ability to form a stable SLN/PLB/SERCA2a ternary complex [54,58]. Two independent reports describe how cardiac-specific overexpression of SLN in mouse reduced the affinity of SERCA2a for Ca^{2+} [54,63]. In these studies on SLN transgenes, a slowed cardiomyocyte relaxation and impaired cardiac function was reported. The studies showed however some discrepancies, which may depend on the SLN species used for overexpression (rabbit versus mouse SLN, which may slightly differ in their ability to inhibit SERCA activity). In one of the SLN transgenes, the impaired SR Ca^{2+} ATPase activity and cardiomyocyte contractility evoked a hypertrophic response [54] (Fig. 1D). Depending on the report, at least three mechanisms were held responsible for the reduced cardiac contractility in SLN transgenic mice: (i) direct inhibition of SERCA2a by SLN [54,63]; (ii) formation of the highly inhibitory SLN/PLB binary complex [54,63] and (iii) a reduction in PLB phosphorylation [54].

Importantly, the β-adrenergic agonist isoproterenol largely restored contractility in SLN transgenes, suggesting that like it is the case for PLB, also SLN inhibition can be reversed by β-receptor activation [54,63]. The de-inhibition of SLN may be a direct action of β-receptor stimulation on SLN, but an enhanced phosphorylation of PLB by PKA in the presence of SLN cannot be excluded [54]. However, the observation that β-agonist stimulation still largely reversed the inhibitory effect of SLN on Ca^{2+} dynamics in transgenic mice expressing SLN but lacking PLB points to a direct effect on SLN [64].

How the inhibition of the SERCA pump by SLN(/PLB) might be regulated in vivo remains unknown. Compared to PLB, the cytosolic regulatory domain of SLN is much smaller, with Ser4 and Thr5 (only Thr5 in human SLN) as putative phosphorylation sites [64,67]. Attempts to phosphorylate SLN by PKA, CaMKII or endogenous kinases in vitro were unsuccessful and mutation of these sites had little or no effect on the regulatory behavior of SLN on SERCA activity in HEK-293 cells [67]. Nevertheless, serine/threonine 16 kinase could bind to and reverse the inhibitory effect of SLN on SERCA1a activity, an effect which is dependent on Thr5 [64]. Therefore, these data suggest that β-adrenergic stimulation of the heart might directly stimulate phosphorylation of SLN involving serine/threonine 16 kinase. The physiological role of serine/threonine 16 kinase in the regulation of SLN remains to be addressed. But it is clear that many questions on the role of this relatively novel SERCA2a regulator in the heart still need to be answered.

4. A reduced Ca^{2+} affinity of SERCA2a in heart failure

4.1. SERCA2a activity is reduced in heart failure

End-stage heart failure is marked with depressed systolic performance and diastolic dysfunction. The impaired contractility of the failing heart is at least partially reflected by a decrease in the intracellular Ca^{2+} transient and a diminished SR Ca^{2+} load. Diastolic dysfunction is associated with a prolonged duration of the Ca^{2+} transient and elevated resting cytosolic Ca^{2+} concentration [68–71]. Reduced Ca^{2+} transport by SERCA2 likely contributes to the altered Ca^{2+} handling. In human heart failure, most findings now indicate that the levels of SERCA2a protein often decrease [70,72–77].

Although reduced SERCA2a expression seems to be a hallmark of heart failure, a direct correlation between a reduced SERCA2a activity and the development of heart failure does not always hold. A number of loss-of-function mutations in the human SERCA2 gene (ATP2A2) result in Darier's disease, an autosomal dominantly inherited skin disorder [11,78,79]. Contrary to expectation, adult Darier disease patients display normal cardiac performance and do not develop hypertrophic cardiomyopathy or heart failure [80,81]. Neither do mice lacking one functional Atp2a2 allele ($SERCA2^{+/-}$) develop cardiac disease, although the SERCA2a content and its maximal pumping rate were reduced by ~35% in the heart [82]. Interestingly, the apparent Ca^{2+} affinity of the pump remained unaltered in the heterozygote mice, which can be explained by the spontaneous reduction in the inhibitory properties of PLB (reduced expression, increased phosphorylation). This compensation might, among others (like the increased NCX activity), help to prevent hypertrophic remodelling [83].

Besides the reduced expression of SERCA2a, most studies indicate that the levels of PLB protein remain unchanged in heart failure patients [72–74]. The resulting increase in the PLB to SERCA2a ratio would thus lead to a lower Ca^{2+} affinity of the pump, which would add up with the lower pump level in causing a prolonged cardiomyocyte relaxation time. In some patients a reduced phosphorylation status of PLB at Ser16 and Thr17 was also observed, further enhancing the inhibition by PLB [77,84]. The reduced phosphorylation status of PLB may partially be explained by the downregulation of β-adrenergic receptors in heart failure patients leading to a reduced activity of PKA. It should be noted that impaired PKA activity may also indirectly lower PLB phosphorylation via inactivation of inhibitor-1 (through reduced phosphorylation at Thr35). This, in turn, reinforces the activity of PP1, the main phosphatase of PLB [85–87]. Dephosphorylation, and thus inactivation of inhibitor-1 may

further occur upon calcineurin activation [88], a Ca^{2+}-calmodulin-dependent phosphatase which plays a pivotal role in the hypertrophic response [89]. In addition, the Ca^{2+}-dependent protein kinase Cα may contribute to enhanced PP1 activity. PKCα has the ability to phosphorylate inhibitor-1 at another site (Ser67) than PKA, thereby depressing inhibitor-1 activity and reinforcing PP1 [90].

The effects of a reduced SR Ca^{2+} uptake on the SR Ca^{2+} content in heart failure would be exacerbated by a leaky Ca^{2+} release channel in the SR (hyperphosphorylation of RyR2 would increase its open probability) [91] and an increase in NCX activity in the plasma membrane [71]. Hyperphosphorylation of RyR2 contrasts strikingly with the reduced phosphorylation of PLB, but may depend on a local rise in cAMP levels, associated with a reduced activity of the phosphodiesterase 4D (PDE4D), which is anchored to RyR2 [92]. These observations underscore that local changes in the activity of kinases and phosphatases play an important role in the altered Ca^{2+} homeostasis in heart failure.

4.2. Detrimental effects of a low Ca^{2+} affinity: studies in humans

Studies in transgenic mice overexpressing normal PLB [37] or gain-of-function PLB mutants [39,40] suggested a causal relationship between a chronically reduced Ca^{2+} affinity of the Ca^{2+} pump, defective SR Ca^{2+} cycling and cardiac remodeling leading to heart failure. This hypothesis was also tested in humans by searching for gain-of-function mutations in the PLB gene which could be associated with familial dilated cardiomyopathy (Fig. 1B).

Two such types of PLB mutations have so far been described: the Arg9Cys and Arg14Del mutants were linked to familial dilated cardiomyopathy and both displayed a dominant inheritance pattern [93,94]. The clinical findings of these mutations were supported by studies in transgenic mouse models of the mutants. Cardiac overexpression of the Arg9Cys mutant caused a chronically reduced phosphorylation status of PLB, lowering SERCA2a's Ca^{2+} affinity, cardiomyocyte Ca^{2+} handling and cardiac function. The lower degree of PLB phosphorylation is due to an impaired PKA effect. The Arg9Cys PLB mutant displays an enhanced affinity for PKA resulting in a stabilized PLB–PKA complex. The trapped PKA fails to phosphorylate not only the mutated PLB but also the wild-type PLB molecules, explaining the dominant effect of the mutation [93]. The strong super-inhibitory effect of the Arg14Del mutant on the Ca^{2+} affinity of the SR Ca^{2+} ATPase is likely related to the altered structure of the mutant. Increased pentameric instability was observed and β-stimulation failed to completely reverse the inhibitory properties [94].

Another mutation in the upstream non-coding region of the PLB gene (A to G at −77 bp) was found in one patient with cardiac hypertrophy. This mutation increased PLB promoter activity by 1.5-fold in neonatal rat myocytes, suggesting that it may lead to depressed SR Ca^{2+} cycling and cardiac hypertrophy in vivo [95].

In conclusion, in line with observations in mice, the reported long-term effects of increased PLB inhibition on the Ca^{2+} transient in human ventricular cardiomyocytes appear to be sufficient to induce cardiac hypertrophy. Dilated cardiomyopathy in humans can develop when endogenous β-agonists no longer can reverse inhibition of SERCA2a by PLB [93,94].

4.3. Therapeutic approaches aimed to improve the activity of SERCA

Given the central role of reduced SR Ca^{2+} uptake, targeting the SERCA2a–PLB interplay may have an important therapeutic potential. Hence, strategies were explored aimed to increase the expression of the SR Ca^{2+} ATPase or to attenuate its inhibition by PLB [96].

4.3.1. Increasing the expression of SERCA

Initial studies provided the proof of concept that increasing the expression and activity of the SR Ca^{2+} pump enhanced cardiomyocyte contractility [97,98]. In addition, cardiac-specific transgenic overexpression of SERCA2a or of the skeletal-muscle-specific SERCA1a isoform was associated with increases in SR Ca^{2+} uptake, content and release, improving cardiac relaxation and contractility [99–103]. Interestingly, SERCA overexpression enhanced the maximal SR Ca^{2+} uptake activity in the heart without changing the apparent affinity for Ca^{2+} [100,101]. PLB expression remained unaffected, suggesting that the inhibitory properties of PLB should be stronger to keep the Ca^{2+} affinity of the SERCA2 uptake unchanged (e.g. reduced PLB phosphorylation or an increased number of PLB monomers).

The group of Hajjar further explored whether gene-transfer of the SR Ca^{2+} ATPase would improve contractility in diseased cardiomyocytes. SERCA2a gene delivery restored the perturbed Ca^{2+} handling and contractility in human failing cardiomyocytes [104]. In vivo adenoviral gene transfer of SERCA2a in aortic-banded rats also improved SR Ca^{2+} uptake and cardiac function in transition to heart failure [105,106]. Systolic and diastolic parameters were restored to normal levels with beneficial effects on survival, and without compromising cardiac energetics [106]. Finally, depressed Ca^{2+} uptake and cardiac relaxation in rats with diabetic cardiomyopathy were also partially restored by transgenic overexpression of SERCA2a [107].

In general, gene-transfer of SERCA2 looks promising to treat heart failure. However, increasing the number of Ca^{2+} pumps is not without danger. First, transgenic SERCA2a overexpression increased the risk of acute arrhythmias and sudden death in rats challenged with myocardial infarction [108]. The associated increase in SR Ca^{2+} content might promote SR Ca^{2+} leakage from the SR and trigger delayed afterdepolarizations [108]. Second, excessive overexpression of SR Ca^{2+} pumps fails to improve cardiomyocyte function and decreases myocyte shortening. In these conditions, the excessive Ca^{2+} uptake activity might result in a maximal SR Ca^{2+} load so that more SERCA cannot further increase SR Ca^{2+} content. The high number of Ca^{2+} pumps might then compete

for Ca^{2+} binding to troponin C and directly interfere with contraction. Together, these observations warn to carefully control the transfection efficiency of SERCA [109].

4.3.2. Enhancing the Ca^{2+} affinity of the pump

Interference with PLB function could be useful to enhance the apparent affinity of the pump for Ca^{2+} and improve cardiomyocyte function. Attenuation of PLB activity can be achieved in several ways: knock-out of the PLB gene [25], expression of antisense RNA of PLB [110], gene-delivery of dominant-negative PLB mutants [111], gene-delivery of a SERCA2 pump which is insensitive to PLB inhibition [112] and gene-transfer of a PLB-targeted antibody [113].

Attenuation of PLB activity increased the rate of the decay of the Ca^{2+} transient in isolated normal cardiomyocytes [5,110,114,115]. More importantly, interference with PLB inhibition restored the contractile parameters in isolated failing cardiomyocytes [111,114–116] and could reverse cardiomyocyte hypertrophy [114]. The beneficial effects of these interventions were further confirmed in vivo in animal models of hypertrophy and heart failure. Genetic ablation of PLB prevented the onset of heart failure in $MLP^{-/-}$ mice [117] and reversed depressed function and hypertrophic growth in cardiomyocytes of calsequestrin-overexpressing hearts [118]. Cardiac hypertrophy was also attenuated in transgenic mice with cardiac-specific overexpression of a SERCA2a mutant deficient in the binding of PLB [112]. In addition, cardiac-specific adenoviral gene-delivery of a pseudophosphorylated PLB mutant (Ser16Glu) in hamsters or rat attenuated the progression of heart failure, showing that PLB inhibition also proved therapeutically efficacious after the onset of cardiomyopathy [119,120]. These studies support that targeting of PLB activity may be therapeutically beneficial. Future studies are needed to translate these findings into larger animal models.

5. A high Ca^{2+} affinity of SERCA and hypertrophic cardiomyopathy

The notion that a reduced SR Ca^{2+} load is at the basis of the reduced cardiac contractility in the failing heart has become widely accepted. However, as is often the case in biology, the story is more complex than initially thought. More recent findings suggest that cardiac hypertrophy and heart failure may be induced by chronic activation of the SR Ca^{2+} pump. In addition, some heart failure models exhibit improved rather than impaired baseline SR Ca^{2+} handling or are refractory to enhance SR Ca^{2+} uptake.

5.1. Not all models of cardiac hypertrophy benefit from targeting PLB

The studies discussed in Section 4 paint the uniform picture that augmentation of cardiomyocyte contractility through intervention with the SERCA2–PLB interaction is of significant benefit to the failing myocardium. However, in several other mouse models of hypertrophic cardiomyopathy and heart failure PLB gene disruption provided no benefit [121–123]. Importantly, PLB ablation in at least some of these models could supernormalize cardiomyocyte Ca^{2+} cycling and contractility, but a concomitant rescue in whole-organ performance or hypertrophy was not observed [122,123]. In fact, the persistent hypertrophy, together with altered chamber geometry and defective intercellular communication, may prevent that improved cardiomyocyte function is translated to the whole heart [123].

Cardiac hypertrophy and heart failure are heterogeneous disorders with diverse etiologies. Defects in Ca^{2+} handling may therefore not represent a universal pathway in heart failure, which points to the requirement of alternative therapeutic interventions.

5.2. Detrimental effects of an excessive Ca^{2+} affinity of SERCA2

5.2.1. Studies in mice

Given the beneficial effects of an increased Ca^{2+} affinity of the SR Ca^{2+} ATPase on cardiac growth and function, it is rather surprising that in the heart a SERCA2 isoform is expressed with a lower affinity for Ca^{2+} (SERCA2a) than in most other cell types (SERCA2b). The expression of SERCA2a is nevertheless essential for normal cardiac development and function [12]. This is clearly shown by studies on $SERCA2^{b/b}$ mice in which the SERCA2a isoform is replaced by the higher Ca^{2+}-affinity variant SERCA2b. As explained before, the primary transcripts of the wild-type Atp2a2 allele can, depending on the cell-type and differentiation status, be alternatively processed to SERCA2b or SERCA2a. The full substitution of the SERCA2a isoform by SERCA2b could therefore be obtained by preventing the alternative splicing of the SERCA2a pre-mRNA through modification of the Atp2a2 gene [12]. The $^{b/b}$ superscript indicates that both Atp2a2 alleles lack some gene sequences necessary to allow SERCA2a specific splicing and hence can only express the SERCA2b protein [12].

$SERCA2^{b/b}$ mice displayed a higher incidence of embryonic and neonatal mortality, associated with structural cardiac malformations (early phenotype). Adult $SERCA2^{b/b}$ animals develop concentric left ventricular cardiac hypertrophy with moderate cardiac dysfunction. [12]. However, the interpretation of the $SERCA2^{b/b}$ phenotype was complicated by its apparently compensatory expression of two-fold lower SERCA2 levels compared to the SERCA2a protein levels in wild-type hearts and by the stronger inhibitory properties of PLB (i.e. increased PLB expression, reduced PLB phosphorylation) [12,13,124]. Importantly, PLB modulates the affinity of SERCA2a and SERCA2b to the same extent [9]. Thus, the increased PLB/SERCA2 ratio in $SERCA2^{b/b}$ mice could in principle contribute to the impaired cardiac phenotype. Still, in contrast to other models of increased PLB inhibition [37], the affinity-modulator PLB plays a protective role in the heart of $SERCA2^{b/b}$ mice by partially reducing the higher affinity of SERCA2b [13]. Ablation of PLB in the SERCA2b background further increased the Ca^{2+} affinity of the pump, but with devastating effects on cardiac function and survival (Fig. 1A). The uncompensated higher Ca^{2+} affinity of SERCA2 in the latter mice aggravated

cardiac hypertrophy provoking diastolic dysfunction and filling restrictions. Moreover, PLB ablation severely reduced the cardiac reserve which is associated with stress-induced acute heart failure and death in these mice [13].

The question remains whether the lower SERCA2 levels in these SERCA2b mice would contribute to the severe cardiac phenotype. Interestingly, SERCA2b activity only becomes limiting at high Ca^{2+} loads [124]. Moreover, ablation of PLB in the SERCA2b background improved SR Ca^{2+} handling and relaxation of the cardiomyocytes compared to wild-type, despite a 3-fold reduction in the number of Ca^{2+} pumps [13]. Together, these observations indicate that due to the enhanced Ca^{2+} affinity of SERCA2b SR Ca^{2+} uptake activity is not limited. The reduced SERCA2 content in $SERCA2^{b/b}$ mice may rather serve the same purpose as the enhanced PLB inhibition, i.e. restrain the activity of the pump in the submicromolar Ca^{2+} concentration range in the cardiomyocyte [13].

In conclusion, protective mechanisms can be recruited that limit the Ca^{2+} affinity of the SERCA2 pump. These compensations take place even if, as a result of the lower SERCA2 levels, it entails a reduced maximal pumping rate at high cytosolic Ca^{2+} levels. Preventing excessive cytosolic Ca^{2+} removal by SERCA2b in the low Ca^{2+} concentration range seems more crucial to avoid excessive cardiac growth/hypertrophy than maintaining a normal maximal pumping capacity [13].

5.2.2. Studies in humans

Interestingly, the findings in SERCA2b mice which in addition also lack PLB, mirror the cardiac consequences of an early-stop mutation in the PLB gene (Leu-39-stop) in human patients with familial dilated cardiomyopathy [125]. In the heterozygous state, the accompanying loss of functional PLB in humans is associated with cardiac hypertrophy, but normal contractile performance. Moreover, individuals homozygous for this mutation develop dilated cardiomyopathy at a young age. The apparent pathological effects caused by the absence of PLB in human hearts contrast strikingly with the benefits observed upon the loss of PLB in mice [125]. It should be taken into account that due to the human-specific substitution in PLB of Asn27 to Lys, PLB exhibits stronger inhibitory properties in human than in other species [126]. This more prominent inhibition by PLB may explain the different impact of PLB deficiency on the heart in man versus mouse. Other fundamental differences between man and mouse in the relative contribution of Ca^{2+} cycled via the SR versus Ca^{2+} cycling through the extracellular medium may also contribute to the species-dependent effect of PLB ablation [125]. Importantly, the studies of SERCA2b mice demonstrate that PLB deficiency in mice can be as devastating as in humans, but only in the context of a pre-established higher Ca^{2+} affinity of the SR Ca^{2+} -ATPase [13].

In conclusion, previous reports highlighted that a chronically reduced Ca^{2+} affinity of the pump impairs cardiac function and may lead to cardiac remodeling (Fig. 1B, D). In contrast, SR Ca^{2+} pumps with a too high Ca^{2+} affinity may have equally detrimental effects (Fig. 1A). The Ca^{2+} affinity of the pump should therefore be tightly controlled and kept in a narrow physiological window (Fig 1).

5.3. High Ca^{2+} affinity, a reduced reserve and heart failure

One widely used mouse model of dilated hypertrophic cardiomyopathy harbors a deficiency in a cytoskeletal protein, the muscle-specific LIM protein (MLP) [127]. At odds with reports on reduced PLB phosphorylation in human heart failure, this model of heart failure is characterized by hyperphosphorylation of PLB and a concomitant higher affinity of the pump for Ca^{2+}, resulting in faster cytosolic Ca^{2+} removal in the cardiomyocyte [128,129]. Hyperphosphorylation of PLB was also suggested to occur in two rat models of cardiac hypertrophy, but these studies did not report a detailed analysis of Ca^{2+} handling [130,131]. Furthermore, an increased phosphorylation status of PLB was observed in a rabbit model of heart failure, although at the same time overall SERCA activity was reduced [132].

Importantly, the enhanced basal SR Ca^{2+}-uptake activity in the $MLP^{-/-}$ cardiomyocytes could not be further elevated at higher stimulation frequencies indicating a reduced frequency-dependent increase of Ca^{2+}-release. This diminished reserve might be a hallmark for the development of heart failure in the $MLP^{-/-}$ mice [128]. Interestingly, similar to $MLP^{-/-}$ mice, the cardiomyocytes of the $SERCA2^{b/b}$ mice lacking PLB also displayed faster SR Ca^{2+} removal and high SR Ca^{2+} content at baseline [13]. Preliminary results support the view that the SR Ca^{2+} content could not be further enhanced by high frequency stimulation, similar to the frequency response observed in the $MLP^{-/-}$ cardiomyocytes. This was also associated with a reduced orthosympathic cardiac reserve in vivo [13] which is likely responsible for the stress intolerance and high mortality of the SERCA2b mice. In humans, a reduced cardiac reserve has been associated with increased morbidity and mortality [133], suggesting that the lack of cardiac reserve in the SERCA2b model might be a risk factor for the acute heart failure in stress conditions [13].

Together, the findings in two non-related mouse models suggest that a reduced reserve for frequency-dependent increase of Ca^{2+} might represent a novel paradigm for altered Ca^{2+} handling in heart failure, underscoring the complexity of Ca^{2+} handling in the normal and failing heart.

5.4. Concluding remarks

In conclusion, the heart exploits several tools which tightly control and adjust the affinity of the pump for Ca^{2+} within a narrow, physiological range. An imbalanced activity of the SR Ca^{2+} pump by profound and long-term changes in its Ca^{2+} affinity has a severe impact on cardiac growth and function. Both a chronically reduced and increased Ca^{2+} affinity can evoke hypertrophic cardiomyopathy in mice and humans. Heart failure is marked by a reduced affinity of SERCA2a for Ca^{2+} which partially underlies the diastolic dysfunction. However, this may not represent a universal hallmark, since recent findings demonstrated that cardiac hypertrophy and heart failure can be associated with a higher Ca^{2+} affinity of the pump and consequently with improved cardiomyocyte function.

In contrast to the well-characterized role of Ca^{2+} in cardiac contraction, only little is known about how Ca^{2+} may drive the vast number of Ca^{2+}-dependent growth signals in the heart. Some models propose that IP3-regulated Ca^{2+} pools could more specifically target the growth/survival branch [134]. Moreover, it is currently unclear how changes in the activity of SERCA2a affect the Ca^{2+} movements that drive cardiac contractility separately from those controlling growth (hypertrophy) and cell survival. Many studies described the beneficial effects of improving SERCA2 activity on cardiac contractility and cardiac remodeling. However, in certain settings attenuation of cardiac hypertrophy and better cardiac function appear to be incompatible phenomena. Together, these observations point to the complex interactions between cardiac contractility and signaling, which are both under the direct control of Ca^{2+}.

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