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Role of PP1 in the regulation of Ca cycling in cardiac physiology and pathophysiology

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1. ABSTRACT

Type 1 protein phosphatase (PP1) is a critical regulator of several cellular processes. In the heart, it mediates restoration of contractility to basal levels by dephosphorylating key phospho-proteins, after beta-adrenergic stimulation. PP1 is a holoenzyme consisting of its catalytic and regulatory subunits, which anchor the catalytic subunit to desired subcellular locations, define substrate specificity and modulate catalytic activity. At the level of the cardiac sarcoplasmic reticulum (SR), PP1 is regulated by Inhibitor-1 (I-1) and Inhibitor-2 (I-2), which modulate its activity, and the striated muscle-specific glycogen-targeting subunit, G_M/R_{GL}, which targets it to the SR vicinity. PP1 regulation is highly important in maintaining cardiac function under physiological conditions. In fact, aberrant Ca handling and depressed contractility in heart failure have been, at least partly, attributed to increases in PP1 activity, mediated by impaired regulation via its inhibitors. Importantly, increases in the level and activity of I-1 and I-2 in animal models have been successful in ameliorating dysfunction and remodeling in heart failure, suggesting that PP1 inhibition may be a plausible therapeutic strategy in heart failure.

2. INTRODUCTION

Despite the recently reported progress, cardiovascular disease remains the leading cause of morbidity and mortality in the United States, with heart failure representing the fastest growing subcategory over the past ten years (1). In fact, 5.3 million Americans were afflicted with heart failure in 2005 (1). According to the 44-year-follow-up of the Framingham Heart Study, 80% of men and 70% of women under the age of 65 suffering from heart failure will die within eight years (2). These statistics are a clear indication of ineffective therapeutic agents. A common clinical characteristic of heart failure is disturbed Ca homeostasis (3) and depressed Ca cycling (4, 5). As such, therapeutic interventions have been targeted at rectifying this impaired Ca handling. The sarcoplasmic reticulum (SR) is the major organelle responsible for proper Ca cycling in the cardiac cell. In general, it regulates Ca dynamics on a beat-to-beat basis through Ca release during contraction and Ca sequestration during relaxation. Importantly, Ca homeostasis is also subject to regulation by the phosphorylation status of key proteins, which is very tightly regulated by the balance of kinases and phosphatases in the cardiomyocyte. This becomes of

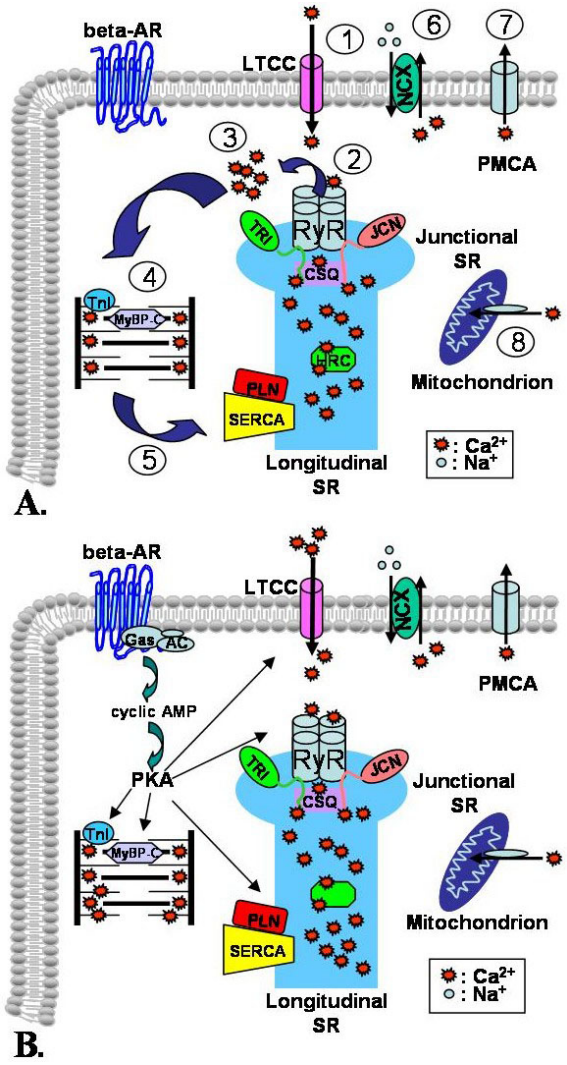

Figure 1. Excitation-contraction coupling in the cardiomyocyte. A. Upon a depolarizing signal, extracellular Ca enters the cell via the LTCC (1), which binds to the RyR (2) and induces release of a greater amount of Ca (3), which initiates contraction at the myofilaments (4). Removal of Ca is primarily facilitated via SERCA2a (5) and to a lesser extent by the NCX (6), the PMCA (7) and the Ca uniporter on the mitochondrial membrane (8). B. Upon beta-adrenergic stimulation, AC is activated, which leads to production of cAMP and PKA activation. PKA then phosphorylates the RyR, TnI, PLN and MyBP-C, which augment contractility. LTCC: L-type Ca channel; RyR: ryanodine receptor; TRI: triadin; JUN: junctin; CSQ: calsequestrin; HRC: histidine-rich Ca binding protein; TnI: troponin I; MyBP-C: myosin binding protein C; SERCA2a: SR/ER Ca-ATPase; PLN: phospholamban; SR: sarcoplasmic reticulum; NXC: sodium-calcium exchanger; PMCA: plasma-membrane Ca-ATPase.

particular importance in the failing heart, where abnormalities in the activity of enzymes in the kinase and phosphatase families disturb this fine equilibrium of phosphorylation.

In this review, we summarize the current views on SR Ca-cycling and provide evidence that the type 1 protein phosphatase (PP1) is an important negative regulator of Ca cycling and contractility in the heart. Furthermore, we discuss the regulation of this enzyme in terms of localization and activity modulation at the level of the SR. Subsequently, we outline the potential contribution of disrupted PP1 regulation to the impaired Ca cycling and dysfunction observed in the diseased myocardium. Finally, we discuss the potential therapeutic benefits of inhibition of SR-coupled PP1 in the failing heart as a novel and perhaps efficacious therapeutic strategy.

3. SR CALCIUM CYCLING AND EXCITATION-CONTRACTION COUPLING IN THE CARDIOMYOCYTE

Increases in intracellular Ca are responsible for initiating cell contraction during systole, while the decay of cytoplasmic Ca causes relengthening in diastole. The cellular events mediating these effects are illustrated in Figure 1A. In response to a depolarizing signal during an action potential, a relatively small amount of Ca enters the cell via the voltage-dependent L-type Ca channel (LTCC). The LTCC are localized in specialized invaginations of the sarcolemma, named transverse tubules (T-tubules), which are in close proximity to the SR. As such, the Ca entering the cell via these channels causes localized increases in the cleft between the sarcolemma and the SR, and subsequently activates the release of a larger amount of Ca from the Ryanodine Receptor (RyR) on the SR membrane. This process is termed Calcium-Induced-Calcium-Release (CICR) (6). The Ca diffuses to the myofilaments where it initiates contraction. Collectively, this process facilitates the transduction of the electrical stimulation to a mechanical result and is known as excitation-contraction coupling. To commence relaxation, the Ca is re-sequestered in the SR by the SR Ca-ATPase pump (SERCA2a) located on the SR membrane, which is regulated by the small phospho-protein, phospholamban (PLN), and to a lesser extent by the Na-Ca exchanger (NCX), the sarcolemma Ca-ATPase and the Ca uniporter found on the mitochondrial membrane.

3.1. Beta-adrenergic stimulation and Ca-cycling

The rate and amplitude of Ca cycling and therefore contractility is enhanced in response to neurohormonal signals in the body under flight-or-fight conditions, in order to accommodate increased bodily demands. The cellular events, which account for these increases are illustrated in Figure 1B. Specifically upon binding of a catecholamine (epinephrine or norepinephrine) to the beta-adrenergic receptors, adenylate cyclase becomes activated (7). This enzyme catalyzes the conversion of ATP to 3'-5'-cyclic-AMP (cAMP), which subsequently binds to the regulatory subunit of protein kinase A (PKA) and allows activation of its catalytic subunit. PKA then phosphorylates several proteins, including the LTCC, the RyR, Troponin I (TnI), Myosin Binding Protein C (MyBP-C) and PLN, which lead to enhanced contractile function. The process of phosphorylation is counter-acted by dephosphorylation, carried out by phosphatases, which

facilitate restoration of contractility to basal levels. The type 1 protein phosphatase PP1, and the type 2 phosphatases, PP2A and PP2B, constitute the majority of phosphatase activity in the heart. Amongst these enzymes, PP1 is of particular importance as it has been implicated as an important negative regulator of cardiac function. In fact, perturbations in PP1 levels or activity have been suggested to contribute to the pathogenesis of heart disease.

4. TYPE 1 PROTEIN PHOSPHATASE (PP1)

Serine/Threonine Protein Phosphatases (PP) are classified into two broad categories, namely the Phospho-Protein Phosphatases (PPP) and the Magnesium-Activated Protein Phosphatases (PPM). These enzymes are further categorized according to their biochemical properties (8). PP1 is classified as a type 1 phosphatase based on its preference for the beta subunit of phosphorylase kinase and the fact that it can be inhibited by okadaic acid at relatively high concentrations (20 nM) and the endogenous inhibitory phospho-proteins, inhibitor-1 (I-1) and inhibitor-2 (I-2). PP1 is a holoenzyme composed of the catalytic subunit (PP1c), which possesses its phosphatase activity, complexed with as many as 100 established or putative regulatory proteins (9, 10). These associated proteins either function as targeting subunits, which guide PP1 to its substrate and to the desired subcellular location or regulate its catalytic activity (11). Importantly, it has also been suggested that these targeting subunits stabilize PP1 and prevent its degradation (12). The regulation of PP1 by its auxiliary proteins at the level of the SR is described in Section 5.

4.1. The catalytic subunit

Mammalian species have four known PP1c isoforms, which are encoded by three independent genes, namely: PP1c-alpha, PP1c-gamma, which gives rise to two splice variants (PP1c-gamma1 and PP1c-gamma2) and PP1c-beta/delta (13-15). These homologues are ubiquitously expressed, and are ~80% identical in amino acid sequence (9). Interestingly, the catalytic subunit of PP1 is one of the most highly conserved proteins, with its catalytic core being the most conserved domain. Importantly, PP1c also shows a high degree of similarity throughout the eukaryotic phylogenetic tree both structurally and functionally. In fact, the PP1c in the early eukaryote *Giardia lamblia* is 72% identical to the isoform in mammalian species (16).

Our understanding of the mechanism by which PP1c catalyzes the removal of phosphate from its substrate targets as well as its interactions with its regulators has been advanced by extensive crystallographic and biochemical studies. The first study to give such mechanistic insights resolved the crystal structure of PP1c complexed with the inhibitory toxin microcystin (17). This seminal study revealed that two bi-metallic ions are positioned in the central vicinity of an alpha- and beta-helical sandwich, which are thought to assist in catalysis. Importantly, these experiments also identified three surface grooves projected from the central scaffold as potential binding sites for regulatory molecules and identified the hydrophobic groove in the beta12-beta13 loop, which is in close proximity to the active site, as the site of interaction for inhibitory toxins. In fact, subsequent studies also implicated the beta12-beta13 loop as the binding site for other endogenous and exogenous inhibitors. Specifically, it has been shown that alterations in the beta12-beta13 loop attenuated inhibition by the endogenous inhibitors, I-1 and I-2, toxins and the commonly used protein phosphatase inhibitors calyculin A and okadaic acid (18-20). In a more recent study, Xie *et al* (21) showed that mutagenesis of individual amino acids in this region severely impaired PP1c activity, suggesting that this loop may also be involved in activity regulation. Interestingly, this region is the least conserved region between PP1 and other enzymes in the PPP family. Substitutions of this part of the molecule with the respective sequence in PP2A (19, 20) or PP2B (21, 22) resulted in chimeric proteins, which displayed an attenuated ability to be inhibited by endogenous inhibitors and toxins, similar to the type 2 enzymes. Overall, these studies implicate the beta12-beta13 loop as an important regulatory site in PP1c and indicate that this region may be responsible for the unique properties of PP1c amongst the PPP family.

In addition to the identification of the beta12-beta13 loop as an important regulatory site in PP1c, several studies have also suggested that the RVXF motif in its binding partners is also important for their interaction (23-25). This RVXF motif is a short, well-conserved motif present in the majority of PP1c-binding proteins, which facilitates their interaction with a hydrophobic groove located at a distance from the catalytic site on the surface of PP1c (23). Subsequent mapping studies showed that the binding between PP1c and other regulatory subunits is mediated by either the RVXF motif or a degenerate variant of it conforming to $[RK]x_{0-1}[VI]\{P\}[FW]$, where x refers to any amino acid and {P} is any residue except proline (26-29). Perturbations in this motif, using site-directed mutagenesis, have shown that this domain is important for the binding of I-1. Specifically these studies have shown that the sensitivity of PP1c to this endogenous inhibitor was drastically altered, when these sites were mutated (25, 29). A more recent study, which solved the crystal structure of the PP1c/I-2 complex, also showed that the KSQKW amino acid sequence in I-2 binds to the same groove that the other binding proteins do in PP1c, suggesting that the sequence degeneracy at the RVXF site is greater than previously indicated (30).Overall, these studies have yielded great mechanistic insights into the regulation of PP1c and may facilitate the design of more specific and potent PP1c inhibitors, which may be used therapeutically (see Section 7).

4.2. The role of PP1 in cardiac contractility

The potential significance of PP1 as an important regulator of cardiac function was realized when PP1 was identified as the major phosphatase responsible for dephosphorylating PLN in SR-enriched preparations in two independent studies (31, 32). Based on the major role of this small phospho-protein in cardiac contractility, it was suggested that PP1 may be an important regulator in the heart. As mentioned previously, PLN regulates the activity

of the Ca-ATPase pump on the SR. Specifically, it inhibits the pump's activity in its dephosphorylated state. Phosphorylation of PLN, by the beta-adrenergic cascade, at its PKA- (Ser16) and its CAMKII- (Thr17) dependent sites, relieves the inhibition and allows for increased Ca influx into the SR. The physiological role of PLN was elucidated through the generation and characterization of genetically altered models. Specifically, mice lacking PLN exhibited enhanced Ca-uptake and augmented contractility (33), whereas transgenic mice overexpressing PLN displayed decreased Ca influx into the SR and depressed function (34). These studies and many others that followed (reviewed in 35-38) established PLN as a key regulator of both basal contractility and the heart's beta-agonist responses. Therefore, the identification of PP1 as a putative regulator of the phosphorylation status of PLN was of great importance.

The first evidence that demonstrated the potential physiological relevance of PP1 came from use of non-specific inhibitors, which inhibit both PP1 and PP2A at low concentrations. Specifically, two independent studies showed that okadaic acid enhanced contractility in guinea-pig isolated ventricular muscles (39, 40) by enhancing phosphorylation of PLN and TnI (39, 40). The positive ionotropic effects of PP1 inhibition were also noted using calycullin A or cantharidin in isolated guinea pig ventricular myocytes (41, 42). Importantly, these studies identified key regulatory phosphoproteins, which were associated with the enhanced function. These included PLN, TnI, myosin light chain 2 (MLC2), C-protein and the LTCC. Even though these studies provided the first evidence of PP1 physiological significance, they were limited by the promiscuous nature of the inhibitors. The generation and characterization of PP1c-alpha overexpressing mice provided the first direct evidence into the functional significance of this enzyme in the heart (43). These mice exhibited significant increases in PP1 activity (almost three-fold), depressed contractile function and diminished phosphorylation of PLN at Ser16. Important insights into the physiological role of this enzyme also came from genetic models, which either overexpress or lack the endogenous inhibitors, I-1 and I-2 (see Section 5). Briefly, mice lacking the endogenous inhibitor I-1 exhibited moderate increases in PP1 activity (23%), impaired contractility and depressed PLN phosphorylation at both sites (43). Conversely, overexpression of constitutively active forms of I-1 (44) or I-2 (45) yielded significantly enhanced function and increased PLN phosphorylation. Collectively, these data suggest that PP1 is an important regulator of cardiac function.

5. REGULATION OF PP1 IN THE SARCOPLASMIC RETICULUM

The tight regulation of protein phosphorylation and dephosphorylation in the cell is enacted by protein kinases and phosphatases. To that end, the human genome encodes ~500 protein kinases and only ~150 phosphatases, of which ~40 are Ser/Thr phosphatases. As such, in order to achieve the high degree of diversity and versatility exhibited by the vast number of kinases, phosphatases are complexed to various regulatory proteins. In fact, the PP1 catalytic subunit has been shown to exist as a holoenzyme complexed with as many as 100 regulators, which dictate its distinctive localization, substrate specificity and activity (9, 10). This section reviews the regulation of PP1 at the level of the SR.

5.1. Inhibitor-1

Inhibitor-1 (I-1) was the first recognized endogenous inhibitor of PP1 (46). Early studies revealed that upon phosphorylation at Thr35 by PKA, I-1 potently inhibits PP1 activity (IC_{50}: 1 nM) (46, 47). The significance of I-1 has been described extensively in neuronal tissue, where it was implicated as an important mediator of synaptic plasticity (48, 49). However, only in more recent years has the physiological role of I-1 in the heart been emerging. Initial studies showed that I-1 is present in the heart and that it is hormonally-regulated in guinea pig ventricles (50, 51) and rat heart slices (52). Gupta and colleagues later showed that I-1 is present in cardiomyocytes (53). Importantly, these studies indicated that I-1 can be phosphorylated upon treatment with isoproterenol (51-53) resulting concomitantly in decreased PP1 activity. These lines of evidence suggested that I-1 may be an important regulator of cardiac function. Indeed, the generation and characterization of a mouse deficient in I-1 gave further insights into its physiological role in the heart. This model was characterized by increased PP1 activity, depressed cardiac function, blunted beta-adrenergic response and reduced PLN phosphorylation (43). In subsequent experiments, El-Armouche *et al* showed that adenoviral delivery of I-1 in engineered heart tissue as well as in neonatal and adult rat cardiomyocytes enhanced contractility and increased PLN phosphorylation, upon stimulation with the beta-agonist, isoprenaline (54). A more recent study showed that expression of a truncated (AA: 1-65), constitutively active (T35D) form of I-1 (I-1c) in a transgenic mouse model, effectively decreased PP1 activity at the level of the SR and enhanced both basal contractility and the heart's beta-adrenergic response (44). Importantly, the authors showed that these effects were specifically attributed to enhanced PLN phosphorylation at both Ser16 and Thr17, while the phosphorylation levels of two other major regulators of contractility, namely the RyR and TnI, were unaffected. As mentioned previously, the role of the regulatory subunits is not only to dictate activity but also substrate specificity. Therefore, these results suggest that I-1 may specifically regulate the PP1/PLN complex in the SR. Collectively, these experiments suggested that I-1 may be acting as a molecular ionotrope by suppressing PP1 activity and allowing for unopposed increases in the phosphorylation of PLN, which amplifies the beta-agonist response. Interestingly, I-1 is itself dephosphorylated by PP2A and PP2B, which allows for restoration of function to basal levels by relieving PP1 inhibition (55), which suggests that the cross-talk between the cAMP and Ca signaling pathways may be partly mediated through I-1.

Even though the role of phosphorylation at Thr35 is now well-accepted, the role of other identified phosphorylation sites in I-1 is less clear and has been the

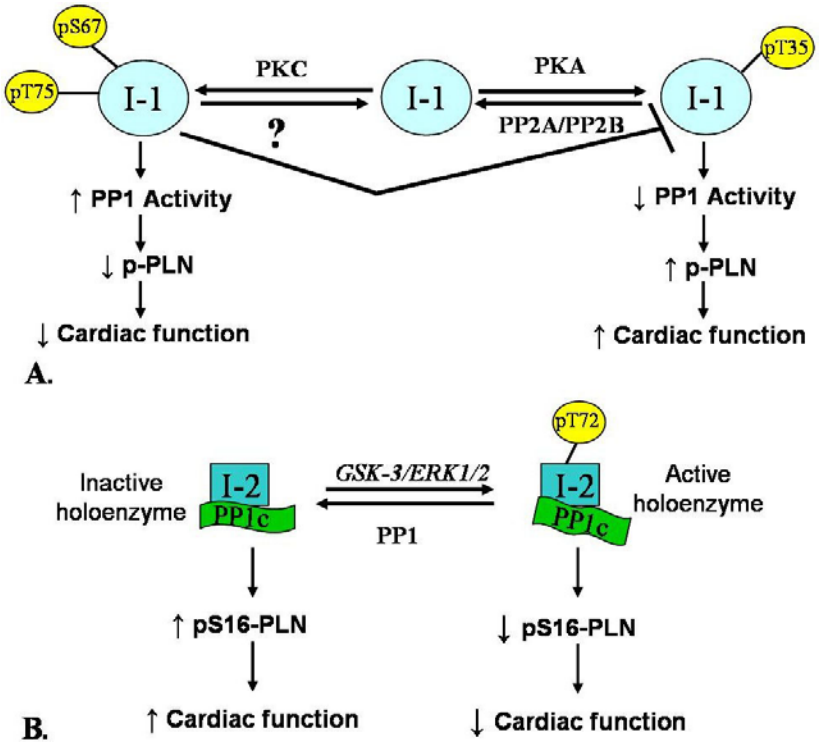

Figure 2. Proposed role of I-1 and I-2 in cardiac function. A. Phosphorylation of I-1 at Thr35, suppresses PP1 activity, which subsequently increases PLN phosphorylation and contractility. Phosphorylation at Ser67 and/or Thr75 depresses phosphorylation at Thr35, which increases PP1 activity and depresses PLN phosphorylation and contractility. B. I-2 forms a latent complex with PP1, which allows for unopposed increases in the phosphorylation of PLN specifically at its S16 site, which leads to augmented contractility. Phosphorylation of I-2 at Thr72 activates the PP1/I-2 holoenzyme, which dephosphorylates pS16-PLN and results in depressed cardiac function. Even though it is not currently known which kinases phosphorylate I-2 at Thr72 in the heart, it has been reported that I-2 is phosphorylated at this site by the ubiquitously expressed kinases GSK-3 and ERK1/2. It is therefore possible that these kinases may also phosphorylate I-2 in the heart. I-1: Inhibitor-1; PP1/2A/2B: protein phosphatase-1/2A/2B; PKA: protein kinase A; PKC: protein kinase C; PLN: phospholamban; I-2: Inhibitor-2; GSK-3: glycogen synthase kinase 3; ERK1/2: mitogen-activated protein kinase extracellular signal-related kinase 1/2; italics: putative kinases.

target of several studies. Aitken *et al* were the first to show that I-1 could be phosphorylated at another site, namely Ser67 in skeletal muscle and that phosphorylation at this site had no effect on PP1's catalytic activity *in vitro* (56). Conversely, Huang *et al* showed that phosphorylation at Ser67 makes I-1 a potent PP1 inhibitor, similar to phosphorylation at Thr35 (57). Bibb *et al* later showed that Ser67 could be phosphorylated *in vitro* by several kinases, namely cyclin-dependent kinase 1 (Cdk1), cyclin-dependent kinase 5 (Cdk5) and mitogen-activated protein kinase, while it was only phosphorylated by Cdk5 in striatal brain tissue *in vivo* (58). Similarly to the study by Aitken *et al*, these authors also noted no changes in PP1 activity by phosphorylation at Ser67. However, they did find that phosphorylation at Ser67 makes I-1 a poor substrate for phosphorylation at the Thr35 PKA site, suggesting a physiological role for phosphorylation of Ser67 *in vivo*. The same group later reported the identification of another phosphorylation site in the rat I-1 at Ser65 by PKC (59), which prevented efficient phosphorylation at Thr35, similarly to their reported results with Ser67. However, Ser65 is substituted by aspartic acid in humans, limiting the significance of this site in human physiology. The first evidence for the functional role of phosphorylation at Ser67 in the heart was shown in a study by Braz *et al*, which indicated that phosphorylation of I-1 at Ser67 by PKC-alpha was associated with increased PP1 activity and depressed contractility *in vivo* (60). Interestingly, an additional PKC-alpha phosphorylation site on human I-1, Thr75, was recently identified and this was associated with increased PP1 activity, depressed PLN phosphorylation and depressed contractile function in isolated myocytes (61). A subsequent study, which compared the Thr75 site to the Ser67 site, showed that phosphorylation at Ser67 and/or Thr75 can depress contractile function to a similar extent in isolated myocytes (62). Importantly, this study also showed that activation of the cAMP pathway was able to only partially reverse the observed depressed contractile parameters and that this may be related to the inability of Thr35 to become phosphorylated efficiently, when the PKC sites are phosphorylated, similarly to previous reports (58, 59). As such, I-1 appears to be an important mediator of the crosstalk between the PKA and the PKC pathways in the heart (Figure 2). Overall, these studies suggest that I-1 is a

more complex regulator than previously thought and may modulate PP1's activity according to cellular conditions.

5.2. Inhibitor-2

Inhibitor-2 (I-2), similarly to I-1, is a thermostable phospho-protein, which was initially isolated from skeletal muscle (46). I-2 forms a stable and latent complex with PP1c (63). Phosphorylation at Thr72 causes a conformational change in I-2, which reactivates the complex (63, 64). Even though the kinases, which phosphorylate I-2 at Thr72, have not been reported in the heart, it has been shown that I-2 is phosphorylated by the ubiquitously expressed kinases, glycogen synthase kinase 3 (GSK3) and mitogen-activated protein kinase extracellular signal-related kinase 1/2 (ERK1/2) *in vitro* or in intact cells (63-66). As such, it is possible that these kinases may also mediate phosphorylation of this site in the heart *in vivo*. Furthermore, I-2 also contains 3 casein kinase-2 phosphorylation sites, which do not in themselves alter its inhibitory activity, but rather enhance phosphorylation by GSK-3 at the Thr72 site (64). Notably, once the holoenzyme is activated, it is dephosphorylated by PP1 in an autocatalytic manner (Figure 2B). The functional significance of I-2 in the heart has only recently been explored. Kirchhefer *et al* (45) generated a mouse model expressing a truncated (AA: 1-140) and constitutively active form of I-2 (I-2*), which could not be inhibited by phosphorylation at Thr72 (67, 68). This study showed that expression of I-2* resulted in depressed PP1 activity, associated with enhanced contractile parameters and increased Ca transient kinetics. Interestingly, the authors demonstrated increased PLN phosphorylation at Ser16 but not at Thr17, suggesting that the PP1c/I-2* complex may preferentially dephosphorylate the PKA site in PLN. Furthermore, overexpression of I-2* was associated with increased PP1 protein levels, while mRNA levels were unchanged, which suggested that I-2 may be able to stabilize the PP1c protein. Indeed, previous reports support a chaperone-like function of I-2. Specifically, mammalian PP1c expressed in bacteria shows altered properties to native PP1c. However, binding of phosphorylated I-2 to PP1c transformed recombinant PP1c properties to those of native PP1c, suggesting that I-2 may be involved in mediating the correct folding of PP1c (69, 70). It is important to note however that the stabilization of PP1c by its regulatory proteins is not unique to I-2. Specifically, increased PP1 levels have also been observed in transgenic mice overexpressing the G_M/R_{GL} subunit in skeletal muscle (71). Overall, these experiments have begun to elucidate the role of I-2 in the heart and suggest that I-2 may regulate PP1 activity in the SR and specifically regulate the PP1/pS16-PNL complex (Figure 2B). Future studies may yield further insights into its role in the heart.

5.3. The G_M/R_{GL} targeting subunit

The study of PP1 in glycogen metabolism has been instrumental in unraveling important insights into the complex regulation of this enzyme. In fact, PP1 was initially identified as the enzyme responsible for dephosphorylating phosphorylase a, which catalyzes the conversion of glycogen to glucose-1-phosphate (72). The regulatory subunits mediating binding between PP1 and glycogen particles were identified in these initial seminal studies. The most well-known regulatory subunits in this family are G_M/R_{GL} and G_L, where G stands for glycogen-targeting subunit. However, it is now recognized that these subunits may promote binding between PP1c and other biological molecules. The subscript denotes the tissue that these G subunits are more abundant in: striated muscle (M) and liver (L), respectively (73, 74).The mammalian genome contains seven genes encoding these regulatory subunits, four of which have been studied at the protein level (16). In the heart, there exists some evidence that implicates a G subunit as a potential PP1 regulatory protein at the level of the SR. Indeed, Hubbard and colleagues showed that PP1c is targeted to the SR by a protein that is highly similar or identical to the G_M/R_{GL} subunit that directs PP1c to glycogen in skeletal muscle (75). In fact, early studies showed that the C-terminus of G_M/R_{GL} had a hydrophobic region, suggesting a potential association with a membrane compartment (73). Interestingly, a study demonstrated that the transmembrane domain of PLN interacts directly with the C-terminus of this subunit (76), raising the possibility that G_M/R_{GL} may also target PP1c to PLN, providing yet another layer of complexity and regulation. Interestingly, a truncation mutation in the region that binds to the SR in human G_M/R_{GL} has been shown to result in aberrant PP1 regulation, impaired glycogen synthase activity and diminished glycogen content in human carriers (77, 78), human muscle cells (79), and in a knock-in mouse model expressing this mutant (78). However, the effects of this mutant were not reported in the heart. Furthermore, it has been suggested that the $PP1/G_M/R_{GL}$ complex is also regulated by phosphorylation of the G_M/R_{GL} subunit at Ser48 and Ser67. Specifically, it has been reported that Ser48 phosphorylation increases the rate at which the $PP1/G_M/R_{GL}$ complex dephosphorylates its substrates (80), while phosphorylation at Ser67 disrupts the interaction between PP1c and the G_M/R_{GL} subunit, which could potentially result in inhibition of dephosphorylation of its protein-targets (81). However, the role of these sites in SR function in the heart is not currently clear. Interestingly, it has also been postulated that these subunits may be responsible for stabilizing the PP1 protein, similarly to I-2 (45). In fact, mice overexpressing (71) or lacking G_M/R_{GL} (82, 83) are characterized by increased and decreased PP1 levels, respectively. Overall, these data suggest that the G_M/R_{GL} subunit is an important modulator of PP1. Although some evidence exists that suggests that G_M/R_{GL} may affect PP1 at the level of the SR, more studies are needed to elucidate its physiological role in the heart.

6. PP1 IN CARDIAC PATHOLOGY

Heart failure is a chronic condition manifested in the inability of the heart to pump blood and perfuse bodily organs efficiently. The failing heart is characterized by ventricular dilation, circulatory congestion as well as systolic and diastolic dysfunction. At the cellular level, failing cardiomyocytes present with depressed contractility, decreased peak systolic Ca and increased diastolic Ca (3-4). These disturbances in Ca homeostasis are at least partly mediated at the level of the SR, where reduced Ca transport into the sarcoplasmic reticulum and depressed SR Ca load

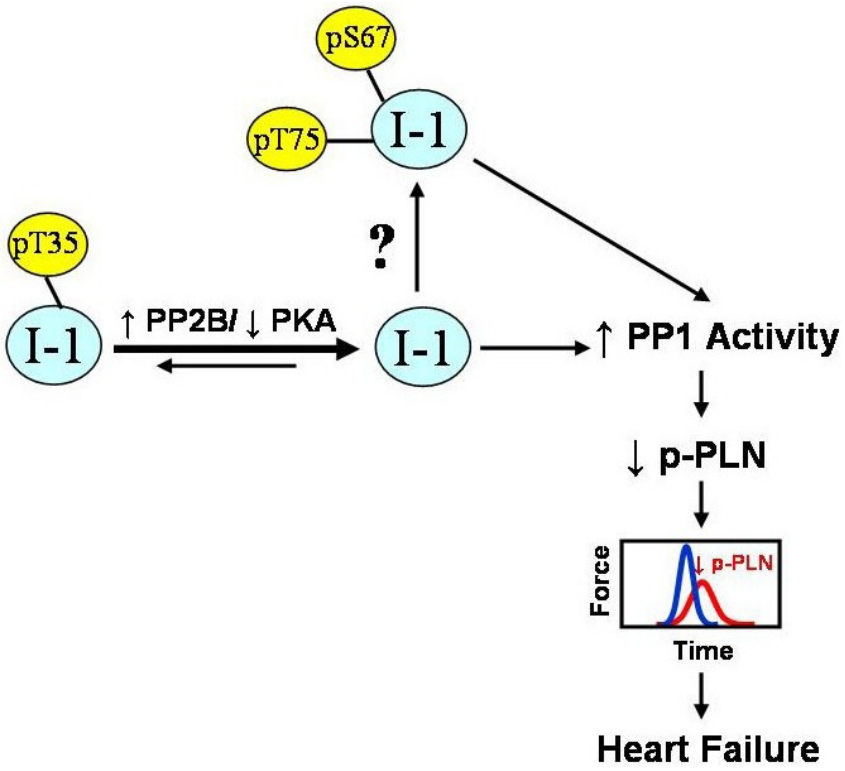

Figure 3. Impaired regulation of PP1 by I-1 in heart failure. The inhibitory effects of I-1 are depressed due to decreased phosphorylation at Thr35, which could be due to altered PKA and/or PP2B activity observed in the failing heart. Altered phosphorylation of this endogenous inhibitor ultimately leads to increased PP1 activity, depressed function and heart failure. Importantly, enhanced phosphorylation at Ser67 and/or Thr75 of I-1 may also contribute to enhanced PP1 activity. Future studies may examine their role in heart failure. I-1: Inhibitor-1; PP1/2B: protein phosphatase-1/2B; PKA: protein kinase A; PLN: phospholamban.

(84) have been reported. A major contributing factor to SR Ca cycling dysfunction is impaired regulation of the Ca-ATPase pump and its regulatory phospho-protein, PLN. Specifically, decreased SERCA2a activity evoked by either reduced protein levels or increased inhibition by PLN in its dephosphorylated state have been linked to SR dysfunction in the failing heart (85, 86). This diminished phosphorylation of PLN can be attributed to an attenuation of the beta-adrenergic cascade due to receptor desensitization, receptor downregulation and uncoupling, which occurs during disease progression (87, 88). Importantly, more recently it has been recognized that activation of phosphatases may play an important role in the diseased myocardium and may also contribute to the dephosphorylation of PLN. This section reviews the contribution of alterations in PP1 levels and activity to the etiopathogenesis of heart failure.

6.1. The role of PP1 in heart disease

Several lines of evidence have shown that PP1 activity is increased in both experimental models of heart failure and in human heart failure, which further exacerbates cardiac function by promoting dephosphorylation of its protein-targets. Neumann and colleagues were the first to demonstrate that membrane vesicles from hearts of patients with end-stage heart failure displayed significant increases in PP1 activity (89). Furthermore, these authors showed that PP1 mRNA levels were increased in these patients, providing a plausible mechanism for the observed increase in activity. Mishra *et al* subsequently showed that protein phosphatase activity was increased in the left ventricular myocardium of patients suffering from idiopathic dilated cardiomyopathy (90). Importantly, these authors correlated the enhanced phosphatase activity, attributed both to PP1 and PP2A, to depressed PLN phosphorylation at Ser16 and reduced Ca-uptake into the SR. Data from several animal models of heart failure later correlated increases in PP1 activity to diminished PLN phosphorylation. Two independent studies examined PP1 activity at different stages of heart disease, in a rat model of myocardial infarction. In particular, Huang *et al* found that PP1 activity was increased during the compensated phase of heart failure and this was associated with depressed PLN phosphorylation at both its Ser16 and its Thr17 phosphorylation sites (91). Furthermore, Sande and

colleagues showed that PP1 levels were upregulated in the decompensated phase of heart failure and this was accompanied by decreased PLN phosphorylation at the PKA site, Ser16 (92). In addition to this, PP1 activity was also shown to be enhanced, with concomitantly depressed PLN phosphorylation at both sites, in a rat model of cardiac hypertrophy induced by long-term beta-adrenergic stimulation (93) and in a different model of congestive heart failure induced by chronic renal hypertension (94). An important extension of these studies was the demonstration that the PP1 activity coupled to the SR was specifically increased in a canine model of heart failure (95). Overall, these sets of experiments show overwhelmingly that PP1 activity is increased during hypertrophy and congestive heart failure, regardless of the model, suggesting that this is a universal characteristic of heart failure and may be associated with cardiac dysfunction. Indeed, Carr *et al* validated this hypothesis in a more direct way (43). Specifically, a genetic model overexpressing PP1c-alpha to similar levels as those seen in human heart failure (89) was characterized by depressed cardiac function, dilated cardiomyopathy and premature mortality. These authors also showed that this increase in PP1 activity leads to depressed PLN phosphorylation. Collectively, these data point to an important role for PP1 in heart failure as a major contributing factor to dephosphorylation of PLN and aggravation of cardiac function.

6.2. PP1 regulation in heart disease

The increases in PP1 activity observed in the failing heart raised the possibility that regulation of this phosphatase via its endogenous inhibitors, I-1 and I-2, may be impaired in the diseased myocardium. As such, recent studies in both human and experimental heart failure sought to identify these potential alterations, focusing mainly on the role of I-1 (Figure 3). Two independent studies showed that the phosphorylation of I-1 at Thr35 was depressed in human failing hearts, which was associated with depressed PLN phosphorylation (43, 96). This decrease in I-1 phosphorylation levels may be a reflection of attenuated PKA activity or increased calcineurin activity observed in the failing heart (97). Similar results have been obtained in an experimental model of heart failure. In particular, Gupta *et al* showed that phosphorylation of I-1 at its PKA site was depressed, whereas I-2 phosphorylation was increased in a rat heart failure model induced by renal hypertension (94). Overall, these changes in phosphorylation were reflected in depressed inhibitory activity for both I-1 and I-2, which was manifested in increased PP1 activity and depressed PLN phosphorylation. In addition to depressed phosphorylation at Thr35, decreased I-1 protein levels have also been observed in the human failing heart (96), in a canine heart failure model (95) and after long-term beta-adrenergic stimulation, mimicking the excessive adrenergic drive observed in heart failure, in a rat model (98), thereby providing another mechanism by which PP1 activity may be enhanced under this pathophysiological setting. Importantly, the contribution of altered phosphorylation at Ser67 and Thr75 of I-1 to increased PP1 activity has yet to be explored. Conceivably, enhanced phosphorylation at these sites would provide another mechanism, which could account for the depressed phosphorylation at Thr35 and enhanced PP1 activity (58, 59, 62) observed in heart failure. A more recent study identified a polymorphism in the human I-1 gene, which entails substitution of a glycine at position 147 with an aspartic acid (99). This substitution resulted in a blunted beta-adrenergic response and depressed PLN phosphorylation in adult rat cardiomyocytes, suggesting that this polymorphism may be a contributing factor to impaired SR Ca cycling in heart failure. Importantly, alterations in PP1 activity have also been associated with another heart disease, namely chronic atrial fibrillation (cAF) in humans (100). Specifically, El-Armouche and colleagues have shown that total PP1 activity is increased in cAF, associated with depressed phosphorylation of MyBP-C at its Ser282 PKA site. Paradoxically, PLN phosphorylation was found to be enhanced, which was attributed to increased phosphorylation of I-1 at Thr35 and possibly decreased SR-coupled PP1 activity. As such, this study demonstrates differential compartmentalization and regulation of kinases and phosphatases in the cell. Furthermore, the authors postulated that hyperphosphorylation of PLN in the atria may enhance leakiness of the RyR and trigger arrhythmogenesis. Overall, these findings have shown that regulation of PP1c by its inhibitors is altered in the diseased myocardium, suggesting that restoration of PP1 regulation may be a plausible therapeutic modality, as discussed in the following section.

7. TARGETING PP1 IN HEART FAILURE

Generally, ionotropic therapies, either at the receptor or intracellular levels, have yielded controversial results as to their efficacy as therapeutic modalities (101). These controversial results could be attributed to the pleiotropic effects mediated by the targeted molecules. Therefore, more specific and more efficacious therapies are needed, which will restore contractile function, using a more targeted approach. Since several lines of evidence implicate PP1 as an important contributor to the cardiac dysfunction observed in heart failure, inhibition of this enzyme was the focus of many studies as a novel therapeutic strategy. Importantly, PP1 is functionally very diverse but is regulated tightly by numerous auxiliary proteins to carry out its role in specific subcellular compartments. This provides us with a unique opportunity to specifically target the PP1 compartmentalized to the SR as a therapeutic strategy in heart failure.

The potential of phosphatase inhibition as a viable therapeutic approach to enhance cardiac

function in heart failure was realized early on with the use of non-specific phosphatase inhibitors. Interestingly, a study by Linck and colleagues showed that the non-specific phosphatase inhibitor, cantharidin induced an ionotropic effect in failing human hearts, which was as strong as the effect of isoprenaline in non-failing hearts (102). Similarly, Huang *et al* showed that another phosphatase inhibitor, namely okadaic acid, enhanced PLN phosphorylation and partially restored the prolonged relaxation of intracellular Ca transients in a myocardial infarction-induced hypertrophy rat model, which was characterized by increased PP1 activity (91). Even though, these studies used non-specific inhibitors, they did raise the possibility that inhibition of phosphatases may alleviate the contractile dysfunction observed in the failing heart. Subsequent studies, described below, explored the therapeutic potential of more targeted strategies to inhibit SR-coupled PP1, using the endogenous inhibitors, I-1 and I-2.

Carr and colleagues demonstrated that the contractile response and the Ca kinetics of human failing myocytes were enhanced by adenoviral-mediated expression of the constitutively active (T35D), truncated form of I-1 (I-1c), described in Section 5 (43). This molecule lacks the Ser67 and Thr75 sites, which could limit I-1's therapeutic value and may thus represent a better therapeutic strategy. To better address any potential beneficial effects of chronic administration of I-c *in vivo*, the same group extended these studies to a transgenic mouse model overexpressing this molecular ionotrope (44). As described in section 5, these mice showed enhanced basal contractility and an enhanced beta-adrenergic response. Importantly, upon aortic stenosis, which recapitulates important clinical features of heart failure, these mice exhibited enhanced function and attenuated progression to heart failure, characterized by a diminished extent of cardiac hypertrophy and no decompensation. Furthermore, this study also showed that adenoviral delivery of I-1c in pre-existing heart failure, in a rat model of pressure overload, restored contractility to non-failing levels. Of special interest is the fact that these beneficial effects were mediated by enhanced phosphorylation of PLN, while phosphorylation levels of the RyR were unchanged. This may have important implications at a therapeutic level, since increased RyR phosphorylation may potentially lead to diastolic leakiness and arrhythmogenic activity (103, 104). A subsequent study, using I-2 as a therapeutic modality yielded similar results (105). Specifically, I-2 was delivered by gene transfer in a cardiomyopathic hamster, a well-established genetic heart failure model. I-2 overexpression during the transition phase from moderate to severe dysfunction successfully prevented heart failure progression both in the short-term, by adenoviral gene transfer of I-2 for one week, and in the long-term, by adeno-associated viral-mediated transfer of I-2 for 3 months, where it also increased survival. These beneficial effects were attributed to decreased PP1 activity and increased PLN phosphorylation at Ser16. Similarly to the I-1 studies, these authors did not note any changes in the phosphorylation state of the RyR. Collectively, these sets of experiments have established that PP1 inhibition, using these endogenous inhibitors may be a promising therapeutic strategy to alleviate the contractile dysfunction and remodeling observed in the failing heart due to their specificity for the SR-coupled enzyme and perhaps PLN.

8. CONCLUSIONS AND PERSPECTIVE

In summary, accumulating evidence implicates PP1 as a key regulator of cardiac function, which is very tightly modulated. At the level of the SR, this regulation is mediated via its association with I-1, I-2 and the GM/RGL targeting subunit, which appear to guide it to this subcellular compartment and perhaps to PLN. Importantly, perturbations in PP1 regulation via its endogenous inhibitors have been suggested to contribute significantly to the impaired function in the failing heart. As such, targeted inhibition of PP1, using I-1 and I-2 has been shown to be beneficial in alleviating the contractile dysfunction and remodeling observed in heart failure, by enhancing PLN phosphorylation. It is important to note that these studies were conducted in rodents, which differ significantly in regards to Ca cycling and motor proteins from humans. Therefore, it is essential to assess the potential beneficial effects of PP1 inhibition in higher species and in a clinical setting. Nevertheless, compelling evidence suggests that SR-coupled PP1 inhibition may be a plausible therapeutic strategy, since it not only alleviates contractile dysfunction but it also attenuates remodeling and progression to decompensation. Importantly, extensive crystallographic, structural and biochemical studies have defined the domains of PP1c, which mediate binding with its regulatory proteins, which may be instrumental in designing short and specific inhibitors, which may be used therapeutically in heart failure.

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Abbreviations: PP1: type 1 protein phosphatase; Ca: Calcium; PP1c: type 1 protein phosphatase catalytic subunit; I-1: inhibitor-1; I-2: inhibitor-2; SR: sarcoplasmic reticulum; LTCC: L-type Ca channel; RyR: ryanodine receptor; SERCA2a: SR/ER-Ca ATPase 2a; PLN: phospholamban; NCX: sodium-calcium exchanger; cAMP: 3'-5'-cyclic-AMP; PKA: protein kinase A; TnI: troponin I; MyBP-C: myosin binding protein C; PPP: phospho-protein phosphatases; PPM: magnesium-activated phosphatases; PP2A: type 2 protein phosphatase A; PP2B: type 2 protein phosphatase B; CaMKII: calmodulin kinase 2; MLC2a: myosin light chain 2a; PKC: protein kinase C; G-subunit: glycogen-targeting subunit; G_M: striated muscle glycogen-targeting subunit; G_L: liver glycogen-targeting subunit; R_{GL}: regulatory glycogen-targeting subunit; Cdk1: cyclin dependent kinase 1; Cdk5: cyclin dependent kinase 5; GSK3: glycogen-synthase kinase 3; ERK1/2: mitogen-activated protein kinase extracellular signal-related kinase 1/2

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