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REVIEW

Phosphatase-1 inhibitor-1 in physiological and pathological β-adrenoceptor signalling

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Abstract Control of protein phosphorylation–dephosphorylation events occurs through regulation of protein kinases and phosphatases. Phosphatase type 1 (PP-1) provides the main activity of serine/threonine protein phosphatases in the heart. Inhibitor-1 (I-1) was the first endogenous molecule found to inhibit PP-1 specifically. Notably, I-1 is activated by cAMP-dependent protein kinase A (PKA), and the subsequent prevention of target dephosphorylation by PP-1 provides distal amplification of β-adrenoceptor (β-AR) signalling. I-1 was found to be down-regulated and hypophosphorylated in human and experimental heart failure but hyperactive in human atrial fibrillation, implicating I-1 in the pathogenesis of heart failure and arrhythmias. Consequently, the therapeutic potential of I-1 in heart failure and arrhythmias has recently been addressed by the generation and analysis of several I-1 genetic mouse models. This review summarizes and discusses these data, highlights partially controversial issues on whether I-1 should be therapeutically reinforced or inhibited and suggests future directions to better understand the functional role of I-1 in physiological and pathological β-AR signalling.

Keywords β-Adrenoceptor signalling • Inhibitor-1 • Kinases • Phosphatases

1. Introduction

The prevalence of heart failure is continuously rising, and heart failure is among the most common causes of morbidity and mortality in Europe.[1] Almost half of heart failure patients die from sudden, probably arrhythmogenic cardiac death,[2] and heart failure is a frequent cause of atrial fibrillation (AF), the most common cardiac arrhythmia.[3] Regardless of the underlying cause, heart failure patients show a hyperactive sympathetic nervous system with elevated plasma catecholamine levels and subsequent chronic activation of the β-adrenoceptor (β-AR) signalling pathway. Accordingly, in a variety of animal models—including transgenic mice—β-AR overstimulation induces cardiac hypertrophy, contractile dysfunction and arrhythmias.[4,5] Conversely, large-scale clinical trials showed that β-AR blockers evoke remarkable beneficial effects and reduce mortality in heart failure patients.[6–8] Thus, β-AR overstimulation—although initially serving to compensate for contractile dysfunction—results in deterioration of cardiac function and structure. Despite significant progress in our understanding of the different components of the β-AR system, the precise mechanisms by which β-AR stimulation and blockade contribute to progression and attenuation of heart failure, respectively, are still unclear. Thus, better insight into the molecular mechanisms involved in physiological and pathological β-AR signalling and their alterations in heart failure is likely to identify key downstream elements that might provide targets for the development of novel therapeutic approaches.

Key components of the β-AR signalling system have been implicated in heart failure pathology, and accumulating evidence suggests a critical role of abnormal kinase/phosphatase signalling in heart failure as well as in atrial and ventricular arrhythmias. Inhibitor-1 (I-1), the major endogenous regulator of the most abundant cardiac serine/threonine type-1 protein phosphatase (PP-1), has recently been associated with heart failure and arrhythmia-associated atrial and ventricular remodelling. Therefore, I-1 has been suggested as a promising therapeutic target in heart diseases. Here, we summarize the physiological role of I-1 in cardiac β-AR signalling and its role in disease-related ventricular and atrial remodelling and critically discuss recent data from genetically modified I-1 mouse lines addressing the therapeutic potential of I-1-based approaches.

2. Physiological and pathological β-adrenoceptor signalling in the heart

The β_1-AR is the most abundant subtype in the normal heart.[9] Its stimulation via neuronally released and circulating catecholamines leads to

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activation of adenylyl cyclases and therefore increased cyclic AMP production. In pacemaker cells, cAMP shifts the voltage dependence of the inward pacemaker current to more depolarized potentials and thereby increases heart rate.[10] In ventricular myocytes, the primary target for cAMP is protein kinase A (PKA), which phosphorylates regulatory proteins that control excitation–contraction coupling: sarcolemmal L-type Ca^{2+} channels (LTCC at Ser1928, increasing Ca^{2+} current), phospholamban [PLB at Ser16, increasing sarcoplasmic reticulum (SR) Ca^{2+} pump rate and Ca^{2+} resequestration into the SR], the myofibrillar proteins troponin I (TnI at Ser23/24) and cardiac myosin-binding protein C (cMyBPC at Ser282, decreasing myofibrillar Ca^{2+} sensitivity and increasing cross-bridge kinetics; for review see[11]). PKA also phosphorylates sarcoplasmic ryanodine receptor channels (RyR2 at Ser2808, which may increase its open probability and SR Ca^{2+} release, the role of which remains controversial; for review see[12]). The concerted phopshorylation of target proteins results in stronger and faster contraction and relaxation of the cardiac muscle.

In addition, activation of β-AR leads to phosphorylation of Ca^{2+}-handling proteins via Ca^{2+}–calmodulin-dependent protein kinase II (CaMKII), e.g. RyR2 at Ser2814 and PLB at Thr17, with similar but not identical consequences to the PKA-mediated phosphorylation of the neighbouring sites (for review see[13,14]). This is in part due to PKA-mediated augmentation of Ca^{2+} cycling (Ca^{2+} influx and efflux), but seems also to occur independently of cAMP and cytosolic Ca^{2+} elevation.[15]

The actions of cAMP and PKA-mediated phosphorylation events are counterbalanced by cyclic nucleotide phosphodiesterases (e.g. PDE3 and PDE4) and Ser/Thr protein phosphatases (e.g. PP-1 und PP-2A), respectively. Their activity, in turn, is primarily controlled by their subcellular localization and/or binding to accessory subunits, which are regularly organized in macromolecular signalling complexes.[16–18]

One hallmark of the failing heart is β-AR desensitization, resulting in blunted β-AR agonist effects on contractile performance. Characteristic molecular alterations include down-regulation of β_1-AR, desensitization and uncoupling from stimulatory Gs proteins, as well as depressed intracellular cAMP synthesis[19] (for review see[5,20]). In parallel, the level and activity of PP-1 were consistently found to be increased in human and in experimental heart failure.[21,22] Consequently, the phosphorylation level of PKA substrates, e.g PLB (Ser16), TnI (Ser23/24) and cMyBP-C (Ser282), is lower in failing hearts.[23–25] However, desensitized β-AR signalling and higher PP-1 activity do not always result in lower phosphorylation of PKA substrates. Marx *et al*. reported higher PKA phosphorylation of RyR2 (Ser2808) in failing human hearts and in experimental heart failure models[26] paralleled by lower amounts of PP-1 and PDE4D3 associated with RyR2. The latter changes were assumed to be a potential molecular mechanism, because local PKA activity was not increased.[26] Therefore, it was concluded that the balance between the activities of PKA, PDEs, and PP-1 within the RyR2 macromolecular complex was changed in heart failure to favour RyR2 PKA hyper-phosphorylation, despite the 'global' increase in PP-1 activity. This concept was further supported by the demonstration that hypo-phosphorylation of PLB and hyper-phosphorylation of RyR2 at the PKA sites (Ser16 and Ser2808, respectively) occur in parallel within the same failing cardiomyocytes.[27] Collectively, these findings suggested that PP-1 activity may differ substantially in the distinct subcellular microdomains of the failing heart.

3. Protein phosphatase-1 and inhibitor-1 in the heart

Given the focus of this review on I-1, we will briefly discuss PP-1 and the regulation of the catalytic subunit PP-1c by association with regulatory subunits and its regulation by endogenous inhibitors.

3.1 Protein phosphatase-1

PP-1 regulates many biological processes, including protein synthesis, cell survival, carbohydrate metabolism, neuronal signalling, and muscle contraction.[16,17,28–30] Currently, there are four known isoforms of PP-1c encoded by three independent genes. With the exception of PP-1cγ2, which is predominantly expressed in testes, the other isoforms (PP-1cα, β, and γ1) are widely expressed in tissues.[17] Recently, Aoyama *et al*. specifically knocked down PP-1c isoforms in cultured cardiomyocytes and reported that amongst the α-, β/δ- and γ-isoforms, the knock-down of the β-isoform most strongly affected SR Ca^{2+} uptake by an enhancement of PLB phosphorylation.[31] This suggests that each isoform may govern a different subset of target phospho-proteins in cardiomyocytes.

In contrast to protein kinases, protein phosphatases do not exhibit consensus sequence selectivity, but dephosphorylate multiple substrates *in vitro* and *in vivo*.[32] Today, it is clear that PP-1c rarely exists in a 'free' monomeric form in the cell and that the functional diversity of PP-1 results from the association of its catalytic subunit with a variety of regulatory or inhibitory subunits.[16,17] The latter include the heat-stable inhibitors I-1, its neuronal homologue DARPP-32 (dopamine- and cAMP-regulated phosphoprotein; molecular weight 32 kDa), and inhibitor-2 (I-2).[33] These inhibitors act generally in a 'PP-1c substrate-independent manner' by blocking the active site of PP-1c, whereas the regulatory subunits (e.g. RGL, the glycogen-binding subunit, or MyPTs, the myosin-binding subunit) localize PP-1c in proximity to particular substrates and modify its activity towards specific substrates ('substrate specifiers'). Thus, it is possible that each regulatory targeting subunit recruits active PP-1c to dephosphorylate a specific Ser or Thr residue of one target protein with considerable selectivity.[16,17,34,35] Importantly, it has also been recognized that both regulatory (e.g. R_{GL}/G_M)[36] and inhibitory subunits (e.g. I-2[37,38]) are capable of stabilizing PP-1c isoforms by preventing their degradation.[17] This could have unforeseeable and confounding consequences and should deserve special attention when overexpressing PP-1c regulatory or inhibitory subunits *in vitro* and *in vivo*.

3.2 Structure, function, and regulation of inhibitor-1

I-1 was first identified in rabbit skeletal muscle by Huang and Glinsmann in 1976 and was the first endogenous molecule found to regulate phosphatase activity.[39] I-1 is a cytosolic protein and is widely expressed in mammalian tissues, including the heart and cardiomyocytes.[40,41] In contrast to most other proteins, I-1 is thermostable and cannot be precipitated by 1% trichloroacetic acid.[42] These features can be used to enrich I-1 from total homogenates (see below). I-1 is a small protein, consisting of 171 amino acids, and has a calculated molecular weight of ~19 kDa;[43] however, the protein migrates at ~26 kDa on SDS–PAGE. This discrepancy has been explained by a low degree of order in the protein.[44] The N-terminal region is highly conserved, and species differences were

noted only in the C-terminus.[43] Interestingly, I-1 is vertebrate specific and represents from an evolutionary point of view a rather recent addition of the fine-tuning interplay between PKA and PP-1 signalling.[17]

Functionally, I-1 is a potent and specific PP-1 inhibitor, but only after being phosphorylated at Thr35 by PKA (IC_{50} ~1 nM[44]). The additional essential elements in I-1 that mediate PP-1 inhibition include a tetrapeptide consensus motif (KIQF), enabling binding to PP-1 irrespective of its phosphorylation status.[34,44] Its inhibitory activity apparently requires that the phosphorylated residue is 'pushed' into the active site of PP-1c, as recently shown by investigating the effect of PKA phosphorylation on I-1 structure with nuclear magnetic resonance spectroscopy[45]). Unfortunately, crystallographic studies on Thr35-phosphorylated I-1/PP-1 complexes with detailed information about the interaction pattern are still missing.

Despite early discovery of I-1, the understanding of its physiological role progressed only slowly, partly because of difficulties in the immunological detection of endogenous I-1 with standard tools in most tissues. Initially, a non-specific band in standard western blots has been misinterpreted in human hearts by our laboratory and by others.[46,47] We have therefore adapted the trichloroacetic acid extraction protocol developed by Foulkes *et al*.,[48] taking advantage of the unusual physical properties of I-1. This allowed us to enrich I-1 >500-fold from heart tissues and subsequently to quantify I-1 protein levels.[49] The protein level in normal hearts is relatively low, being ~120 fmol/mg protein, which probably reflects the strong potency of I-1. Extraction of I-1 requires more than 100 mg cardiac tissue samples as starting material. Thus, methodological improvement for the detection of endogenous I-1, e.g. a high-sensitivity ELISA, is clearly warranted.

While exogenously applied or overexpressed I-1 can be used to test the participation of PP-1 in specific cellular functions, the results obtained do not necessarily imply physiological involvement of endogenous I-1. This has been elegantly documented by the observation that the expression of mammalian I-1 in yeast reduced the amount of protein of a number of endogenous PP-1 holoenzymes, although yeast itself does not express an I-1 homologue.[50] Nevertheless, the role of I-1 has been studied extensively in the brain, the skeletal muscle and most recently in the heart, where it was implicated in neuronal plasticity, glycogen metabolism and cardiac contractile function, respectively. This was achieved in particular due to the availability of I-1 knock-out mice (see section 5.1).

3.3 Inhibitor-1 in the heart

Early work indicated that I-1 Thr35 phosphorylation and thus its activity are subject to hormonal regulation, primarily by the sympathetic nervous system. Cohen and colleagues showed in skeletal and heart muscle that the *in vivo* phosphorylation of I-1 is ~30% in basal conditions, but increases up to ~70% after intravenous injection of adrenaline.[42,48] This was associated with a reduction in PP-1 activity. Subsequent studies by Iyer *et al*. demonstrated that the PP-1 activity towards PLB is solely decreased by phosphorylated (activated) I-1.[40]

We have overexpressed I-1 adenovirally in isolated cardiac myocytes and in engineered heart tissue (EHT) to test more directly the proposed role of I-1 in β-AR signalling. Indeed, I-1 overexpressing cardiac myocytes displayed enhanced PLB Ser16 phosphorylation as well as increased cell shortening in response to a submaximal concentration of isoprenaline. Likewise, EHTs overexpressing I-1 exhibited a five-fold higher sensitivity to the force-increasing effect of isoprenaline.[47] These experiments provided direct evidence that I-1 amplifies β-AR signalling via PP-1 inhibition, resulting in a stronger phosphorylation of at least one PKA substrate (*Figure 1A* and *B*). Moreover, Carr *et al*.[46] showed in diseased cardiomyocytes from human hearts that the diminished inotropic response to isoprenaline could be normalized by adenoviral overexpression of a truncated, constitutively active form of the I-1 protein (I-1c; for details on this mutant see *Figure 1C* and[44]).

Dephosphorylation and thus deactivation of I-1 at Thr35 is essential for returning its function back to the basal level. It seems to be a general feature that dephosphorylation of phosphatase inhibitors (e.g. I-1, DARPP-32, and I-2) is reversed by phosphatases other than the phosphatase they inhibit.[33] This allows a cross-talk, in which one phosphatase regulates the function of another phosphatase via dephosphorylation of an inhibitor. Indeed, we showed in isolated cardiac myocytes that blocking the Ca^{2+}-dependent phosphatase calcineurin (PP-2B) with cyclosporine A or PP-2A with ocadaic acid increased phosphorylation of I-1 at Thr35 and its downstream target PLB (PKA site Ser16).[51] These results indicated that PP-2B and PP-2A act to maintain a low basal level of phosphorylated (active) I-1 in cardiac myocytes (*Figure 1B*).

I-1 can also be phosphorylated at Ser67. Huang and Paudel have shown in brain tissue that Ser67 phosphorylation by neuronal cyclin-dependent-like kinase 1 CDC2-like kinase activates I-1 comparable to Thr35 phosphorylation, even if the Thr35 phosphorylation site was lacking (Thr35Ala).[52] In contrast, Bibb *et al*. observed no effect of I-1 phosphorylation at Ser67 by proline-dependent kinases on PP-1 inhibition in neurons.[53] More recently, Braz *et al*. demonstrated that phosphorylation of I-1 at Ser67 by PKCα attenuates its inhibitory activity towards PP-1[54] (*Figure 1B*). The PKCα-dependent regulation of I-1 has been associated with higher PP-1 activity, lower PLB-Ser16 phosphorylation, and depressed cardiac function in PKCα transgenic mice, whereas PKCα knock-out mice displayed exactly the opposite phenotype. By using adenovirally delivered short hairpin RNAs in EHTs we confirmed the principal finding on improved contractility through specific PKCα knockdown.[55]

4. Protein phosphatase-1 and inhibitor-1 in heart failure and arrhythmias

4.1 Heart failure

Human and experimental heart failure is associated with maladaptive changes within the β-AR signalling system and increased global and/or SR-associated PP-1 activity (see section 2). Consistent with these findings, chronic infusion of isoprenaline in rats was associated with cardiac hypertrophy and higher PP-1 activity, indicating that the augmentation of PP-1 is a consequence of β-AR overstimulation.[56] Interestingly, we observed that cardiac atrophy induced by mechanical unloading in rats (heterotopic heart transplantation) was also associated with higher PP-1 activity, to an extent similar to that in heart failure.[57] Assuming that mechanical stress—independent of its direction—causes a common response in cardiac myocytes, including a reactivation of the fetal gene programme, it seems plausible that increased PP-1 activity is a consequence of this and/or represents a common myocardial response to stress.[58] This could be considered

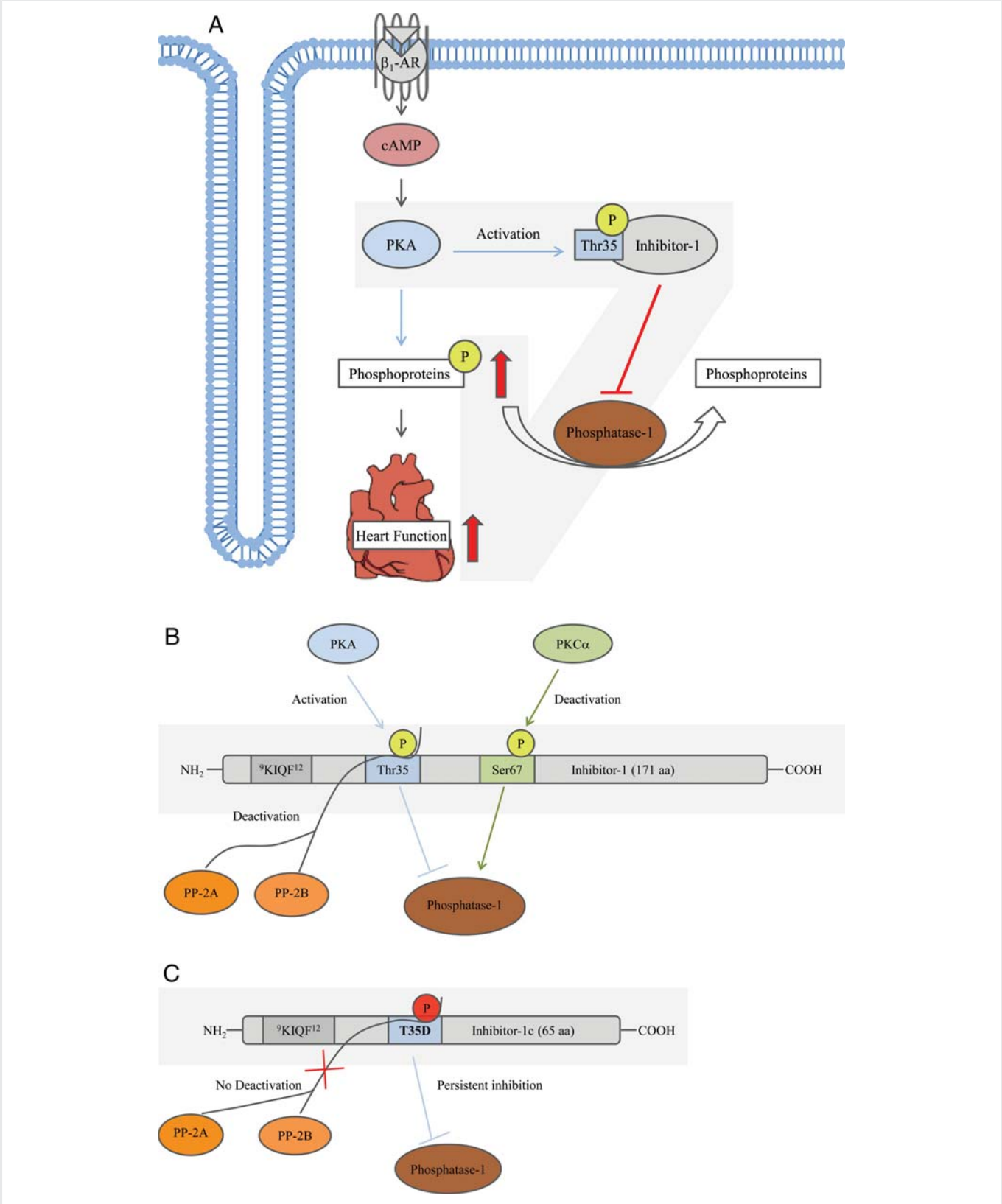

Figure 1 Function and regulation of inhibitor-1 (I-1) and I-1c. (*A*) I-1 represents a distal element of the β-adrenoceptor signalling, which allows amplification of protein kinase A (PKA)-mediated effects on the phosphorylation state of regulatory proteins by type-1 protein phosphatase inhibition. (*B*) I-1 consists of 171 amino acids (aa) and an N-terminal consensus motif (KIQF), which is essential for I-1 binding to protein phosphatase-1 (PP-1). I-1 becomes activate upon phosphorylation by cAMP-dependent PKA at Thr35, resulting in a potent and selective inhibition of PP-1. Phosphorylation at Ser67 by PKCα attenuates the inhibitory activity of I-1 towards PP-1. PP-2A and Ca^{2+}-dependent phosphatase-2B (PP-2B) dephosphorylate (deactivate) I-1. (*C*) Constitutively active I-1 form (I-1c) with replacement of Thr35 by phosphomimetic aspartic acid (T35D) and C-terminal truncation to 65 aa.

a maladaptive response because heart-specific overexpression of PP-1cα (approximately three-fold) was associated with depressed cardiac function, pathological hypertrophy and premature mortality in mice.[46] In contrast, by using the chemical phosphatase inhibitors, Ladilov *et al.* provided evidence that higher PP-1 activity protects isolated rat hearts from ischaemia–reperfusion injury and contractile dysfunction,[59] which may also protect against ischaemia-induced arrhythmias. Moreover, in a broader sense, PP-1 has been described as a 'green' molecule in mammalian physiology, which controls a balanced use of energy and plays an important role in the recovery from stress, but accelerates apoptosis, for example, when cells are damaged beyond repair (for review see[17]).

Thus, whether the activation of PP-1 is a beneficial or detrimental response of the failing heart is still not clear, and isoform-specific conditional knock-out models are needed to define ultimately the role of PP-1c isoforms in physiological and pathological conditions.

The finding of hyperactive PP-1 in failing human myocardium raised the possibility that I-1 might be dysregulated in heart failure. Indeed, I-1 mRNA and total I-1 protein amount were reduced by ∼60%, and its PKA-dependent phosphorylation level even by ∼80%, in failing human hearts.[47,49] Decreased I-1 phosphorylation presumably reflects desensitization of β-AR signalling with decreased cAMP levels and PKA activation in failing myocardium. An increase in PP-2B (calcineurin) activity, particularly in conditions of increased cytosolic Ca^{2+} levels, is likely to contribute to diminished I-1 phosphorylation as well. These data suggest that down-regulation and inactivation of I-1 could contribute at least partly to increased PP-1 activity and decreased PLB phosphorylation. Indeed, the degree of dephosphorylation of I-1 and dephosphorylation of PLB in the very same heart samples correlated, indicating a causal relationship.[49] Subsequent studies in experimental heart failure models, including a large animal model in dogs, confirmed the marked down-regulation and deactivation of I-1 in failing hearts.[60–62]

These data suggest that I-1 down-regulation is part of the stereotypic desensitization of the β-AR pathway in heart failure and secondary to the excessive adrenergic drive. Indeed, rats treated chronically with isoprenaline showed decreased I-1 mRNA and protein levels, albeit to a lower extent than in failing human hearts, indicating that other factors may also contribute to I-1 down-regulation.[60] It would be of great interest to test whether β-blocker therapy normalizes I-1 expression and phosphorylation, as it has been shown for $β_1$-AR and other components of the β-AR pathway.[63,64]

4.2 Ventricular arrhythmias

Almost half of heart failure patients die from sudden cardiac death, most probably due to ventricular tachyarrhythmias.[2] Generally, these arrhythmias are initiated and maintained by re-entry or focal (ectopic) mechanisms. At the cellular level, the latter have been linked to increased diastolic SR Ca^{2+} leak from RyR2 with subsequent activation of Na^{+}/Ca^{2+}-exchanger (NCX), which, because of its electrogenicity, generates depolarizing current that may cause arrhythmogenic delayed after-depolarizations. In this context, the role of PKA and CaMKII RyR2 phosphorylation for diastolic SR Ca^{2+} leak has been the subject of intense research and debate in recent years and has been extensively reviewed elsewhere.[12,65,66] While general consensus exists that β-AR stimulation increases SR Ca^{2+} leak, the role of RyR2 phosphorylation remains controversial.[12] Marx and colleagues suggested that PKA-dependent RyR2 phosphorylation at Ser2808 is the major mechanism, which destabilizes RyR2 via the loss of calstabin, increasing diastolic SR Ca^{2+} leak to cause triggered arrhythmias.[26] Recently, other studies showed that inhibition of CaMKII rather than of PKA reduces arrhythmogenic SR Ca^{2+} leak in experimental heart failure models.[15,67] van Oort *et al.* generated a knock-in mouse model, in which RyR2 at Ser2814 was replaced by aspartic acid (S2814D), simulating CaMKII-dependent 'constitutively activated' RyR2. These mice showed sustained ventricular tachycardia and a higher incidence of sudden cardiac death upon catecholaminergic or programmed electrical provocation, substantiating the important role of this phosphorylation site in arrhythmogenesis and sudden cardiac death.[68]

By contrast, only little attention has focused on the physiological and pathological role of RyR2 dephosphorylation by phosphatases. Both PP-1 and PP-2A are coupled to the RyR2 macromolecular complex by the leucine zipper adaptor proteins spinophilin and PR130, respectively.[26] The PP-1/PP2-A inhibitor okadaic acid increases the RyR2 open probability, and phosphorylated RyR2 subunits have been shown to be dephosphorylated by recombinant PP-1.[69] Unfortunately, the RyR2 phosphorylation states have not been reported in PP-1c or in PP-2Ac overexpressing mice. In heart failure, Marx and colleagues showed that both PP-1 and PP-2A protein levels were decreased in the RyR2 macromolecular complex, potentially contributing to RyR2 hyperactivity in heart failure, despite globally increased phosphatase activity.[26] In this scenario, the role of I-1, which is down-regulated and deactivated in heart failure, was difficult to implicate. Nevertheless, the potential role of I-1 in RyR2 phosphorylation, diastolic Ca^{2+} leak and the related arrhythmogenesis has recently been studied extensively in I-1 knock-out mice and I-1c transgenics. These data are discussed in detail in section 5.2.

4.3 Atrial fibrillation

Multiple studies have shown that abnormal SR Ca^{2+} handling plays a central role in initiation and/or maintenance of chronic AF (cAF) in humans.[70] Enhanced dephosphorylation of LTCC subunits and myofibrillar MyBP-C is supposed to contribute to an AF-associated reduction of LTCC current and atrial hypo-contractility.[3] In contrast, hyper-phosphorylation of RyR2 at PKA (Ser2808) and CaMKII (Ser2814) sites was shown to predispose to spontaneous SR Ca^{2+} releases in atrial myocytes from cAF patients.[71] The enhanced incidence of spontaneous SR Ca^{2+} releases is accompanied by an increase in NCX expression and function,[71,72] which may trigger ectopic focal discharges or facilitate micro-re-entry circuits promoting AF. The hyper-phosphorylation of RyR2 and PLB occurred despite globally increased PP-1 and PP-2A activities,[72,73] highlighting the importance of local differences in phosphatase activity/targeting within discrete cellular microdomains of diseased hearts. In cAF patients, SR Ca^{2+} content was preserved,[71] despite reduced SR Ca^{2+}-ATPase 2a (SERCA2a) protein levels, probably because of concomitant hyper-phosphorylation of PLB at both PKA (Ser16) and CaMKII (Thr17) sites.[72]

The molecular mechanisms of enhanced steady-state phosphorylation of RyR2 and PLB in cAF are poorly understood. Although PP-1 and PP-2A activities are increased in atria of cAF patients,[72] their activities within the RyR2 and PLB macromolecular complexes might not follow the global increases of phosphatase function. The expression and autophosphorylation (activity) of CaMKII at Thr287 are higher in cAF patients,[74] presumably contributing to the higher CaMKII phosphorylation of RyR2 (at Ser2814) and PLB (at Thr17).[72,74] The mechanism of enhanced steady-state PKA

phosphorylation of RyR2 (at Ser2808) and PLB (at Ser16) in cAF patients is less clear, because basal and cAMP-induced PKA activities were 50% lower in goats with sustained AF,[73] rendering an increase in PKA activity unlikely. In contrast to failing human hearts (see section 4.1), protein levels of I-1 as well as PKCα phosphorylation of I-1 at Ser67 and PKCα protein levels were unchanged in cAF patients.[72] More surprisingly, PKA-dependent Thr35 phosphorylation (activation) of I-1 was 10-fold higher in cAF patients,[72] which is sufficient to completely suppress SR-bound PP-1 activity,[75] indirectly increasing phosphorylation of PLB (Ser16) and RyR2 (Ser2814).[72,74] Since CaMKII may also phosphorylate Ser2808,[76] it could be speculated that within the SR compartment the AF-related increase in CaMKII activity[74] overcomes the enhanced PP-1 activity,[72] causing greater steady-state RyR Ser2808 phosphorylation.[74,77]

Hyper-phosphorylation of I-1 at Thr35 may contribute to AF-related atrial arrhythmogenesis, because inhibition of CaMKII reduces SR Ca^{2+} leak and Ca^{2+} spark frequency,[71] and genetic inhibition of CaMKII-mediated phosphorylation of RyR2 in RyR2 Ser2815Ala knock-in mice prevents carbachol-induced AF.[74] I-1 in cAF may amplify the CaMKII-induced hyper-phosphorylation of RyR2 at Ser2814[78,79] and could contribute to the increase in Ser16 phosphorylation of PLB,[72] thereby playing a permissive role in diastolic SR Ca^{2+} leak by preventing depletion of SR Ca^{2+} content. Thus, the identification of the molecular mechanisms underlying abnormal RyR2 and PLB phosphorylation, in which I-1 appears to play a critical role, is expected to uncover novel therapeutic options for AF treatment based on normalization of SR function.

The signalling pathways leading to PKA hyper-phosphorylation of I-1 at Thr35 during AF remain to be determined. Mice that overexpress the PKA catalytic subunit exhibit hyper-phosphorylation of PLB and RyR2 and develop AF,[80] supporting the arrhythmogenic potential of hyper-phosphorylated PLB and RyR2, and possibly I-1, in AF. However, high stimulation frequencies, as during AF, do not affect PKA phosphorylation at Ser16 of PLB or at Ser2808 of RyR2, and the β-AR density, Gs protein abundance, and adenylyl cyclase activity are preserved in AF patients,[81] excluding a pathologically enhanced β-AR signal transduction in cAF patients. Increased atrial angiotensin II[3] may activate presynaptic angiotensin II type 1 receptors and increase noradrenaline release from sympathetic nerve endings,[82] causing local PKA increases with subsequent PKA hyper-phosphorylation of I-1, which might not be reflected by measurements of global PKA activities. In cultured cardiac myocytes from neonatal ventricles, the major phosphatases that dephosphorylate I-1 at Thr35 are PP-2A and calcineurin,[72] but whether Thr35 phosphorylation of atrial I-1 is controlled by PP-2A and calcineurin is unknown, and the higher global PP-2A[72,83] and calcineurin[84] activities in cAF patients suggest either a locally distinct control of I-1 phosphorylation by these phosphatases or involvement of different phosphatases in I-1 dephosphorylation in the atria. More work is needed to verify these hypotheses.

5. Consequences of inhibitor-1 deletion and heart-specific I-1c overexpression

5.1 Deletion of inhibitor-1

The generation and subsequent investigation of a mouse model with I-1 deletion[85] provided the first evidence that I-1 is of important significance *in vivo* in the heart. In work-performing Langendorff-perfused hearts, I-1 deletion was associated with a mild decrease in basal contractile parameters but a significantly blunted β-adrenergic response.[46] Isolated left atria from I-1 knock-out hearts displayed normal isometric force in basal and maximally stimulated conditions but a rightward shift of the concentration–response curve of isoprenaline. While both results point to a less than normal β-adrenergic response of I-1-deficient hearts, two studies of cardiac function in the whole animal came to different conclusions. Carr *et al.*[46] demonstrated depressed basal heart function, whereas we[78] found normal basal contractile function. The reasons for this discrepancy are unknown, but could be related to differences in the depth of anaesthesia during echocardiography and/or the degree of catecholaminergic stimulation, as well as environmental factors in mouse husbandry. Importantly, neither the β-AR density nor the PP-1c protein levels differed from wild-type littermates, indicating no obvious major compensatory changes in the I-1 KO hearts.[46,78] Moreover, systemic deletion of I-1 was not associated with an apparent disease and did not negatively affect life expectancy and/or heart structure in elderly mice.[46,78] However, I-1 knock-out mice were partly protected against structural (hypertrophy, dilatation and fibrosis) and functional effects (loss of inotropic response to dobutamine) of chronic β-AR stimulation via minipump isoprenaline infusions.[78] Moreover, injections of increasing doses of isoprenaline with continued ECG monitoring revealed marked protection from fatal catecholamine-induced arrhythmias in I-1 knock-out mice. Notably, the protection against β-AR-mediated cardiotoxicity in I-1 knock-out mice was not associated with changes in heart rate regulation.

Immunoblot analysis of I-1 knock-out mouse hearts revealed preferential reduction of PLB phosphorylation at Ser16 and Thr17.[46] A subsequent study confirmed these findings and reported formerly unrecognized lower RyR2 phosphorylation at Ser2814, whereas the neighbouring RyR2 Ser2808 was unaltered.[78] The preferential regulation of Ser2814, rather than Ser2808 of RyR2 in I-1 knock-out mice was previously not described and suggested regulation of CaMKII phosphorylation by I-1. Indeed, PKA-activated I-1 can get control over the phosphorylation state of proteins which are phosphorylated by other kinases via PP-1 inhibition (see above). Autophosphorylation (activation) of CaMKII at Thr287 maintains the enzyme active for minutes even after intracellular [Ca^{2+}] declines to normal levels.[13] In neurons, I-1 appears to participate in a cross-talk between PKA and CaMKII by inhibiting PP-1-mediated dephosphorylation (inactivation) of CaMKII at Thr287.[86] Whether this mechanism exists in cardiac myocytes is currently unknown. Collectively, these findings substantiated in the whole animal that PLB and RyR2 are controlled by PP-1c and I-1 and that I-1 mediates its effects preferentially on the SR by specifically regulating PKA (PLB Ser16) and CaMKII (RyR2 Ser2814) phosphorylation sites.

Other typical PP-1 substrates, such as the myofibrillar TnI, MyBP-C and myosin light chain II, were not affected by I-1 ablation.[46,78] Likewise, although recent studies have shown that PP-1 is a key player in the regulation of LTCC, neither I-1 ablation[46] nor adenoviral overexpression in isolated cardiomyocytes (A. El-Armouche and G. Vandecasteele; and own unpublished data) had significant effects on the activity of the LTCC. The lack of I-1 effects on LTCC and key myofibrillar proteins exemplifies the paradigm that I-1 controls only a subset of cellular PP-1 targets with preferential localization at the SR.

It is likely that hypo-phosphorylation of RyR2 and PLB contributes directly to the protection against catecholaminergic stress, as we

noted in I-1 knock-out mice. Interestingly, Sossalla *et al.* recently reported that acute CaMKII inhibition by KN-93 or AIP improves contractility in trabeculae from failing human hearts, which was associated with reduced SR Ca^{2+} leak and increased SR Ca^{2+} load.[87] Thus, the lower phosphorylation at Ser2814 of RyR2 in I-1 knock-out mice is a potential candidate for the protective mechanism against arrhythmias and the preserved contractile response after chronic isoprenaline treatment in these animals. Normally, the concomitant hypophosphorylation of PLB is expected to cause less efficient diastolic SR Ca^{2+} re-uptake. This is thought to be an adverse effect due to an impaired relaxation, lower SR Ca^{2+} load, and therefore a decreased SR Ca^{2+} release during systole.[88] However, the combination of lower PLB and RyR2 phosphorylation could primarily lead to a reduced SR Ca^{2+} turnover, mimicking a lower degree of adrenergic stimulation. In particular, in situations of a high β-adrenergic drive or in the failing heart, this could be an attractive protective mechanism against arrhythmias and contractile dysfunction.

5.2 Overexpression of inhibitor-1 and constitutively active inhibitor-1 (I-1c)

Conventional transgenic mice overexpressing full-length I-1 under control of the alpha-myosin heavy chain promoter were generated to study the effects of enhanced I-1 function *in vivo*. These mice developed spontaneous cardiac hypertrophy and cardiac dysfunction already at a younger age (three months),[78] but at the same time also displayed a serious compensatory increase in PP-1c abundance and activity (three-fold). Thus, qualified conclusions based on data obtained from mice lines with more than 200-fold overexpression levels of I-1 were not possible.

By contrast, transgenic mice with ~25-fold overexpression of the truncated, constitutively active I-1c (*Figure 1C*) showed no compensatory increases of PP-1c levels.[89] This could be due to the lower expression and/or the truncation. Interestingly, these mice showed a hyper-contractile phenotype similar to PLB knock-out mice, identifying I-1c as a positive inotropic molecule *in vivo*.[89] In subsequent studies using models of pressure overload and chronic isoprenaline infusion-induced heart failure, I-1c overexpression was associated with a preserved cardiac function, an attenuated development of cardiac hypertrophy, and a lower degree of fibrosis and apoptosis.[89,90] Furthermore, inducible cardiac I-1c expression (Tet-Off system) protected against ischaemia–reperfusion-induced injury and was associated with both attenuated myocardial infarct size and apoptotic injuries[91] (see *Figure 2* for an overview). Consistent with the I-1 knock-out data, conventional and conditional I-1c transgenic hearts showed higher PLB phosphorylation at Ser16, whereas the PKA phosphorylation sites of RyR2 (Ser2808), TnI (Ser23/24), and MyBP-C (Ser282) were not affected. Thus, I-1c appeared as a specific regulator of PLB, suggesting that I-1c could be an interesting new target for gene therapy in heart failure.[92]

The beneficial effects of I-1c overexpression against heart failure progression and ischaemia–reperfusion-induced injury on the one hand and the protection against catecholamine-induced arrhythmias and remodelling in I-1 knock-out mice on the other hand created an apparently paradoxical situation. A potential explanation was that I-1c may confer specific protection because it lacks the inactivating PKCα site,[44] which may have detrimental consequences during catecholamine infusions in wild-type mice. Thus, loss of I-1 may have an overall benefit in these conditions.[78,92] In addition, I-1 and I-1c

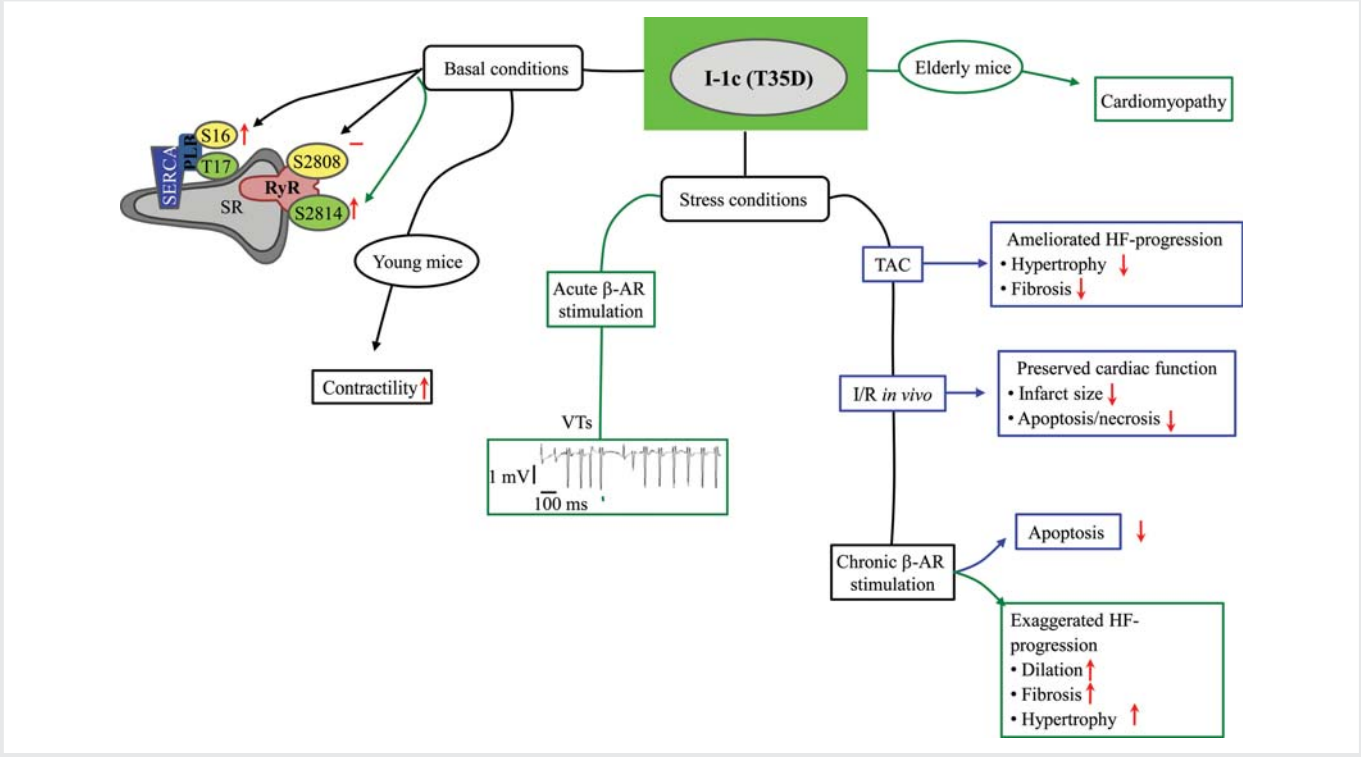

Figure 2 Consequences of I-1c overexpression *in vivo*. This scheme summarizes the outcome on the cardiac phenotype of I-1c transgenic mice at rest and in various stress models addressed by independent groups. Arrows/symbols: green arrow, I-1c on an I-1 knock-out background;[79] blue arrow, I-1c in the presence of endogoneous I-1;[89–91] black arrow;[79,89–91] (↑/↓) increase/decrease in function/phosphorylation; –, no effect.

may display different subcellular localizations and thus may target different pools of PP-1c and consequently differently located phosphoproteins.[78,92]

To further dissect the effects of I-1c vs. I-1 in the heart and the role of its PKCα phosphorylation, inducible transgenic mice (Tet-Off system) with cardiac-restricted expression of I-1c and a full-length mutant lacking the PKCα phosphorylation site (I-1 Ser67Ala) were generated and investigated in parallel.[79] To avoid unforeseeable or confounding consequences of competition between endogenous I-1 and I-1c/I-1 Ser67Ala, the lines were crossed into an I-1 null background (see discussion below and[93]). Given the above-mentioned potential of I-1c as a therapeutic target in heart failure, a disease of the elderly with increased risk for arrhythmias and sudden cardiac death, consequences of I-1c expression were not only studied at rest, but in particular in acute and chronic catecholaminergic stress, as well as in ageing mice. In line with previous reports,[89,91] the expression of both variants improved cardiac contractility in young mice at rest *in vivo*. However, both lines showed exaggerated structural consequences of chronic catecholaminergic stress (see *Figure 2* for an overview).[79] Moreover, telemetric ECG recordings in I-1c/I-1 Ser67Ala mice revealed that acute catecholaminergic stress caused partially lethal catecholamine-induced ventricular tachycardias, whereas they were absent in the control animals. Importantly, all abnormalities were reversed by shutting off I-1c/I-1 Ser67Ala transgene expression, proving a causal relationship. Moreover, aged I-1c and I-1 Ser67Ala mice spontaneously developed a cardiomyopathic phenotype. At the molecular level, I-1c/I-1 Ser67Ala hearts mirrored the effects observed in I-1 knock-out hearts, showing higher PLB Ser16 and RyR2 Ser2814 phosphorylation.[79] This indicated that I-1 and the mutated forms, I-1c and I-1 Ser67Ala, control similar subcellular PP-1c pools. In addition, isolated cardiomycytes from I-1c and I-1 Ser67Ala hearts showed higher Ca^{2+} spark frequency when challenged with isoprenaline,[79] offering a plausible link between hyper-phosphorylated RyR2 and the higher diastolic SR Ca^{2+} leak that underlies the higher risk for triggered arrhythmias. As proposed by Eisner *et al*., a higher spontaneous SR Ca^{2+} release should result in a self-limited event due to lowering of the SR Ca^{2+} content.[66] However, coincident PLB hyper-phosphorylation in I-1c/I-1 Ser67Ala mice would accelerate refilling of the SR, thereby promoting a permanent SR Ca^{2+} leak, which may lead to triggered activity and ventricular arrhythmias (see section 4.2).

The findings in I-1c and I-1 Ser67Ala mice of improved contractile function in young mice but accelerated age-related cardiomyopathy were reminiscent of earlier reports on mouse models overexpressing other stimulatory elements of the β-AR system, e.g. β_1-AR,[94] the α-subunit of stimulatory G proteins,[95] or the catalytic subunit of PKA.[80]

How can one reconcile the different studies with apparently opposing results? On the one hand, the propensity for stress-induced ventricular arrhythmias and the consequences of long-term expression on age-related cardiomyopathy have not been explicitly addressed in the studies showing beneficial effects of I-1c overexpression.[89–91] On the other hand, the two laboratories used different mouse strains, FVB/N vs. C57Bl/6J. However, it seems unlikely that this alone explains the higher susceptibility to catecholaminergic stress in the conditional I-1c/I-1 Ser67Ala mice, especially when considering the fact that the C57Bl/6J strain efficiently down-regulates cardiac β-AR density and thus exhibits lower cardiac toxicity in response to catecholamines.[96,97] The most relevant difference between both approaches is the factor that I-1c was expressed either on top of endogenous I-1,[89–91] or in the absence of endogenous I-1.[79] The rationale for expressing I-1c on an I-1 knock-out background in the absence of endogenous I-1 was that in the presence of endogenous I-1, the less potent (∼eight-fold higher IC_{50},[44] but stoichiometrically dominating I-1c, 25-fold overexpressed) would compete for binding to PP-1c with the phosphorylated (active) and the non-phosphorylated (inactive) I-1. The coexistence of I-1c and I-1 would thus attenuate the maximal effect of catecholamines due to a higher amount of I-1c, which binds to PP-1c but exerts less inhibitory activity (making I-1c a partial antagonist). However, in the absence of endogenous I-1, I-1c would increase basal activity, making I-1c a partial agonist. Therefore, the PP-1-inhibiting effect of I-1c will depend on endogenous I-1 levels and its phosphorylation status and thus on the degree of cellular catecholaminergic stress. This may indeed explain why the results from the I-1c studies differ substantially in pathophysiological conditions. Finally, the I-1c and I-1 Ser67Ala lines showed qualitatively and quantitatively super-imposable detrimental results, with: (i) an improved cardiac function in young mice; (ii) a higher susceptibility to catecholamine-induced arrhythmias, associated with an increased SR Ca^{2+} spark frequency; (iii) a hyper-phosphorylation of PLB Ser16 and RyR Ser2814; and (iv) exaggerated cardiac dysfunction after pathological β-adrenergic stimulation as well as with ageing.[79] These data argue against the concept that the PKCα phosphorylation of I-1 accounts for the adverse part of I-1 effects. Thus, identifying the functional impact of this residue *in vivo* remains a further challenge.

6. Conclusions and perspective

At present, we face the unusual situation that two contradictory therapeutic approaches are proposed by two groups, both based on solid experimental data in mouse models. On the one hand, it is intended to overexpress I-1c by adeno-associated virus (AAV) gene therapy, and on the other hand, to develop an I-1 antagonist strategy for the treatment of heart failure. The first strategy aims at improving Ca^{2+} re-uptake into the SR (via increased phosphorylation of PLB) and thereby increasing force of contraction and diastolic properties in patients with heart failure. The second strategy aims at protecting the heart from acute and chronic catecholamine toxicity in a manner similar to β-blockers but devoid of heart-rate lowering. The first strategy is based on the idea that I-1c specifically targets PLB and leaves RyR2 and thus Ca^{2+} release properties from the SR unmodified. This is an important difference to the action of cAMP-increasing drugs (which all tend to increase mortality) and is the rationale for SERCA gene therapy. Our data somewhat argue against this restricted action of I-1c because we have found RyR2 phosphorylation at Ser2814 (the CaMKII site) to be increased in I-1c overexpressing mice. However, the argument is well taken that differences may relate to the different genetic background, I-1 knock-out mice in our study[79] and wild-type mice in the studies from Dr Kranias's group.[89–91] If I-1c indeed acts as a partial agonist (and thereby as a partial antagonist in situations of stress), then I-1c gene therapy in patients with heart failure may indeed have predominantly beneficial effects. A final answer to the question of whether one should treat chronic heart failure by overexpressing I-1c or by I-1 antagonists will have to await further studies. Such studies would need a rigorous analysis of how I-1c overexpression on a wild-type background (in the presence of endogenous I-1) affects RyR2

phosphorylation (at the CaMKII site), Ca^{2+} spark frequency, and arrhythmogensis in various pathological models of heart failure and in advanced ageing. Another important step would be to establish AAV I-1c or anti-I-1 short hairpin RNA long-term gene transfer in large-animal models in the context of pre-existing structural heart disease and failure. Finally, translational proof-of-concept studies with either small potent 'I-1-like' PP-1 inhibitors or I-1 antagonists should be performed in 'clinically relevant' animal models. Crystallographic studies on Thr35-phosphorylated I-1/PP-1 complexes with detailed structural information will be essential for the design of such novel I-1-modulating drugs. These novel agents should be studied in large-animal models and particularly on top of existing pharmacological therapy (including β-blockers and angiotensin-converting enzyme inhibitors) with hard end-points, such as survival and sudden death.

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